CELL-FREE BIOSYNTHESIS OF ABSCISIC ACID (ABA) IN EXTRACTS OF FLAVEDO FROM *CITRUS SINENSIS* (L.) OSBECK.

by

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In memory of

Douglas George Richardson 1938-1983

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ABSTRACT

The biosynthetic origin of the plant growth regulator abscisic acid remains equivocal and almost nothing is known about the enzymes involved in this process. The present research programme describes the development of a cell-free system, capable of synthesizing abscisic acid and attempts to provide further information about the biochemistry and enzymology of this important biosynthetic pathway.

Cell-free extracts were prepared either directly from the flavedo (crude) or from an acetone powder derived from flavedo, of mature coloured fruits of *Citrus sinensis* L. cv. Midknight and incubated with mevalonic acid, isopentenyl pyrophosphate, farnesyl pyrophosphate, geranylgeranyl pyrophosphate, β-carotene and 1',4'-*trans*-abscisic acid diol. The neutral and acidic products formed were purified by thin-layer chromatography and high performance liquid chromatography, and quantified by high performance liquid chromatography, gas chromatography-electron capture and unequivocally identified by combined gas chromatography-mass spectrometry.

Abscisic acid, 1',4'-*trans*-abscisic acid diol and phaseic acid were unequivocally identified as the major acidic products formed in this cell-free system. The acid fraction also contained xanthoxin acid. Labelled and unlabelled β-carotene was converted into the neutral compounds xanthoxin and xanthoxin alcohol. In addition, high performance liquid chromatography-photodiode array analysis of the oxy-carotenoid fraction revealed the complete spectrum of β,β-carotenoids including zeaxanthin, antheraxanthin and violaxanthin with accumulation of an oxygenated carotenoid tentatively identified as 9-*cis*-violaxanthin. Identification of putative C_{15} intermediates was achieved by either UV spectrophotometry and combined capillary gas chromatography-mass spectrometry or microchemical analysis and co-chromatography. Refeeding studies using (±)-[2-^{14}C]-abscisic acid diol as substrate revealed that abscisic acid was not metabolized to abscisic acid diol, suggesting that it was/is produced as an intermediate rather than as a catabolite of ABA in this system.

Stigmasterol, and to a lesser extent cholesterol reduced conversion of β-carotene to abscisic acid but did not influence transformation of 1',4'-*trans*-abscisic acid diol to

abscisic acid. AMO1618 stimulated formation of abscisic acid and appeared to exert its effect at the level of conversion of 1',4'-*trans*- abscisic acid diol. Zeatin and the cytokinin analogue, ancymidol inhibited the biosynthesis of abscisic acid whereas dithiothreitol increased incorporation of label from β-carotene into abscisic acid suggesting involvement of a cytochrome P450-type mixed function oxidase in this reaction sequence. Sodium dodecylsulphate polyacrylamide gel electrophoresis of the enzyme extract derived from *Citrus flavedo* revealed the presence of a 53 kD protein with peroxidase activity characteristic of a cytochrome P-450.

Abscisic acid biosynthesizing activity was always greater in extracts from acetone powder and abscisic acid biosynthesis was enhanced in the presence of AMO 1618, NAD^+, NADH, NADPH, $MgCl_2$ and Molybdate but was inhibited by FAD. Activity was further enhanced by the addition of (R,S)-abscisic acid as a cold-pool trap and by including 0.1% w/v of either Tween 20 or Triton X 100 in the extraction buffer. When *cis*-β-carotene was used as substrate, no abscisic acid was produced. Conversely when either *all-trans*-β-carotene or a mixture of the two isomers was used, incorporation into abscisic acid occurred.

Lipoxygenase activity in cell-free extracts of Citrus flavedo increased with increasing protein concentration. As the ability of lipoxygenase to make xanthoxin from violaxanthin, had been reported, increased activity in the cell-free system implied that carotenoid cleavage was being brought about by a non-haem oxygenase with lipoxygenase-like properties.

Reports had implicated phoshorylation in the activation of many catalytic enzymes (Hanks *et al.*, 1985). Phosphorylation of the enzymes in this cell-free system proved unsuccessful. Further, it had been reported that *in vitro* phosphorylation of several membrane polypeptides and soluble polypeptides from corn, had been promoted by the addition of Ca^{2+}. In this cell-free system Ca^+ did not have a stimulatory effect on protein phosphorylation.

Dioxygenases generally occur as soluble enzymes, where they catalyse many oxygenation reactions in metabolic pathways. The addition of 2-oxo-glutarate, a requirement of most soluble oxidases, did not affect the activity of the cell-free system.

CHAPTER 1

INTRODUCTION

Abscisic acid (ABA Fig. 1.1), is a plant growth regulator that has an important role to play in normal plant growth and development. Since its discovery, about 30 years ago, an enormous amount of information relating to the biochemistry and physiology of this important natural product has been forthcoming. ABA was originally thought to be a growth inhibitor but it is now known that, like other plant growth regulators, it is involved in many aspects of growth and development (Zeevaart and Creelman, 1988). ABA has therefore been implicated as a key regulator in the mediation of processes such as dormancy, abscission, senescence and fruit ripening. More importantly though, is its role in the response of plants to stress and in particular, drought stress. Unfortunately much of the early evidence supporting such roles for ABA was inconclusive as many experiments were based on external application of high concentrations of ABA to intact plants or excised tissues. It becomes difficult to interpret the physiological significance of any response to such abnormal distributions and concentrations of ABA. Correlations between ABA levels and physiological changes were thus, often based on unreliable and inaccurate techniques for quantification of ABA (Neill and Horgan, 1987).

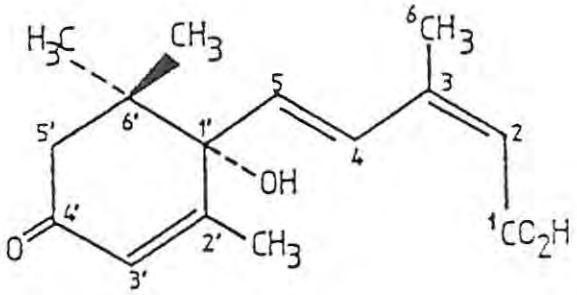


Figure 1.1 *The structure of naturally-occurring (+)-S- abscisic acid.*

More recent experiments have employed greater accuracy in the procedures used for the measurement of ABA levels and have made use of mutants deficient in ABA in order to determine the molecular mechanisms mediated by this growth substance

at the cellular level (Brenner *et al.*, 1977; Duckham *et al.*, 1991; Fong *et al.*, 1983a; 1983b; Karssen *et al.*, 1987; Moore *et al.*, 1985; Moore and Smith, 1984; 1985; Parry *et al.*, 1991; Robichaud *et al.*, 1980; Rock *et al.*, 1992; Smith *et al.*, 1978). Thus the most reliable information regarding the mode of action of ABA has come from studies of the following processes viz.(1) stomatal closure, (2) inhibition of precocious germination of embryos and (3) tolerance to stress (Hetherington and Quatrano, 1991).

When plants are exposed to conditions of water stress endogenous levels of ABA increase and, this increase is localised to the area surrounding stomatal guard cells (Davies and Mansfield, 1983; Hartung, 1983a; 1983b). Associated with the increase in ABA is rapid stomatal closure brought about by the redistribution of intracellular ABA (Hartung and Slovik, 1991; Slovik *et al*, 1992a; 1992b; 1992c). In addition, it has been shown that roots also synthesise ABA in increased amounts as they are exposed to drying soil. Correlative evidence for the control of shoot growth and physiology by a root signal has been provided by the increasing number of reports of a clear relationship between leaf conductance and soil water status and an apparent relationship between both of these variables and the concentration of ABA in the xylem (Davies *et al.*, 1994; Hetherington and Quatrano, 1991; Janssen and Markhart, 1993; Khalil and Grace, 1993; Tardieu *et al.*, 1992a; 1992b).

Perhaps the best understood role of ABA at the cell level is its involvement in the control of stomatal aperture. Stomatal aperture is generally controlled by osmotic adjustment in the two opposing guard cells surrounding each stoma and is directly related to the apoplastic concentrations of ABA. Apoplastic levels of ABA increase as a result of dehydration-induced release of ABA from leaf mesophyll cells and production of ABA by roots in drying soil, which moves via the transpiration stream and accumulates at or near the guard cells. The active site of ABA action for stomatal regulation has been shown to be the cell wall face of the guard cell plasmalemma (Hartung and Slovik, 1991), and this ABA may interact directly with membrane phospholipids to alter membrane permeability mediated by phosphoinositide metabolism by regulating cytosolic Ca^{2+} levels (Blatt *et al.*, 1990; Gilroy *et al*, 1990; 1991). The rapid decrease in the levels of phosphatidylinositol 4,5-bisphosphate [$PI(4,5)P_2$] in guard cell protoplasts in response to ABA, might be

the result of hydrolysis to produce inositol 1,4,5- trisphosphate [Ins(1,4,5)P_3] (Brearley and Hanke, 1994; Cotè and Crain, 1993; Lee *et al.*, 1993).

Although ABA induces closure of stomata under stress (Zeevaart and Creelman, 1988), the total ABA content per unit leaf area does not increase before stomata close (Dörffling, 1983). Consequently there must be a redistribution of ABA between different leaf compartments. As ABA redistributes almost ideally according to the anion trap concept (Baier and Hartung, 1988; Hartung, 1983a; Slovik *et al.*, 1992; Slovik and Hartung 1992a; 1992b), there must be compartmental pH-shifts, and these have been observed in different compartments under osmotic stress, drought stress and possibly frost stress (Anderson *et al.*, 1994b; Baier and Hartung, 1988; Berkovitz and Gibbs, 1983; Chen and Gusta, 1983; Daeter and Hartung, 1990; Hartung *et al.*, 1981; 1988; Hartung and Radin, 1989; Isawari and Palta, 1989; Slovik and Hartung, 1992a, 1992b).

Most evidence suggests that the site of ABA action in guard cells is located on the outer membrane, where specific ABA-binding proteins are located. (Anderson *et al.*, 1994a; Gilroy and Jones, 1994; Hartung, 1983b; Hornberg & Weiler, 1984). The physiological cascade initiated by the binding of ABA either to a receptor (or transport protein) or through an interaction with membrane lipids (Hetherington and Quatrano, 1991) prevents stomatal opening by arresting H^+ extrusion and K^+ influx, and initiates closure by facilitating the release of K^+, Cl^-, and malate$^-$. This suggests that ABA may influence the H^+-ATPase that regulates proton movement across the membrane. Furthermore changes in ABA are known to occur coincidental with changes in intracellular Ca^{2+} and this impacts on metabolism by influencing the phosphorylation of regulatory proteins and enzymes such as the well characterised Ca^{2+}-calmodulin pathway (Shimazaki *et al.*, 1992; Veluthambi and Poovaiah, 1984).

An increase or decrease in concentration of plant hormones, or other regulatory chemicals, is achieved by enzyme synthesis. and hence alterations in both anabolic and catabolic enzyme activities and by import and export of the hormone from cells and tissues. Biochemical studies have shown that ABA can induce and inhibit the synthesis of specific proteins (Bray, 1991a; 1991b; Dommes and Northcote, 1985a, 1985b; Lin and Ho, 1986; Walbot and Bruening, 1988). ABA-deficient mutants have been used to conclusively demonstrate that elevated levels of ABA

are required for the expression of specific drought-induced genes (Bray, 1993), as it has been shown that a key role of ABA in developmental and physiological processes appears to be its ability to induce and suppress the expression of specific genes (Skriver and Mundy, 1990; Zeevaart and Creelman, 1988) and numerous clones of ABA-responsive genes have been isolated (Bray, 1991a; Cohen and Bray; 1990, Kurkela and Franck, 1990; Skriver and Mundy, 1990). Although the function of the products of these genes remains to be elucidated, evidence from amino acid sequence analysis and homologies with known proteins suggest possible roles as seed storage proteins, osmoprotectants and regulatory proteins (Parry, 1993). Although it is well documented that plant hormones such as ABA act by modulating the levels of mRNA transcripts, the pathway from ABA to this level of control is not known (Hetherington and Quatrano, 1991). Some of the enzymes involved in ABA metabolism could also be induced by increased intracellular ABA content (Lin and Ho, 1986).

1.1 Biochemistry of Abscisic Acid.

The catabolism of ABA in plants has been extensively investigated (Cowan and Railton, 1987b; Gross, 1972; Loveys and Milborrow, 1984; Milborrow, 1968; Murphy, 1984; Sembdner *et al.*, 1980; Tietz *et al.*, 1979; Zeevaart and Creelman, 1988; Zeevaart and Milborrow, 1976). When (±)-[2-^{14}C] ABA was fed to petiolar segments of bean (*Phaseolus vulgaris*) and sycamore (*Acer pseudoplatanus*), two labelled metabolites were identified (Milborrow, 1968). The one was identified as the glucose ester of ABA (ABAGE Fig. 1.2, structure 1; Milborrow, 1970), and the other as an oxidation product. This oxidation product was identified by Milborrow (1968) as a 6'-hydroxymethyl derivative of ABA (6'-HM-ABA; Fig. 1.2, structure 2) and has subsequently been renamed 8'-OH-ABA in accordance with the revised numbering system for ABA and its metabolites (Boyer *et al.*, 1985), which was spontaneously rearranged to phaseic acid (PA; Milborrow, 1969). The original structure proposed for PA (Fig. 1.2, structure 3), was incompatible with isomerisation from a hydroxylated form of ABA, and an alternative structure (Fig. 1.2, structure 4) for PA was proposed (Milborrow, 1969). This was later confirmed by synthesis (Abrams *et al.*, 1990; Takahashi *et al.*, 1986). Studies by Walton and Sondheimer (1972) demonstrated that excised embryonic axes of *Phaseolus vulgaris* rapidly catabolized ABA into two acidic products viz. PA and 4'-

dihydrophaseic acid (4'-DPA; Fig. 1.2, structure 5; Tinelli *et al.*, 1973). The absolute configuration of PA and 4'-DPA were later determined by Milborrow (1975) as being (-)-3-methyl-5[8{1(R), dimethyl-8(8)-hydroxy-3-oxo-6-oxabicyclo (3,2,1)-octane}] 2-*cis*-4-*trans*-pentadienoic acid and (-)-3-methyl-5[8{3(5),8(5)-dihydroxy-1(R),5 (R)-dimethyl-6-oxabicyclo-3(3,2,1)-octane}] 2-*cis*-4-*trans*-pentadienoic acid, respectively. Later work has shown that ABA and its acidic catobolites undergo oxidative degradation, and that both ABA and the oxidized metabolites are conjugated with glucose (Loveys and Milborrow, 1984).

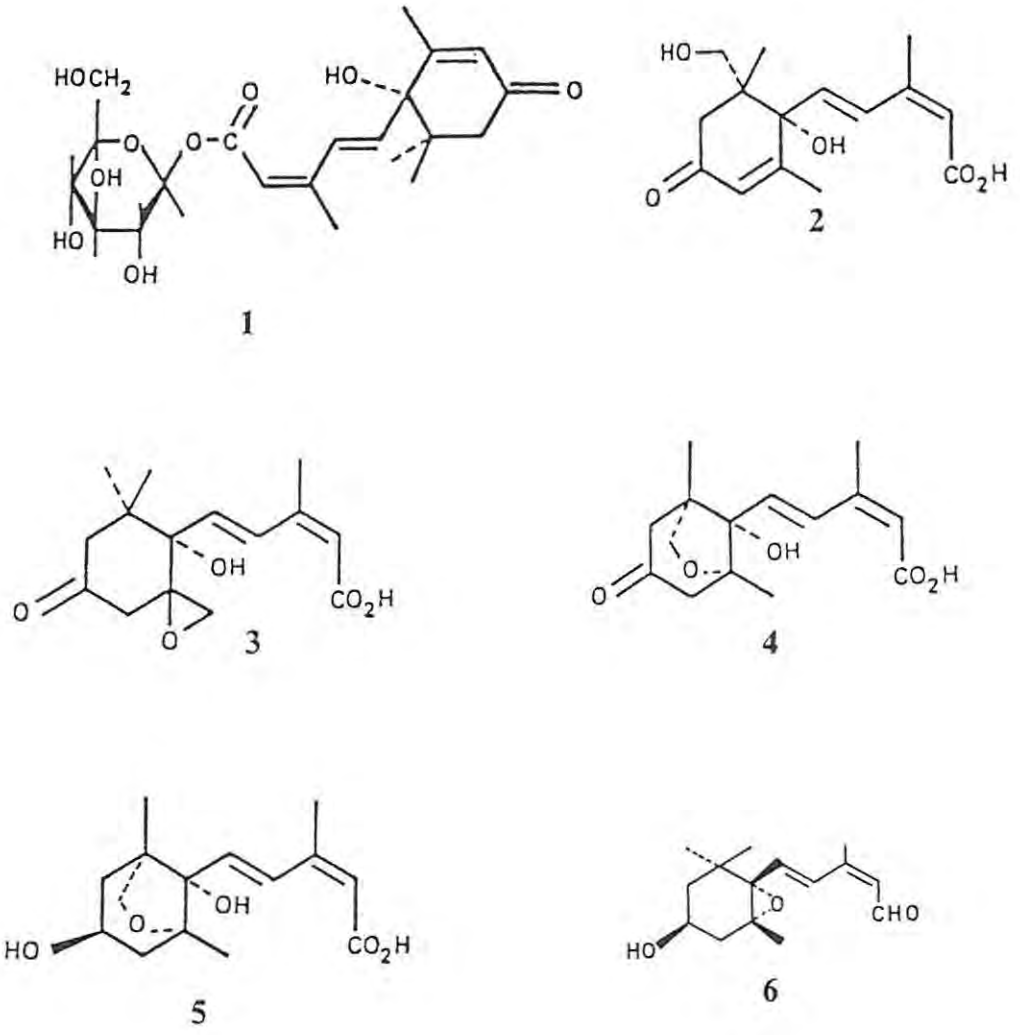


Figure 1.2 *Structural formulae of compounds implicated in ABA biosynthesis.* 1) ABA glucose ester, 2) 6'-HM-ABA now 8'-OH-ABA, 3) Phaseic acid (original structure, 4) Phaseic acid (presently accepted formula), 5) 4'-DPA, 6) Xanthoxin.

In contrast, the pathway of synthesis is less well defined. Although our understanding of ABA synthesis in plants has progressed greatly in the past 10 years, the pathway has still not been completely elucidated. ABA has the structure of a sesquiterpenoid and as all terpenoids are derived from melavonic acid (MVA) by a well characterised pathway (Goodwin, 1971; Goodwin and Mercer, 1983; Popjack and Cornforth, 1966) it would be expected that ABA should be similarly produced in higher plants (Figure 1.3). However, based on the structure of ABA and its close resemblance to carotenoids, Taylor and Smith (1967) postulated that ABA might be derived by carotenoid breakdown. Taylor and Burden (1970a), demonstrated that photolytic cleavage of violaxanthin, a C_{40} carotenoid, resulted in the production of a C_{15} intermediate characterised as xanthoxin (Fig. 1.2, structure 6). Subsequently, Firn and Friend (1972) showed that xanthoxin could also be produced by enzymatic cleavage of violaxanthin with lipoxygenase. This work lent some credence to the hypothesis that ABA might be an apo-carotenoid and thus derived from MVA via the carotenoid biosynthetic pathway. In addition, Burden and Taylor (1976) demonstrated the conversion of xanthoxin to ABA in shoots of bean (*Phaseolus vulgaris*) and tomato (*Lycopersicon esculentum*) which also supported the carotenoid origin of ABA (Fig. 1.4). However, research into this avenue declined in the mid seventies largely because Robinson (cited in Milborrow, 1974a) showed that when [^{14}C]-phytoene (the precursor of carotenoids) and [^{3}H]-MVA (general precursor of terpenoids) were applied to plant tissues simultaneously, only tritiated ABA could be recovered. The result of this study implied that carotenoids were not the precursors of ABA in higher plants. However, what Robinson had failed to do was determine the distribution of radioactivity in the xanthophyll fraction and so these results remain inconclusive. Thus in the 1970's and 1980's it was considered that ABA could be derived from either of two probable pathways. The first, or "direct" pathway suggested that ABA was formed from farnesyl pyrophosphate (FPP), the precursor of sesquiterpenoids, possibly via xanthoxin whereas the alternative or "indirect" pathway indicated that ABA was formed as an apo-carotenoid by enzymatic (photolytic) cleavage of a C_{40} carotenoid which was then converted to ABA via xanthoxin.

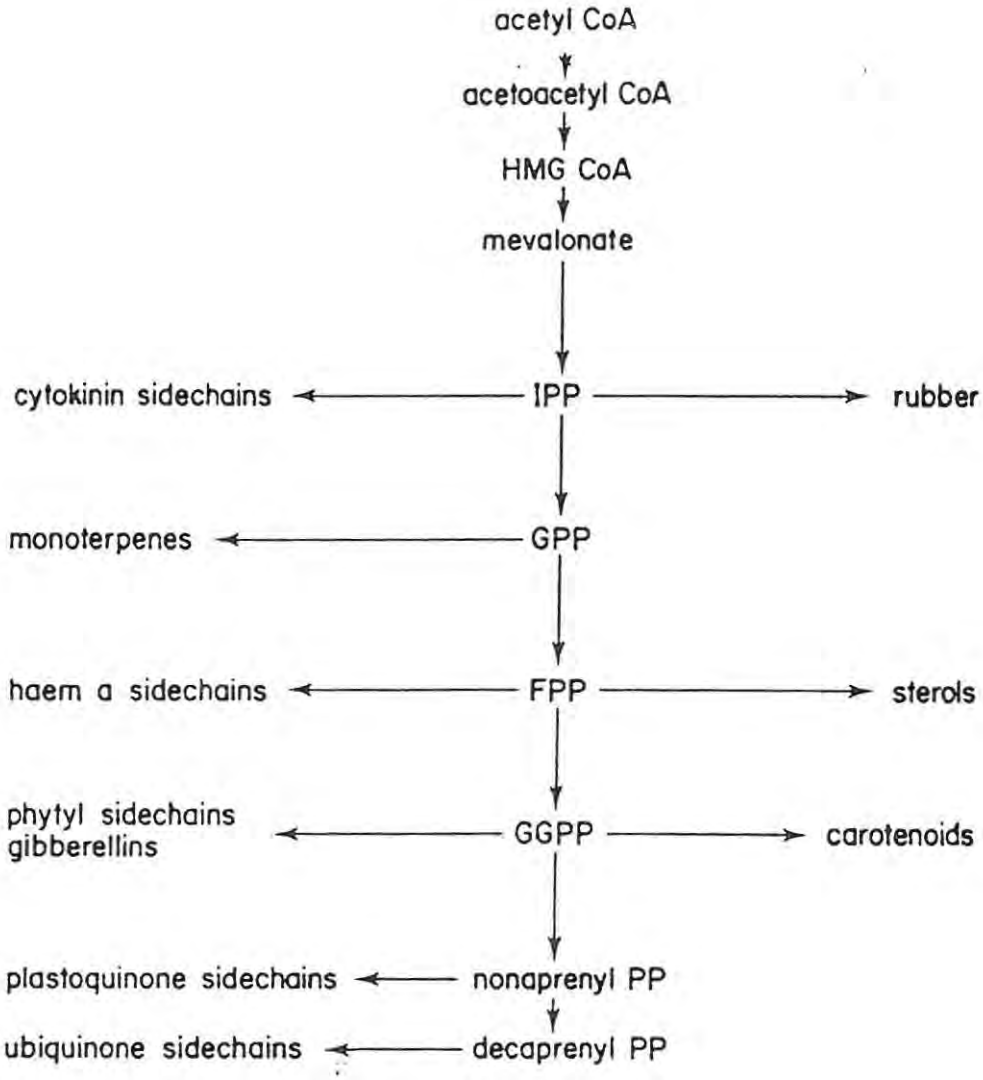


Figure 1.3 *Scheme for the biosynthesis of isoprenoid compounds from a common pathway (from Gray, 1987).*

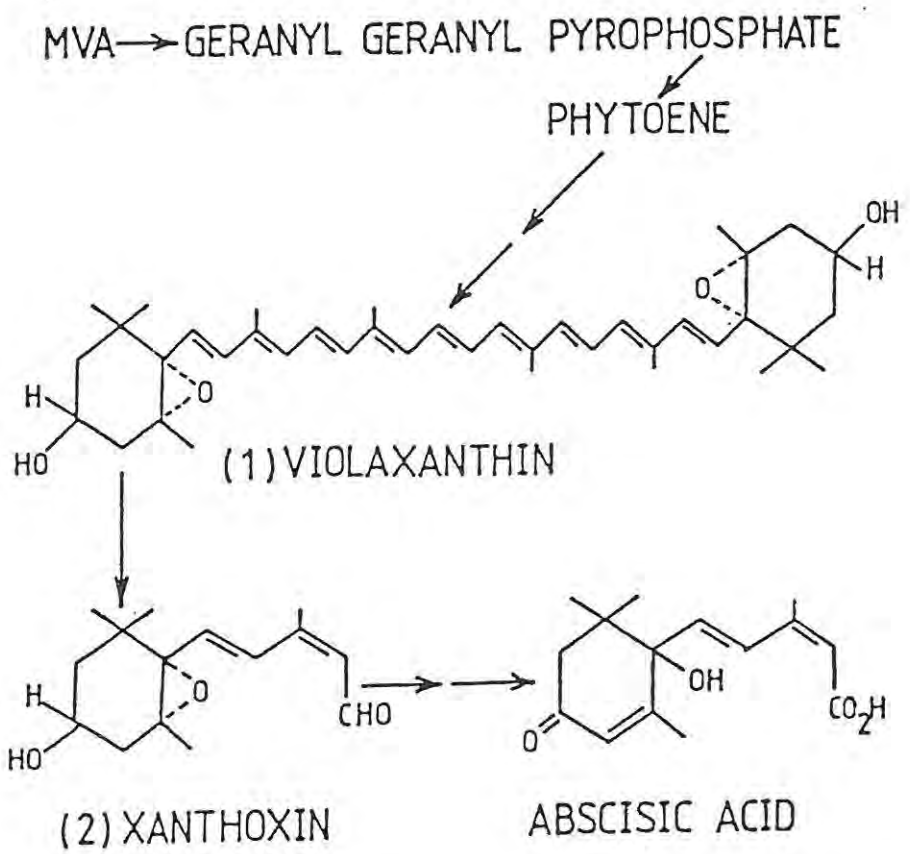


Figure 1.4 *The hypothetical carotenoid (C-40) pathway for abscisic acid biosynthesis.*

1.1.1 Direct pathway of ABA biosynthesis.

ABA can be produced directly from a C_{15} precursor derived from FPP. Initially, Noddle and Robinson (1969) reported that label from MVA was incorporated into ABA in fruits of *Lycopersicon esculentum* and *Persea americana*. In addition, subsequent investigations demonstrated that label from MVA was also incorporated into ABA in wilted leaves of *Triticum aestivum* (Milborrow and Noddle, 1970), stems, leaves and cotyledons of *Persea americana* (Milborrow and Robinson, 1973), lysed chloroplasts from *Persea americana,* fruit and leaves of *Phaseolus vulgaris* (Milborrow, 1974a) and in sterile liquid suspension cultures prepared from *Vitis vinifera* pericarp tissue (Loveys *et al.*, 1975). MVA was also incorporated into ABA in several other tissues (Cowan and Railton, 1986; Hirai *et al.*, 1986; Milborrow, 1983) but the yield was always very low. This could be as a result of competition for MVA by other terpenoids in the pathway (e.g. sterols) and extensive dilution of the radioactive MVA by a large precursor pool of ABA.

The biosynthesis of sesquiterpenes from labelled sodium acetate and MVA has been widely demonstrated in fungal preparations (Arigoni, 1975; Baker *et al*, 1975; Bradshaw *et al*, 1978; Cane *et al*, 1981; Cane, 1983; Evans and Hanson, 1975). In addition, FPP has been implicated as the key intermediate in the biosynthesis of these compounds (Banthorpe and Charlwood, 1980; Loomis and Croteau, 1980). In addition, studies employing MVA as substrate have demonstrated the synthesis of numerous sesquiterpenoids in higher plants (Banthorpe *et al.*, 1985; Coolbear and Threlfall, 1985; Croteau *et al.*, 1972; Croteau and Loomis, 1972; Gliezes *et al.*, 1984; Loomis and Croteau, 1980). It was therefore, not unreasonable to expect that ABA could be similarly derived (see Fig. 1.3). In addition, Robinson and Ryback (1969) suggested that ABA was biosynthesized from a C_{15} precursor having a *trans*-double bond corresponding in position to the *cis*-2,3 double bond of ABA, based on the retention of ^{3}H at C-2 and C-5' in the ABA molecule, when [(4R)-4-^{3}H]-mevalonate was used as substrate. Attempts to elucidate a C_{15} (direct) pathway of ABA biosynthesis in plants relied heavily on feeding of synthetic analogues. For example, Milborrow and Noddle (1970) demonstrated the conversion of the epoxide 5-(1,2-epoxy-2,6,6-trimethyl cyclohexyl)-3-methylpenta-*cis*-2-*trans*-4-dienoic acid into ABA in *Lycopersion esculentum* fruit and that water stress increased

incorporation of this putative precursor into ABA in leaves of *Triticum aestivum*. Furthermore, Milborrow and Garmston (1973), demonstrated that plants converted this epoxy acid to (-)-1',2'--2-*cis*-xanthoxin acid which is not converted to ABA in plants. Similarly, the α-ionylideneacetic acids which were supplied to intact plant tissues could not be converted to ABA (Lehmann and Shütte, 1976). It was later shown that whereas (+)-(2Z,4E)-4'-hydroxy-β-ionylideneacetic acid was converted to ABA in *Oryza sativa* (Oritani and Yamashita, 1979), (±)-(2Z,4E)-α-ionylideneacetic acid was converted to 4'-oxo-α-ionylideneacetic acid only (Oritani and Yamashita, 1987). These authors did however show that (±)-(2Z,4E)-1'-hydroxy-α-ionylideneacetic acid was metabolized to the β-dehydro derivative and (+)-ABA in *Lycopersicon*. Although these studies remain inconclusive, it was the discovery of ABA-producing fungi (Assante *et al.*, 1977) that enabled researchers to study in more detail, aspects of the direct pathway of ABA biosynthesis. Much of this work was probably motivated by the success that had been achieved with elucidation of the gibberellin biosynthetic pathway in the GA-producing fungus *Gibberella fujikuroi* (Hedden *et al.*, 1978). Even so, the vast amount of information obtained from biosynthetic studies using ABA producing fungi has unfortunately also yielded inconclusive findings, with respect to this process in higher plants (Zeevaart & Creelman, 1988).

On the other hand, it was suggested that ABA might be biosynthesized by photolytic cleavage (Burden and Taylor, 1970) or enzymatic cleavage (Firn and Friend. 1972), with either lipoxygenase or linoleate, of the C_{40} carotenoid violaxanthin (see Fig. 1.4). One of the resultant products from this reaction was a C_{15} intermediate, *cis*-xanthoxin which was converted to ABA when supplied to shoots of *Phaseolus vulgaris* and *Lycopersicon esculentum* (Burden and Taylor, 1976; Taylor and Burden, 1972; 1973). This series of investigations thus, suggested the synthesis of ABA from MVA *via* the carotenoid biosynthetic pathway. The low yields (±1%) of xanthoxin produced, coupled with the high light intensities required for photolysis led many workers to favour the synthesis of ABA by the direct route common to all terpenoids, although attempts to distinguish between the two, by means of experiments using stereospecifically labelled MVA indicated that three residues of the natural R-enantiomer were incorporated into ABA. The stereochemical retention, in ABA, of hydrogens from MVA was however, similar to that observed with

carotenoid biosynthesis (Milborrow, 1983). It was thus not possible to distinguish between the C_{15} and C_{40} pathways with this approach.

ABA has been identified in a great variety of species and appears to be of universal occurrence among vascular plants (Addicott and Carns, 1983). It is present in mosses and some unicellular algae (Boyer and Dougherty, 1988; Tietz and Kaspric, 1986; Tietz *et al.*, 1987) but absent from liverworts and bacteria. Several genera of fungi have been described that can produce ABA as a secondary metabolite (Assante, *et al.*,1977; Dahiya *et al.*, 1988; Dörffling, *et al.*, 1984; Marumo, *et al.*, 1982; Okamoto, *et al.*, 1987; Oritani *et al.*, 1982; Oritani and Yamashita, 1985a). Of these fungi, especially *Cercospora rosicola*, *Cercospora cruenta*, and *Botrytis cinerea* have been used to study the biosynthesis of ABA. Intensive studies were undertaken in the hope that the pathway in these organisms could be elucidated, and that it would be similar or identical to the one in higher plants. Mycelial suspensions of *Cercospora rosicola* have been shown to incorporate label from [1,2,-$^{13}C_2$]-sodium acetate (Bennett *et al.*, 1981), [2-^{3}H]-MVA (Neill *et al.*, 1982a), farnesyl-[1-^{14}C] phosphate and farnesyl-[1-^{14}C] pyrophosphate (Bennett *et al.*, 1984; Norman *et al.*, 1982; 1986), ^{2}H-α-ionylidene ethanol and ^{2}H-α-ionylideneacetic acid (Neill *et al.*, 1982a; Neill and Horgan, 1983; Norman *et at.*, 1985) into ABA and possible, hypothetical routes for the later stages of ABA synthesis in this fungus are depicted in Figure 1.5. Biosynthesis of ABA has been studied in *Cercospora rosicola*, *Cercospora cruenta*, *Cercospora pini-densiflorae* and *Botrytis cinerea* (Zeevaart and Creelman, 1988) and current knowledge concerning ABA biosynthesis in these organisms is depicted in Figure 1.6. No universal pathway seems to exist but all are variations on a C_{15} pathway involving α- or -γ ionylidene derivatives. These pathways were deduced following the isolation of intermediates from cultures and through the feeding of putative labelled intermediates.

As shown in Fig 1.5 the biosynthesis of ABA in *Cercospora rosicola* involves successive oxidations of α-ionylidene ethanol, through α-ionylideneacetic acid, 4'-hydroxy-α-ionylideneacetic acid and 1'-desoxy-ABA the latter being the immediate precursor of ABA (Neill *et al.*, 1981; 1982a; 1982b; 1987; Neill and Horgan 1983). A second minor pathway may proceed via α-ionylidene ethanol and α-ionylideneacetic

acid through 1'-hydroxy-α-ionylideneacetic acid to ABA (Neill *et al.*, 1987; Norman *et al.*, 1985).

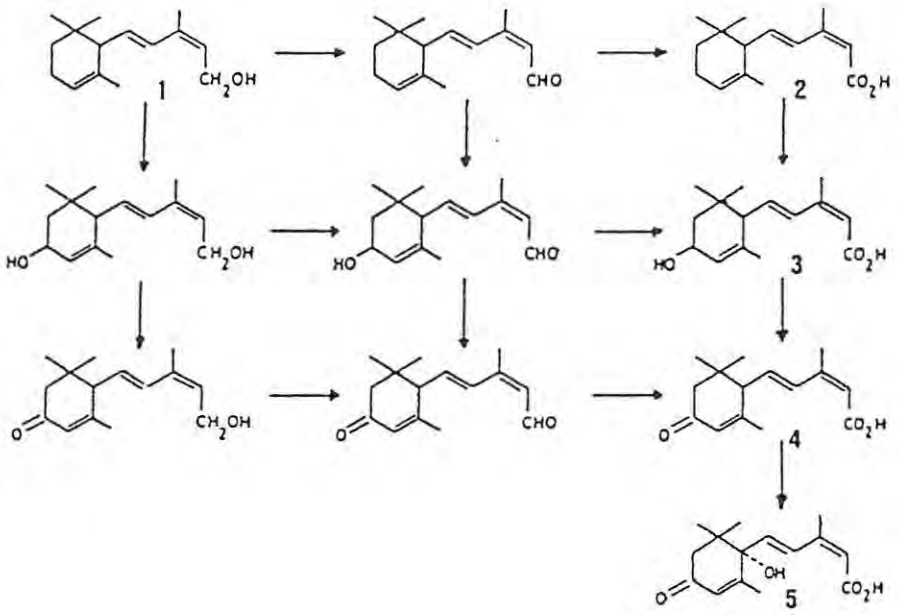


Figure 1.5 *Hypothetical routes for the latter stages of abscisic acid biosynthesis in* Cercospora rosicola *(after Neill et al, 1984).* 1),α-ionylidene ethanol; 2),4'-deoxy-α-ionylideneacetic acid; 3),1'-desoxy ABA; 4),4'-hydroxy-α-ionylideneacetic acid; 5), ABA.

Although cultures of *Cercospora pini-densiflorae* contained small amounts of α-ionylidene ethanol, 4-hydroxy-α-ionylideneacetic acid, 1'-desoxy-ABA and ABA they also contained large amounts of 1',4'-*trans*-ABAdiol (Okamoto *et al.*,1988a). This suggested that the main pathway to ABA in this fungus was from α-ionylidene ethanol through 4-hydroxy-α-ionylideneacetic acid to 1',4',-*trans*-ABAdiol which is the immediate precursor of ABA (Okamoto *et al.*, 1988a; 1988b). Cultures of *Botrytis cinerea* also contained 1',4'-*trans*-ABAdiol and were able to convert labelled diol into ABA (Hirai *et al.*, 1986). The situation in *Cercospora cruenta* is more complex as this fungus possesses the ability to convert α-ionylideneacetic acid through 4-hydroxy-α-ionylideneacetic acid and 1'-desoxy-ABA to ABA in the same

way as *Cercospora rosicola*, and 1'desoxy-ABA has been identified as a natural metabolite in *Cercospora cruenta* (Ichimura *et al.*, 1983; Oritani *et al.*, 1982; 1984; 1985a; 1985b).

However the major ABA pathway is thought to involve a series of γ-ionylidene derivatives (Fig. 1.6). γ-Ionylidene ethanol, 4'-hydroxy-γ-ionylideneacetic acid and 1',4'-dihydro-γ-ionylideneacetic acid are all present in cultures of *Cercospora cruenta* (Oritani *et al.*, 1984; 1985a; Oritani and Yamashita, 1987).

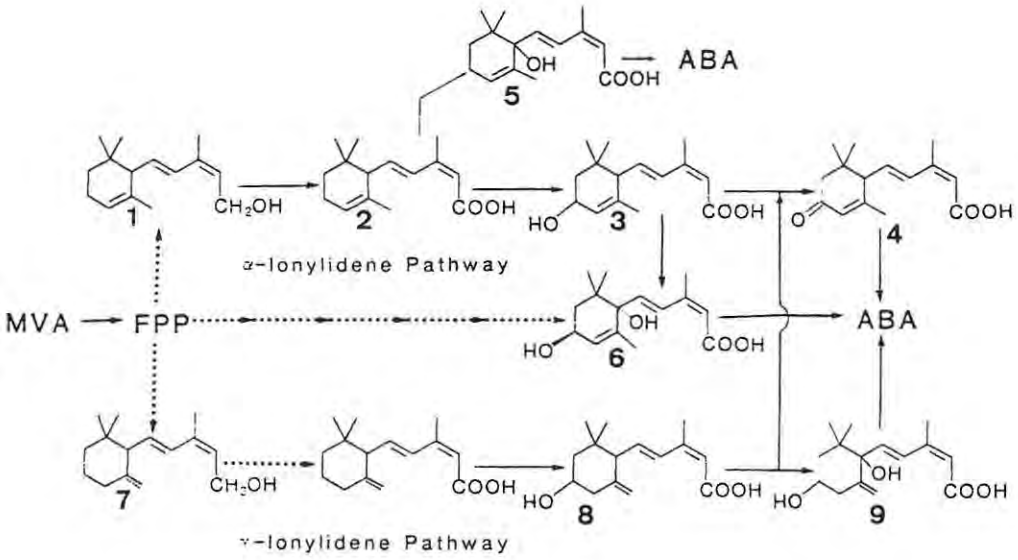


Figure 1.6 *Later stages of the ABA biosynthetic pathway in the fungi* Cercospora rosicola *(α-ionylidene pathway),* Cercospora cruenta *(γ-ionylidene pathway),* Cercospora pini-densiflorae, *and* Botrytis cinerea. A single arrow between two compounds does not necessarily indicate that only one enzymatic step is involved. Dotted lines: Conversions not unequivocally demonstrated (from Zeevaart & Creelman, 1988). 1) α-Ionylidene ethanol, 2) α-ionylideneacetic acid, 3) 4'-hydroxy-α-ionylideneacetic acid, 4) 1'-desoxy-ABA, 5) 1'-hydroxy-α-ionylideneacetic acid, 6) 1',4'-*trans*-ABAdiol, 7) γ-ionylidene ethanol, 8) 4'-hydroxy-γ-ionylideneacetic acid and 9) 1',4'-dihydroxy-γ-ionylideneacetic acid.

Labelling experiments using labelled 4'-hydroxy-γ-ionylideneacetic acid implicated 1',4'-dihydro-γ-ionylideneacetic acid as the normal precursor to ABA (Kato *et al.*, 1987; Oritani *et al.*, 1988).

The various hypothetical precursors to ABA, found in fungi, have been fed to higher plant tissues but with little success. Mallaby and Milborrow (cited in Milborrow, 1974a) demonstrated that 2,4,6-*trans*-dehydrofarnesol, added as a "cold-pool trap" to fruits of *Persea americana* that were synthesizing ABA from [^{14} C]-MVA, accumulated label and that [^{14}C] from [^{14}C]-dehydrofarnesol was incorporated into ABA. However, both these experiments can be criticized; the former because different cellular compartments could be involved and the latter because neither the [^{14}C]-2-*trans*-dehydrofarnesol incorporated into ABA nor the labelled trap had been degraded to ensure that the label was in the expected positions. Thus these results must be considered inconclusive. In addition, as none of these hypothetical precursors have been reported in higher plants, the question arises; does the FPP actually get into the fungus and are the ionylidene derivatives actual products of ABA biosynthesis or are they products of biotranstermation (i.e. extracellular)?

In an attempt to narrow the range of possible pathways Bennett and co-workers (1990) fed several [1-^{14}C]-labelled substrates to *Cercospora rosicola*. Their results suggested that (E,E,E)-dehydro-farnesyl pyrophosphate and either (E,E,Z)-dehydro-farnesyl pyrophosphate or trans-α-ionylidene ethyl pyrophosphate were the true intermediates on the pathway, which was indicative of the existence of a C_{15} pathway for ABA biosynthesis. However Daub and Payne (1989) reported that *Cercospora beticola, Cercospora nicotianae* and *Cercospora zeaemaydis* accumulated β-carotene and other minor carotenes in mycelial cultures. Also, Norman (1991) reported that ABA biosynthesis in *Cercospora rosicola* occurred coincident with synthesis of carotenoids in this fungus, the main one being *all-trans*-β-carotene. Thus, although ABA biosynthesis in *Cercospora rosicola* was thought to take place *via* the C_{15} pathway, the occurrence and rapid increase in β-carotene content concurrent with rapid ABA production seemed to indicate that at least some of the ABA could arise *via* the C_{40} carotenoid pathway. A similar observation was reported for stressed cells of the unicellular green algae *Dunaliella salina* (Cowan and Rose, 1991).

1.1.2 Indirect pathway of ABA biosynthesis.

The similarity between ABA and the end groups of certain xanthophylls was first noted by Taylor and Smith (1967).

Earlier research had suggested that light could stimulate the production of ABA and so studies were undertaken to investigate the possibility that illumination of carotenoids could produce ABA. Pigments were extracted from dried nettle (*Urtica dioica*) leaves, purified and exposed to light. Photo-oxidation of xanthophylls possessing 5,6 epoxy- and 3 hydroxy- groups such as violaxanthin (Fig. 1.7, structure 1) and neoxanthin (Fig. 1.7, structure 2) did give rise to (a) substance(s) that inhibited cress seed germination (Taylor, 1968; Taylor and Smith, 1967).

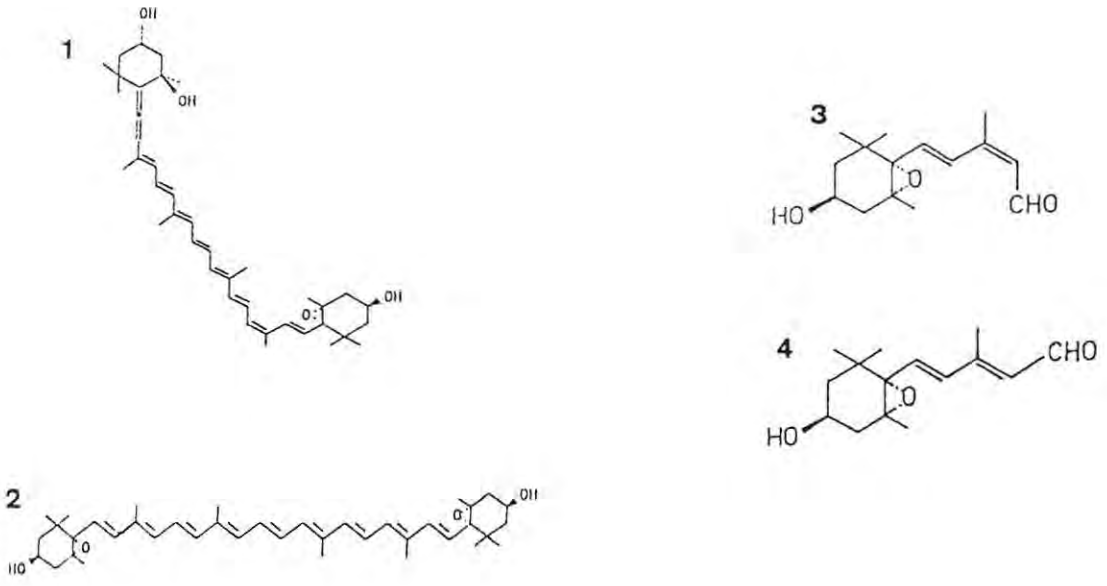


Figure 1.7 *Structural formulae of xanthophylls and neutral compounds implicated in ABA biosynthesis 1) Violaxanthin, 2) Neoxanthin, 3) Xanthoxin, 4) t-Xanthoxin).*

However, although this substance was inhibitory, it was found to be neutral (Taylor, 1968) and thus, not ABA. It was theorised that the inhibition was due to the formation of a compound that was closely related to ABA, perhaps an alcohol or an aldehyde which was converted to ABA by the cress seeds (Taylor, 1968). Subsequent identification of the active component as a neutral C_{15} compound revealed two structural isomers were present xanthoxin (Xan) and 2-*trans*-xanthoxin (*t*-Xan) and these are shown in Fig. 1.7, structures 3 and 4 (Taylor and Burden 1970a; 1970b). In a wide range of bioassays Xan possessed activity similar to that of ABA with *t*-Xan being almost biologically inactive (Burden *et al.*, 1971; Taylor and Burden, 1972). A chemical conversion of Xan to ABA was also described in 1970 (Burden and Taylor, 1970).

Xan and *t*-Xan were shown to be naturally occurring compounds when Taylor and Burden (1970b) isolated both isomers from dwarf bean (*Phaseolus vulgaris*) and wheat (*Triticum vulgare*) leaves. Firn *et al.*, (1972) analysed a range of tissues and found Xan and *t*-Xan in leaves and shoots of several higher plants and the fronds of two ferns. Strangely, they did not find Xan in some tissues which were known to contain ABA (such as tomato leaves).

Based on the above work (Taylor, 1968; Taylor and Smith, 1967) it seemed likely that Xan would be converted to ABA following uptake by cress seeds. Bioassays indicated that cress seeds pre-treated with Xan did contain greater amounts of ABA (Burden and Taylor, 1972). Similarly feeding unlabelled Xan to tomato shoots produced a large increase in the concentration of ABA (Taylor and Burden, 1972). The possibility that Xan was stimulating ABA biosynthesis without acting as a precursor was discounted by feeding ^{14}C-labelled Xan to tomato and dwarf bean shoots. ABA labelled with ^{14}C was extracted (Taylor and Burden, 1973; 1974). Unfortunately no intermediates between Xan and ABA were detected.

Considerable evidence now favoured the existence of an ABA biosynthetic pathway originating from one or more of the xanthophylls and including Xan as an intermediate. Production of Xan seemed to require light and yet ABA could be synthesized in the dark (Milborrow 1974b). This problem was resolved when Firn and Friend (1972) incubated violaxanthin with lipoxygenase (LOX) and linoleic acid. Xan and *t*-Xan were produced in yields comparable to those obtained photolytically.

Therefore by the early 1970's Xan was regarded as a likely *in vivo* precursor of ABA arising via either photolytic or enzymic xanthophyll breakdown.

More than a decade elapsed with little work carried out on the biochemistry of Xan and its relationship to ABA, due mainly to a lack of synthetic unlabelled and labelled Xan. However, the resurgence of interest in ABA biosynthesis began in the mid-1980's. The next major developments occurred with the introduction of inhibitors of carotenoid biosynthesis, carotenogenic and wilty mutants and stable isotopes.

1.1.2.1 Use of inhibitors of carotenogenesis.

Inhibitors of carotenogenesis have been found to inhibit ABA accumulation. Fluridone (1-methyl-3-phenyl-5-(3-trifluoromethyl]-phenyl)-4-(1H)-pyridinone) and norflurazon (SAN 9789; 4-chloro-5-(methylamino)-2-(α,α,α-trifluoro-*m*-tolyl)-3-(2H)-pyridazione) both block the dehydrogenation of phytoene to phytofluene. (Ridley 1982). Developing wild-type maize seeds sprayed with fluridone adopted phenotypes identical to the mutant *vp5*, which possesses a lesion between phytoene and phytofluene and is ABA deficient, in that they are white and viviparous. Normal seed development, but not pigmentation, could be restored if the fluridone-treated seeds were sprayed with ABA (Fong *et al.*, 1983a; 1983b). ABA levels in maize seedlings treated with fluridone were reduced below detectable limits (Moore *et al.*, 1985; Moore and Smith 1984). Seedlings of barley and pearl millet (*Pennisetum americanum*) grown in the presence of norflurazon were incapable of synthesising ABA in response to stress (Henson 1984; Quarrie and Lister 1984).

Furthermore, when green tissue is treated with inhibitors of carotenogenesis ABA accumulation is not affected (Gamble and Mullet 1986; Henson 1984; Li and Walton 1987; Quarrie and Lister 1984). The pool size of any putative xanthophyll ABA precursor in green tissue is likely to be of sufficient size to support ABA biosynthesis even if carotenogenesis is inhibited (Gamble and Mullet 1986; Walton *et al.*, 1985). Li and Walton (1987) incubated green *Phaseolus* leaves, pre-treated with fluridone or water, in $^{14}CO_2$ environment for 24 hours and then, followed by stress, in air for 14 hours. A similar reduction of specific activity (>90%) in both xanthophylls and ABA occurred in fluridone-treated leaves, although ABA

accumulation was not reduced. This indicated that the conversion of ABA from a preformed precursor was not inhibited by fluridone but that the incorporation of $^{14}CO_2$ into both ABA and the xanthophylls was equally inhibited providing strong circumstantial evidence for a carotenoid origin of ABA in higher plants

1.1.2.2 Carotenogenic and wilty mutants.

Further evidence in favour of the C_{40} pathway is available in the form of carotenoid-deficient mutants that show a pleiotropic deficiency in ABA (Duckham *et al.*, 1991; Parry *et al.*, 1991; Rock and Zeevaart, 1991; Zeevaart and Creelman, 1988). The best characterised carotenoid-deficient mutants are the viviparous (*vp*) *Zea maize* strains, some of which have 'blocks' in the biosynthetic pathway between phytoene and the xanthophylls which result in the embryos having a much reduced ABA content (Neill *et al.*, 1986a). The ABA mutant of *Arabidopsis thaliana (aba)* is not only deficient in ABA but also contains very low levels of antheraxanthin, violaxanthin and neoxanthin, presumably due to an inability to convert zeaxanthin, which accumulates in the mutant, to antheraxanthin (Duckham *et al.*, 1991; Rock and Zeevaart, 1991).

The most likely C_{40} ABA precursor is usually assumed to be violaxanthin because it has two "ABA-like" end groups and would therefore be biochemically "economical" in such a role (Taylor 1987). The cleavage of all-*trans*-violaxanthin could produce two molecules of *t*-Xan. Evidence to date is consistent with Xan being an ABA precursor and against it arising by isomerisation of *t*-Xan *in vivo* (Zeevaart and Creelman 1988). It therefore seems probable that any C_{40} ABA precursor would be a 9,10 (9',10') *cis*-xanthophyll. Oxidative cleavage of 9,9'-di-*cis*-violaxanthin. across the 11,12 and 11',12' double bonds, would produce two Xan molecules and a central dialdehyde C_{10} residue (2,7-dimethyl-2,4,6-octatrienedialdehyde; Taylor 1987). The presence of such an ABA biosynthetic "by-product" in plant tissue would, it has been suggested (Taylor 1987), provide additional circumstantial evidence in support of a C_{40} pathway. Taylor (1987) proposed that *in vivo* the C_{10} dialdehyde would be oxidised to the corresponding dicarboxylic acid (2,7-dimethyl-2,4,6-octatrienedioic acid; OTA). Both the dialdehyde and OTA have been identified as products of *in vitro* β-carotene oxidation (Wendler *et al* 1950). Attempts to detect

either of these compounds in tomato leaves have been unsuccessful to date (Taylor 1987).

An unknown compound did accumulate in the leaves of *flacca*, one of the wilty tomato mutants, and this was eventually identified as 2,7-dimethyl-2,4-octadienedioc acid (ODA; Linforth *et al.*, 1987a; 1987b; Taylor *et al.*, 1987). This has been described as an ABA biosynthetic by-product on the basis of its structural similarity to OTA and the pattern of ODA concentrations found in wilty tomato mutants (Taylor *et al* 1984; Linforth *et al* 1987a; 1987c; Taylor 1987). The wilty tomato mutants, *notabilis* (*not*), *flacca* (*flc*) and *sitiens* (*sit*) are all ABA deficient as a result of inhibited ABA biosynthesis (Parry *et al.,* 1988). Elevated levels of ODA in non-stressed and stressed leaves of *flc* and *sit* were said to result from increased rates of C_{40} cleavage, caused by positive feedback in response to blocks in the biosynthetic pathway at the C_{15} level (Parry *et al.*, 1986; Taylor, 1987). Reduced ODA concentration in *not* was said to result from a block in the ABA biosynthetic pathway at the C_{40} level (Parry *et al.*, 1986; Taylor 1987).

The origin of ODA is unknown but if it is a by-product of ABA biosynthesis then it must arise either *via* reduction of OTA (i.e. post cleavage) or *via* cleavage of a "dihydrocarotenoid" (Fig. 1.8; Taylor, 1987). It has been postulated that reduction at the C_{40} level would produce a more flexible molecule which in some way might facilitate a "dual cleavage event" (Taylor, 1987).

It has been questioned why ODA should accumulate in the *flc* and *sit* single-gene mutants when its further metabolism should be independent of impaired ABA biosynthesis (Koorneef, 1986; Neill and Horgan, 1985).

Milborrow *et al.*, (1988) incubated wild-type tomato shoots in ^2H_2O for 6 days prior to severe wilting, which caused an accumulation of both ABA and ODA. Analysis of extracted ABA revealed the presence of up to 11 ^2H atoms while no label was detected in ODA. It was concluded that ODA could not be a by-product of ABA biosynthesis.

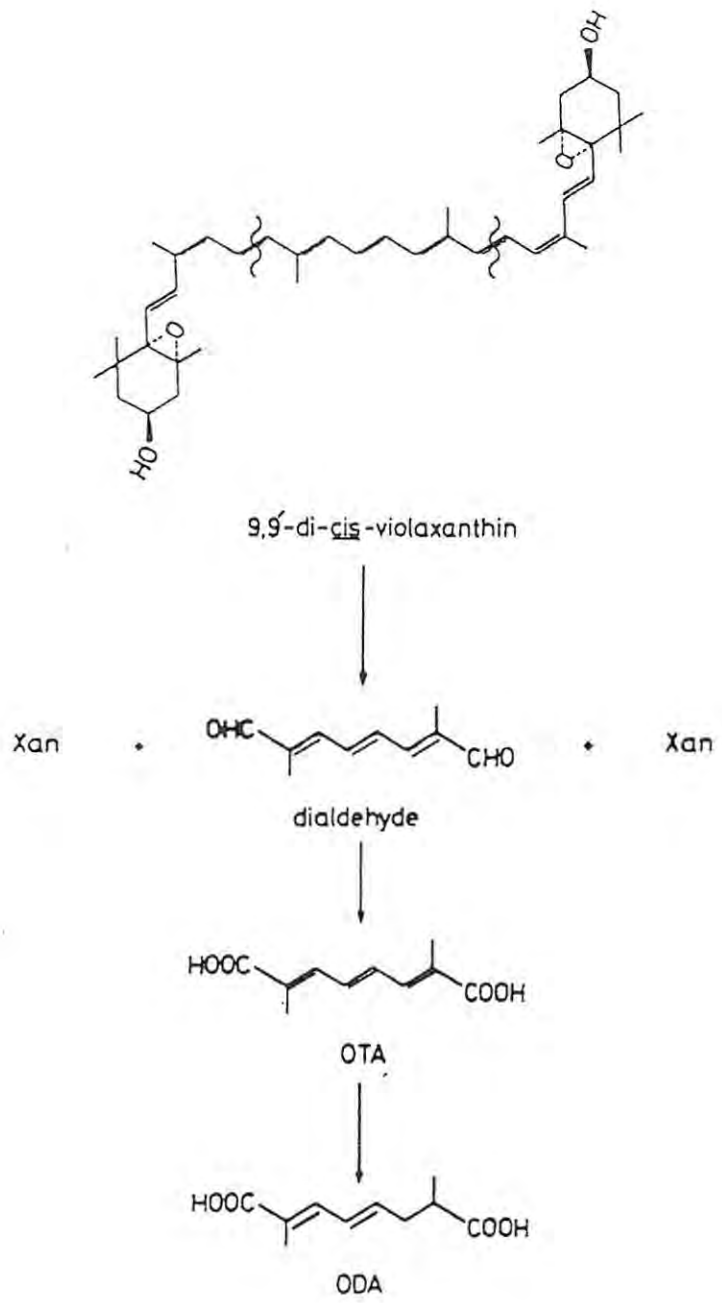


Figure 1.8 *Possible origin of ODA, the C_{10} ABA biosynthetic by-product (after Taylor, 1987).*

Although the above data involving carotenoid-deficient mutants and inhibitors of carotenogenesis are consistent with a role of carotenoids as ABA precursors they do not constitute conclusive proof for the indirect pathway. In addition, a recent investigation into the biosynthetic origin of ABA has revealed firstly, that there are two precursor pools involved in ABA synthesis and that neither consists of carotenoids (Willows *et al.*, 1994) and secondly, that the endogenous precursor of stress-induced ABA is an as yet unidentified compound (Netting and Milborrow, 1994).

1.1.2.3 Use of stable isotopes in ABA biosynthesis.

"Knowledge of the synthesis and degradation of ABA is essential if one is to understand how the concentration of this plant growth substance is regulated at the tissue and cellular level" (Zeevaart *et al.*, 1991). It was thus envisaged that the use of $^{18}O_2$ would help to unravel the ABA biosynthetic pathway as the position of the ^{18}O could be traced by MS. When stressed leaves of *Phaseolus vulgaris* and *Xanthium strumarium* were incubated in the presence of $^{18}O_2$, the ABA extracted was labelled with only one ^{18}O in the carboxyl group (Creelman and Zeevaart, 1984). Incorporation into the ring oxygens also occurred but to a lesser degree (Creelman *et al.,* 1987). More recent work by Creelman, (1989) showed a similar labelling pattern when ABA was extracted from other tissues (non-stressed leaves, roots, fruits and embryos) incubated in the presence of $^{18}O_2$. Isotopic enrichment was determined by MS with electron impact (EI) ionization (Zeevaart *et al.*, 1991). They did not however, find this method sufficiently sensitive and for later experiments (Zeevaart *et al.*, 1991) negative chemical ionization (NCI) was used. Although fragmentation in the NCI spectrum was limited, the few ions observed provided complete information about the location of the ^{18}O atoms in the molecule (Heath *et al.*, 1990; Netting *et al.*, 1988). Thus a considerable body of work (Creelman *et al.*, 1987; Creelman and Zeevaart, 1984; Li and Walton, 1987; Linforth *et al.*, 1987b; Parry *et al.*, 1990; Parry and Horgan, 1991a; 1991b; 1992) has been taken as evidence that carotenoids are precursors of ABA, and together these results suggest a universal ABA biosynthetic pathway is operating in higher plants (Creelman *et al.*, 1987; Gage *et al.*, 1989; Parry, 1993; Zeevaart *et al.*,

1989;) and that the "pre-cleavage" precursor is a xanthophyll such as 9-*cis*-violaxanthin or 9-*cis*-neoxanthin.

In an attempt to isolate a possible specific xanthophyll precursor Li and Walton (1987) carried out $^{18}O_2$ labelling experiments in which violaxanthin was specifically labelled with ^{18}O. De-epoxidation of violaxanthin, *via* antheraxanthin to zeaxanthin was followed by their epoxidation back to violaxanthin when exposed to air and darkness. Excised *Phaseolus* leaves were exposed to N_2 in the light and 70 % of the violaxanthin was de-epoxidated within 15-30 min (Li and Walton 1990). These leaves were then transferred to an $^{18}O_2$ atmosphere in the dark where epoxidation and ^{18}O incorporation occurred. After the leaves had been stressed both the ABA and violaxanthin were extracted. Although between 40-45 % of the violaxanthin was labelled only 10-15 % of the ABA contained ^{18}O at the 1' position. This suggested that only a portion of the stress-induced ABA was derived from the ^{18}O-labelled violaxanthin.

In the same way deuterium-oxide labelling experiments were carried out in an attempt to determine whether ABA was produced from a carotenoid precursor. Parry *et al.*, (1990) showed that deuterium from deuterium oxide (D_2O) was incorporated into carotenoids in etiolated *Phaseolus* seedlings. As water-stress induced ABA was labelled with deuterium to a similar extent as neoxanthin and violaxanthin, they considered this to be confirmatory evidence for the apo-carotenoid nature of ABA. To further investigate the relationship between violaxanthin, neoxanthin, lutein and ABA, extracts were made from etiolated leaves of *Phaseolus vulgaris* grown on 50 % D_2O. They were found to be 15-18 % deuterated (Parry *et al.*, 1990). Although these results did not exclude the possibility of a C_{15} pathway, they were consistent with the operation of a C_{40} pathway. In addition, the close similarity between the extent of deuteration of ABA and neoxanthin appears to support a product-precursor relationship. These results are very different from those obtained by Nonhebel and Milborrow (1987) using tomato shoots. These authors did not detect neoxanthin. However, they are in agreement with the results obtained by Li and Walton (1987), who also suggested that 9'-*cis*-neoxanthin, synthesised from all-*trans*-violaxanthin, is the major precursor of ABA in higher plants.

If, as now seems likely, 9'-*cis*-neoxanthin and/or 9'-*cis*-violaxanthin are ABA precursors, then by implication Xan should be an intermediate in the ABA biosynthetic pathway. The *in vitro* transformations of xanthoxin acid (Xan acid) ABA aldehyde and 1',4'-*trans*-ABAdiol have been demonstrated in extracts prepared from *Phaseolus vulgaris* leaves and turgid and water-stressed leaves of wild type *Lycopersicon esculentum* and the wilty mutants *sit, not* and *flc* (Sindhu et al., 1990; Sindhu and Walton, 1987; 1988). Insignificant conversion was obtained when Xan acid and 1',4'-trans-ABAdiol were used as substrates. However, Xan was converted to ABA in good yield in extracts of *Phaseolus vulgaris* leaves, and both Xan and ABA-aldehyde were transformed into ABA in extracts from leaves of wild type *Lycopersicon esculentum* and the wilty mutant, *not.* While these results are consistent with a role for Xan as an intermediate en route to ABA and suggest that ABA-aldehyde is the final ABA precursor, (Fig. 1.9) the biosynthetic origin of these compounds has yet to be unequivocally established.

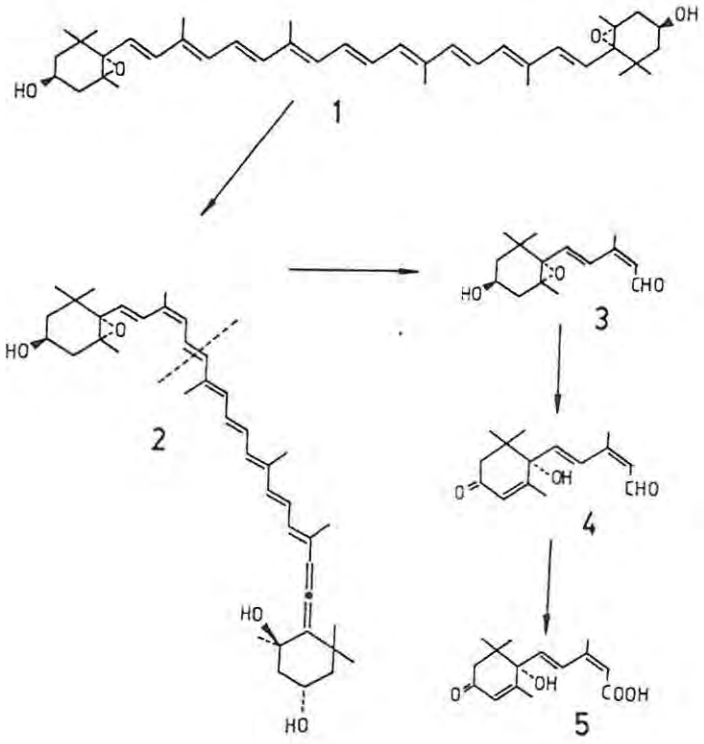


Figure 1.9 *Scheme for the biosynthesis of ABA from all-*trans*-violaxanthin, involving cleavage of 9'-*cis*-neoxanthin.* 1) all-*trans*-violaxanthin; 2) 9'-*cis*-neoxanthin; 3) xanthoxin; 4) ABA-aldehyde; 5) ABA.

1.2 Cell-free studies on ABA metabolism.

Another avenue which could be followed in attempting to elucidate the biosynthetic pathway of ABA, is the use of cell-free systems. Cell-free systems eliminate the difficulty of penetration of mevalonate and other putative precursors into the site of ABA biosynthesis although they do have the disadvantage of loss of compartmentalization which could impact on enzyme activity.

Nevertheless, the development of reliable, cell-free ABA biosynthesizing systems from plants are essential for a better understanding of ABA biogenesis and its regulation. Furthermore, this method has been successfully employed to study the biosynthesis (Graebe, 1987) and enzymology (Lange and Graebe 1993) of gibberellins.

1.2.1 Cell-free studies in ABA-producing fungi.

Early breakthroughs in gibberellin biosynthesis were made using cell-free systems of the fungus *Gibberella fujikuroi* (Hedden *et al.*, 1978). It is therefore surprising that no similar cell-free systems have been produced from the ABA-synthesising fungi. A recent breakthrough in this area was the conversion of 1'-desoxy-^{2}H-ABA to ^{2}H-ABA in cell-free extracts from *Cercospora rosicola* (Al-Nimri and Coolbaugh, 1991) which will now make it possible to characterize the enzyme responsible for 1'-desoxy-ABA oxidation.

1.2.2 Cell-free studies in plant extracts

The cell-free biosynthesis of several sesquiterpenoids in *in vitro* systems has been well studied, for example, limonene in citrus (George-Nascimento and Cori, 1971) the partial purification of prenyltransferases from the flavedo of *Citrus sinensis* (De La Fuente *et al.*, 1981); the biosynthesis of sesquiterpene alcohols and aldehydes by cell-free extracts from orange flavedo (Chayet, *et al.*, 1973); hydrolysis of allylic phosphates by enzymes from the flavedo of *Citrus sinensis* (Perez *et al.*, 1980); formation of isopreniod pyrophosphates from mevalonate by orange enzymes (Potty and Bruemmer, 1970); isopentenyl pyrophosphate isomerase and prenyltransferase

from tomato fruit plastids (Spurgeon *et al.*, 1984); the biosynthesis of mono- and sesquiterpenes in peppermint from glucose ^{14}C and $^{14}CO_2$ (Croteau *et al.*, 1972) the synthesis of carotenoids and polyterpenoids (Bramley, 1985; Britton, 1976a; 1976b; 1984; Davies and Taylor, 1976; Goodwin 1971; Goodwin and Mercer, 1983; Spurgeon and Porter, 1980) and the gibberellins (GAs) and intermediates in the GA biosynthetic pathway (Bearder, 1983; Coolbaugh, 1983; Graebe, 1986, 1987; Graebe and Ropers, 1978; Hedden *et al.*, 1978; Hedden and Graebe 1982; MacMillan, 1983, 1984; Railton, 1976; 1982; Sembdner *et al.*, 1980)

Surprisingly, only a few studies have attempted to demonstrate the cell-free biosynthesis of ABA and for the most part these have met with little success. A cell-free system of lysed chloroplasts was used to demonstrate ABA biosynthesis from MVA but activity was low and no intermediates were reported (Milborrow, 1974a). Unfortunately all subsequent attempts to demonstrate ABA biosynthesis in chloroplast isolates, whether intact or lysed, have proved unsuccessful (Cowan and Railton, 1986; Hartung *et al.*, 1981). More recently, a cell-free system derived from imbibed embryos of *Hordeum vulgare* was shown to synthesize a range of terpenyl pyrophosphates from labelled MVA and to transform MVA and isopentenyl pyrophosphate (IPP) into an acid with similar chromatographic properties to those of ABA (Cowan and Railton, 1987a). Activity was however low and the identity of the acid as ABA was not unequivocally established. By comparison greater success has been achieved using putative post-FPP intermediates as substrates in ABA-biosynthesizing cell-free systems (Cowan and Richardson, 1993b).

1.3 Enzymology of ABA biosynthesis.

As an almost complete pathway for ABA biosynthesis has now been elucidated. and likely control points identified (Parry and Horgan, 1991a; 1991b; Parry, 1993), a discussion of the putative enzymology is possible. Most evidence indicates that ABA is derived from either 9'-*cis*-neoxanthin or 9'-*cis*-violaxanthin with xanthoxin as an intermediate. Li and Walton (1990) showed that when turgid *Phaseolus* leaves were treated with cycloheximide there was a 50 % reduction in ABA levels, which was not the result of catabolism. Yet the amount of "Xan oxidising" activity isolated from plant tissues by Sindhu and Walton (1987) was unaffected by pre-treatment with

cycloheximide. It therefore, appeared that the biosynthesis of ABA was regulated by the activity of an inducible enzyme, probably a lipoxygenase which acts specifically to cleave 9'-*cis*-neoxanthin, and has a high turnover rate.

Creelman *et al.* (1992) used a number of soybean (*Glycine max.* L) lipoxygenase inhibitors to inhibit stress-induced ABA accumulation in soybean cell culture and soybean seedlings. All lipoxygenase inhibitors significantly inhibited ABA accumulation in response to stress. These results suggested that the *in vivo* oxidative cleavage reaction involved in ABA biosynthesis requires activity of a non-haem oxygenase having lipoxygenase-like properties. Several studies have implicated a molybdenum-requiring soluble oxidase as the terminal enzyme in ABA-biosynthesis (Sindhu *et al.*, 1990; Walker-Simmons *et al.*, 1989). Likewise, the present work has shown that molybdate stimulates ABA-biosynthesizing activity in cell-free extracts prepared from *Citrus* flavedo. This requirement for molybdate supports current conjecture that a molybdenum-containing oxidase enzyme catalyses the terminal step in ABA biosynthesis. Furthermore, a requirement for NAD^+ or NADP (Sindhu and Walton, 1987) suggests that the enzyme concerned is a molybdenum-containing hydroxylase possibly of the $NAD(P)^+$-dependent dehydrogenase form (Wootton *et al.*, 1991).

Objectives.

In the current research programme, experiments were therefore carried out in order to:

1. develop a cell-free system in which to study ABA biochemistry and enzymology in more detail and;
2. to identify, where possible, the intermediates en route to ABA and to explore aspects of the enzymology of this process.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Chemicals

2.1.1 Radioactive substrates

(R,S,)-[2-^{14}C]-ABA marketed as DL-*cis,trans*-[2-^{14}C]-ABA (sp. act. 833 MBq mmol^{-1}, (R,S)-[G-^{3}H]-ABA marketed as DL-*cis,trans*-[G-^{3}H]-ABA (sp. act. 4.26 TBq mmol^{-1}), R-[2-^{14}C]-MVAL (sp. act. 1.92 GBq mmol^{-1}), R-[1-^{14}C]-isopentenyl pyrophosphate (IPP), ammonium salt (sp. act.1.96 GBq mmol^{-1}), sodium [^{14}C] bicarbonate (sp. act. 1.92 GBq mmol^{-1}) and [2-^{14}C]-acetic acid, sodium salt (sp. act. 2.11 GBq mmol^{-1}) were purchased from Amersham International, Buckinghamshire, England, Adenosine-5'-triphosphate [gamma-^{32}P] (sp. act. 167 TBq mmol^{-1}) was purchased from ICN Pharmaceuticals Inc., Radiochemicals Division, Irvine, California.

2.1.2 Plant growth regulators

(R,S) Abscisic acid methyl ester (ABAMe), (±) *cis,trans* ABA, benzyladenine,6-(benzylamino) purine (BAP), ancymidol (α-cyclopropyl- α-(p-methoxy phenyl 0-5 - pyrimidine methyl alcohol) and gibberellic acid (GA_3) were purchased from Sigma Chemical Company, St Louis, MO, USA. AMO 1618 (2-isopropyl-4'-trimethylammonuim chloride)-5'-methyl phenyl piperidine-1'-carboxylate) was obtained from Calbiochem, Behring Corp., La Jolla, Ca, USA.

2.1.3 Cofactors

Adenosine-5'-triphosphate (ATP) the disodium salt ($C_{10}H_{14}N_5O_{13}P_3Na_2$); and glutathione were purchased from Sigma Chemical Co., St Louis, MO, USA. B-Nicotinamide-adenine dinucleotide (NAD), the free acid ($C_{21}H_{21}N_7O_{14}P_2$); B-nicotinamide-adenine dinucleotide reduced (NADH), the disodium salt ($C_{21}H_{27}N_7O_{14}P_2Na_2$); B-nicotinamide-adenine dinucleotide phosphate reduced (NADPH), the tetra-sodium salt ($C_{21}H_{26}N_7O_{17}P_3Na_4$); and flavine adenine

dinucleotide (FAD), the disodium salt ($C_{27}H_{31}N_9O_{15}P_2Na_2$) were purchased from Boehringer Mannheim, West Germany.

2.1.4 General Chemicals

Stigmasterol (3β-hydroxy-24-ethyl-5,22-cholestadiene); lanosterol (3β-hydroxy-8,24-lanostadiene); cholesterol (5-cholesten-3β-ol); trans-4,5-dihydroxy-1,2-dithiane (oxidized DDT); DL-Dithiothreitol (DTT); DL-MVAL; FPP (3,7,11-trimethyl-2,6,10-dodectrien-1-ol pyrophosphate), ammonium salt; GGPP (3,7,11,15-tetramethyl-2,6,10,14-hexadeatetraenyl pyrophosphate), ammonium salt; *all-trans*-β-carotene; linoleic acid; N-methyl-N'-nitro-N-nitrosoguanidine; hydrogen peroxide (H_2O_2); bovine serum albumin (BSA) and Coomassie brilliant blue G-250 were purchased from Sigma Chemical Company, St Louis, MO, USA. Serva Blue G was purchased from GmbH & Co, Heidelberg, Germany.

3,3',5,5'-tetramethylbenzidine (TMBZ) and Triton X 100 were purchased from Aldrich Chemical Company, Milwaukee, USA. Tween 20 (Polyoxyethylene sorbitan monolaurate) and ethylene diamine tetra-acetic disodium salt (EDTA) were purchased from Saarchem, Krugersdorp, SA. Sodium borohydride and 2,6-di-tert-butyl-p-cresol (BHT) was obtained from B.D.H. Laboratory Chemicals Division, Poole, England. Pico Fluor 40 scintillation cocktail was purchased from Packard Instrument Company, USA.

2.1.5 Solvents.

HPLC grade solvents (Acetonitrile, UV cutoff 189 nm, Ethyl Acetate, UV cutoff 253 nm, methanol, UV cutoff 210 nm and Hexane, UV cutoff 193 nm) were obtained from Baxter Healthcare Corporation, McGraw Park, Illinois, USA; Bio-lab Laboratories Ltd, Jerusalem, Israel; Romil Chemicals Limited, Loughborough, Leicester, England and B.D.H. Laboratory supplies, Poole. England. All other solvents were of analytical grade and were glass-distilled at 67.7 °C (methanol), 77 °C (ethyl acetate) and 55.5 to 56.5 °C (acetone)

2.1.6 Chromatographic Media

Sep-Pak C_{18} cartridges were purchased from Waters Chromatography Division, Millipore Corporation, Milford, MA, USA. For thin layer chromatography (TLC), kieselgel 60 GF_{254} and DC-Alufolien kieselgel 60 GF_{254}, 20 x 20 cm (0.2 mm thick), ready prepared plates were obtained from Merck, Darmstadt, Germany. Thin layer plates (0.25 mm thickness) of silica gel GF_{254} (Merck) were also prepared by adding 60 mL of distilled water to 30 g of silica gel which was then shaken vigorously for 2 min and 5, 20 x 20 cm thin layer plates prepared using a DESAGA TLC spreader and the plates allowed to air dry overnight. DC-Fertig-platten sil G-25 UV_{254} ready-prepared plates were purchased from Macherey-Nagel, Düren, Germany. A capillary column (15 mm x 0.53 mm i.d.) of SPB-1 purchased from SUPELCO, Bellefonte, CA, USA, was used for Gas Chromatography-Electron Capture (GC-EC). High Performance Liquid Chromatography (HPLC) columns were as follows: for analysis of pigments a 5 mm C_{18} Nucleosil column (250 x 4.6 mm i.d.) was purchased from Macherey-Nagel, Düren, Germany, and for analysis of abscisic acid and related compounds a Bondaclone 10 mm C_{18} (150 x 3.9 mm i.d.) column purchased from Phenomenex, Torrance, CA was used.

2.2 General Techniques

2.2.1 Preparation of ethereal diazomethane

Ethereal diazomethane was generated at room temperature without co-distillation, by the hydrolysis of N-methyl-N'-nitro-N- nitrosoguanidine with NaOH (5 N) in a Wheaton Diazomethane Generator (Pierce Chemical Co., Rockford, ILL, USA) using the small scale procedure described by Fales *et al.*, (1973). 133 mg N-methyl-N'-nitro-N-nitrosoguanidine and 0.5 mL distilled water, to dissipate any heat generated, were placed in the inside tube, 3.0 mL dry diethyl ether was placed in the outer tube and the two parts assembled and held together with a pinch-type clamp. The apparatus was then placed in an ice-bath and 0.6 mL 5 N NaOH injected through the teflon rubber septum and the reaction allowed to proceed for ±45 min or until the ether developed a deep yellow colour.

2.2.2 Preparation of aqueous-buffered carotene-linoleate solution.

This solution, prepared immediately before use, contained 0.75 mg linoleic acid (*cis*-9,*cis*-12-octadienoic acid), 0.01 mg β-carotene, 66 x 10^{-5} mL Tween 20 and 0.50 mg EDTA mL^{-1} as described by Ben-Aziz *et al.,* (1971). 1 mL Aqueous linoleate (7.5 %, w/v) linoleic acid in ethanol mixed with 0.3 mL (10 %, v/v) Tween 20 in ethanol to which was added 5 mL 0.5 % aqueous EDTA and the pH adjusted to 9.0 with 0.1 N NaOH and the final volume made up to 10 mL with distilled water) was mixed with I mL aqueous carotene (25 mg β-carotene and 0.9 mL Tween 20 dissolved in 25 mL chloroform. 1 mL of this solution was evaporated to dryness under nitrogen, and the residue dissolved in 10 mL 0.25 % EDTA solution), and the volume adjusted to 10 mL with 0.05 M potassium (K) phosphate buffer, pH 7.0.

2.2.3 Protein determination

The concentration of protein in plant tissue cell-free extracts was determined spectrophotometrically using the Bradford dye-binding assay (Bradford, 1976). To 0.1 mL of the protein solution, or any dilution thereof, 5.0 mL of Bradfords Reagent (100 mg Coomassie Brilliant Blue G-250 or Serva Blue G ($C_{47}H_{48}N_3O_7S_2Na$) was dissolved in 50 mL 95 % ethanol to which was added 100 mL 85 % (w/v) phosphoric acid and the solution was diluted to a volume of 1 L with distilled water) was added, the samples thoroughly mixed and the absorbance at 595 nm determined using a Beckman DU 65 spectrophotometer. Protein concentration of the samples were determined from a standard curve for BSA.

2.3 Lipoxygenase assay

Lipoxygenase (LOX) activity was assayed as described by Ben-Aziz *et al.*, (1971). Acetone Powder homogenates (see Section 2.4.3) were used as a source of enzyme activity. Incubations for LOX activity contained 7.5 mL buffered carotene-linoleate, 2 mL distilled water and 0.5 mL supernatant. Reactions were initiated by addition of supernatant and allowed to proceed for 1 min at 25 °C before being terminated by the addition of BHT in ethanol (0.106 mg/mL) to give a final

concentration of 480 μM. The degree of bleaching was determined spectrophotometrically at 460 nm using a Beckman DU 65 spectrophotometer.

2.4 Plant material

Mature fruits of *Citrus sinensis* L.Osbeck cvs Midknight, Palmer Navel, Moss Seedless, Bahianinha, Lane Late and Washington Navel were kindly supplied by Riverside Enterprises, Fort Beaufort and Amanzi Estates, Uitenhage, South Africa. Fruit was harvested between late April and August for endogenous ABA studies and between June and August, following development of colour, for cell-free studies.

2.4.1 Preparation of Acetone Powder

Acetone powder was prepared according to the method of Perez *et al.*, (1980). Fruits were thoroughly washed with 1 % Sodium Hypochlorite solution then cleaned with running tap water followed by distilled water. The flavedo (exocarp) was peeled off and collected at 0 °C. It was homogenized in a Waring Blender in a coldroom with 2 L acetone kg^{-1} flavedo. The suspension was filtered through Whatman No. 1 filter paper in a Buchner funnel and the residue was washed with ± 15 L acetone followed by 6 L diethyl ether (Et_2O) kg^{-1}. The powder obtained was dried *in vacuo* and stored at 4 °C.

2.4.2 Determination of background ABA in extracts of acetone powder

Acetone powder was analysed to determine the presence, if any, of background ABA . A known amount of powder was homogenised with ice-cold methanol/ethyl acetate (50:50, v/v), containing BHT as antioxidant, filtered through Whatman No. 1 filter paper, centrifuged and the supernatant was decanted into a suitable flask and extracted. Tritiated ABA was added in order to correct for recovery and the extracts filtered, dried *in vacuo* and resuspended in 0.5 mol m^{-3} phosphate buffer and partitioned against diethyl ether (Et_20) containing BHT as antioxidant. The pH was then reduced to pH 2.5 and repartitioned. The pH 2.5 fraction was dried *in vacuo* at 35 °C, separated on TLC and the zone co-chromatographing with authentic ABA was eluted and quantified by GC-EC in order to determine the amount of

background ABA. The results are shown in Figure 2.1. The total ABA in the powder was calculated by peak integration after calibration with standard ABA, as 0.012 μg per 10 g powder.

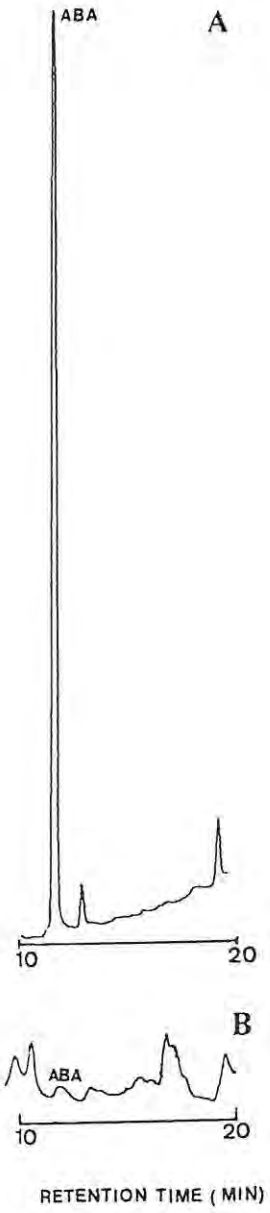


Figure 2.1 ***(A)*** *GC-chromatogram of standard ABA showing retention time.* ***(B)*** *GC-chromatogram of extract from acetone powder homogenate of* Citrus sinensis *flavedo.* Acids were analysed using a Perkin-Elmer 8310 instrument fitted with an electron capture detector (^{63}Ni source) using a fused-silica capillary column (15 mm x 0.53 mm i.d.) of SPB-1 programmed from 120 °C to 5 °C per min with N_2 as carrier gas at the flow rate of 17 mL min. The retention time of Std ABA was 19.5 min.

2.4.3 Preparation of cell-free extracts

Crude extracts were prepared as follows: the flavedo (exocarp) was grated off the whole fruit and the gratings homogenized at 0 °C for 3 min, using an Ultra-Turrax top-drive homogenizer, in 0.1 M Tris-HCl buffer (pH 7.4) equivalent to 1.5 times the fresh weight. Acetone powder cell-free extracts were prepared by homogenizing amounts of the dried powder for 5 min at 0 °C in 0.1 mol m^{-3} Tris-HCl buffer (pH 7.4, 2 mL g^{-1} powder) followed by sonication for 30 sec (3 by 10 sec bursts). Homogenates were filtered through two layers of cheesecloth and centrifuged at 2 °C for 30 min at 23 500 g in a Sorvall-RC5 or a Hitachi CR20B2 refrigerated centrifuge. The supernatant was used as a source of enzyme activity following determination of protein concentration (see section 2.2.3). Where specified, the 23 500 g supernatant from crude tissue homogenates was dialysed for 2 h against 20 mol m^{-3} Tris-HCl buffer (pH 7.4) at 0 °C.

2.4.4 Incubation procedure

Incubations were carried out essentially as described by Cowan and Richardson (1993a). Routinely 2.5 mL of the 23 500 g supernatant (equivalent to 0.25-2.5 mg protein) was incubated in 50 mol m^{-3} Tris-HCl buffer (pH 7.4) containing glutathione (10 mol m^{-3}), Sodium fluoride (5 mol m^{-3}), ATP (5 mol m^{-3}), $MgCl_2$ (12.5 mol m^{-6}), AMO 1618 (1 mol m^{-3}) and various combinations of NAD^+, NADH, NADPH, FAD (12.5 mol m^{-6}) and either sodium or ammonium molybdate (10 mol m^{-3}) in a total volume of 5 mL. Where specified, non-labelled (R,S)-ABA (0.2 mol m^{-3}) was included as a cold-pool trap. Following the addition of substrate (either R-[2-^{14}C]-MVA, [1-^{14}C]-IPP, FPP, GGPP, [^{14}C]-β-carotene or synthetic all-*trans*-β-carotene and 1'4'-*trans*-ABAdiol and ABA), reactions were initiated by adding enzyme and allowed to proceed for 2 h (unless otherwise stated) at 37 °C under continuous low-light illumination (40 μmol m^{-2} s^{-1}) in a shaking water bath. Reactions were terminated by heating to 100 °C for 3 min and protein precipitated by addition of an equal volume of ice-cold methanol, after incubates had been cooled to room temperature. Heat-inactivated (100 °C for 10 min) extracts were routinely included as controls.

2.5 Protein phosphosylation

Reaction mixtures (final volume 100 µl) contained 50 mM Tris-HCl buffer (pH 7.4), 10 mM glutathione, 5 mM ATP, 1.25 mM $MgCl_2$, 1.25 x 10^{-2} mM of each NAD^+, NADH and NADPH, 1 mM AMO 1618, 5 mM NaF, 1 x 10^{-3} mM Na_2MoO_4, and 10 mCi [gamma-^{32}P]-ATP (specific activity 167 TBq $mmol^{-1}$) with or without ABA as a cold-pool trap. Reactions carried out at 37 °C, were initiated by the addition of *Citrus* enzyme extract (equivalent to 4.6 µg/100 µl) and stopped after 2 h by the addition of either 100 µl of 4 x SDS sample loading buffer (0.2 M Tris-HCl pH 6.8, 8 % (w/v) SDS, 40 % (v/v) glycerol, 0.5 % (w/v) bromophenol blue) or by the addition of 100 µl 10 % Trichloroacetic acid (TCA). The former were immediately loaded onto the gel while the latter were left on ice for 30 min to precipitate the proteins, centrifuged and washed with 50 mM Tris-HCl (pH 7.4), resuspended in SDS sample loading buffer and loaded onto the gel. These procedures resulted in visible differences in protein profiles obtained following electrophoresis (Fig. 2.2).

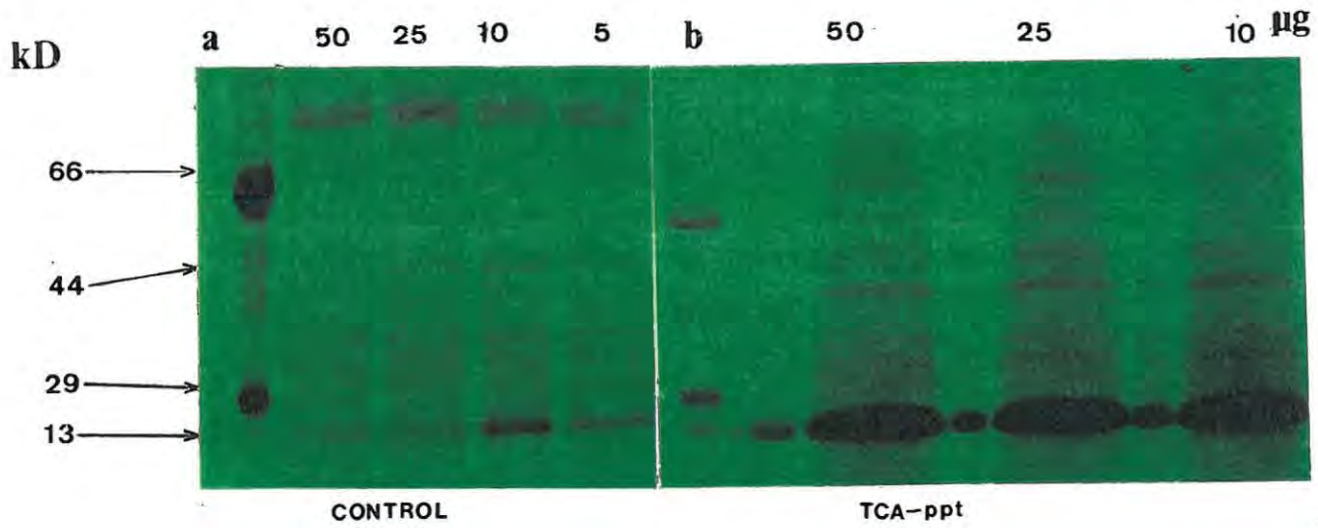


Figure 2.2 *Electrophoretic profile of proteins in supernatant of acetone powder cell-free extracts with or without TCA precipitation and analysed on 12.5 % gels.* **(A)** control; **(B)** TCA-ppt. Molecular weight markers are shown on the left hand side.

2.6 Extraction and analysis of products

2.6.1 Extraction and purification of plant pigments (carotenoids).

Precipitated protein was removed by centrifugation and the pellet extracted three times with 5 mL volumes of ice cold methanol/ethyl acetate (50: 50, v/v) containing BHT as an antioxidant. The supernatant was combined and the extracts were reduced to dryness *in vacuo* at 35 °C, redissolved in 70 % aqueous methanol and applied to pre-rinsed Sep-pak C_{18} cartridges. Pigments were eluted from the cartridge with 90% methanol followed by 100 % acetone as described in section 2.7.2.1. Methanol and acetone eluates were filtered through 0.2 μm Spinex™ disposable centrifugal filters, reduced to dryness under nitrogen and resuspended in acetonitrile/water (9:1, v/v) and analysed by reversed-phase HPLC.

2.6.2 Extraction and purification of neutral compounds (Xanthoxin and Xanthoxin alcohol).

The aqueous residue was resuspended in 0.5 mol m^{-3} phosphate buffer pH 8.0 and partitioned against diethyl ether containing BHT as antioxidant to remove neutral and basic compounds. This neutral fraction was dried *in vacuo* at 35 °C. The residue was resuspended in a known volume of methanol/ethyl acetate (50:50, v/v) and chromatographed three times on thin layers of silica gel GF_{254} in light petroleum/acetone (3:1, v/v). The UV absorbing bands were eluted with water-saturated ethyl acetate and dried *in vacuo*. The residue was rechromatographed in the solvent system *n*-Hexane/ethyl acetate (1:1, v/v) and the zones corresponding to the published Rf values (Burden and Taylor, 1972) of xanthoxin (Rf 0.21) and xanthoxin alcohol (Rf 0.12) eluted with water saturated ethyl acetate, centrifuged, evaporated *in vacuo* at 35 °C and the residue resuspended in methanol for analysis by UV spectrophotometry, HPLC and GC-MS.

2.6.3 Extraction and purification of endogenous ABA and acidic products from cell-free incubates.

The flavedo (exocarp) (± 30 g fr wt) was grated and the gratings homogenised in methanol/ethyl acetate (1:1, v/v) and allowed to extract overnight at -20 °C. Tissue homogenates were then filtered through Whatman No. 1 filter paper using a Buchner funnel and reduced to a small aqueous volume *in vacuo* at 35 °C.

For cell-free incubations precipitated protein was removed by centrifugation and the pellet extracted three times with 5 mL volumes of ice-cold methanol/ethyl acetate (50:50, v/v) containing BHT as an antioxidant. Where non labelled products were to be analysed, a small amount of [^{3}H]-ABA was added to correct for losses during purification.

Combined organic extracts were reduced to a small aqueous volume *in vacuo* and partitioned against diethyl ether to remove neutral and basic compounds. The pH of the aqueous fraction was then adjusted to 2.5 and the acids partitioned into diethyl ether. Diethyl soluble acids were purified on Sep-pak C_{18} cartridges as described in section 2.7.1. Purified acid fractions were analysed on thin layers of silica gel GF_{254} in toluene/ethyl acetate/acetic acid (25:5:2, v/v) and/or by HPLC as described in section 2.8.4. Distribution of radioactivity was determined by liquid scintillation spectrometry as described in section 2.8.5. Extracts containing non-labelled products were likewise separated by thin-layer chromatography or reversed phase HPLC and zones co-chromatographing with authentic 1',4'-*trans*-ABAdiol, ABA and PA were eluted, methylated with excess diazomethane and rechromatographed on silica gel in *n*-hexane/ethyl acetate (1/1, v/v). The UV-absorbing band co-eluting with authentic 1',4'-*trans*-ABAMe diol, ABAMe and PAMe were recovered into diethyl ether and quantified by GC-EC as described in section 2.7.4.2. Unequivocal identification was established by GC-MS as described in section 2.7.4.4.

2.7 Sep-Pak C_{18} cartridge purification

2.7.1 Sep-pak C_{18} cartridge purification of pigments.

Pigment fractions were resuspended in 5 mL 70 % methanol and loaded onto pre-rinsed (2 mL absolute ethanol followed by 5 mL distilled water) Sep-pak C_{18} cartridges. Pigments were eluted from the cartridge with sequential washings of 90 % methanol in H_2O to remove xanthophylls, followed by 100 % methanol to remove chlorophylls and finally with 100 % acetone which contained the carotenes.

2.7.2 Sep-pak C_{18} cartridge purification of ABA and related compounds.

The diethyl ether (Et_2O)-soluble acids from partially purified plant extracts were redissolved in 200-500 µl of 32% methanol in 20 mM K_2HPO_4/KH_2PO_4 buffer, pH 8.0. The samples were then applied to prewashed (2 mL absolute methanol followed by 5 mL distilled water) Sep-pak C_{18} cartridges using a glass syringe with Luer end-fitting. ABA and related compounds were eluted from the Sep-pak cartridge with a single 6.0 mL aliquot of 32 % methanol in 20 mM K_2HPO_4/KH_2PO_4 buffer, pH 8.0 at a flow rate of 1-2 mL/min. The eluates were reduced to dryness *in vacuo* at 35 °C.

Diethyl ether-soluble acids, from cell-free extracts were purified on pre-rinsed Sep-pak C_{18} cartridges (pre-rinsed as above) by sequential washing with 1 % aqueous acetic acid followed by 20 % methanol/1 % aqueous acetic acid and ABA and related compounds eluted from the cartridge with 65 % methanol/1 % aqueous acetic acid (Pierce and Raschke, 1981).

2.8 Analytical procedures

2.8.1 Thin Layer Chromatography (TLC)

ABA and related compounds were separated by TLC (silica gel GF_{254}) using the solvent system toluene/ethyl acetate/acetic acid (50:30:4. v/v) developed (2 x) to 15 cm (Zeevaart and Milborrow, 1976). The relative mobilities of authentic ABA and

related compounds in this solvent system are presented in Table 2.1. In biosynthetic studies, diethyl ether acid fractions containing [^{14}C]-ABA were chromatographed on TLC (silica gel GF_{254}) using the above solvent system containing BHT (10 mg L^{-1}) as an antioxidant and developed 2 x to 15 cm (Milborrow and Robinson, 1973), as described above. Radioactive zones, detected by liquid scintillation spectrometry (see Section 2.8.5), and non-radioactive zones, detected under UV light, with Rf values corresponding to those of authentic (R,S)-ABA (Sigma) and biosynthetically generated 1',4'- *cis*- and 1',4'-*trans*-ABAdiol (see Section 2.9.2) were immediately scraped from the TLC plates into short glass columns plugged with glass wool which was covered with a 5 mm layer of glass beads. Compounds were then eluted from the silica gel using H_2O-saturated ethyl acetate. Likewise previously unidentified metabolites of ABA, detected as either UV-quenching bands or radioactive zones, were similarly eluted from the silica gel. ABAMe and the 1',4'-*cis* and 1',4'-*trans* diols of ABAMe were routinely separated on TLC (silica gel GF_{254}) using the solvent system, *n*-hexane/ethyl acetate (1/1, v/v) developed (1 x) to 15 cm (Loveys *et al.*, 1975; Milborrow and Noddle, 1970; Noddle and Robinson, 1969). The relative mobilities of the standard methyl esters are presented in Table 2.2. Where efficient separation relied on more than one development of the chromatogram, TLC plates were briefly dried, using a hair-dryer, between each run. Following chromatography the TLC plates were air dried in a fume cupboard for a maximum of 12 h. In all cases, when radioactive samples were chromatographed, an aliquot of the radioactive sample was removed, prior to chromatography, and the absolute amounts of radioactivity determined using liquid scintillation spectrometry to allow for correction of elution efficiency.

TABLE 2.1 *Relative mobilities of authentic ABA and related compounds on silica gel GF_{254} (0.25 mm thickness) in the solvent system toluene/ethyl acetate/acetic acid (50:30:4, v/v) developed (2 x) to 15 cm.*

Compound	Rf x 100
ABA	66
t-ABA	76
1',4'-*cis*-ABAdiol	40
1',4'-*trans*-ABAdiol	60
PA	50

TABLE 2.2 *Relative mobilities of standard methyl esters of ABA and related compounds on silica gel GF_{254} (0.25 mm thickness) in the solvent system n-Hexane/ethyl acetate (1:1, v/v) developed 1 x to 15 cm.*

Compound	Rf 100
ABAMe	80
PAMe	56
1',4'-*cis*-diol of ABAMe	60
1',4'-*trans*-diol of ABAMe	70

2.8.2 Quantification of pigments by reversed-phase HPLC

Aliquots of each sample were chromatographed using either an isocratic system of methanol/acetronitrile (9:1, v/v) at a flow rate of 0.8 mL per minute or a linear gradient of 0-100 % ethyl acetate in acetonitrile/water (9:1, v/v) at a flow rate of 0.8 mL per minute. For the isocratic system detection was at 410 nm using an SSI UV-Vis detector coupled to a TSP Data Jet Integrator, whereas for the linear gradient peaks were detected at 450 nm using a linear UV-Vis detector coupled to a Spectra Physics SP 4290 integrator. Pigments were quantified by peak integration after calibration with standard lutein, α-carotene and β-carotene as external standards (Fig. 2.3).

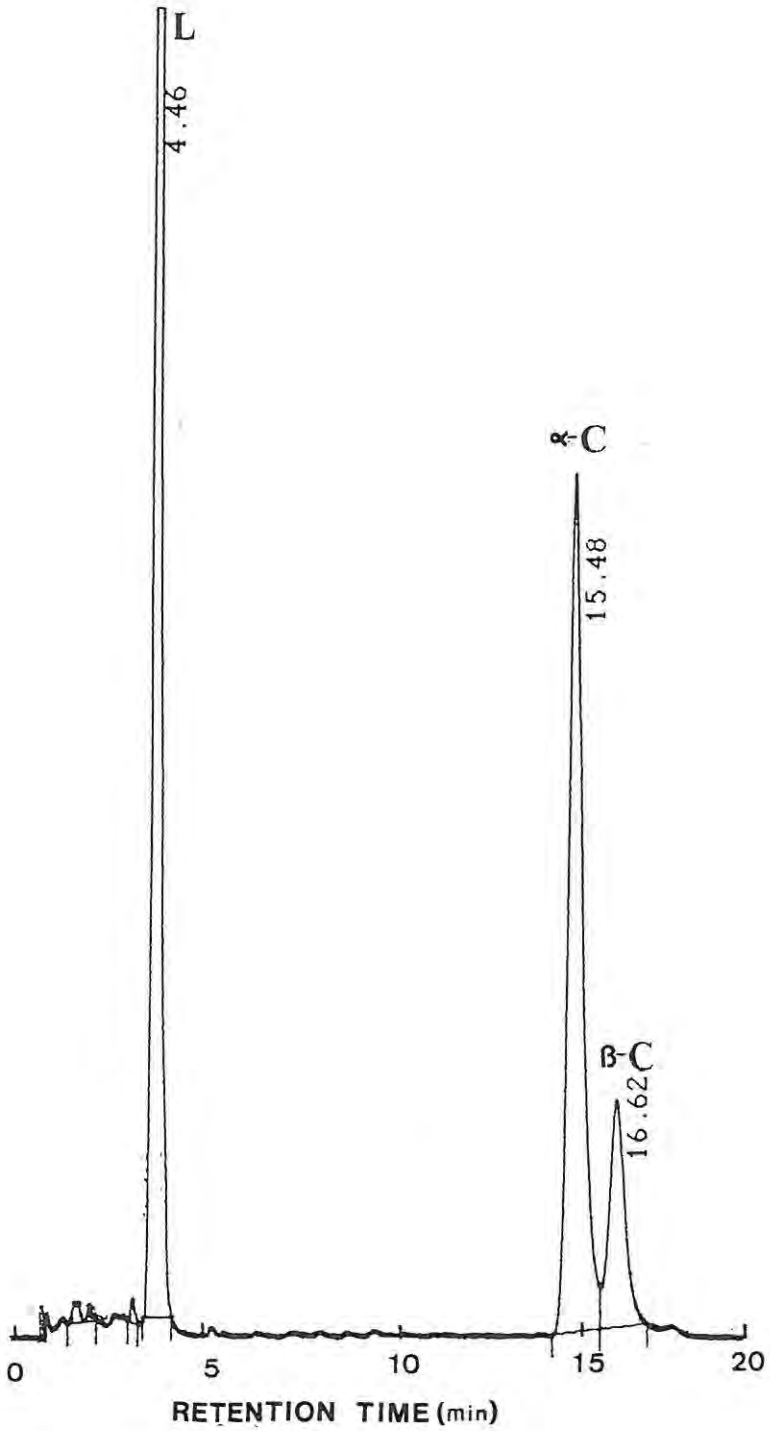


Figure 2.3 *Chromatogram of standard lutein, α-carotene and β-carotene showing retention times.* HPLC was carried out isocratically using a 5 μm C_{18} Nucleosil column with a mobile phase of methanol/acetonitrile (9:1, v/v) at 0.8 mL min^{-1} over 25 min. L = lutein, α-C = α-carotene, β-C = β-carotene,

2.8.3 Identification of Plant Pigments (Carotenoids).

Pigments were extracted from the leaves of *Petunia* plants (which had been growing in sunlight), and purified using Sep-Pak C_{18} cartridges as described by Eskins and Dutton (1979). The pigments (xanthophylls, chlorophylls and carotenes) were fractionated by reversed-phase HPLC either isocratically using the solvent system methanol/acetonitrile (9:1, v/v) or a linear gradient of 1-100 % ethyl acetate in acetonitrile/water (9:1, v/v) containing 0.1 % triethylamine. A flow rate of 0.8 mL min^{-1} over 25 min was used in both cases. These solvent systems separated all the major peaks within 25 min. Figure 2.4 shows the isocratic separation of pigments from *Petunia* leaves.

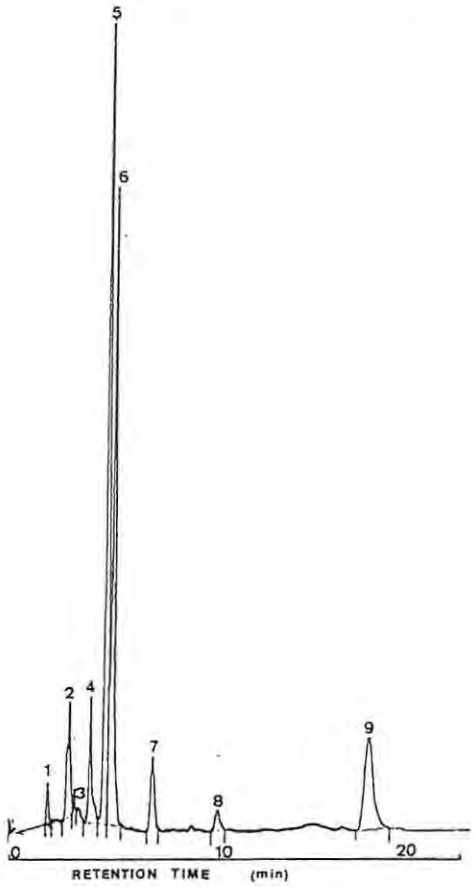


Figure 2.4 *Chromatogram of plant pigments from* Petunia *leaves* . Carotenoids were separated on a 5 μm C_{18} Nucleosil (250 x 4.6 mm i.d.) isocratically with methanol: acetronitrile (9:1, v/v/) as mobile phase at 0.8 mL^{-1} over 25 min. Peaks: 1) Neoxanthin; 2) Violaxanthin; 3) Antheraxanthin; 4) Lutein-5,6-epoxide; 5) Lutein; 6) Zeaxanthin; 7) Chlorophyll a; 8) Chlorophyll b; 9) β-Carotene.

Eluates corresponding to each peak in the chromatogram were collected and reduced to dryness under nitrogen. Peaks were identified by measuring their absorption spectra in appropriate solvents (Davies, 1976) using a Shimadzu, UV-160A recording spectrophotometer, and by comparing the spectral characteristics (Table 2.3) with spectra published in the literature (Young and Britton, 1993).

Table 2.3 *Spectrophotometric analysis of* Petunia *pigment factions resolved isocratically by reversed-phased HPLC using the solvent system methanol/acetonitrile (9:1, v/v) as described in Fig. 2.2.* Compounds were identified by comparing spectral characteristics with spectra of each pigment published in the literature (Davies 1976; Young & Britton 1993).

Peak No	Retention time min	Pigment	λ_{Max} (Hexane)	λ_{Max} (Ethanol)
1	3.12	Neoxanthin	412 436 465	416 438 466
2	3.52	Violaxanthin	407 434 463	416 444 475
3	4.38	Antheraxanthin	415 440 467	420 440 470
4	4.56	Lutein 5,6-epoxide	420 445 475	422 447 470
5	5.3	Lutein	421 449 475	424 449 476
6	5.75	Zeaxanthin	425 445 473	428 450 473
7	7.37	Chlorophyll *a*	410 650	
8	9.13	Chlorophyll *b*	470 630	
9	18.34	β-carotene	425 450 477	427 449 475

2.8.4 Quantification of ABA and related compounds by reversed-phase HPLC

ABA and related compounds were analysed by reversed-phase HPLC eluted either isocratically with methanol/water/acetic acid (40:60:1, v/v) at 1 mL min^{-1} or using a linear gradient of 0-100 % methanol containing 0.5 % acetic acid throughout at 2 mL min^{-1} after recovery of compounds of interest from silica gel. Detection was at 254 nm using either a Linear UV-Vis detector coupled to a Spectra-Physics SP4290 integrator or an SSI UV-Vis detector coupled to a Thermo Separation Products Data Jet Integrator. ABA and related compounds were quantified by peak integration after calibration with reference compounds.

2.8.5 Liquid scintillation spectrometry

TLC plates, used for the separation of radioactive ABA and related compounds, were each divided into 30 equal strips which were then scraped into scintillation vials and eluted with methanol (0,5 mL). The level of radioactivity was determined using either a Beckman LS 2800 scintillation spectrometer, with a counting efficiency of 98 % for [^{14}C], a Beckman LS 5801 or a Packard 1500 scintillation spectrometer, programmed for automatic quench correction, following the addition of 10 mL cocktail [either 2,5-dipenyloxazole pyrophosphate (PPO) in toluene, 5 g/l, or Pico Fluor 40 (Packard)]. Where required, radioactivity in aqueous and water-containing HPLC fractions was determined using Brays scintillant prepared by dissolving naphthalene (30 g) and 2,5-diphenyl oxazole pyrophosphate, PPO (2 g) in 50 mL methanol, and the solution diluted to 500 mL with 1,4-dioxan (Bray, 1960).

2.8.6 Gas chromatography-electron capture quantification of ABA and related acids.

Methyl ester derivates were analysed using a Perkin-Elmer 8310 instrument fitted with an electron capture detector (^{63}Ni source) using a fused-silica capillary column (15 mm x 0.53 mm i.d.) of SPB-1 programmed from 120 °C at 5 °C per minute with N_2 as carrier gas at a flow rate of 17 mL min^{-1}. The retention time for ABAMe (Figure 2.5) was 7.80 minutes.

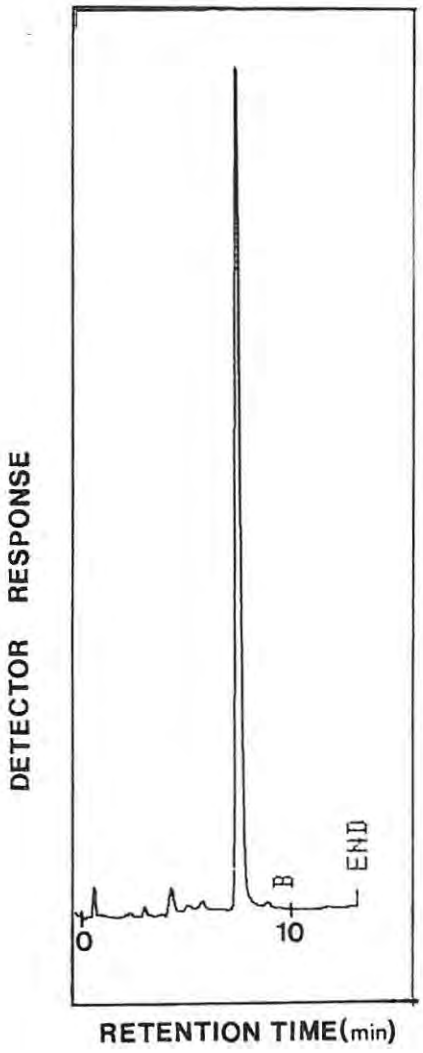


Figure 2.5 *GC-EC chromatogram of standard ABA methyl ester.* Gas chromatography was carried out using a capillary column (15 mm x 0.53 mm i.d.) of SPB-1 programmed from 120 °C at 5 ° per minute with N_2 as carrier gas at a flow rate of 17 mL m^{-1}.

2.8.6.1 Combined capillary gas chromatography - mass spectrometry.

Methylated samples were analysed using a Hewlett-Packard 5890 gas chromatograph fitted with a fused-silica capillary column (12 m x O.32 mm i.d.) of HP-1 programmed from 120 °C at 5 °C min^{-1} with He as carrier gas (1.5-2.0 mL min^{-1}). Electron impact mass spectra were recorded at 70 eV and a source temperature of 250 °C using a Hewlett-Packard 5988A MS system. Alternatively methylated samples were analysed using a Hewlett-Packard 5890 gas chromatograph fitted with a fused silica capillary column (50 m x 0.2 mm i.d.) of HP Ultra 1 programmed from 50-260 ° at 5 ° min^{-1}, with He as carrier gas (0.5 mL min^{-1}) The compounds were identified from published mass spectra (Figs. 2.6, 2.7, 2.8 Gray *et al.*, 1974: Dörffling and Tietz, 1983).

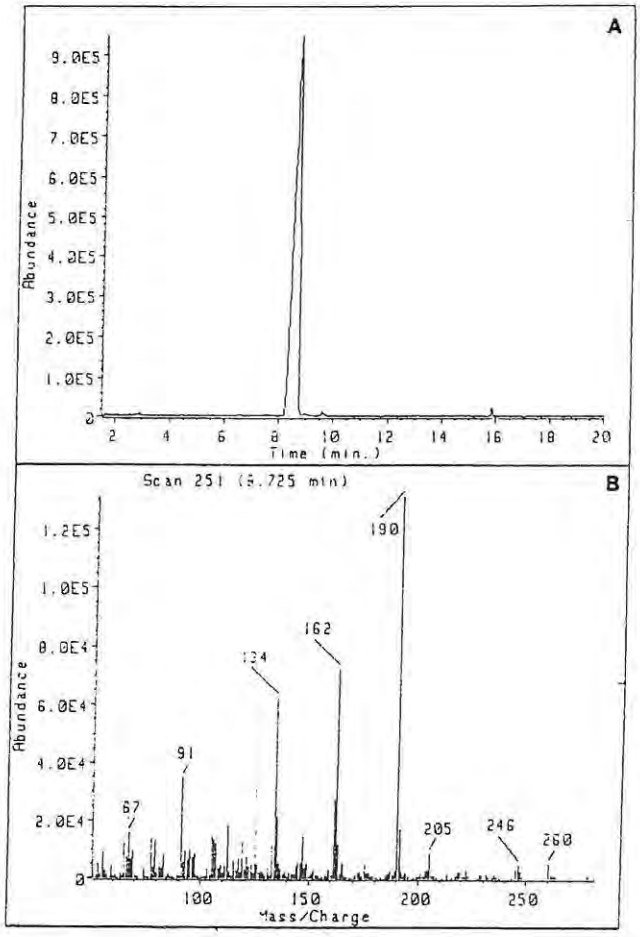


Figure 2.6 **(A)** *Total ion chromatogram of authentic ABA methyl ester.* ***(B)*** *Electron impact mass spectrum of authentic ABA methyl ester.* Conditions were as described in Section 2.8.6.1

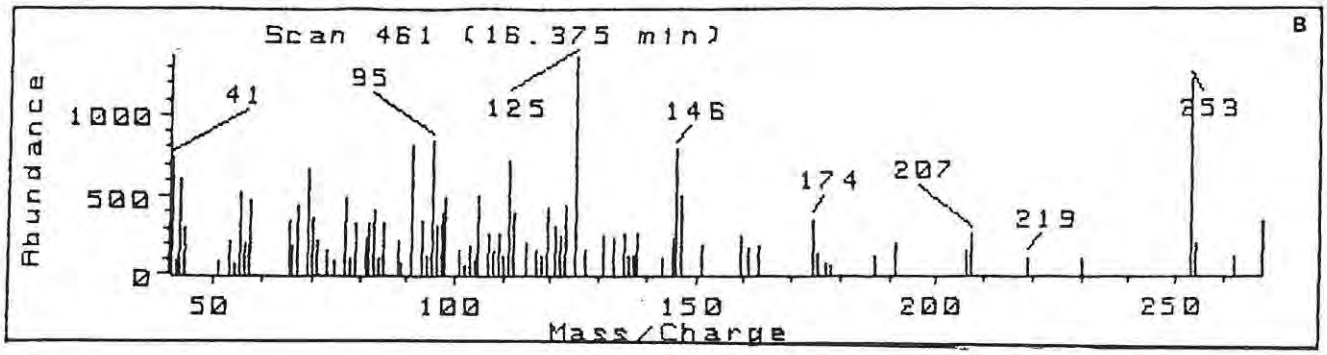


Figure 2.7 *Electron impact mass spectrum of the 1',4'-trans diol of ABAMe.* Mass spectrum was recorded at 70 eV with source temperature of 250 °C using a Hewlett-Packard 5988 A mass spectrometer coupled to a Hewlett-Packard 5890 gas chromatograph fitted with a fused-silica capillary column (12 m x 0.32 mm i.d.) of HP 1 programmed from 120 °C at 5 °C min^{-1} with He as carrier gas (1.5 - 2.0 mL min^{-1}).

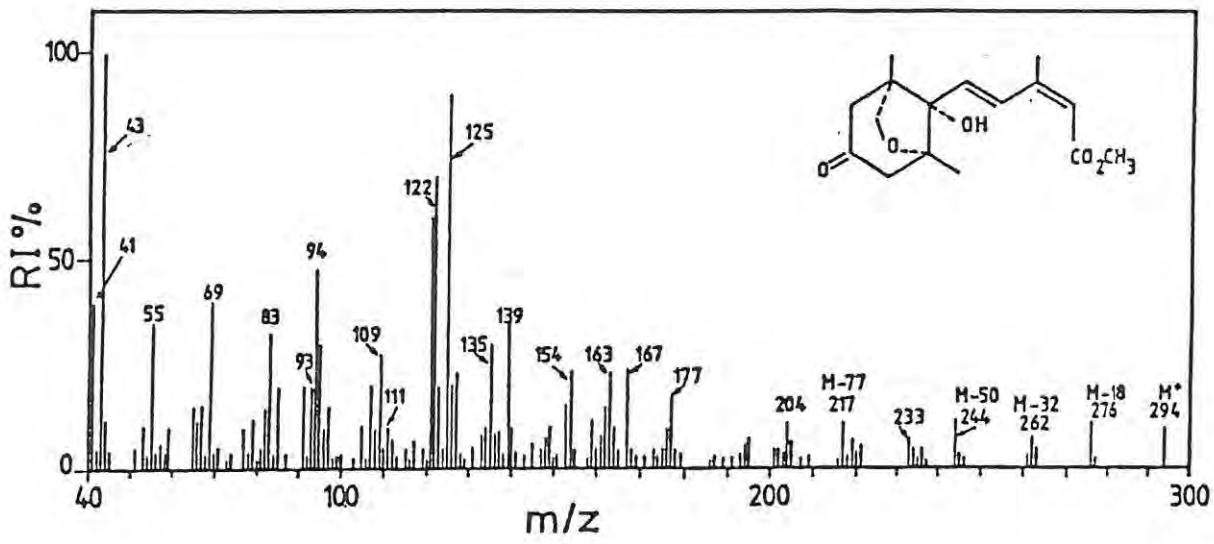


Figure 2.8 *Electron impact mass spectrum of standard PAMe of authentic stock prepared from barley* (Hordeum vulgare). Mass spectrum was recorded at 70 eV with source temperature of 250 °C using a Hewlett-Packard 5988A mass spectrometer coupled to a Hewlett-Packard 5890 gas chromatograph fitted with a fused-silica capillary column (12 m x 0.32 mm i.d.) of HP-1 programmed from 120 ° at 5 °C min^{-1} with He as carrier gas (1.5-2.0 mL min^{-1}.).

2.9 Preparation of standards

2.9.1 Preparation of radio-active β-carotene

Substrate [^{14}C]-all-*trans*-β-carotene was prepared by feeding 74 MBq [^{14}C]-sodium bicarbonate (NaH[^{14}C]O_3 (Sp. act. 1.92 GBq mmol^{-1}), to an axenic culture of *Dunaliella salina* which was then exposed to high light intensity (1000 μmol m^{-2} s^{-1}) for 5 d at 27 °C, conditions which cause the alga to accumulate massive amounts of β-carotene (Cowan and Richardson 1993b; Lers *et al.* 1990). Cells of *Dunaliella salina* were harvested by centrifugation at 4 °C in a Sorvall RC-5 refrigerated centrifuge at 5000 g for 10 min and the cell debris precipitated. The supernatant was discarded. The algal material thus harvested was extracted by sonication in ice- cold acetone containing BHT (20 mg L^{-1}). After sonication, the β-carotene was extracted by centrifugation at 14 000 g and repeated washing of cells in equal volumes of acetone. Combined supernatant extracts were filtered through Whatman No. 1 filter paper, and the residue washed with excess solvent. The filtrate was reduced to dryness *in vacuo* at 35 °C and resuspended in 70 % aqueous methanol and separated into xanthophylls, chlorophylls and β-carotene using the Sep-Pak procedure described in Section 2.7.1. Acetone was evaporated under nitrogen and the residue analysed isocratically by reversed-phase HPLC using the solvent system methanol/acetonitrile (9:1, v/v) at a flow rate of 0.8 mL min^{-1}. The β-carotene was separated into its *trans*- and 9-*cis*-isomers by HPLC fractionation, (Fig. 2.8). The fractions were collected and evaporated under nitrogen and the

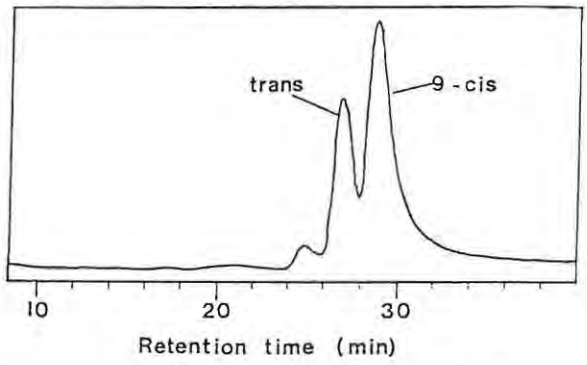


Figure 2.9 *HPLC chromatogram showing separation of β-carotene into the* cis *and* trans *isomers.* β-carotene was separated isocratically by reversed-phase HPLC using the solvent system methanol/acetonitrile (9:1, v/v) at 0.8 mL min^{-1}.

2.9.2 Preparation of labelled and unlabelled 1',4'-*trans*- and *cis*- ABAdiol

Both labelled and unlabelled 1',4'-*trans*- and *cis* - ABAdiol were prepared by a procedure modified from that described by Vaughan and Milborrow, 1987. A known amount of either (R,S)-[2-^{14}C]-ABA (sp. act. 833 MBq mmol^{-1}) or (±) *cis,trans* ABA was resuspended in 1 mL ice-cold methanol water (2:1, v/v), excess sodium borohydride ($NaBH_4$) was added and the reaction allowed to proceed overnight at -20 °C. The methanol was removed under nitrogen and the remaining water made up to 1 mL. The 1',4'-ABAdiol was partitioned into ethyl acetate, dried under nitrogen and separated into *cis*- and *trans*-ABAdiol by thin layer chromatography (TLC) 2x in the solvent system toluene/ ethyl acetate/ acetic acid (50:30:4, v/v). The zones corresponding to authentic 1',4'-*cis*- and *trans*-ABAdiol were removed from the gel using water-saturated ethyl acetate, dried under nitrogen and used immediately.

2.10 Polyacrylamide gel electrophoresis (PAGE)

2.10.1 Sample preparation for SDS-Polyacrylamide gel electrophoresis.

Extracts from crude or acetone powder homogenates of *Citrus* flavedo were incubated for 2 h at 37 °C and the samples were stored in a low ionic strength buffer (0.1 M Tris-HCl) in water as recommended by Schuler and Zielinski (1989). Protein concentration of the samples was determined by the Bradford assay (Bradford 1976, Section 2.2.3), and varying amounts of protein were loaded onto the gels as specified in Results (Chapter 6).

2.10.2 Molecular weight markers.

The protein standards (molecular weight in Kilodalton) routinely included on the gels were albumin, bovine *(66)*; ovalbumin *(44)*; carbonic anhydrase *(29)* and myoglobin *(13)*.

2.10.3 Electrophoretic techniques.

The separation of polypeptides by SDS-polyacrylamide gel electrophoresis was achieved using a 12.5 % acrylamide running gel and 4 % acrylamide stacking gel, in the buffer system of Laemmli (1970). Electrophoresis was carried out using either SE 250 Mighty small gels at 18 mA per gel for 1½ h or SE 600 gels at 25 mA per gel for 7 h. Gels were stained for 4 h in 0.125 % (w/v) Coomassie brilliant blue, destained overnight in 50 % (v/v) methanol, 10 % (v/v) glacial acetic acid and stored in 10 % methanol, 7 % glacial acetic acid.

2.10.4 Detection of Cytochrome P450 on SDS gels.

Peroxidase activity of Cytochrome-P450 was checked by SDS-polyacrylamide gel electrophoresis. The presence of peroxidase activity was checked using the following staining procedure. A 6.3 mM 3',3',5,5'-Tetramethylbenzidine (TMBZ), solution was freshly prepared in methanol. Immediately before use, 3 parts of the TMBZ solution were mixed with 7 parts of 0.25 M sodium acetate, pH 5.0 (as described by Thomas 1976). The gels were immersed in this mixture at room temperature in the dark. After 1 to 2 h on a slow shaker, H_2O_2 was added to a final concentration of 30 mM. The staining was visible within 3 minutes and increased in intensity over the next 30 minutes. At this time or a few hours later the gels were placed in isopropanol: 0.25M sodium acetate, pH 5.0, at a ratio of 3:7. The acetate buffered 30 % isopropanol solution was replaced once or twice with fresh solution to remove any precipitated TMBZ. The replacement of the staining solution with the acetate-buffered 30 % isopropanol also served to clear the gel background and enhance the staining intensity (Thomas *et al.*, 1976).

CHAPTER THREE

RELATIONSHIP BETWEEN ABSCISIC ACID AND COLOUR DEVELOPMENT IN FLAVEDO OF *CITRUS SINENSIS*.

3.1 Introduction

Colour development in *Citrus* fruit peel involves temporal changes in chloroplast ultrastructure and the metabolism of both chlorophylls and carotenoids. For example, at colour break thylakoid degeneration and the transition of chloroplasts to chromoplasts is accompanied by a decrease in carotenogenesis and enhanced chlorophyll breakdown followed by an increase in carotenoid biosynthesis (Gross, 1981; Gross, et al., 1983). Early suggestions were that ABA may play a role in colour development in fruit, particularly as application of ABA enhanced coloration in tomato (Khudairi and Arboleda, 1971) and grape (Coombe and Hale, 1973). However, all attempts to demonstrate an effect of ABA on coloration of *Citrus* have thus far proved unsuccessful (Goldschmidt, 1988). Even so, content of ABA increases throughout the course of *Citrus* fruit development (Aung *et al.,* 1991; Hofman, 1990; Sagee and Erner, 1991) and concentrations in mature fruit peel are among the highest reported in the literature (Goldschmidt, 1976).

There is however no published information on changes in carotenoid composition, which in all likelihood, occur during development of colour. Without such information no conclusions can be drawn about the origin of ABA in *Citrus sinensis* flavedo. With regard to tangerine, β-carotene content decreases with development of colour from 14.6 to 0.3% of total carotenoids. In the same study it was demonstrated that the violaxanthin level increased from 1.6 to 39.8% of total carotenoids while the neoxanthin level decreased gradually over the same period (Gross 1981). This suggests that changes in β, β-carotenoid composition may account for increases in ABA content.

The goal of this study was to investigate the change in endogenous ABA concentration and attempt to relate these changes to changes in pigmentation and carotenoid composition.

3.2 Results

3.2.1 Changes in ABA content

The relationship between ABA accumulation and colour development in flavedo of the six varieties examined is illustrated in Figure 3.1. It is apparent that ABA content of mature fruit increased throughout the duration of colour development (Fig. 3.1A) Only after the development of colour was there an appreciable decline in ABA content, presumably, due to enhanced catabolism and formation of ABA-conjugates (Harris and Dugger, 1986). Although the time taken to reach maximum ABA content was similar for all varieties examined (37-39 weeks post anthesis), it is noteworthy that the common (sweet) oranges (viz. Midknight and Moss Seedless) accumulated substantially more ABA, and colour development in these varieties occurred visibly later (Fig. 3.1B). This observation may suggest that elevated endogenous ABA levels contribute to the apparent retardation of colour development in fruit of these varieties.

3.2.2 Changes in carotenoid composition

Analysis of the carotenoid composition of flavedo tissue at colour break (June) and after colour development revealed considerable differences and these are shown in the chromatograms presented in Figure 3.2. The pigment composition of flavedo tissue at colour break resembled closely that of photosynthetically active leaves and the major components were identified as neoxanthin, violaxanthin, antheraxanthin, lutein, zeaxanthin, β-cryptoxanthin, chlorophyll b, chlorophyll a and β-carotene (Fig. 3.2A). Also present was β-citraurin, a characteristic component of extracts of *Citrus* flavedo (Gross, 1981). Following full development of colour, major differences in pigment composition included loss of chlorophyll and a substantial decline in levels of neoxanthin, violaxanthin, lutein and β-carotene, with an accompanying increase in antheraxanthin and the formation of violaxanthin monoesters and other unidentified xanthophyll acyl esters (Fig. 3.2B). Quantification of these differences in pigment composition is presented in Table 3.1. Contrary to findings by other workers (Goldschmidt, 1976) results from this experiment reveal that an increase in endogenous ABA levels coincides with onset of colour. Analysis of coloured fruit flavedo revealed an accumulation of xanthophyll acryl esters at the expense of free xanthophylls.

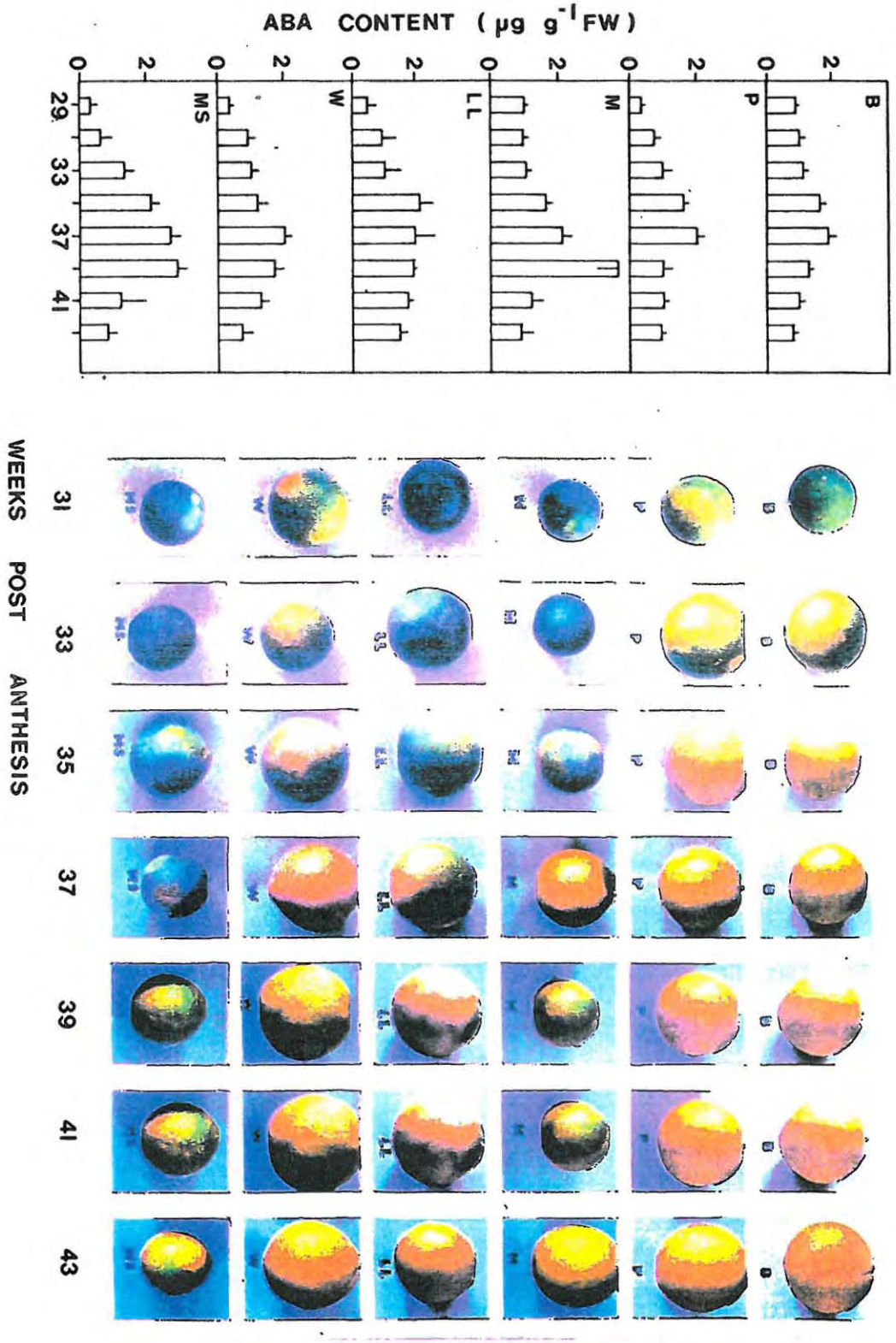


Figure 3.1 *The abscisic acid content in relation to colour development in flavedo from fruit of* Citrus sinensis cvs. Bahianinha, Palmer, Midknight, Lane Late, Washington and Moss Seedless. Changes in abscisic acid content **(A)** and visual colour development **(B)** of mature *Citrus* fruit. Abscisic acid data are expressed as the mean ± SE of quantifications made during the 1991 and 1992 seasons.

Furthermore, these results show that the transition from colour break to full colour is accompanied by an accumulation of carotenoids indicative of enhanced carotenogenesis.

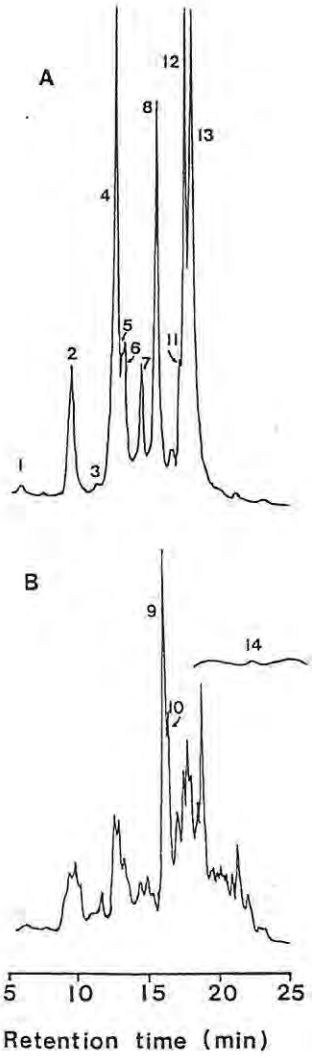


Figure 3.2 *Representative high performance liquid chromatograph profiles illustrating the pigment composition of flavedo from* Citrus sinensis *cv. Midknight fruit at* ***(A)*** *colour break and* ***(B)*** *after development of full colour.* Peaks: (1) neoxanthin, (2) violaxanthin, (3) antheraxanthin, (4) lutein, (5) zeaxanthin, (6) β-cryptoxanthin, (7) chlorophyll *b*, (8) chlorophyll *a*, (9) violaxanthin ME1, (10) violaxanthin ME2, (11) β-citraurin ME1, (12) β-citraurin ME2, (13) β-carotene and (14) unidentified xanthophyll acyl esters.

Table 3.1 *Pigment concentration of* Citrus sinensis *cv.* Midknight *flavedo at colour break and after development of colour.* Only data for identified pigments is presented.

Pigment	Concentration	
	Colour Break	Orange
	($\mu g\ g^{-1}$ fresh weight)	
Neoxanthin	0.50	tr.[a]
Violaxanthin	6.91	1.26
Antheraxanthin	0.10	1.78
Lutein	15.82	3.14
Zeaxanthin/	6.73	6.46
β-cryptoxanthin	-	-
Chlorophyll *b*	49.32	ND[b]
Chlorophyll *a*	164.91	ND
Violaxanthin ME	ND	17.99
β-citruarin ME	8.21	9.11
β-carotene	1.41	0.28
Total carotenoids	48.32	90.18

[a]trace amounts only

[b]not detected

CHAPTER 4

DEVELOPMENT OF AN ABA-BIOSYNTHESIZING CELL-FREE SYSTEM FROM FLAVEDO OF *CITRUS SINENSIS* (L.) OSBECK CV MIDKNIGHT FRUIT.

4.1 Introduction

Various studies using stable isotopes, inhibitors of carotenogenesis and mutants deficient in both carotenoids and ABA have attempted to resolve the controversy surrounding the origin of ABA in higher plants. The success achieved in these studies has been limited as evidenced by two recent publications (Netting and Milborrow, 1994; Willows *et al.*, 1994). This suggests that an alternative method be sought. One possibility is to develop cell-free systems, capable of synthesising ABA from "simple" precursors, in which to study more detailed aspects of the biochemistry and enzymology of the post-FPP transformations. This approach has been successfully employed to study the biosynthesis (Graebe, 1987) and enzymology (Lange and Graebe, 1993) of gibberellins.

Reactions in sesquiterpenoid synthesis occur slowly and the enzymes involved are usually at low intracellular concentrations (Alonso and Croteau, 1993). Thus it might be expected that it would be difficult to detect putative post-FPP intermediates such as xanthoxin, xanthoxin acid, ABA-aldehyde, 1',4'-trans-ABAdiol, and product ABA, in *in vitro* systems. Attempts to resolve this problem were made by utilizing, as a source of enzyme, *Citrus* flavedo.

ABA was first detected in *Citrus* fruits by optical rotatory dispersion (Milborrow, 1967) and later unequivocally characterized in extracts of *Citrus* flavedo by combined gas chromatography-mass spectrometry (Goldschmidt et al., 1973). Since ABA concentrations in *Citrus* flavedo are of the order of 2.5 mg kg^{-1} fresh weight (Goldschmidt, 1976), among the highest reported for a higher plant tissue, it was envisaged that flavedo might be a useful tissue in which to study aspects of ABA biosynthesis *in vitro*.

Recent studies suggest a universal pathway for ABA biosynthesis in plants involving cleavage of an oxygenated-carotenoid, possibly 9'-*cis*-neoxanthin to yield xanthoxin

which is then converted to ABA *via* ABA-aldehyde (Rock et al., 1992; Rock and Zeevaart, 1991). This pathway is dependent upon isoprenoid synthesis and the production of carotenoids. *Citrus* flavedo is a rich source of chromoplasts and these organelles represent a major site of carotenogenesis in plants (Beyer et al., 1985; 1989). Unfortunately, the interrelationship between carotenogenesis and ABA biosynthesis in isolated chromoplasts has yet to be investigated. Nevertheless, a suggestion by Attaway et al. (1967) that linolyl pyrophosphate, a tertiary isomer of NPP and GPP, could be an intermediate *en route* to limonene biosynthesis allowed George-Nascimento and Cori (1971) to demonstrate terpenoid biogenesis from GPP and NPP in extracts derived from *Citrus sinensis* flavedo. In subsequent studies these authors demonstrated the synthesis of sesquiterpenoid alcohols and aldehydes using similar cell-free extracts (Chayet et al., 1973). More recently, a prenyltransferase activity (E.C. 2.5.1.1.) has been partially purified from flavedo of *Citrus sinensis* (De la Fuente et al., 1981). This enzyme was shown to catalyze the condensation of isopentenyl pyrophosphate with either dimethylallyl pyrophosphate or geranyl pyrophosphate, and the (2*E*, 6*E*)- and (2*Z*, 6*E*)-farnesyl pyrophosphates were identified as the predominant products. This accumulated information suggested that cell-free extracts of *Citrus sinensis* flavedo should contain the necessary enzymes to demonstrate ABA biosynthesis *in vitro*.

Of the six varieties examined in Chapter 3, cv Midknight contained substantially more ABA than the other varieties at or after colour break and it was therefore considered that tissue from this cultivar would be a rich source of ABA biosynthetic enzymes.

4.2 Results

4.2.1 ABA synthesis from MVA in crude extracts of *Citrus sinensis*.

Cell-free enzyme preparations derived from *Citrus sinensis* flavedo incorporated 2R-[2-^{14}C]-MVA into acid compounds that were chromatographically similar to ABA, 1'4'-*trans*-ABAdiol and PA. (Table 4.1)

Table 4.1 *Incorporation of radioactivity into acidic products in cell-free extracts of* Citrus sinensis *flavedo supplied with R-[2-^{14}C]-MVA (370 kBq).* Reaction conditions were as described in "Material and Methods". Radioactive acids were analysed either by TLC (silica gel GF_{254} in toluene/ethyl acetate/acetic acid, 25: 15: 2, v/v) or by reversed-phase HPLC (linear gradient of 0-100 % methanol containing 0.5 % acetic acid throughout at 2 mL min^{-1}) and the distribution of radioactivity determined by liquid scintillation spectrometry as described in Section 2.8.5

	Distribution of Radioactivity[a]		
	PA	1',4'-*trans*-ABA-diol	ABA
	kBq mg protein^{-1} (% incorporation)		
Experiment 1	1.18 (0.32)	3.23 (0.87)	3.46 (0.94)
Experiment 2	1.41 (0.38)	2.92 (0.79)	4.22 (1.14)
Experiment 3	1.50 (0.41)	3.49 (0.94)	5.83 (1.58)

[a] Identification of metabolites based on co-chromatography with authentic standard.

The identity of these acids was tentatively established by micro-chemical analysis of the methyl ester derivatives and by co-chromatography on thin layers of silica gel according to the criteria proposed for the identification of ABA and related compounds (Milborrow and Noddle, 1970) and the results are presented in Table 4.2.

Table 4.2 *Microchemical characterization and chromatographic behaviour of products formed from R-[2-^{14}C]-MVA by crude enzyme preparations as described in Table 4.1*

Treatment	Putative		Authentic	
	ABA	1',4'-*trans*-ABA-diol	ABA	1',4'-*trans*-ABA-diol
	Rf values			
Untreated[1]	0.63	0.53	0.63	0.53
Diazomethane[2,3]	0.63	0.83	0.63	0.83
$NaBH_4$[2,5]	0.20, 0.40	NR[4]	0.20. 0.40	NR
MnO_2[2,5]	NR	0.63	NR	0.63

[1] TLC on silica gel in toluene/ethyl acetate/acetic acid (50:30:4. v/v) developed 2 x to 15 cm.
[2]TLC on silica gel in *n*-hexane/ethyl acetate (1:1, v/v) developed 1 x 15 cm.
[3]TLC on silica gel in *n*-hexane/ethyl acetate (1:1, v/v) developed 3 x 15 cm.
[4]No Reaction.
[5]Methyl ester derivatives treated.

In attempts to enhance incorporation of radioactivity into the acidic products, the contribution of developmental stage of *Citrus* fruits and the kinetics of the reaction were determined. Extracts prepared from chromoplast-containing flavedo, that is from fruits that had already undergone colour change, were better able to convert R-[2-^{14}C]-MVA into ABA and related acidic products (Fig 4.1). Thus all further studies were carried out using fruits at this developmental stage. Kinetic studies revealed that production of putative ABA and PA was sustained for 2 h and thereafter declined, presumably due to product catabolism (Fig. 4.2 a and b). A decline in radioactivity associated with both ABA and PA occurred concomitant with an increase in putative 1',4'-*trans*-ABAdiol (Fig. 4.2 c).

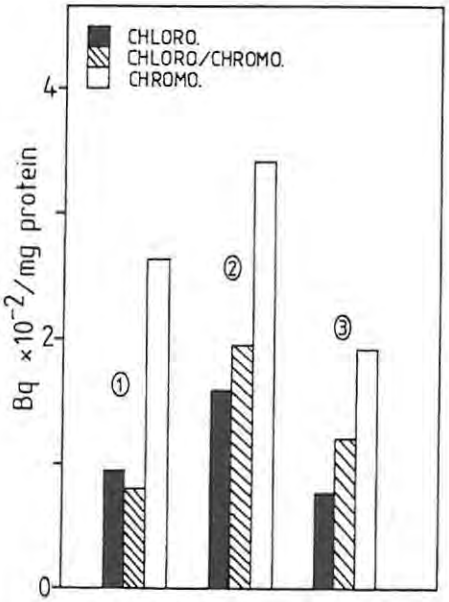
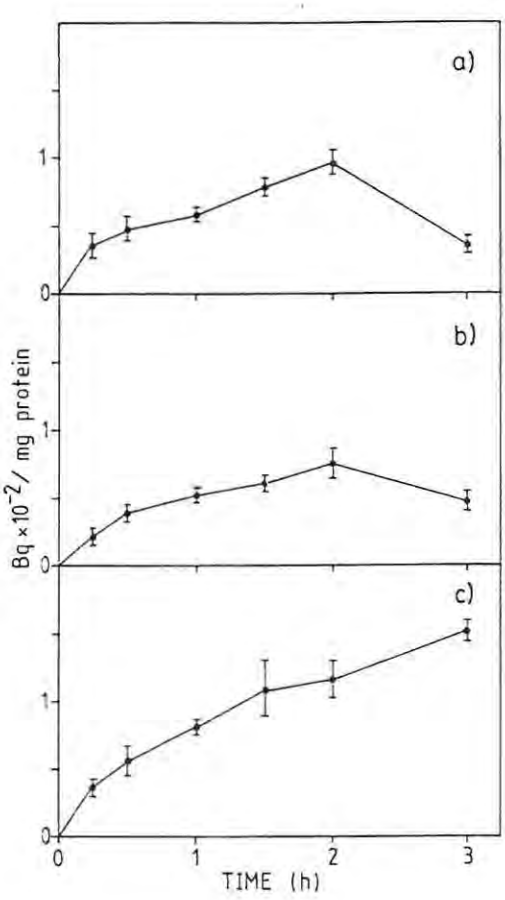


Figure 4.1 *The distribution of label between ABA(3), 1',4'-*trans*-ABA-diol (2) and PA (1) obtained from cell-free enzyme systems of* Citrus sinensis *flavedo.* Extracts were prepared from chloroplast-containing flavedo and from exocarp of fruits during colour change. Enzyme preparations were incubated with *R*-[2-^{14}C]-MVA (50 kBq) as described in Section 2.4.4 and the products analysed as described in Table 4.1.

Figure 4.2 *Kinetics of the incorporation of label from R-[2-^{14}C]-MVA (50 kBq) into ABA(a), PA (b) and 1',4'-*trans*-ABA-diol (c) by cell-free extracts of* Citrus sinensis *flavedo.* Reaction conditions were as described in Section 2.4.4. Data represent the mean of three replicates ±S.E.



4.2.2 ABA synthesis from [1-^{14}C]-IPP in crude extracts of *Citrus sinensis*

Cell-free enzyme preparations derived from *Citrus* flavedo incorporated [1-^{14}C]-IPP into acidic products that were chromatographically similar to ABA and 1',4'-*trans*-ABAdiol. A third acid which was not chromatographically similar to PA was also present (Table 4.3).

Table 4.3 *Incorporation of radioactivity into products in cell-free extracts of* Citrus sinensis *flavedo supplied with [1-^{14}C]-IPP (370 kBq).* Reaction conditions were as described in Section 2.4.4. Radioactive acids were analysed either by TLC (silica gel GF_{254}) in toluene/ethyl acetate/acetic acid (25:15:2, v/v) or by reversed-phase HPLC (linear gradient 0-100 % methanol containing acetic acid throughout at 2 mL min^{-1}) and the distribution of radioactivity determined by liquid scintillation spectrometry as described in section 2.8.5.

	Distribution of Radioactivity		
	Unknown acid	1',4'-*trans*-ABAdiol	ABA
	kBq mg protein^{-1} % incorporation		
Experiment 1	1.98 (0.53)	4.61 (1.24)	3.98 (1.08)
Experiment 2	1.87 (0.50)	4.06 (1.09)	4.87 (1.32)
Experiment 3	1.90 (0.51)	4.76 (1.28)	6.27 (1.70)
Mean	1.92 (0.51)	4.48 (1.20)	5.04 (1.37)
S.E.	±0.05 ±0.12	±0.30 ±0.08	±0.94 ±0.26

Kinetic studies revealed that production of putative ABA, 1',4'-*trans*-ABAdiol and another radioactive acid was sustained for 2 h and thereafter declined, presumably due to product catabolism. A decline in radioactivity associated with both ABA and the unknown acid occurred concomitant with an increase in putative 1',4'-*trans*-ABAdiol (Fig 4.3)

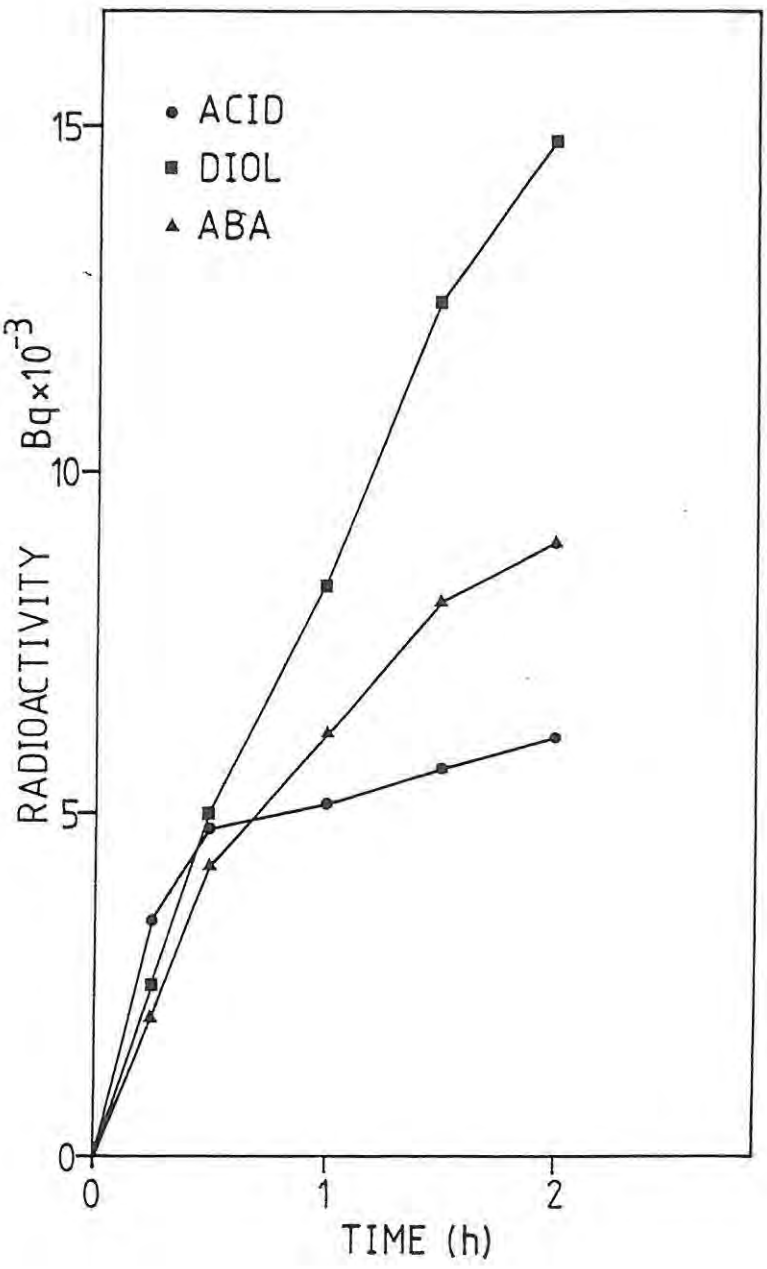


Figure 4.3 *Kinetics of the incorporation of label from [1-^{14}C]-IPP (50 kBq) into ABA, Xan acid and 1',4'-*trans-*ABAdiol by cell-free extracts of* Citrus sinensis *flavedo.* Reaction conditions were as described in Section 2.4.4.

4.3 Identification of products in the crude cell-free system from *Citrus sinensis* flavedo.

4.3.1 TLC separation of the acids from MVA

The acids in the acid fraction obtained from the cell-free system of *Citrus* flavedo were separated on TLC (silica gel GF_{254}) in toluene/ethyl acetate/acetic acid (25: 15: 2, v/v) developed 2 x to 15 cm (Zeevaart and Milborrow, 1976). A typical separation is shown in Figure 4.4.

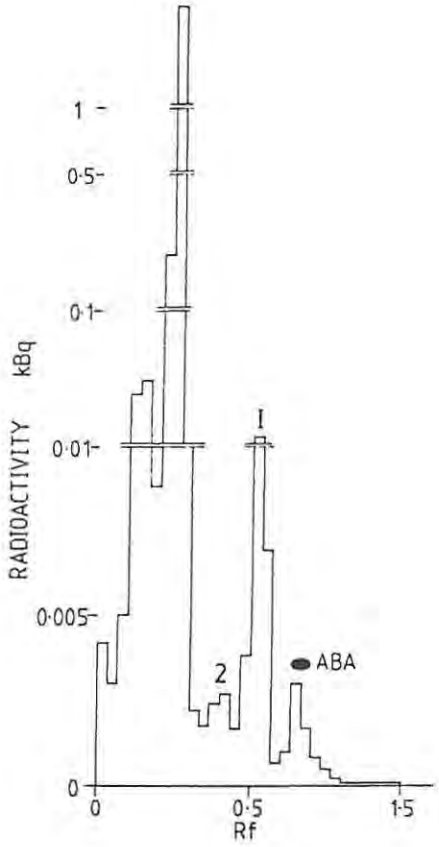
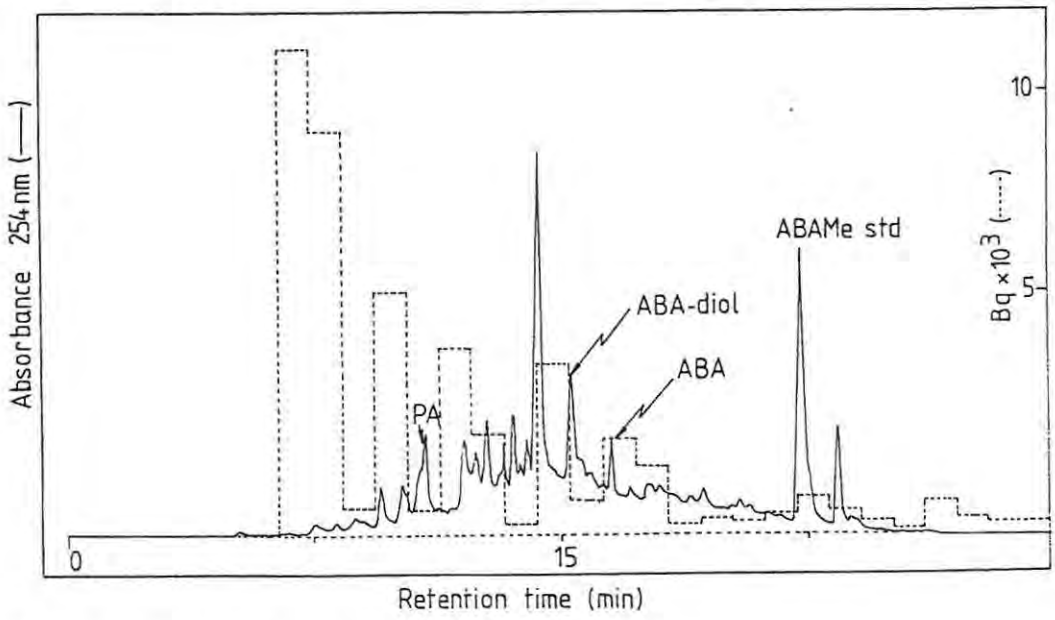


Figure 4.4 *Thin layer chromatographic separation of (R,S)-[2-^{14}C]-ABA from cell-free extracts of* Citrus *flavedo incubated as described in section 2.4.4.* 1) ABAdiol; 2) PA.

4.3.2 HPLC separation of the acids from IPP

The acid fraction obtained from cell-free extracts of *Citrus* flavedo was analysed by reversed phase HPLC using a linear gradient of 0-100 % methanol containing acetic acid throughout at 2 mL min^{-1}. The typical elution profile obtained is shown in Figure 4.5.

Figure 4.5 *Typical elution profile obtained when the acid fraction from cell-free extracts of* Citrus *flavedo with [1-^{14}C]-IPP as substrate was analysed by reversed-phase HPLC (linear gradient 0-100 % methanol containing acetic acid throughout at 2 mL min^{-1}).*



4.3.3 Identification of acids by GC-MS

After purification by HPLC the radioactive fractions corresponding to authentic ABA, PA and 1',4'-*trans*-ABAdiol were collected. The individual components were methylated with ethereal diazomethane and further analysed by GC-MS using a Hewlett-Packard 5890 gas chromatograph fitted with a fused-silica capillary column (12 m x 0.32 mm i.d.) of HP1 programmed from 120 °C at 5 °C min^{-1} with He as carrier gas (1.5 - 2.0 mL min^{-1}). The electron-mass spectra are shown in figures 4. 6, 4.7 and 4.8. The compounds were identified from published mass spectra (Dörffling and Tietz, 1983; Gray *et al.*, 1974; Tietz *et al.*, 1979).

Figure 4.6 *Electron impact mass spectrum of the methyl ester derivative of ABA synthesised in a cell-free system from* Citrus sinensis *flavedo supplied with either* R-*[2-*^{14}*C]-MVA (1.83 MBq, 120 μg) or* R-*[1-*^{14}*C]-IPP.* Conditions of incubations were as described in Section 2.4.4. The mass spectrum was obtained at 70 eV and a source temperature of 250 °C using a Hewlett-Packard 5988A MS system and compounds identified from published mass spectra (Dörffling and Tietz, 1983; Gray et al., 1974)

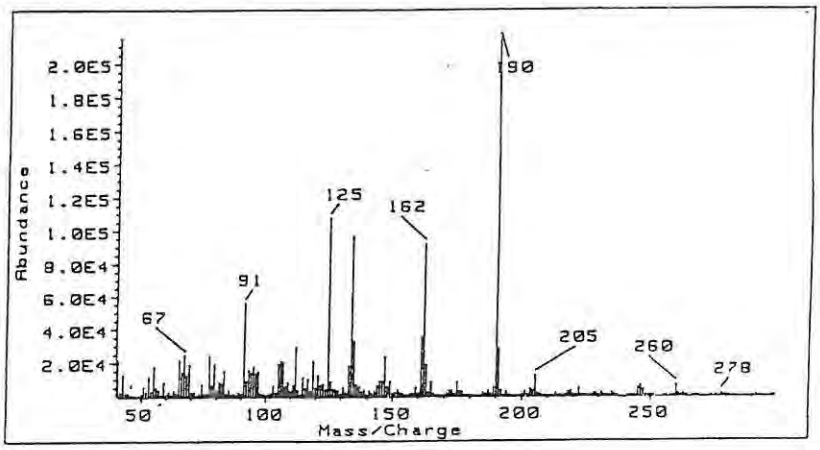
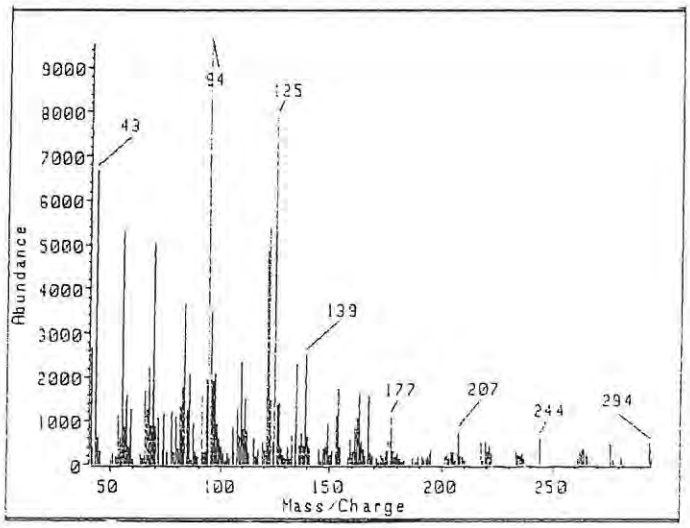
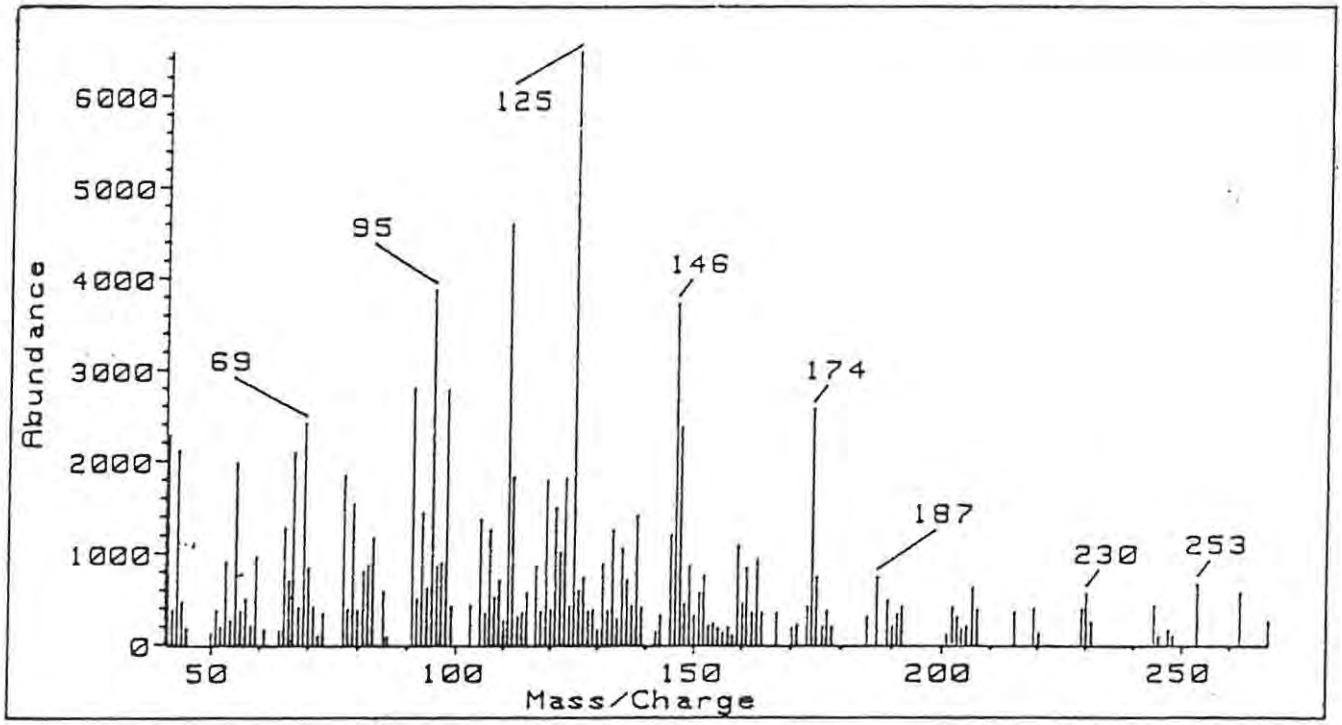


Figure 4.7 *Electron impact mass spectrum of the methyl ester derivative of PA synthezised in a cell-free extract from* Citrus sinensis *flavedo supplied with* R-*[2-*^{14}*C]-MVA (1.83 MBq, 120 μg) or R-[l-*^{14}*C]-IPP.* Conditions of incubations were as described in Section 2.4.4. The mass spectrum was obtained at 70 eV and a source temperature of 250 °C using a Hewlett-Packard 5988A MS system and PAMe identified from its published mass spectrum (Tietz *et al.*, 1979). No labelled PA was detected due to cold-pool trap conversion to PA.



This spectrum is identical to that of authentic PAMe (Tietz *et al.*, 1979) with a characteristic molecular ion at 294 (Fig. 4.7)

Figure 4.8 *Electron impact mass spectrum of the dehydration product of 1',4'-trans-ABAMe-diol sythesized in a cell-free system of* Citrus *flavedo supplied with R-[2-^{14}C]-MVA (1.83 MBq, 120 μg).* Conditions of incubations were as described in Section 2.4.4. Conditions for GC-MS were as described in Figure 4.7.



The cracking pattern with a molecular ion (m\z) at 262 was identical to, and could be identified as 4'-desoxy-ABAMe, characterized previously by Tietz *et al.*, (1979) and later shown to be the product of dehydration of 1',4'-*trans*-ABAMe diol during GC analysis (Milborrow 1983). Based on the positions at which label from R-[2-^{14}C]-MVA occurs in ABA (Milborrow, 1975), an apparent ion at m\z 268 (3.84 % relative to b.p. m\z 125) may be attributable to 6 atomic mass units extra due to incorporation of [^{14}C] but no radioactivity was detected due to dilution with cold-pool ABA. The cold-pool trap of (R,S)-ABA increased the amount of radioactivity associated with ABA but diluted the available radioactivity for incorporation into PA and 1'4-trans-ABA-diol.

4.4 ABA synthesis in acetone powder homogenates

4.4.1 ABA biosynthesis from *R*-[2-^{14}C]-MVA, [1-^{14}C]-IPP and β-carotene

In an attempt to assess the efficiency of the *Citrus* cell-free system in the biosynthesis of ABA and related compounds, both crude enzyme extracts (which contain soluble enzymes) and acetone-powder extracts (which contain membrane-bound enzymes) were used. Results in Table 4.4 provide a comparative assessment of the ABA-biosynthesizing activity of cell-free extracts prepared from crude, dialysed crude and acetone powder homogenates of *Citrus* flavedo supplied with 3R-[2-^{14}C]-MVA as substrate.

Table 4.4 *ABA-biosynthesizing activity in cell-free preparations from* Citrus *flavedo.* Incubations contained glutathione, NaF, ATP, $MgCl_2$, NAD+, AMO 1618 and cold ABA at the concentrations specified in Section 2.4.4, and were carried out at 37 °C for 2 h. Incorporation of label from 3R-[2-^{14}C]-MVA (150 kBq) into 1',4'-*trans*-ABAdiol, ABA and PA was determined by liquid scintillation spectrometry following separation of the diethyl ether-soluble acids by TLC and/or reversed phase HPLC. Enzyme activity is expressed as radioactivity incorporated into 1',4'-*trans*-ABAdiol + ABA + PA mg protein^{-1} h^{-1}.

Extract	Activity (Bq mg protein^{-1} h^{-1})
Undialysed Crude	158.48
Heat inactivated[a]	ND[b]
Dialysed Crude	211.91
Heat inactivated	ND
Acetone Powder	335.45
Heat inactivated	ND

[a]100°C for 10 min. [b]Not detected

The activity was enhanced almost 2-fold in extracts prepared from acetone powder homogenates. Heat inactivated extracts showed no detectable enzyme activity.

When either radioactive IPP or β-carotene was used as substrate in acetone powder homogenates, incorporation into radioactive acids that co-chromatographed with 1',4'-*trans*-ABAdiol, ABA and PA respectively was enhanced (Table 4.5). ABA biosynthesizing activity was increased nearly 7-fold with β-carotene as substrate (Table 4.5) which indicates that ABA may be derived from carotenoids.

Table 4.5 *ABA-biosynthesis in cell-free extracts of acetone powder homogenates of* Citrus *flavedo.* Complete acetone powder incubates were prepared as described in Section 2.4.4 and reactions carried out at 37 °C for 2 h with either 3R-[2-^{14}C]-MVA (50 kBq), [1-^{14}C]-IPP (50 kBq) or [^{14}C]-*all-trans*-β-carotene (10 kBq) as substrate. Incorporation of label into 1',4'-*trans*-ABAdiol, ABA and PA was determined after separation of the diethyl ether-soluble acids first by TLC and then by reversed phase HPLC analysis of the individual acids.

Substrate	Distribution of radioactivity			
	1',4'-*trans*-ABAdiol	ABA	PA	All Three
	(kBq mg protein^{-1} incorporation)			
MVA	6.15 (1.65 %)	3.21 (0.86 %)	4.13 (1.11 %)	13.49 (3.62 %)
IPP	8.76 (2.35 %)	3.81 (1.02 %)	5.26 (1.41 %)	17.83 (4.78 %)
β-carotene	38.13 (10.23 %)	19.90 (5.34 %)	26.85 (7.21 %)	68.26 (22.78 %)

4.4.2 Substrate specificity of β-carotene

In an attempt to determine which isomer (*cis* or *trans*) of β-carotene was involved in the biosynthesis of ABA, substrate specificity was tested by using either *cis*- or *trans*-β-carotene, or a mixture of the two isomers, as substrate. When only 9-*cis*-β-carotene was used as substrate no ABA or related compounds were detected indicating that ABA was not derived from *cis*-β-carotene. . Use of *all-trans*-β-carotene lead to the incorporation of radioactivity into putative ABA, 1',4'-*trans*-ABAdiol and PA. However, a mixture of the two isomers greatly enhanced incorporation of radioactivity into ABA and related acids. This suggested that the *cis*-β-carotene isomer served to solubilize *all-trans*-β-carotene thus making it more readily available for the biosynthesis of ABA. The results are shown

in Table 4.6. This result was confirmed using the detergents, Tween 20 and Triton x 100 (see Table 4.7).

Table 4.6 *Substrate specificity for conversion of β-carotene to abscisic acid and related compounds in Citrus cell-free extracts.* Complete acetone powder incubates were supplied with [^{14}C]-9-*cis*-β-carotene, *all-trans*-β-carotene or *trans/cis*-β-carotene as substrates. Conditions of incubation and analysis were as described in "Materials and Methods". Data are expressed as [^{14}C] incorporated into ABA and related compounds. (Bq mg protein^{-1}). Results are mean results of 3 experiments.

	Distribution of radioactivity		
Substrate	1',4'-*trans*-ABAdiol	ABA (Bq mg protein^{-1})	PA
trans			
Mean ± SE	41.51 ± 1.20	69.99 ± 2.57	20.08± 1.40
cis*	ND	ND	ND
trans/cis			
Mean ± SE	102.84 ± 1.12	185.07 ± 1.80	45.0 ± 0.48

*Results from 3 experiments showed no incorporation

4.4.3 Effect of detergents

The majority of carotenogenic enzymes are membrane proteins and solubilization is essential for catalytic activity. Although acetone powders routinely yield soluble, enzymatically active preparations (Bramley, 1985) attempts were nevertheless made to increase ABA-biosynthesizing activity using detergents. The results in Table 4.7 show that both Tween 20 and Triton X100 increased catalytic activity at 0.1% w/v. However, at a concentration of 0.05 % w/v these detergents were much less effective.

Table 4.7 *Effect of detergents on ABA-biosynthesizing activity of acetone powder homogenates of Citrus flavedo.* Complete acetone powder incubates were supplied with synthetic *all-trans*-β-carotene (1 mg) and reactions carried out at 37°C for 2 h. ABA was extracted and quantified by GC-EC as described in Section 2.8.6.

Assay conditions	Activity ($\mu g\ mg\ protein^{-1}\ h^{-1}$)
Complete	1.005
+0.1% w/v Tween 20	4.152
+0.05% w/v Tween 20	2.464
+0.1% w/v Triton X100	5.326
+0.05% w/v Triton x100	2.332

4.5 Identification of ABA as a product of radioactive β-carotene metabolism

In order to confirm the identity of putative ABA as a product of *all-trans*-β-carotene metabolism labelled β-carotene (0.88 MBq, 15 mg, Sp. act. 31 MBq m mol^{-1}) was supplied as substrate. The diethyl ether soluble acids were initially purified on TLC and the zone co-migrating with authentic ABA was eluted. Analysis of this putative ABA by reversed-phase HPLC revealed a compound of 277 μg which was chromatographically identical to authentic ABA at Rt=7.5 min (Fig 4.9).

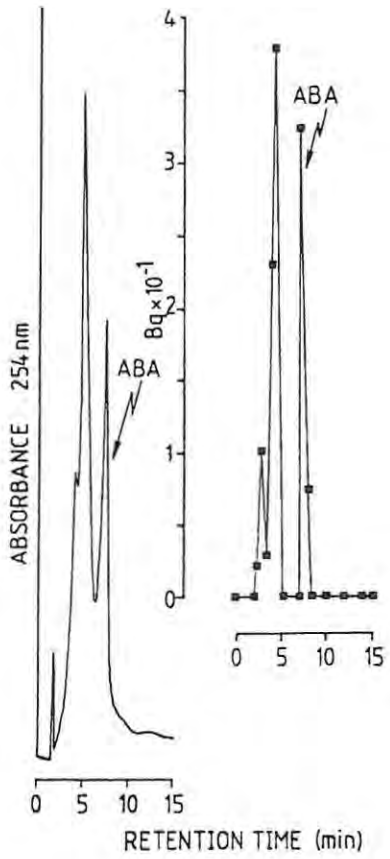


Figure 4.9 *Analysis by HPLC of the acidic products of incubation of [^{14}C]-all-*trans*-β-carotene with cell-free extracts from* Citrus sinensis *exocarp.* Chromatography was performed on a Bondaclone 10 μm C_{18} column (150 x 3.9 mm i.d.). The column was eluted isocratically with methanol/ water/ acetic acid (40: 60: 1, v/v) at 1 mL min $^{-1}$.

After purification by HPLC, the component tentatively identified as ABA was derivatized in ethereal diazomethane and further analysed by GC-MS and the electron impact mass spectrum is shown in Figure 4.10. This spectrum is almost identical to that of authentic ABA methyl ester (Gray *et al.*, 1974) with characteristic ions at 222, 191, 190 (base peak), 162, 134, 125, 112 and 91. Under the conditions described (Fig. 4.10) authentic ABA methyl ester displayed a molecular ion at m/z 278.95. Since mevalonate and β-carotene are uniformly labelled via randomly labelled acetate, radio labelled ABA produced from [^{14}C]-β-carotene should contain six atomic mass units extra (Milborrow 1975). Unfortunately no molecular ion at M+ 284.95 was present in the EI spectrum (Fig. 4.10). Furthermore none of the characteristic fragment ions of ABA appeared to carry [^{14}C] which may reflect the relative insensitivity of the analytical procedure used.

Alternatively the low level of incorporation (3.64 %) coupled with the low specific activity of the product (29 Bq μmol^{-1}) may have contributed to the observed result. Nevertheless, the characterization of ABA by combined capillary GC-MS as a major product in the cell-free system prepared from an acetone powder of *Citrus* flavedo, provides strong evidence for its origin from *all-trans*-β-carotene.

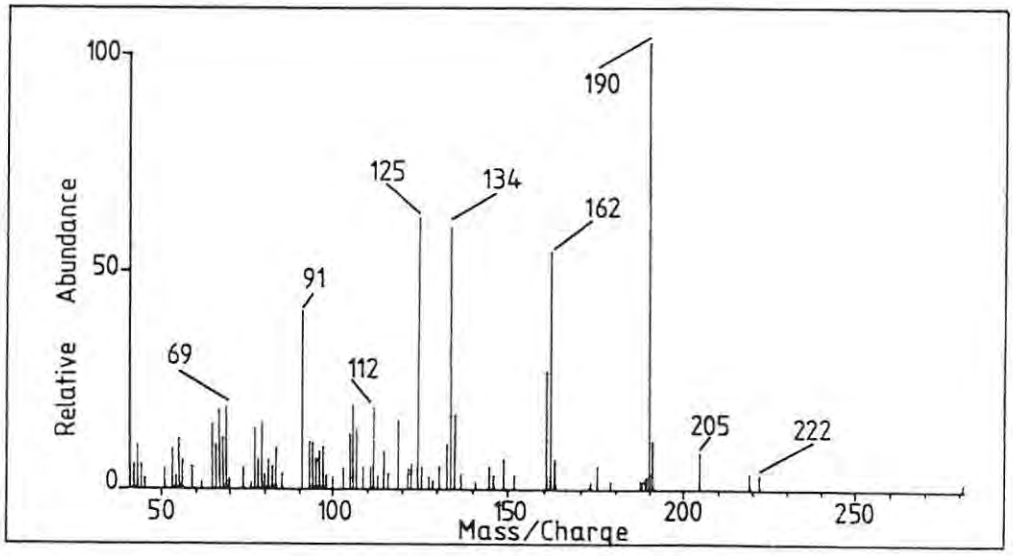


Figure 4.10 *Electron impact mass spectrum of the methyl ester derivative of ABA (277 μg; Sp. act. 29 Bq μmol^{-1}) prepared biosynthetically.* Mass spectra were recorded at 70 eV and a source temperature of 250 °C using a Hewlett-Packard 5988A mass spectrometer coupled to a Hewlett Packard 5890 gas chromatograph, fitted with a fused-silica capillary column (12m X 0.32 mm i.d.) of HP-1 programmed from 120 °C at 5 °C min^{-1} with He as carrier gas (1.5 - 2.0 mL min^{-1}).

4.6 Properties of the ABA biosynthesizing cell-free system

4.6.1 The influence of protein concentration, pH and kinetics on ABA biosynthesis

As expected, the effect of protein concentration and pH followed the same trends as were seen in crude extracts. The results are shown in Figure 4.11A and B. Activity increases linearly as protein concentration increases to a maximum of 2 mg per incubate. Maximal incorporation of label from radioactive MVA occurred at a pH of 7.4

which was not surprising as this is the pH which is favoured in the synthesis of terpenoids (Rogers *et al.*, 1967; Cowan and Railton, 1987a; Bramley, 1993; Cowan and Richardson 1993a; 1993b). Kinetics of the reaction indicated that formation of putative ABA and PA was sustained for 2 h, as in crude extracts, but declined rapidly after 2 h probably as a result of product catabolism (Fig 4.11C).

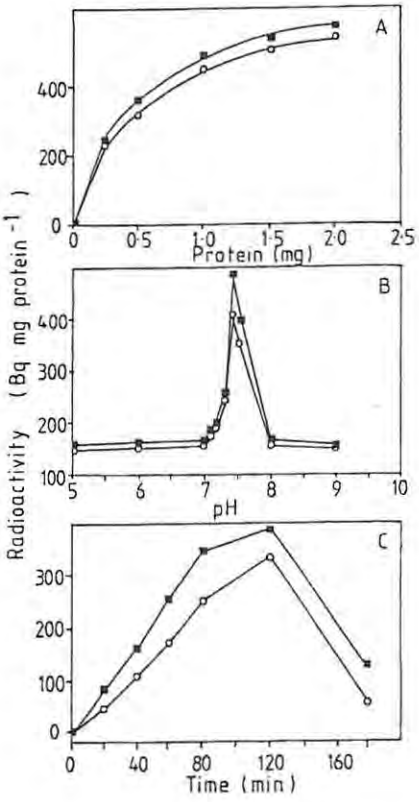


Figure 4.11 *Effect of protein concentration (A) and pH (B) and the reaction kinetics (C) of incorporation of label from 3R-[2-^{14}C]-MVA into ABA and related acids (1',4'-*trans*-ABAdiol + ABA + PA) in crude (o) and acetone powder (■) homogenates prepared from flavedo of* Citrus sinensis. Conditions of incubation and analysis were as described in Sections 2.4.4 and 2.6.3.

4.6.2 Cofactor requirements

Reduced nicotinamide nucleotides enhanced ABA-biosynthesizing activity of crude extracts (Table 4.8). Addition of molybdate further increased enzyme activity in crude cell-free preparations although the stimulating effect was less marked in the acetone powder homogenates. Several studies have implicated a molybdenum-requiring soluble

oxidase as the terminal enzyme in ABA biosynthesis (Walker-Simmons *et al.*, 1989; Sindu *et al*,. 1990). Likewise, the present work shows that molybdate stimulates ABA-biosynthesizing activity in cell-free extracts prepared from *Citrus* flavedo (Table 4.8). The functional regions in the amino acid sequence of molybdenum enzymes comprise both Mo-pterin and FAD binding domains (Wootton *et al.*, 1991). Since the addition of molybdate enhanced ABA biosynthesis from MVA and IPP (Table 4.8), it was important to determine the effect of FAD on enzyme activity.

Table 4.8 *Effect of reduced nicotinamide nucleotides and molybdate on ABA biosynthesis in crude cell-free preparations and acetone powder homogenates from* Citrus *flavedo.* Complete incubations contained glutathione NaF, ATP, $MgCl_2$, NAD+, AMO 1618 and cold ABA at the concentrations specified in Section 2.4.4. NADH (12.5 mol m^{-6}), NADPH (12.5 mol m^{-6}) and MoO_4^{-2}(1 mol m^{-6}), were included in the reaction mixture containing either 3R-[12-^{14}C]-MVA or [1-^{14}C]-IPP (50 kBq) as substrates. Conditions of incubation and analysis were as described in Sections 2.4.4 and 2.6.3. Data are expressed as [^{14}C] incorporated into ABA mg protein^{-1} h^{-1}.

Extract	Assay Conditions	Substrate	Activity (Bq mg protein^{-1} h^{-1})
Undialysed	Complete	MVA	55.60
Crude	Complete	IPP	204.59
	+NADH	MVA	90.24
	+NADPH	MVA	102.10
	+NADH + NADPH	MVA	123.65
	+ NADH + NADPH + MoO_4^{-2}	MVA	157.76
	+NADH = NADPH +MoO_4^{-2}	IPP	404.29
Acetone Powder	Complete	MVA	94.90
	Complete	IPP	249.58
	+NADH	MVA	104.97
	+NADPH	MVA	111.96
	+NADH + NADPH	MVA	173.88
	+NADH + NADPH + MoO_4^{-2}	MVA	201.95
	+NADH + NADPH + MoO_4^{-2}	IPP	449.38

Results in Table 4.9 show that the addition of FAD inhibited incorporation of label from both MVA and IPP into ABA. Furthermore, the inhibitory effect of FAD was concentration dependent. FAD is an essential co-factor in the desaturation of phytoene to lycopene and for cyclization of the latter during the formation of β-carotene (Bramley, 1985; Cunningham *et al.*, 1994). Thus it was important to determine whether reduced ABA levels occurred as a result of carotenoid accumulation.

Table 4.9 *Effect of FAD on ABA biosynthesis in acetone powder homogenates prepared from* Citrus *flavedo.* Complete acetone powder incubates contained glutathione, NaF, ATP, $MgCl_2$, AMO 1618, cold ABA (all at the concentrations specified in Section 2.4.4) and NAD^+, NADH, NADPH and MoO_4^{-2} as specified in Table 4.8 and either [2-^{14}C]-MVA (150 kBq) or [1-^{14}C]-IPP (150 kBq) as substrates. FAD was added at the concentrations indicated below. Reactions were for 2 h at 37° C. Data are expressed as [^{14}C] incorporated into ABA mg protein^{-1} h^{-1}.

Assay Conditions	Substrate	Activity (kBq mg protein^{-1} h^{-1})
Complete	MVA	0.16
+ 0.1 mol m^{-3} FAD	MVA	0.09
+ 1.0 mol m^{-3} FAD	MVA	0.02
Complete	IPP	10.70
+ 0.1 mol m^{-3} FAD	IPP	2.45
+ 1.0 mol m^{-3} FAD	IPP	0.08

Analysis of the β-carotene-containing fraction by reversed phase HPLC and liquid scintillation spectrometry revealed accumulation of radioactivity in a 450 nm absorbing compound with identical chromatographic properties to those of authentic β-carotene. Unfortunately, insufficient mass precluded unequivocal identification of this pigment as β-carotene. Nevertheless as shown in Table 4.10 incorporation of radioactivity into the β-carotene-like component was substantially enhanced in the presence of FAD. While this observation may account for the reduced incorporation of label into ABA in the

presence of FAD, it suggested more importantly that β-carotene might be a precusor en route to ABA in this cell-free system.

Table 4.10 *Effect of FAD on incorporation of radioactivity from MVA and IPP into β-carotene.* Assay conditions were as described for Table 4.9. The neutral diethyl ether fraction was analysed for β-carotene by reversed-phase HPLC using a 5 μm Nucleosil C_{18} column (250 x 4.0 mm i.d.) eluted isocratically with acetonitrile/methanol (9:1, v/v) at a flow rate of 0.8 mL min^{-1}. The eluate was monitored at both 287 and 450 nm and the distribution of radioactivity determined by liquid scintillation spectrometry.

Assay Conditions	Substrate	Radioactivity Incorporated (Bq)
Complete	[2-^{14}C]-MVA	19.0
+ 0.1 mol m^{-3} FAD	[2-^{14}C]-MVA	58.6
+ 1.0 mol m^{-3} FAD	[2-^{14}C]-MVA	56.0
Complete	[1-^{14}C]-IPP	1866.7
+ 0.1 mol m^{-3} FAD	[1-^{14}C]-IPP	4800.0
+ 1.0 mol m^{-3} FAD	[1-^{14}C]-IPP	3866.7

This series of investigations suggested that all further incubations should contain the following cofactors: glutathione, NaF, ATP, AMO 1618, NAD^+, NADH, NADPH, $MgCl_2$ and Molybdate; contain not more than 2 mg protein and be carried out at pH 7.4. In addition, protein extraction buffer should contain either Tween 20 or Triton X 100 at 0.1 % v/w and cold ABA must be added as a cold-pool trap.

CHAPTER 5

THE BIOCHEMISTRY OF ABA BIOSYNTHESIS *IN VITRO*

5.1 Introduction

The preceding chapters (Chapters 3 & 4) have illustrated that colour development in *Citrus*, a process involving loss of β-carotene with a concomitant rise in violaxanthin, occurs coincident with an increase in endogenous ABA and that cell-free extracts prepared from mature coloured exocarp produce ABA from labelled MVA, IPP and β-carotene. In addition, 1',4'-*trans*-ABAdiol was isolated and characterized as a major product of MVA metabolism in this cell-free system and was similarly formed when either IPP or β-carotene were used as substrate. Whether 1',4'-*trans*-ABAdiol was produced en route to ABA in this cell-free system, or formed as a catabolite of ABA was unknown at the time. Even so published data did suggest that 1',4'-*trans*-ABAdiol was a catabolite (Rock and Zeevaart, 1990) and that ABA was in fact synthesized from oxygenated carotenoids via xanthoxin and ABA-aldehyde (Parry 1993). In order to confirm this pathway of ABA biosynthesis in cell-free extracts of *Citrus* flavedo it was necessary to demonstrate carotenogenic activity as well as the formation of C_{15} neutral and acidic intermediates from both terpenyl pyrophosphates and C_{40} carotenoids.

Carotenoid biosynthesis in *in vitro* systems has been reviewed recently (Bramley, 1985) and there are good indications that membrane fractions from both chloroplasts and chromoplasts show high carotenogenic activity. For example, studies by Kleinig's group have shown that cell-free extracts of daffodil chromoplasts synthesize carotenoids (Beyer *et al.*, 1985; 1989; 1991; Kleinig and Beyer, 1985; Mayer *et al.*, 1990). Similarly Camara's group have demonstrated that cell-free preparations from *Capsicum* chromoplasts also synthesize carotenoids (Camara and Moneger 1982; Camara *et al.*, 1982). Furthermore, Milborrow's group have shown that carotenogenic cell-free systems are capable of hydroxylating carotenoids (Milborrow 1982) and synthesizing xanthophylls (Swift *et al.*, 1982), in particular neoxanthin. Thus there is adequate evidence to suggest that both carotenes and xanthophylls can be produced in *in vitro* systems. However, the production of ABA in these systems has not apparently been explored. The ability of the *Citrus* cell-free system (developed in Chapter 4) to produce

ABA from MVA, IPP and β-carotene provided an ideal opportunity therefore, to investigate in detail the biochemistry of ABA biosynthesis *in vitro* in order to elucidate the post-FPP intermediates in this reaction sequence.

5.2 Results.

5.2.1 Carotenogenic activity of the ABA-biosynthesizing cell-free system

It was previously demonstrated (Chapter 4, section 4.6.2) that addition of FAD reduced incorporation of label from both MVA and IPP into ABA and that this reduction in ABA-biosynthesizing activity occurred concomitant with an increase in a radioactive neutral compound with similar chromatographic properties to β-carotene (Fig. 5.1).

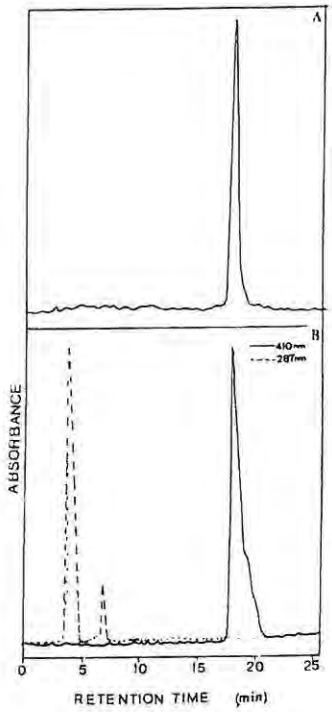


Figure 5.1 *HPLC chromatogram of the major product of [^{14}C]-IPP metabolism in extracts of* Citrus sinensis *flavedo.* Carotenoids were separated on a 5 μm C_{18} Nucleosil column (250 x 4.6 mm i.d.) using a linear gradient of 0-100 % ethyl acetate in acetonitrate/ water (9: 1, v/v) containing 0.1 % trimethylamine at 0.8 mL min^{-1} over 50 minutes. **(A)** standard [^{14}C]-*all-trans*-β-carotene and **(B)** major metabolites of [^{14}C]-IPP metabolism. (a= phytoene; b= unidentified; c= β-carotene).

This observation strongly suggested that acetone powder homogenates of *Citrus* flavedo contained carotenogenic activity. In order to investigate this possibility further, both FPP and GPP were used as substrates and the carotenoid fraction analysed by reversed-phase HPLC. As shown in Figure 5.2 both FPP and GGPP were converted into a range of neutral pigments with similar chromatographic properties to those of the carotenoids derived from light-grown leaves of *Petunia*. This cell-free system also transformed all-*trans*-β-carotene into oxygenated carotenoids (Fig 5.3). The relationship between substrate and concentration of product is shown in Table 5.1.

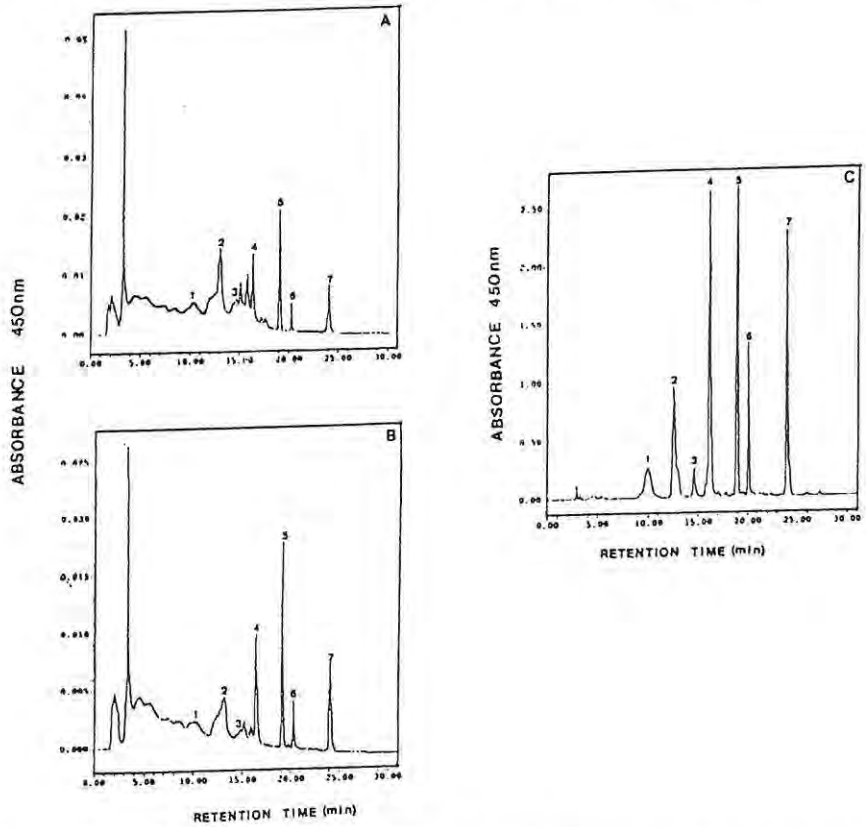


Figure 5.2 ***(A)*** *Representative HPLC chromatogram of the products of FPP metabolism in extracts of Citrus sinensis flavedo.* Carotenoids were separated on a 5 μm C_{18} Nucleosil column (250 x 4.6 mm i.d.) using a linear gradient of 0 - 100 % ethyl acetate in acetonitrile/water (9:1, v/v) containing 0.1 % triethylamine at 0.8 mL^{-1} over 25 min. Peaks (1) Neoxanthin; (2) Violaxanthin; (3) Antheraxanthin; (4) Zeaxanthin; (5) β-cryptoxantin; (6) 5/6-β-carotene epoxide; (7) β-carotene. ***(B)*** *HPLC chromatogram of the products of GGPP metabolism in extracts of Citrus sinensis flavedo.* Conditions and Peaks were as described in (A). Peaks were identified by cochromatography with authentic pigments from leaves of *Petunia* (See Fig. 5.2 C)

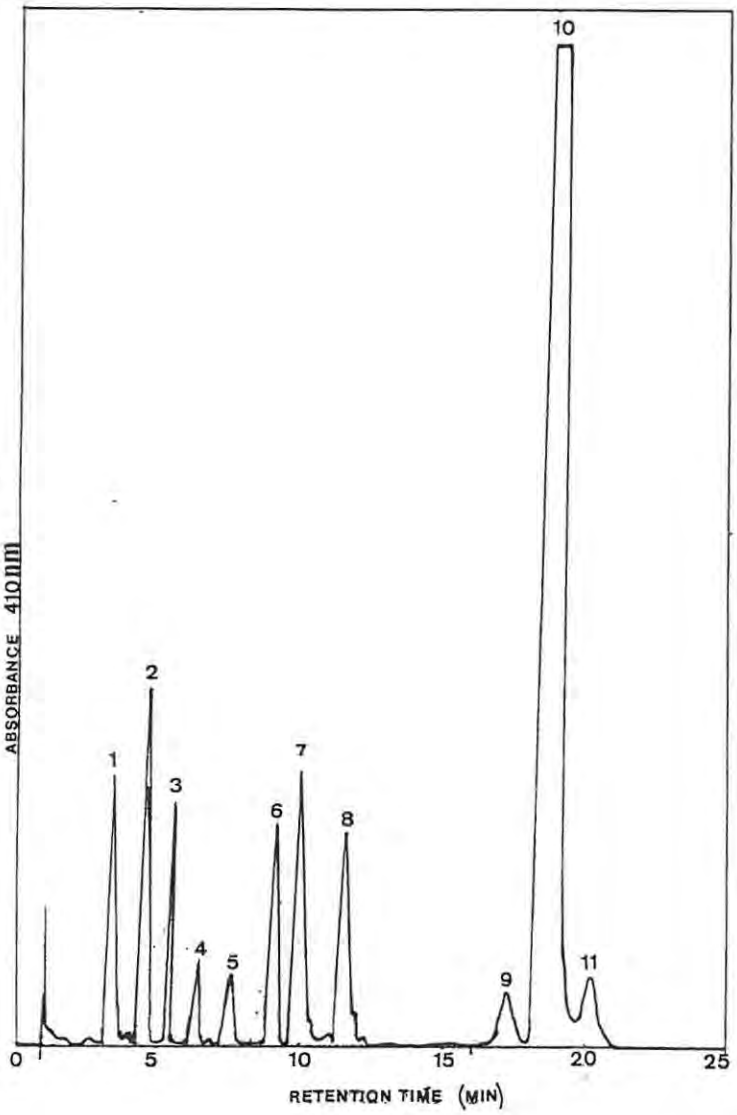


Figure 5.3 *HPLC chromatogram of the products of cold all-*trans*-β-carotene metabolism in extracts of* Citrus sinensis *flavedo.* Carotenoids were separated on a 5 μm C_{18} Nucleosil column (250 x 4.6 mm i.d.) isocratically with methanol/acetonitrile (9:1, v/v) as mobile phase at 0.8 mL min^{-1} over 25 min. Peaks: 1) 9-*cis*-neoxanthin; 2); neoxanthin; 3) violaxanthin; 4) antheraxanthin; 5) zeaxanthin; 6) β-cryptoxanthin; 7) unidentified; 8) β-zea-carotene; 9) α-carotene; 10) *all-trans*- β-carotene; (11) 9-*cis*-β-carotene.

Table 5.1 *Concentration of oxy-carotenoids produced by the cell-free system of* Citrus sinensis *flavedo when FPP, GGPP and β-carotene were used as substrates.* Incubation conditions were as described in Section 2.4.4.

Peak Number	Pigment	Substrate		
		FPP	GGPP	β-carotene
		Concentration (mg mL^{-1})		
1	Neoxanthin	0.142	0.283	0.366
2	Violaxanthin	0.697	0.963	0.675
3	Antheraxanthin	0.142	0.401	0.113
4	Zeaxanthin	0.652	1.388	1.558
5	β-cryptoxanthin	1.133	2.805	2.833
6	β-citraurin	0.283	0.680	0.708
7	β-carotene	0.425	1.218	1.238

Analysis of the pH 8.5 fraction by reversed-phase HPLC revealed that cold-*all-trans*-ß-carotene had been metabolized into several oxygenated derivatives including zeaxanthin, antheraxanthin and violaxanthin (Figure 5.3). Identical results were obtained when [^{14}C]-*all-trans*-ß-carotene was used as substrate. The identity of the carotenoids produced from [^{14}C]-*all-trans*-ß-carotene in the cell-free system from *Citrus sinensis* flavedo, was established by determining their absorption maxima in both hexane and benzene and by cochromatography. Briefly, the presence of 5,6-epoxide groups was confirmed by monitoring the spectral shift following the addition of 1.0-M HCl to ethanolic solutions of each purified carotenoid. The isomerization state was established by iodine-catalysed photomerization. HPLC-PDA analysis of the oxycarotenoid fraction revealed accumulation of a metabolite which was identified as 9-*cis*-violaxanthin (Fig. 5.4). In addition, where possible, UV-Vis spectra were recorded as described in Section 2.8.3 in order to confirm the identity of the respective oxy-carotenoids. The absorbance maxima are shown in Table 5.2.

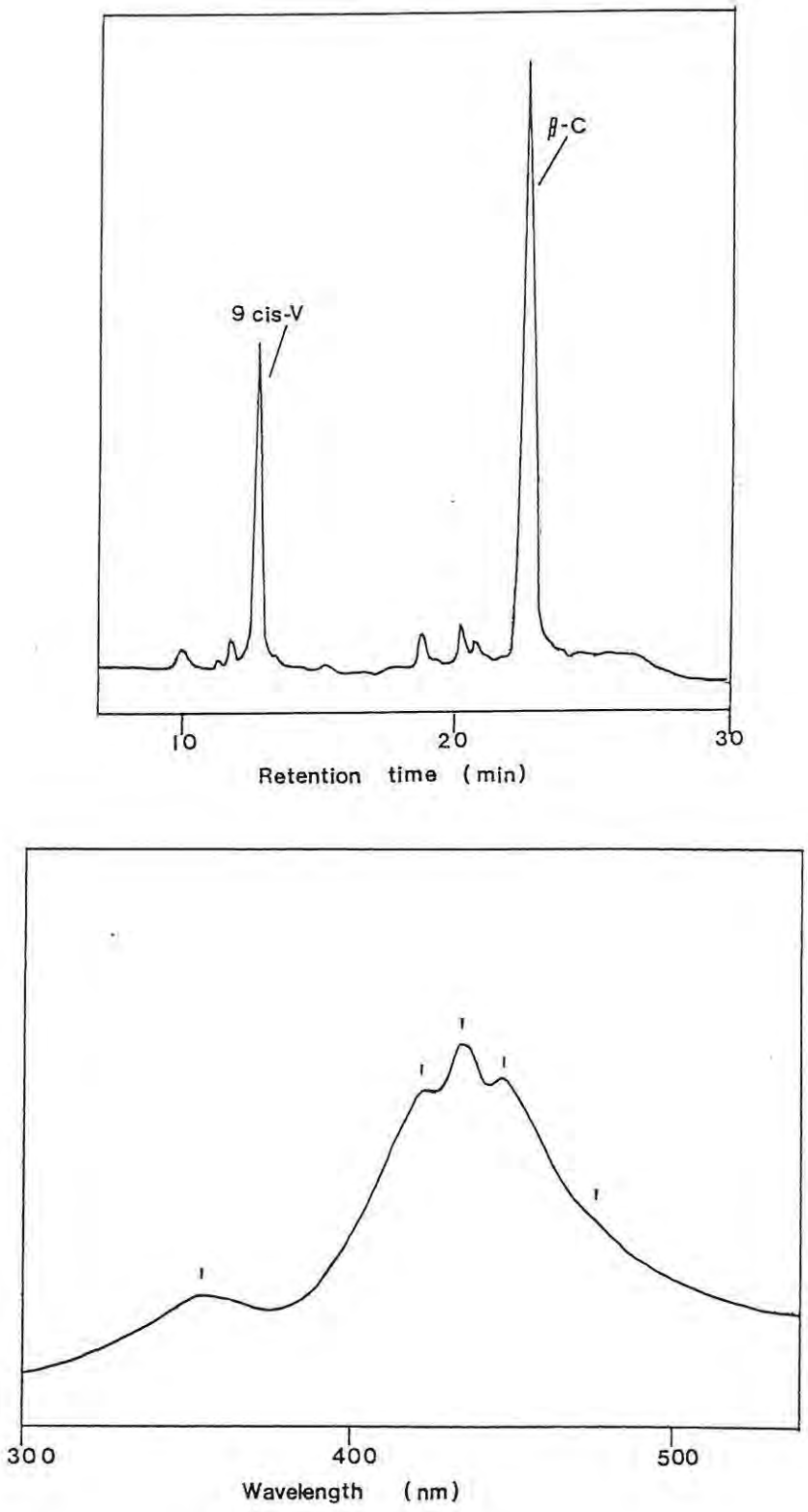


Figure 5.4 ***(A)*** *HPLC chromatogram showing accumulation of 9-cis-violaxanthin from cell-free acetone powder homogenates when [^{14}C]-β-carotene was used as substrate* ***(B)*** *Absorption spectrum of a carotenoid co-eluting with violaxantin on HPLC and which was detected by PDA.* The presence of a *cis* peak at 340 nm suggests that this is in all probability 9' -*cis*-violaxanthin.

Table 5.2 *Specrophotometric analysis of the oxy-carotenoids produced in the cell-free system of* Citrus sinensis *flavedo.* Absorbance spectra were recorded in ethanol.

Oxy-Carotenoid	λ_{max}	Published
all-*trans*-violaxanthin	470	471
Zeaxanthin	476	478
Neoxanthin	466	467
β-carotene	477	476

5.2.2 Conversion of FPP, GGPP and β-carotene to C-15 neutral compounds

Two UV-absorbing neutral compounds were produced when FPP, GGPP and β-carotene were used as substrates in the cell-free system. These compounds were detected following TLC analysis of the diethyl ether fraction obtained by partitioning the aqueous phase at pH 8.5 and the distribution of radioactivity between these products from cell-free incubates supplied with [^{14}C]-β-carotene, is shown in Table 5.3

Table 5.3 *Distribution of radioactivity in Xan and Xan alc when β-carotene was used as substrate in acetone powder homogenates of* Citrus sinensis *flavedo.* Complete acetone powder incubates were prepared as described in Section 2.4.4. Reactions were carried out at 37 °C for 2 h with [^{14}C]-*all-trans*-ß-carotene (10 kBq) as substrate. Incorporation of label into xanthoxin and xanthoxin alcohol was determined after separation of the neutral fraction first by TLC and then reversed-phase HPLC analysis of the individual components. Data are representative of three independent experiments ±SE.

	Distribution of radioactivity (Bq mg protein^{-1})	
	Xan-alc	Xan
Mean ±SE	12.93 ±1.19	21.44 ±0.81

These products were tentatively identified as xanthoxin alcohol (Xan-alc) and Xan by co-chromatography, following feeds of non-labelled β-carotene, and by UV absorbance in methanol with maxima at 240 and 281 nm respectively (Table 5.4 and Fig. 5.5, Taylor

and Burden, 1972). Quantification of these products was done by reversed-phase HPLC and the concentration of Xan and Xan alc produced is shown in Table 5.5

Table 5.4 *Spectophotometric analysis of Xan and related compounds.* Complete acetone powder homogenates were supplied with cold *all-trans*-β-carotene and reactions were carried out at 37 °C for 2 h and Xan and related compounds extracted as described in Section 2.6.2.

Metabolite	Max	Published
Xan-alcohol	240	239
t-Xan-alcohol	236	237
Xanthoxin	281	281.5
t-Xanthoxin	ND*	283.5
ABA-aldehyde	ND*	283

*Not detected.

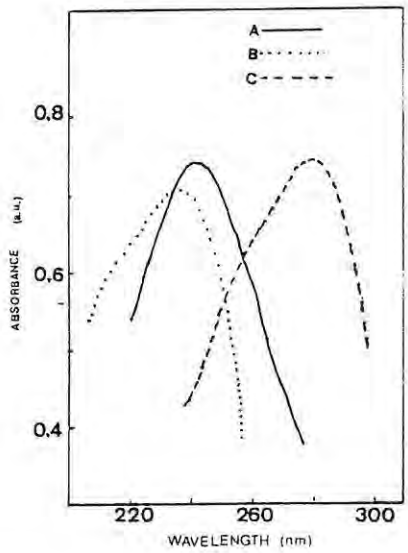


Figure 5.5 *UV absorbance spectra (recorded in methanol) of* ***(A)*** *Xanthoxin alcohol;* ***(B)*** *trans-Xanthoxin alcohol; C) Xanthoxin*

Unequivocal identification of putative Xan and Xan alc was achieved by GS-MS analysis of the neutral pH 8.5 fraction, containing Xan and Xan alc synthesized from *Citrus sinensis* flavedo. The electron impact mass spectrum of putative Xan alc (Fig. 5.6A) gave a molecular ion at m/z = 252 + 6 amu due to [^{14}C], while the one generated by putative Xan (Fig. 5.6B) gave a molecular ion at m\z = 250 + 6 amu due to [^{14}C]. the mass spectrum of Xan was compared with mass spectra of authentic Xan (Parry 1989).

Table 5.5 *Conversion of FPP, GGPP and β-carotene to Xan and Xan alc.*

Complete acetone powder homogenates were supplied with FPP, GGPP or β-carotene and reactions were carried out at 37 ^{0}C for 2 h and Xan and Xan alc extracted as described in Section 2.6.2. Data are representative of three independent experiments ±SE.

Substrate	Xan	Xan Alc
	Concentration (ng mg protein^{-1})	
FPP		
Mean ± S.E	0.25 ±0.06	4.18 ±1.52
GGPP		
Mean ± S.E	0.52 ±0.24	6.69 ±0.29
β-carotene		
Mean ± S.E	0.63 ±0.17	8.05 ±0.26

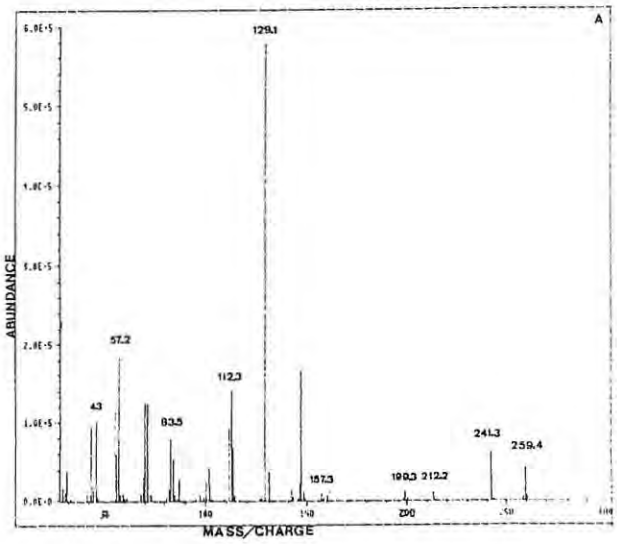


Figure 5.6 (A) *Electron impact mass spectrum of putative Xan alc synthesised in a cell-free system from* Citrus sinensis *flavedo supplied with [^{14}C]-all-*trans*-β-carotene (0.88 MBq, Sp. act. 31 MBq m mol^{-1}).* Conditions of incubations were as described in Section 2.4.4. The mass spectrum was obtained using a Hewlett-Packard 5890 gas chromatograph fitted with a fused silica capillary column (50 m x 0.2 mm i.d.) of HP Ultra 1 programmed from 50-260 °C at 5 °C min^{-1} with He as carrier gas (0.5 ml min^{-1}).

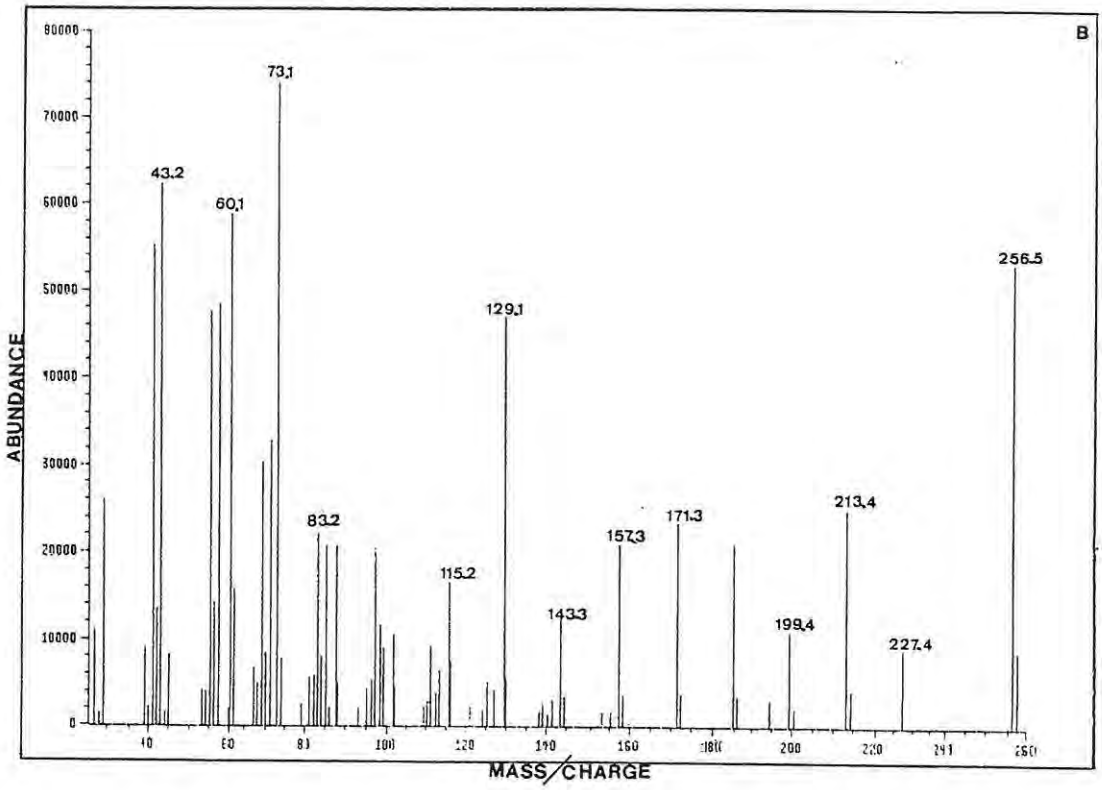


Figure 5.6 (B) *Electron impact mass spectrum of putative Xan synthesised in a cell-free system from* Citrus sinensis *flavedo supplied with [^{14}C]-all-*trans*-β-carotene (0.88 MBq, Sp. act. 31 MBq m mol^{-1}).* Conditions of incubations were as described in Section 2.4.4. The mass spectrum was obtained using a Hewlett-Packard 5890 gas chromatograph fitted with a fused silica capillary column (50 m x 0.2 mm i.d.) of HP Ultra 1 programmed from 50-260 °C at 5 °C min^{-1} with He as carrier gas (0.5 ml min^{-1}).

5.2.3 Conversion of FPP, GGPP and β-carotene to acid compounds

When either FPP, GGPP or β-carotene was used as substrate a series of acid compounds identified as PA, 1',4'-*trans*-ABAdiol, ABA and an unknown acid, compound X, were produced. Compound X was tentatively identified as xanthoxin acid (Xan acid) acid by UV-absorbance in methanol with maximum absorbance at 270 (Table 5.6 and Fig. 5.7, Taylor and Burden 1972) and later unequivocally identified by GC-MS and the electron mass spectrum is shown in Figure 5.8. This electron impact mass spectrum

gave a molecular ion at m/z = 280 + 6 amu due to [^{14}C]. Comparison of this mass spectrum with that of authentic Xan acid methyl0 ester (Parry 1989) confirmed the identity of compound X as Xan acid. 1',4'-*trans*-ABAdiol, ABA and PA were unequivocally identified by GC-MS (Chapter 4, Section 4.3.3). Incorporation into PA, ABA, 1',4'-*trans*-ABAdiol and Xan acid increased substantially when β-carotene was used as substrate (Table 5.7).

Table 5.6 *Spectrophotometric analysis of Xanthoxin acid.* Complete acetone powder homogenates were supplied with cold *all-trans*-β-carotene and reactions were carried out at 37 °C for 2 h and Xan acid extracted as described in Section 2.6.3.

Metabolite	Max	Published
Xan-acid	270	266.5
t-Xan-acid	ND	264.5

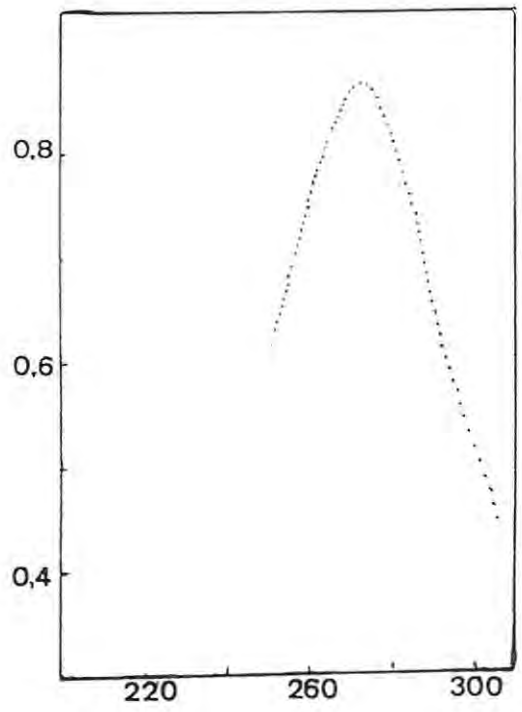


Figure 5.7 *UV Absorbance spectrum (recorded in methanol) of Xan acid with maximum absorbance at 270 nm.*

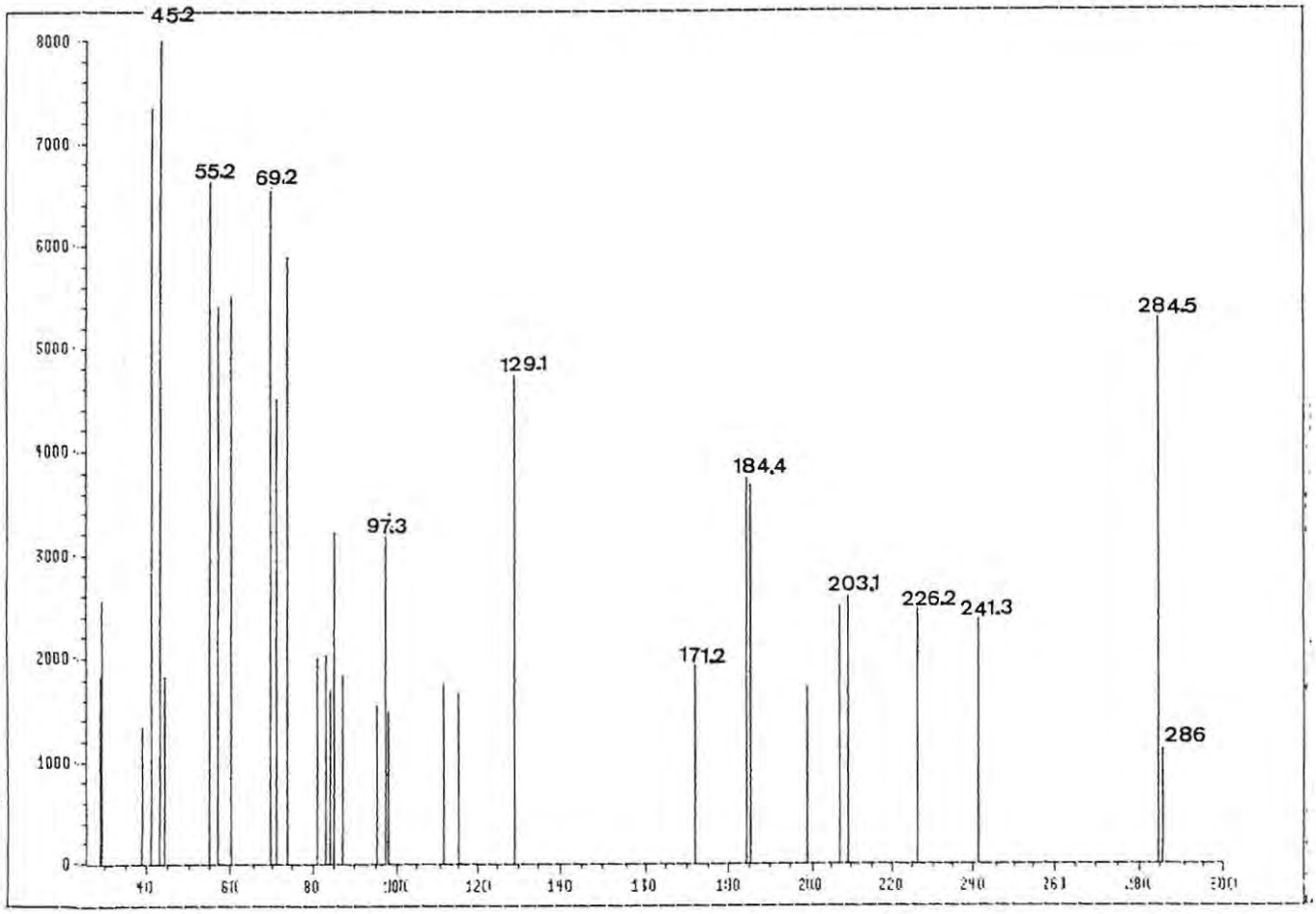


Figure 5.8 *Electron impact mass spectrum of the methyl ester of Xan acid synthesised in a cell-free system from* Citrus sinensis *flavedo supplied with [^{14}C]-β-carotene (0.88 MBq Sp. act. 31 MBq m mol^{-1})*

Table 5.7 *Conversion of FPP, GGPP and β-carotene to acid compounds in the cell-free system of* Citrus sinensis *flavedo.* Complete acetone powder incubates were prepared as described in Section 2.4.4 and reactions were carried out for 2 h with FPP, GGPP or β-carotene as substrate. Incorporation of substrate into 1',4'-*trans*-ABAdiol, ABA, Xan acid and PA were determined after separation of the diethyl ether-soluble acids first by TLC and then reversed-phase HPLC analysis of the individual acids. Data represent the mean of three independent experiments ±SE.

Substrate	Xan Acid	ABAdiol	ABA	PA
		Concentration (ng mg protein^{-1})		
FPP				
Mean ±SE	1.17±0.05	20.14±1.53	20.62±1.73	1.04 0.05
GGPP				
Mean	1.36 ±0.24	31.46±1.74	32.48±0.78	1.03±0.04
β-carotene				
Mean	1.86 ±0.10	44.95 ±2.05	100.90±1.79	1.790.08

5.2.4 1',4'-*trans*-ABAdiol: precursor or product?

In attempting to define the origin of 1',4'-*trans*-ABAdiol, the metabolism of both [2-^{14}C]-ABA and [2-^{14}C]-1',4'-*trans*-ABAdiol was investigated and the results are shown in Table 5.8. When [^{14}C]-ABAdiol was used as substrate it was readily converted to ABA in extracts of acetone powder homogenates, a process that was enhanced by AMO 1618. Results show that approximately 78 % of the applied label was incorporated into ABA during the 2 h incubation procedure. The balance of radioactivity was distributed between substrate 1',4'-*trans*-ABAdiol and PA. Omission of non-labelled ABA decreased the yield of product [2-^{14}C]-ABA. In contrast when 2-[^{14}C]-ABA was used as substrate very little conversion to 1',4'-*trans*-ABAdiol was detected. Furthermore omission of either AMO 1618 or unlabelled ABA appeared to have no significant effect on this reaction. These results suggest that 1',4'-*trans*-ABAdiol is formed as an intermediate en route to ABA in extracts prepared from acetone powder homogenates.

Table 5.8 *Metabolism of [^{14}C]-1',4'-*trans*-ABAdiol to 2-[^{14}C]-ABA in acetone powder homogenates of* Citrus *flavedo*. Complete acetone powder incubates were supplied with either (R,S)-2-[^{14}C]-ABA (333 Bq) or [^{14}C]-1',4'-*trans*-ABAdiol as substrate and reactions allowed to proceed at 37 °C for 2 h. The acids were partitioned into diethyl ether at pH 2.5 and the distribution of radioactivity determined by liquid scintillation spectrometry following separation on thin layers of silica gel GF_{254} in toluene/ethyl acetate/acetic acid (25:15:2, v/v).

Assay Conditions	Substrate	Radioactivity in product [kBq mg protein^{-1} (%)]	
		1',4'-*trans*-ABAdiol	ABA
Complete	ABA	0.06(0.3)	-
	ABAdiol	-	15.68(78.4)
-cold ABA	ABA	0.05(0.2)	-
	ABAdiol	-	7.26(36.3)
-AMO 1618	ABA	0.05(0.2)	-
	ABAdiol	-	2.84(14.4)
-cold ABA,-AMO 1618	ABA	0.05(0.2)	-
	ABAdiol	-	1.11(5.5)

The conversion of 1',4'-*trans*-ABAdiol to ABA involves oxidation whereas the reverse reaction requires reduction of the 4' keto function. Since biosynthesis of ABA is apparently dependent on molybdenum as a co-factor (Walker-Simmons *et al.*, 1989) and conversion of 1',4'-*trans*-ABAdiol to ABA would be expected to involve an oxidase enzyme, the co-factor requirements of this conversion were examined. The results shown in Table 5.9 indicate that conversion of 1',4'-*trans*-ABAdiol to ABA is stimulated by molybdate. Omission of NADPH had little or no effect on enzyme activity. Likewise addition of FAD, a prosthetic group of some molybdenum-requiring enzymes, did not appear to influence enzyme activity appreciably.

Table 5.9 *Cofactor requirements for conversion of 1',4'-*trans*-ABAdiol to ABA in acetone powder homogenates of* Citrus *flavedo.* Complete acetone powder homogenates were supplied with [^{14}C]-1',4'-*trans*-ABAdiol (10 kBq) and reactions carried out at 37 °C for 2 h and [^{14}C]-ABA extracted and analysed as described in Table 5.8.

Assay Conditions	Activity (kBq mg protein-1 h^{-1})	% Change[a]
Complete	3.58	0.00
-MoO_4^{-2}	1.51	-57.82
-MoO_4^{-2},-NADPH	1.54	-56.98
+FAD[b]	3.59	0.28
+FAD, -MoO_4^{-2}	0.78	-78.21

[a] % change relative to complete assay conditions

[b] FAD included at 1 mol m^{-3}

5.2.5 Chemical modification of ABA biosynthesis *in vitro*

5.2.5.1 Effect of sterols and gibberellic acid on ABA biosynthesis.

The ability of the kaurene synthetase and squalene oxide cyclase inhibitor, AMO 1618, to enhance incorporation of radioactivity from either MVA or IPP into terpenyl pyrophosphates and ABA, both *in vivo* and *in vitro* (Cowan and Railton, 1987a; Davies *et al.*, 1975; Milborrow 1976), coupled with the apparent requirement for this inhibitor during conversion of 1',4'-*trans*-ABAdiol to ABA suggested the possibility that either gibberellins or sterols, or intermediates en route to these compounds, interfere with ABA biosynthesis. In order to investigate this possibility the effect of stigmasterol, cholesterol and gibberellic acid (GA_3) on the conversion of *all-trans*-β-carotene and 1',4'-*trans*-ABAdiol to ABA was examined. The results in Table 5.10 show that stigmasterol dramatically reduced incorporation of label from β-carotene into ABA. Furthermore, inhibition by stigmasterol was apparently concentration dependent. Cholesterol, a minor component of thylakoid membranes (Hartmann and Benveniste, 1987), was a much less effective inhibitor. GA_3 had no effect on conversion of β-carotene to ABA. By

comparison, none of the compounds tested influenced conversion of 1',4'-*trans*-ABAdiol to ABA.

Table 5.10 *Effect of sterols and gibberellic acid on conversion of β-carotene and 1',4'-trans-ABAdiol to ABA in acetone powder homogenates of* Citrus *flavedo.* Complete acetone powder homogenates were supplied with either [^{14}C]-β-carotene or [^{14}C]-1',4'-*trans*-ABAdiol in the presence or absence of stigmasterol, cholesterol and GA_3. Reactions were carried out at 37 °C for 2 h and [^{14}C]-ABA extracted and analysed as described in "Materials and Methods".

Treatment	% inhibition relative to control	
	[^{14}C]-β-carotene	[^{14}C]-1',4'-*trans*-ABAdiol
Control	0.00	0.00
+10 mol m^{-6} stigmasterol	-	0.00
+50 mol m^{-6} stigmasterol	76.35	0.00
+100 mol m^{-6}stigmasterol	4.22	0.00
+100 mol m^{-6} cholesterol	8.86	0.00
+100 mol m^{-6} GA_3	0.00	0.00

5.2.5.2 Effect of ancymidol and zeatin on ABA biosynthesis.

Several reports have indicated that cytokinins and the cytokinin analogue, ancymidol, inhibit ABA biosynthesis both *in vivo* and *in vitro* (Cowan and Railton, 1987a; 1987b; Norman *et al.*, 1983a; 1983b). Likewise, in the present study both ancymidol and zeatin reduced incorporation of label from β-carotene into ABA (Table 5.11).

Table 5.11 *Effect of ancymidol and zeatin on ABA biosynthesis in cell-free preparations from* Citrus *flavedo acetone powder homogenates.* Complete acetone powder homogenates were supplied with [^{14}C]-*all-trans*-β-carotene in the presence or absence of ancymidol and zeatin. Reactions were carried out at 37 ^{0}C for 2 h and [^{14}C]-ABA and related acids extracted and analysed as described in "Materials and Methods". Xanthoxin alcohol and xanthoxin were detected following TLC analysis of the neutral fraction, obtained by partitioning the aqueous phase against diethyl ether at pH 8.5 on silica gel GF_{254} in petroleum ether/acetone 3:1 (v/v) developed three times to 15 cm.

	Distribution of Radioactivity				
Treatment	Xan-alc	Xan	1',4'-*trans*-ABAdiol	ABA	PA
			(Bq mg protein $^{-1}$)		
Control	17.41	27.92	34.01	123.80	16.40
+0.1 mol m^{-3}ancymidol	45.86	68.17	10.60	34.53	12.46
+0.5 mol m^{-3}ancymidol	32.27	82.23	11.79	35.47	6.18
+0.1 mol m^{-3}zeatin	24.43	59.75	22.48	61.99	15.39

The formation of PA was not substantially influenced by the lower concentrations of both ancymidol and zeatin but was markedly inhibited by ancymidol at 0.5 mol m^{-3}. Interestingly, reduced incorporation of label into ABA, PA and 1',4'-*trans*-ABAdiol in extracts supplied with ancymidol and zeatin, occurred concomitant with increased incorporation of label into the two neutral compounds, Xan and Xan alc that were detected following TLC analysis of the diethyl ether fraction obtained by partitioning the aqueous phase at pH 8.5. These products were identified as Xan and Xan alc by GC-MS (Section 5.2.2).

5.2.5.3 The effect of Dithiothreitol (DTT), an inducer of cyt P-450 on ABA biosynthesis

As it had previously been shown that inhibitors of cyt P-450 generally inhibited ABA biosynthesis, (Norman *et al.*, 1988), it was of interest to test if inducers of cyt P-450 would enhance ABA biosynthesis in cell-free extracts from *Citrus* flavedo. Analysis of the pH 2.5 fraction by reversed-phase HPLC revealed that the addition of DTT to the

cell-free system, resulted in increased incorporation of [^{14}C]-β-carotene into ABA. Quantification of the ABA was done by peak integration with authentic ABA as external standard (Table 5.12).

Table 5.12 *The effect of Dithiothreitol (DTT) on the incorporation of label from β-carotene into ABA in cell-free systems from* Citrus *flavedo acetone powder extracts, analysed and quantified on HPLC using the solvent system methanol: water: acetic acid (40:60:1, v/v)*

Assay	ABA ($\mu g\ mg\ protein^{-1}\ h^{-1}$)
- DTT	0.8
+ DTT	1.5

These results are in agreement with those of Norman *et al.*, 1988, which showed that DTT, a known enhancer of cyt P-450 activity increased incorporation of label into ABA by 31 %. The same result was obtained in the fungus *Cercospora rosicola* (Norman *et al.*, 1983a; 1983b; 1988)

CHAPTER 6

ASPECTS OF THE ENZYMOLOGY OF ABSCISIC ACID BIOSYNTHESIS *IN VITRO*.

6.1 Introduction

ABA biosynthesis is accelerated in response to water stress and the stimulus for this appears to be loss of cell turgor (Comish and Zeevaart, 1985a; 1985b; Parry, 1993; Pierce and Raschke, 1980,1981). As yet nothing is known about how loss of turgor stimulates ABA biosynthesis. However the accumulation of ABA can be eliminated by inhibitors of nuclear transcription like cordycepin and actinomycin D as well as by inhibitors of protein translation eg. cycloheximide (Guerrero and Mullet, 1986; Parry, 1993; Stewart *et al.*, 1986). Li and Walton (1990) showed that when turgid leaves were treated with cycloheximide, there was a 50 % reduction in ABA levels within 36 h, which was not the result of increased catabolism. On the other hand Sindhu et al., (1990) showed that the amount of 'Xan oxidising' activity isolated from plant tissues was unaffected by pre-treatment with cycloheximide. It therefore appears that ABA biosynthesis is regulated by the activity of an inducible enzyme, probably a dioxygenase which acts specifically to cleave 9'-*cis*-neoxanthin and has a high turnover rate (Parry, 1993).

Changes in gene expression as a result of water stress are now much researched and many such changes have been observed (Bray, 1991a; Chandler & Robertson, 1994; Quarrie, 1990; Skriver and Mundy, 1990). These changes may be directly due to water stress, such as those leading to increased levels of ABA, or they may be secondary, such as those observed in response to increased ABA levels (Ho, 1983; Skriver and Mundy, 1990).

Quarrie (1990) used a rice genotype with a high capacity for water-stress-induced ABA synthesis in an attempt to identify the proteins responsible and which would include the cleavage enzyme. He showed that ABA accumulation began about 30 min after the imposition of stress. Thus only proteins which had been synthesised prior to this were of interest. Preliminary results indicated that a 38.8 kD protein accumulated following stress. In addition, it was shown that this protein was not induced by ABA.

Several lines of evidence point to ABA being derived from 9'-*cis*-neoxanthin or 9'-*cis*-violaxanthin with xanthoxin as an intermediate (Duckham *et al.*, 1991; Parry and Horgan, 1992; Rock and Zeevaart, 1991). The enzymology of the reaction however, is poorly understood. ^{18}O-labelling experiments show incorporation primarily into the side chain carboxyl group of ABA (Zeevaart *et al.*, 1989). This suggests that oxidative cleavage occurs at the 11,12 (11',12') double bond of violaxanthin or neoxanthin. Creelman *et al* (1992) showed that carbon monoxide, a strong inhibitor of *haem*-containing P-450 monooxygenases, did not inhibit ABA accumulation but inhibitors of lipoxygenase significantly inhibited ABA accumulation in response to stress. They concluded that the oxygenase-catalysing carotenoid cleavage was most probably a non-haem oxygenase with lipoxygenase-like properties. Using a cell-free system derived from imbibed barley embryos Cowan and Railton (1987a) were able to demonstrate that incorporation of label from MVA into ABA was inhibited by both anaerobiosis and CO suggesting the participation of mixed function oxidases.

Many catalytic enzymes require phosphorylation for activation (Hanks, *et al.*, 1988; Hunter, 1987 cited in Harper et al., 1993). Although several genes predicted to encode protein kinases have been cloned, only one protein kinase has been biochemically characterised and purified to homogeneity from extracts of higher plants (Harmon, *et al.*, 1987 cited in Harper *et al.*, 1993). This enzyme, calcium-dependent protein kinase (CDPK) is present in significant levels in root and shoot tissues. There is biochemical and immunocytochemical evidence that CDPKs are associated with the plasma membrane (Schaller *et al.*, 1992) and cytoskeletal system (Putnam-Evans *et al.*, 1989). Although the biological functions of CDPKs are unknown, they may include a broad spectrum of phenomena which implicate free calcium as a second messenger, such as cytoplasmic streaming and stress adaptation (Harper *et al.*, 1993).

Current thinking is that cytochrome P-450 may also undergo phosphorylation in order to assume activity although this has only been demonstrated for animal proteins. In higher plants protein phosphorylation has been reported for nuclear proteins (Arfmann and Willmitzer, 1982; Erdmann, 1982; Sasaki and Sugita, 1982; Trewavas, 1979) and for ribosomal proteins (Gowda and Pillay, 1980; Keates and Trewavas, 1974). Two plant enzymes whose activities are controlled by phosphorylation and dephosphorylation have been reported viz. pyruvate dehydroganase (Rao and Randall, 1980) and quinate:

NAD^+ oxidoreductase (Refeno *et al.*, 1982). Several reports have been published indicating the regulatory effect of Ca^{2+} on plant protein phosphorylation (Harper *et al.*, 1993; Hetherington and Trewavas, 1982; Salimath and Marme, 1983;). An interesting aspect which has been uncovered in the present work is that in the presence of an ABA cold-pool trap incorporation of radioactivity into product ABA was stimulated (Chap. 4), whereas in the absence of non-labelled ABA activity was markedly reduced. Since plant hormones contribute to signal response coupling by protein phosphorylation it was considered that the presence of non-labelled ABA may alter the phosphorylation of proteins important for ABA production. Alternatively, ABA may induce the synthesis of novel proteins.

6.2 Results.

6.2.1 SDS-PAGE Electrophoresis of proteins from the cell-free system of *Citrus* flavedo.

The protein profile obtained following polyacrylamide gel electrophoresis (SDS-PAGE) of the 23 500 g supernatant from both acetone powder and crude homogenates is shown in Figure 6.1. Better resolution is obtained with the acetone powder although both extracts contain 8 major protein bands. The results presented in Figure 6.2 illustrate the protein pattern obtained following electrophoresis of the 23 500 g supernatant from homogenates of acetone powder. A major protein band of 90 kD was resolved, with minor bands of 66 kD, 55 kD, 44 kD, 35 kD, 29 kD 18 kD and 13 kD (Fig. 6.2A) When the homogenate was resolved under reducing conditions, the protein pattern changed. The major band at 90 kD was denatured and two strong bands became visible, one at 25 kD and another at 53 kD (Fig. 6.2B). In addition there were a number of minor bands. The protein profile obtained under reducing conditions was similar to that obtained with trichloroacetic acid (TCA) precipitation (Fig. 2.2, Chap. 2)

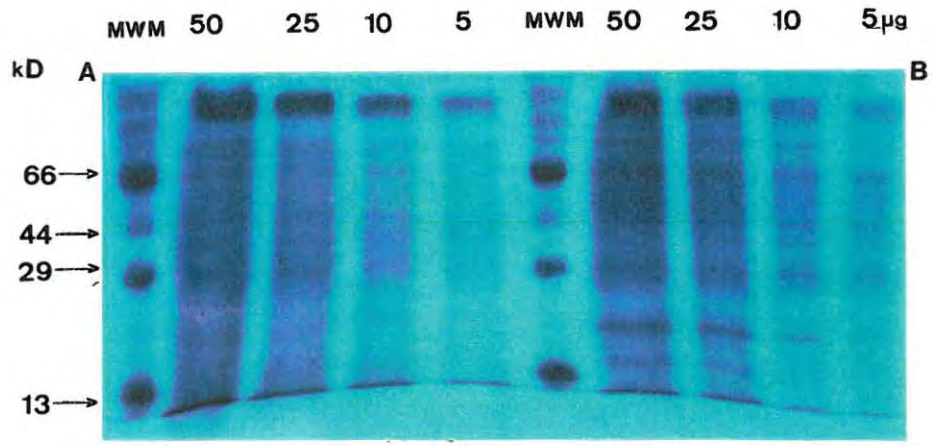


Figure 6.1 *Electrophoretic profiles of proteins in supernatant of* ***(A)*** *acetone powder;* ***(B)*** *crude cell-free extracts from* Citrus sinensis *flavedo, analysed on 12.5 % gels and stained with Coomassie Brilliant Blue as described in Section 2.10.3*

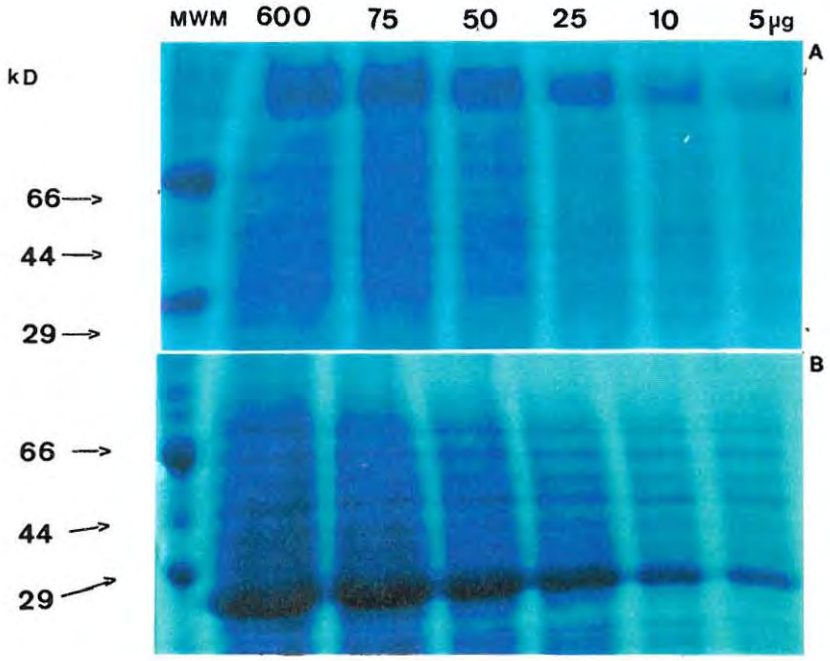


Figure 6.2 *Electrophoretic profiles of proteins in supernatant of acetone powder cell-free extracts from* Citrus sinensis *flavedo under* ***(A)*** *non-reducing conditions and* ***(B)*** *reducing conditions on 12.5 % gels.* Gels were stained with Coomassie Blue as described in Section 2.10.3.

6.2.2 Effect of ABA as a cold-pool trap

Previous work (Cowan and Railton, 1987a) had shown that ABA biosynthesis was increased when ABA was added as a "cold-pool trap". It was thus important to establish whether ABA was responsible for inducing new proteins. Acetone powder homogenates were, therefore, prepared as in section 2.4.3 and incubated with or without ABA as a cold-pool trap (Section 2.4.4), for 2 h at 37 °C.

The electrophoretic profiles obtained with or without ABA differed in that the upper zone at 66 kD was enhanced by ABA. This was especially noticeable in the 5 µg fraction. Additional bands between 44 and 66 kD were seen when ABA was added as a cold-pool trap (Fig. 6.3B). These bands were not visible in the electrophoretic profile from the fraction without an ABA cold-pool trap (fig 6.3A) This implies that new proteins are induced by ABA.

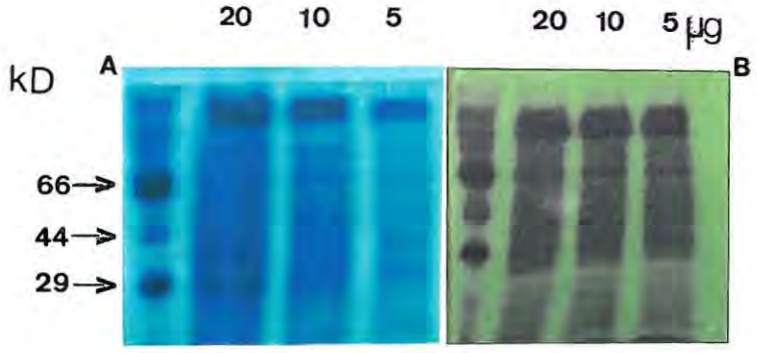


Figure 6.3 *Electrophoretic profiles of proteins in supernatant of acetone powder extracts from* Citrus sinensis *flavedo (A) without cold ABA and* ***(B)*** *with cold ABA in the incubation medium.* Extracts were incubated *as described in section 2.4.4.* Samples were analysed on 12.5 % gels and stained with Coomassie Brilliant Blue as described in Section 2.10.3.

6.2.3 Lipoxidase Activity in cell-free systems from *Citrus* flavedo.

A lipoxygenase enzyme has been implicated in ABA biosynthesis, therefore it was important to determine whether the extracts contained lipoxidase activity and whether any of the proteins co-eluted with standard lipoxidase. The protein profiles obtained following SDS-PAGE of pure lipoxidase from soybean and acetone powder homogenate are shown in Figure 6.4. Under non-reducing conditions poor resolution made interpretation difficult. However under reducing conditions there were 66 kD proteins in the acetone powder homogenate which co-eluted with lipoxidase. There were also protein bands between 44 and 66 kD which co-eluted with lipoxidase. In order to confirm the presence of a lipoxygenase enzyme, extracts of *Citrus* were assayed by the procedure described by Ben-Aziz *et al.*, (1971). The results presented in Figure 6.5 show that lipoxygenase activity increased linearly with increase in protein concentration, which indicates that this is an enzyme catalysed reaction. Thus the enzyme is present and active in extracts of *Citrus*. This result eliminates any doubt with the SDS-PAGE gels.

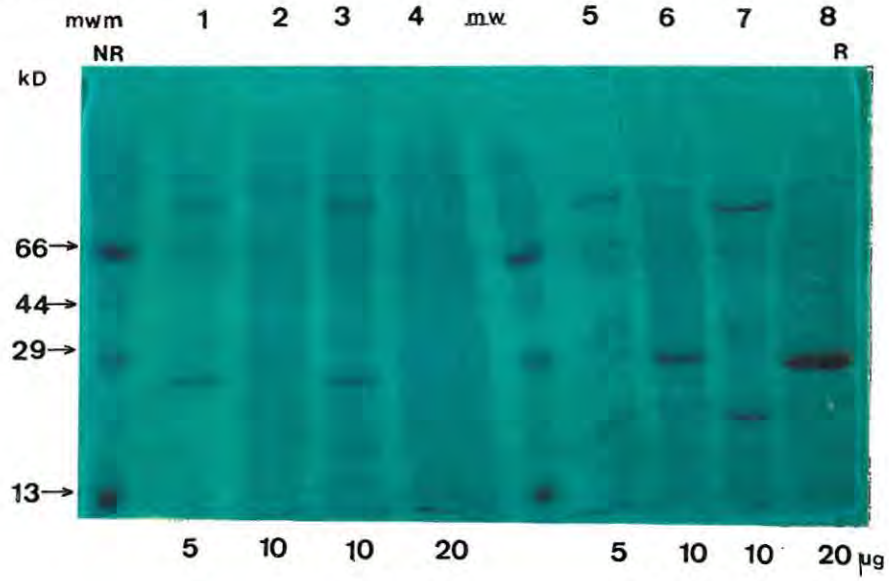


Figure 6.4 *Electrophoretic profiles of proteins in acetone powder cell-free extracts from* Citrus sinensis *flavedo* *1 *and pure lipoxygenase* *2 under non-reducing (NR) and reducing (R) conditions. Incubations were as described in Section 2.4.4. Samples and standard lipoxygenase were analysed on 12.5 % gels and stained with Coomassie Brilliant Blue as described in Section 2.10.3. Molecular weight markers are shown on the left side. *1 (Lanes 2,4,6, 8); *2 (Lanes 1, 3, 5, 7)

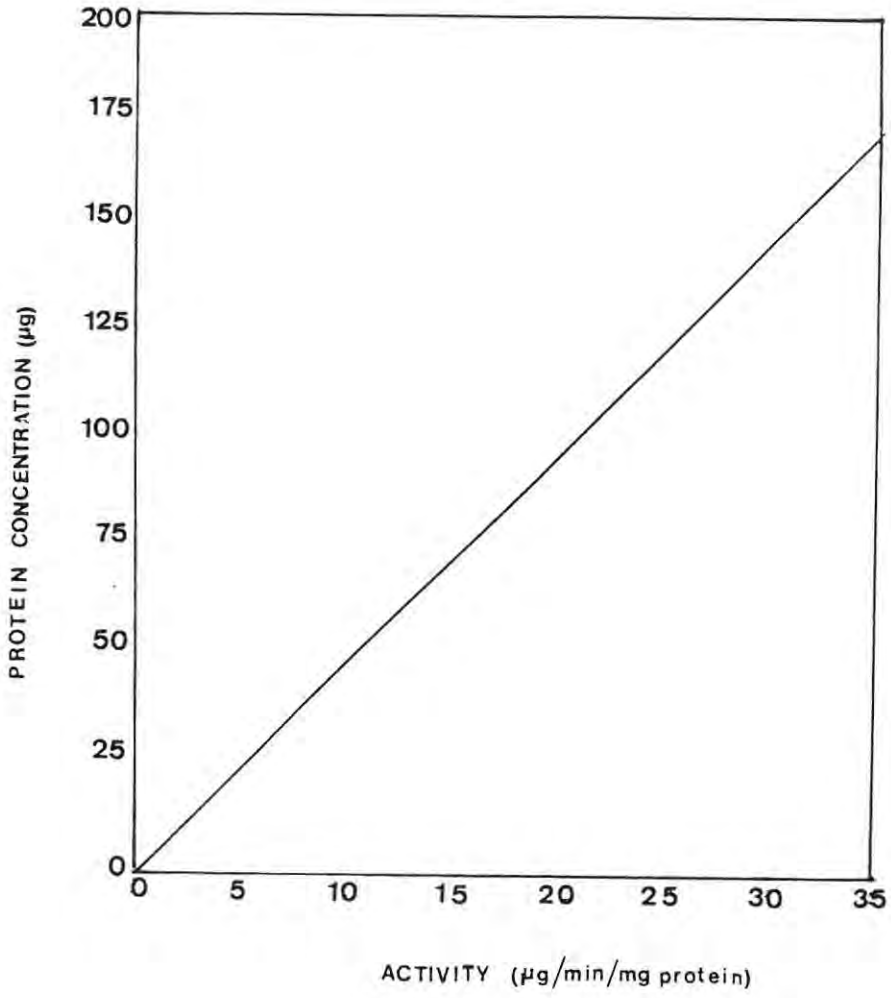


Figure 6.5 *Effect of protein concentration on lipoxygenase activity in cell-free extracts of* Citrus sinensis *flavedo.* Incubations were as described in Section 2.3

6.2.4 Detection of cyt P-450 in cell-free extracts of *Citrus* flavedo using polyacrylamide gel electrophoresis (PAGE)

A cyt-P450 enzyme has been implicated in the biosynthesis of ABA (Gillard and Walton, 1986). In addition work with ancyimidol, a known sterol cytochrome P-450-dependent enzyme inhibitor, resulted in reduced incorporation of label from *all-trans*-β-carotene into ABA. This suggests the involvement of a cytochrome-P450. As peroxidase activity is characteristic of cytochrome P-450, it was decided to use 3',3',5',5'-

tetramethylbenzidine-H_2O_2 (TMBZ) which had been successfully used to stain for the peroxide activity of Cyt-P-450 in SDS-PAGE gels (Thomas et al., 1976). Acetone powder homogenates were incubated for 2 h at 37 °C as described in Section 2.4.4 and then loaded onto the gels. Treatment of unstained gels with TMBZ-H_2O_2 revealed the presence of a 53 kD protein with peroxidase activity characteristic of cytochrome P-450. (Fig. 6.6). The molecular weight of this enzyme is within the range of molecular weights published for cytochrome P-450. It is not clear whether this protein is involved in ABA biosynthesis but it is certainly present and active in this cell-free system from *Citrus sinensis* and is perhaps induced by ABA (see Fig. 6.3B).

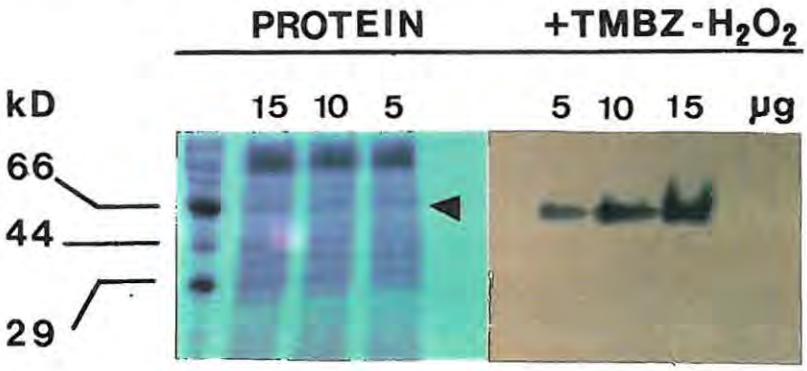


Figure 6.6 *SDS-PAGE analysis of proteins in extracts prepared from acetone powder homogenates of* Citrus sinensis *flavedo stained with Coomassie Brilliant Blue and detection of cytochrome P-450 by straining for peroxidase activity with TMBZ-H_2O_2.* Aliquots of the extract, equivalent to 5, 10 and 15 µg protein, were analysed by polyacrylamide gel electrophoresis. Molecular weight markers are shown on the left side, while the arrow indicates the position of the protein band corresponding to cytochrome P-450.

6.2.5 The effect of 2-oxo-glutarate on the activity of *Citrus* enzymes

The latter stages of gibberellin biosynthesis are catalysed by a series of soluble dioxygenases with an absolute requirement for oxo-glutarate (De Carolis and De Luca 1994). Reported molecular weights of gibberellin dioxygenases are between 26 and 44 kD (Graebe 1987). As SDS-PAGE had resolved bands in both the 26 and 44 kD regions, it was decided to test the requirement for 2-oxoglutarate. The results obtained are shown in Table 6.1.

Table 6.1 *Effect of Fe, Ascorbate and 2-oxo-glutarate on the incorporation of label from 1',4'-*trans*-ABAdiol, into ABA.* Complete acetone powder incubates were supplied with [^{14}C]-*trans*-ABAdiol (10 kBq) in the presence or absence of Fe, ascorbate and 2-oxo-glutarate.

Assay conditions	Radioactivity in product		
	Bq mg protein^{-1}	Control	% of Control
Complete (Control)		685.93	100%
- Molybdate		498.99	72%
+ Ascorbate + 2-oxo-glutarate		226.87	38.9%
-Mo + Fe + Ascorbate + 2-oxo-glutarate		693.04	100%
-Mo + Fe		698.32	100%

The results presented in Chapter 4 indicated a possible requirement for molybdate in the conversion of MVA, IPP 1',4'-*trans*-ABAdiol and β-carotene to ABA (Table 4.4) in this *Citrus* cell-free system. The results in Table 6.1 showed that when molybdate was omitted there was a reduction in incorporation into ABA from 1',4'-*trans*-ABAdiol. However when iron (Fe) was substituted for molybdate there was no apparent loss in incorporation. The addition of ascorbate and 2-oxo-glutarate with molybdate however, caused a loss in efficiency and incorporation into ABA dropped to only 38.9 %. In contrast to this, the addition of these cofactors with Fe did not affect the efficiency of the enzyme. This seemed to indicate that in the absence of Fe^{+} ascorbate acts as an inhibitor of enzyme activity as it prevents oxidation from taking place. In additon ascorbate inhibits the conversion of zeaxanthin to violaxanthin (Demming-Adams *et al.*, 1992) whereas DTT stimulates this reaction (Yamamoto 1979, Yamamoto and Kalmite 1972).

6.2.6 Possible involvement of protein phosphorylation in the biosynthesis of ABA

Many of the effects of cytoplasmic calcium can be traced to a modulation of the levels of specific enzymes (Hetherington and Trewavas, 1984). Amongst these, protein kinases, because of their pleiotropic function, rank as one of the most important. Many reports have indicated the presence of calcium-activated protein kinases in plants (Polya and

Davies, 1982; Salimath and Marme, 1983). As it had been reported that the phosphorylation of some protein species could be increased some 20-fold in the presence of calcium, (Hetherington and Trewavas, 1984), the effect of calcium was tested in cell-free extracts from *Citrus* flavedo. The results are presented in Table 6.2.

Table 6.2 *The effect of Ca^{2+} on the incorporation of radioactivity into ABA from 1) β-carotene and 2) 1',4'-*trans*-ABAdiol into ABA.*

Assay Condition	Substrate	incorporation (kBq mg protein^{-1})	% change
Complete	β-carotene	1.48	100
+ $CaCl_2$	β-carotene	0.54	36.5
Complete	1',4'-*trans*-ABAdiol	7.84	100
+ $CaCl_2$	1',4'-*trans*-ABAdiol	3.00	38.3

In the presence of Ca^{2+} incorporation of label from either β-carotene or 1',4'-*trans*-ABAdiol was substantially reduced. The presence of a cold-pool trap suggests that a reduction in formation of product [^{14}C]-ABA was not due to Ca^{2+} stimulated catabolism. The ability of Ca^{2+} to inhibit ABA production is not surprising given that these two components are antagonistic (Roberts and Harmon 1992; Salimath and Marme, 1983).

In view of the above it was considered that protein phosphorylation might contribute to the down regulation of an enzyme(s) involved in ABA biosynthesis. Unfortunately all efforts to demonstrate protein phosphorylation in cell-free extracts of *Citrus* flavedo acetone powder were unsuccessful (data not shown).

CHAPTER 7

Discussion and Conclusions

Although the pathway of ABA biosynthesis in higher plants remains equivocal, there is considerable evidence to support the hypothesis that ABA is an apo-carotenoid (Creelman *et al.*, 1987; Gage et al., 1989; Li and Walton, 1987; 1990; Parry et al., 1987; 1991; Parry and Horgan 1991a; 1991b; Rock et al, 1991; 1992; Rock and Zeevaart 1991; Sindhu and Walton, 1988; 1987; Zeevaart et al., 1989; 1986). The currently accepted pathway appears to involve cleavage of a 9'-*cis* xanthophyll to yield Xan which is then oxidised to ABA via ABA-aldehyde (Sindhu et al., 1990; Rock and Zeevaart 1990; Taylor et al., 1988). Even so, evidence has been presented to suggest that ABA biosynthesis in plants does not involve carotenoids. For example, Wagner and Elstener (1989) showed that oxidative xanthophyll degradation was not a major biosynthetic pathway for the formation of Xan. Furthermore, Milborrow and co-workers have recently re-evaluated the carotenoid origin of ABA in plants and concluded that the precursor pool of ABA does not involve carotenoids and that ABA-aldehyde is not the endogenous precursor of stress-induced ABA synthesis (Netting and Milborrow, 1994; Willows, *et al.*, 1994). These observations cast some doubt as to the validity of the currently accepted biosynthetic pathway for ABA in higher plants.

When the relationship between ABA accumulation and colour development was examined, it was apparent that ABA content increased throughout the duration of colour development. ABA content only declined after development of colour, presumably due to enhanced catabolism and formation of ABA-conjugates (Harris and Dugger, 1986). This may suggest that elevated endogenous ABA levels contribute to the retardation of colour development which is perhaps not surprising given that ABA participates in the regulation of terpenoid metabolism by feed-back inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA) reductase (Moore and Oishi 1994), a key enzyme in the biosynthesis of isoprenoids and carotenoids. Following full development of colour there was an accumulation of xanthophyll acryl esters at the expense of free xanthophylls, the proposed progenitors of ABA. This implies that a decline in free ABA due to conjugation (Harris and Dugger, 1986) coupled with a loss of unesterified xanthophylls would have

the overall effect of reducing the pool of free ABA available for down regulation of HMGCoA-reductase activity and hence the synthesis of isoprenoids and carotenoids.

Several approaches have been adopted in studies which have attempted to elucidate the biosynthesis of ABA in higher plants. These include feeding of labelled precursors to intact tissue, incubation of extracts in the presence of $^{2}H_2O$ and $H_2{}^{18}O$ and in $^{18}O_2$-containing environments, the use of ABA and/or carotenoid-deficient mutants and putative chemical inhibitors of ABA/carotenoid biosynthesis (see Chapter 1). The one major avenue that has not been explored is that employing cell-free biosynthesizing systems. In fact, at the conclusion of a review on ABA biochemistry and physiology Walton (1980) stated that further advances would depend largely on the isolation and characterization of novel intermediates and the development of ABA-metabolizing cell-free systems. It is therefore surprising that this latter aspect has not been pursued more vigorously particularly given the recent advances on the *in vitro* synthesis and enzymology of sesquiterpenoid biosynthesis (Alonso and Croteau, 1993). What is even more surprising is that carotenogenic cell-free systems do not appear to have been considered in studies on ABA biosynthesis particularly given the ability of some of these systems to produce β,β-xanthophylls (Bramley, 1993)

Acetone powder homogenates from Citrus flavedo contain membrane-bound enzymes whereas crude enzyme extracts contain soluble enzymes. The efficiency of the cell free-system was enhanced by the use of acetone powder homogenates. As the majority of carotenogenic enzymes are membrane-bound, this implied that ABA was produced via the carotenoid biosynthetic pathway.

The cell-free system developed from *Citrus sinensis* flavedo in this research programme, synthesizes ABA from MVA, IPP, FPP and GGPP and all-*trans*-β-carotene, and does so apparently via 1',4'-*trans*-ABAdiol. This represents the first report of a cell-free system capable of synthesizing ABA from both terpenyl pyrophosphates and carotenoids and one in which unequivocal identification of the products has been possible. These results therefore support a carotenoid origin for ABA and suggest that the pathway proceeds from all-*trans*-β-carotene via 1',4'-*trans*-ABAdiol. Although a previous report concluded that 1',4'-*trans*-ABAdiol was a catabolite of ABA in similar cell-free preparations (Cowan and Richardson, 1993a), refeeding studies using both labelled ABA and 1',4'-*trans*-

ABAdiol were not carried out to confirm this finding. The present study has revealed that 1',4'-*trans*-ABAdiol was readily converted to ABA and that this reaction was not the result of spontaneous oxidation in the *Citrus* cell-free system. 1',4'-*trans*-ABAdiol may therefore be the immediate precursor to ABA in this cell-free system. Furthermore, a requirement for molybdate supports current conjecture that a molybdenum-containing oxidase enzyme catalyses the terminal step in ABA biosynthesis. The present study has shown that this enzyme is apparently unaffected by NADPH and FAD, inhibited by FAD in the absence of molybdate and dependent upon NAD^+. A requirement for NAD^+ or NADP (Sindhu and Walton, 1987) suggests that the enzyme concerned is a molybdenum-containing hydroxylase possibly of the $NAD(P)^+$-dependent dehydrogenase form (Wootton *et al.*, 1991).

Thus although it now seems likely that there exists a universal indirect pathway for ABA biosynthesis in higher plants, the question still arises as to whether the "pre-cleavage" precursor is 9-*cis*-violaxanthin or 9'-*cis*-neoxanthin. The work presented in this thesis implicates 9-*cis*-violaxanthin as the precursor of ABA, with Xan, Xan alc and ABAdiol as intermediates. If this is indeed the case, a cleavage enzyme should be present in this system. The ability of lipoxygenase (LOX) to make xanthoxin from violaxanthin (Parry and Horgan, 1991) suggested that a lipoxygenase-like enzyme could be the cleavage enzyme that was involved in ABA biosynthesis. It had previously been shown that lipoxygenase inhibitors significantly inhibited ABA accumulation in response to stress. This observation, together with the fact that extracts of enzymes assayed for the presence of LOX showed an increase in activity (which was concentration dependant), suggested that the *in vivio* oxidative cleavage reaction involved in ABA biosynthesis required activity of an oxygenase having lipoxygenase-like properties.

It had previously been suggested that ABA-ald was the immediate precursor to ABA (Sindhu and Walton 1988; Taylor et al., 1988). In this research, no ABA-ald was found. When Netting and Milborrow 1994, fed (±)-[$^2H_9$]ABA-ald to tomato shoots, they found that there was little incorporation of ABA-ald into stress-induced ABA. This lead them to conclude that (±)-[$^2H_9$]ABA-ald was oxidized by a different enzyme system from that which synthesized stress-induced ABA and that it was unlikely that ABA-ald was the endogenous precursor of stress-induced ABA. No ABA-ald was detected in this series of experiments.

Parry (1989) reported that feeding of [^{13}C]-Xan and t-Xan to wild type and wilty mutants of tomato was consistent with Xan being a normal ABA precursor, and with the ability of the wild type to convert Xan to ABA being constitutive. This suggests that the regulation of ABA biosynthesis occurs prior to the synthesis of Xan. The routine appearance of Xan and Xan-alc as products of β-carotene metabolism in the *Citrus* cell-free system suggested involvement of a cytochrome P450-type mixed function oxygenase that catalyses the successive oxidation of the side chain of the product of carotenoid cleavage (Firn and Friend, 1972; Willows *et al.*, 1994). Coolbaugh (1984) and Coolbaugh and Hamilton (1976) have shown that both cytokinins and ancymidol can interact with cytochrome P450-mixed function oxidases in gibberellin biosynthesis to inhibit kaurene oxidation in plants. Similarly, ancymidol and cytokinins, both inhibitors of ABA biosynthesis *in vivo* and *in vitro* (Cowan and Railton, 1987a: 1987b), significantly reduced incorporation of label from [^{14}C]-β-carotene into 1',4'-*trans*-ABAdiol and ABA with a concomitant rise in Xan-alc and Xan whereas DTT a known inducer of cyt-P450 activity enhanced incorporation of label.

It has been postulated that the inhibitory effect of AMO 1618 and related growth retardants is, in addition to interference with the activity of kaurene synthetase (Graebe, 1987), due partly to inhibition of sterol biosynthesis (Douglas and Paleg, 1981). Also, plant sterols inhibit ABA-induced perturbations in phospholipid bilayers (Stillwell *et al.*, 1990) suggesting that ABA and sterols are antagonistic. Results from the present work demonstrate that stigmasterol, an abundant plant membrane sterol (Hartman and Benveniste, 1987), markedly reduced ABA biosynthesis from β-carotene but was ineffective when 1',4'-*trans*-ABAdiol was used as substrate. This is perhaps not surprising, given that the latter reaction would be expected to involve a soluble enzyme whereas the former interconversions are probably catalysed by membrane-bound enzymes. A possible point of interaction between AMO 1618, sterols and ABA could be cytochrome P-450. Both sterol and ABA metabolism involve at least one cytochrome P-450-catalysed reaction. For example, the enzyme catalyses the three oxygen-requiring stages during demethylation of 14α-methylsterols (Burden *et al.*, 1989) and the conversion of ABA to PA (Gillard and Walton, 1976). Furthermore, sterol cytochrome P-450-dependent enzyme inhibitors inhibited ABA biosynthesis in the fungus, *Cercospora rosicola* whereas inducers of cytochrome P-450 (e.g. dithiothreitol) enhanced ABA synthesis in this organism (Norman *et al.*, 1988). In fact, to obtain Xan oxidase activity

from leaves of *Lycopersicon esculentum* it was necessary for Sindhu and Walton (1988) to include DTT, in the extraction buffer which suggests that conversion of Xan to ABA involves a cytochrome P-450 catalysed reaction. Similarly, in the present study ABA biosynthesis was inhibited when cytochrome P450-dependent enzyme "inhibitors" were included in the incubation medium while activity was enhanced by the addition of DTT (Section 5.2.5.3)

Evidence has been presented to suggest that activity of plant cytochrome P-450 is dependent on developmental stage of the tissue with high cytochrome P-450 activity in young and/or non-photosynthetic tissue such as fruit (Hendry, 1986). Mature, coloured *Citrus* flavedo would therefore be expected to be a rich source of cytochrome P-450. A minor protein detected following analysis of *Citrus* cell-free extracts by SDS-PAGE was shown to contain haem-associated peroxidase activity, characteristic of cytochrome P-450 (P-420). Whether or not this protein is intimately involved in the biosynthesis of ABA remains to be determined. Nevertheless, inhibition of ABA synthesis by ancymidol and stigmasterol coupled with detection of a cytochrome P-450 protein in *Citrus* cell-free extracts suggests the involvement of a membrane-bound enzyme-catalysed reaction sequence, typical of mixed function oxidases, in ABA biosynthesis.

AMO 1618 which has long been recognised as an inhibitor of *ent*-kaurene synthetase and squalene 2,3-oxidocyclase, (Douglas and Paleg, 1978), increased incorporation of label into ABA. The cyclizations of oxidosqualene and GGPP are mechanistically similar and cationic compounds such as AMO 1618 would be expected to inhibit these reactions (Hedden, 1990). The cyclization of lycopene to β-carotene might similarly be effected thereby reducing incorporation of labelled MVA and terpenyl pyrophosphates into ABA, particularly since ABA appears to be derived from a carotenoid origin. However, this research has demonstrated that the *Citrus* cell-free system converts both FPP and GGPP to ABA in the presence of AMO 1618 and that it did, in fact, enhance ABA biosynthesis irrespective of which substrate was used. Milborrow (1976) demonstrated that addition of AMO 1618 substantially increased the incorporation of MVA into ABA *in vivo* in avocado mesocarp. In addition, AMO 1618 stimulated production of ABA in *Botrytis cinerea*, an ABA-producing fungus from which 1',4'-*trans*-ABAdiol was isolated in mass amounts and shown to be converted into ABA (Hirai *et al.*,

1986). This suggests that the growth retarding property of AMO 1618 is related to its ability to facilate enhanced biosynthesis of ABA.

If the operation of this system is indeed dependent on the involvement of cyt P450 mixed function oxygenases, the sequence of intermediates might be expected to be alcohol →aldehyde→acid. Xan acid was routinely identified in the acid fraction. In addition Parry (1989) also identified Xan acid in extracts of tomato shoots fed with ^{13}C-Xan and he further found that all of the extracted Xan acid was labelled with ^{13}C. No traces of an endogenous compound were found, implying that all the Xan acid extracted was sythesised from ^{13}C-Xan en route to ABA.

Thus while the identity and metabolic interrelationship of intermediates between β-carotene and 1',4'-*trans*-ABAdiol remain obscure, the following sequence is proposed based on analysis of products formed when non-labelled FPP, GGPP and β-carotene were used as substrates; 9-cis-violaxanthin, Xan-alc, Xan, Xan-acid, 1',4'-trans-ABAdiol, ABA and PA (Figure 7.1).

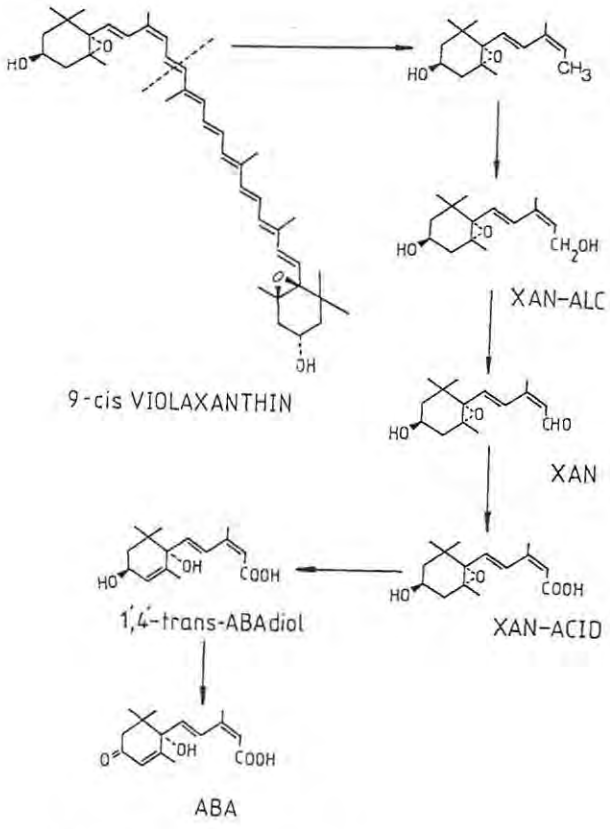


Figure 7.1 *Proposed ABA biosynthetic pathway*

Although Olson (1993), is in agreement with ABA being produced from violaxanthin, he suggests that the action of a 13:14 dioxygenase would yield the C_{18} ketone of violaxanthin, which by a sequence of several oxidative steps, would lose CO_2 and be converted to the *all-trans* 17 carbon aldehyde. This would in turn be converted to 9-*cis*-xanthoxin. 9-*cis*-xanthoxin would undergo a rearrangement in the 6:6 epoxide to produce 1',4'-*trans*-ABAdiol. Dehydrogenation of the C_4 hydroxy to a keto group would then yield ABA (Fig. 7.2). He does however, acknowledge that the sequence of biosynthetic steps may be different.

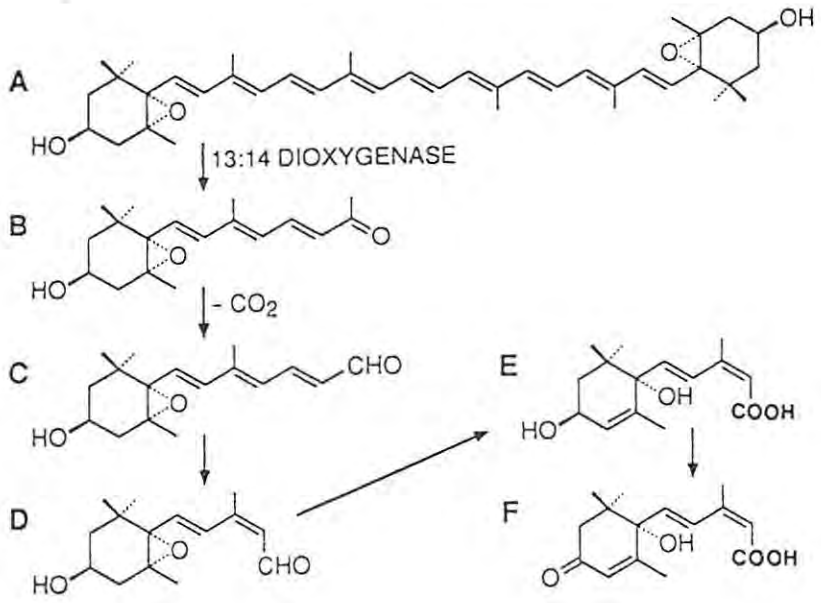


Figure 7.2 *A possible pathway for the formation of abscisic acid from carotenoids.* (A) violaxanthin, (B) C18 ketone of violaxanthin, (C) C17 aldehyde intermediate, (D) 9-cis-xanthoxin, (E) 1',4'-*trans*-ABAdiol, (F) abscisic acid.

Although it has been shown that the enzymes required for the biosynthesis of ABA are present and active in the cell-free system from *Citrus sinensis*, it now remains for these enzymes to be purified and characterized. In light of the recent reports on the effect of Ca^{2+} and calmodulin on protein kinase activity (Hetherington and Trewavas, 1982; Polya and Davies 1982), further attempts to phoshporylate the enzymes in the *Citrus*

cell-free system should be undertaken. Possible reasons for the failure to achieve phosphorylation in this research could be: (1) the temperature at which incubations were carried out and (2) the inclusion of either 0.1 % Tween 20 or Triton X 100 in the incubations. Hetherington and Trewavas (1984) reported good phosphorylation of a pea membrane protein kinase using an incubation temperature of only 25 °C. In addition, as activity of the cell-free system was increased by the addition of 0.1 % Tween 20 or Triton X 100 in the extraction buffer, this was routinely added. However, it has been shown that the addition of Triton X 100 to the incubations caused considerable solubilization of the protein kinase at a detergent concentration of 0.1 %, together with membrane disruption and changes in enzyme activity (Hetherington and Trewavas, 1984). Thus further research of protein phosphorylation of the enzymes present in the cell-free system of *Citrus* flavedo need to be undertaken.

The question arises as to whether 9-*cis*-violaxanthin or 9-*cis*-neoxanthin is the precursor to ABA. One way of answering this question would be to use both 9-*cis*-violaxanthin and 9-*cis*-neoxanthin as substrates in the cell-free system of *Citrus*. It is a simple procedure to extract the *trans*-isomers from green leaves. However, it would then be necessary to purify these isomers further and to examine the extracts for the presence of *cis* isomers, as it is the *cis* isomers which are implicated in ABA biosynthesis.

Nuclear magnetic resonance (NMR) spectroscopy is the most valuable technique for the elucidation of the geometric structure of carotenoids. ^{13}C-NMR spectra can be obtained from less than 1 mg of a pure carotenoid (Goodwin and Britton, 1988). Unfortunately access to such instruments was not possible during these investigations. It should however, be used as it may be a means of obtaining more accurate measurements for violaxanthin and neoxanthin. The compartmentation of these xanthophylls within the chloroplasts should also be examined. Unfortunately lack of time precluded this work from being included in this research project.

Thus although this cell-free system from *Citrus sinensis* flavedo successfully converted MVA, IPP, FPP, GGPP, β-carotene and 1',4'-trans-ABAdiol to ABA and its putative intermediates, the mass amounts of the products was very small. This made identification of the intermediates difficult but not impossible.

It has been shown that the synthesis of ABA by stressed leaves required continued transcription and translation (Quarrie and Lister, 1984; Cowan and Railton, 1986; 1987a), this avenue should be followed in an attempt to identify the products of the genes encoding the ABA biosynthetic enzymes. In additon, much research in recent years has been directed towards regulation of gene expression by endogenous ABA during drought stress (Bray 1991, Benson *et al.,* 1988, Thomas *et al.*, 1991). It is not yet known if the ABA-regulated gene products promote drought resistance.

Although the role of ABA in plant growth and development is not fully understood, ABA is a likely candidate for a signal transducer in the control of some classes of plant genes in response to changes in water potential and to select developmental cues (Thomas *et al.,* 1991). This avenue needs to be explored further as it is only once the function of the ABA-regulated gene products are understood that a full understanding of ABA biosynthesis will be possible. This work was outside the scope of this research project but it is hoped that with the development of this cell-free system which is capable of synthesising ABA and its intermediates from both terpenoid precursors and β-carotene, identification of ABA-regulated genes and gene-products will follow.

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APPENDIX 1

A KEITH COWAN AND GAYNOR R RICHARDSON

1',4'-*Trans*-[^{14}C]-Abscisic Acid Diol: A Major Product of *R*-[2-^{14}C]-Melvalonic Acid Metabolism in Extracts of *Citrus sinensis* Exocarp.

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1′,4′-*Trans*-[^{14}C]-Abscisic Acid Diol: A Major Product of *R*-[2-^{14}C]-Mevalonic Acid Metabolism in Extracts of *Citrus sinensis* Exocarp

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Summary

A cell-free system prepared from *Citrus sinensis* exocarp transformed *R*-[2-^{14}C]-mevalonic acid into abscisic acid, phaseic acid and 1′,4′-*trans*-abscisic acid diol. 1′,4′-*Trans*-abscisic acid diol was unequivocally characterized as the major product of mevalonic acid metabolism in the *Citrus* cell-free system. The addition of (*R,S*)-abscisic acid as a «cold-pool trap» increased incorporation of radioactivity into putative abscisic acid but reduced the levels of [^{14}C] associated with 1′,4′-*trans*-abscisic acid diol, negating a role for this metabolite as a precursor to abscisic acid.

Key words: Citrus sinensis, cell-free system, abscisic acid, 1′,4′-trans-abscisic acid diol.

Abbreviations: ABA = abscisic acid; PA = phaseic acid; MVA = mevalonic acid; GC-MS = gas chromatography-mass spectrometry.

Introduction

Abscisic acid (ABA) was first detected in *Citrus* fruits by optical rotatory dispersion (Milborrow, 1967) and later unequivocally characterized in extracts of *Citrus* flavedo by combined gas chromatography-mass spectrometry (Goldschmidt et al., 1973). Since ABA concentrations in *Citrus* flavedo are of the order of 2.5 mg/kg fresh weight, (Goldschmidt, 1976), among the highest reported for a higher plant tissue, it was envisaged that flavedo might be a useful tissue in which to study aspects of ABA biosynthesis *in vitro*.

Recent studies suggest a universal pathway for ABA biosynthesis in plants involving cleavage of an oxygenated-carotenoid, possibly 9′-*cis*-neoxanthin to yield xanthoxin which is then converted to ABA *via* ABA-aldehyde (Rock et al., 1992; Rock and Zeevaart, 1991). This pathway is dependent upon isoprenoid synthesis and the production of carotenoids. *Citrus* flavedo is a rich source of chromoplasts and these organelles represent a major site of cartoenogenesis in plants (Beyer et al., 1985; 1989). Unfortunately, the interrelationship between carotenogenesis and ABA biosynthesis in isolated chromoplasts has yet to be investigated. Nevertheless, a suggestion by Attaway et al. (1967) that linalyl pyrophosphate, a tertiary isomer of NPP and GPP, could be an intermediate *en route* to limonene biosynthesis allowed George-Nascimento and Cori (1971) to demonstrate terpenoid biogenesis from GPP and NPP in extracts derived from *Citrus sinensis* flavedo. In subsequent studies these authors demonstrated the synthesis of sesquiterpenoid alcohols and aldehydes using similar cell-free extracts (Chayet et al., 1973). More recently, a prenyltransferase activity (E.C. 2.5.1.1.) has been partially purified from flavedo of *Citrus sinensis* (De la Fuente et al., 1980). This enzyme was shown to catalyze the condensation of isopentenyl pyrophosphate with either dimethylalyl pyrophosphate or geranyl pyrophosphate, and the (2*E*, 6*E*)- and (2*Z*, 6*E*)-farnesyl pyrophosphates were identified as the predominant products. This accumulated information suggested that cell-free extracts of *Citrus sinensis* flavedo should contain the necessary enzymes to demonstrate ABA biosynthesis *in vitro*.

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In this paper we describe the identification of 1′,4′-*trans*-ABA-diol, by capillary GC-MS, as the major product of mevalonic acid metabolism in extracts of *Citrus* exocarp and provide evidence to suggest that it is a catabolite and not a precursor *en route* to ABA.

Materials and Methods

Preparation of Cell-free Extracts

Fruits of *Citrus sinensis* L. var. Midknight, grown in the Fort Beaufort region, South Africa, were harvested (June–August) and surface sterilized in 1% sodium hypochlorite and then rinsed with several washings of distilled water. The flavedo (exocarp) was grated off from the whole fruit and the gratings homogenized at 0 °C for 3 min, using an Ultra-Turrax top-drive homogenizer, in 0.1 M Tris-HCl buffer (pH 7.4) equivalent to 1.5 times the fresh weight. The homogenates were filtered through two layers of cheesecloth and centrifuged for 30 min at 23,500 g in a Sorvall-RC5 refrigerated centrifuge. The 23,500 g supernatant was filtered through glass wool and the concentration of protein measured using the Bradford reagent (Bradford, 1976).

Incubation Procedure

Incubations were carried out essentially as described by Chayet et al. (1973). Routinely, 2.5 mL of the 23,500 g supernatant (equivalent of 0.5–1 mg protein) was incubated in 50 mM Tris-HCl buffer (pH 7.4) containing 10 mM glutathione, 5 mM NaF, 5 mM ATP, 1.25 mM $MgCl_2$, 1.25×10^{-2} mM NAD^+, 1 mM AMO 1618 and *R*-[2-^{14}C]-MVA (Sp. act. 2.03 GBq/mmol) in a total volume of 5 mL. Where specified, non-labelled (*R,S*)-ABA (0.2 mM) was included in the incubation medium. Reactions were initiated by the addition of protein and allowed to proceed for 2 h (unless otherwise stated) at 37 °C under constant illumination (42 μmol $m^{-2} s^{-1}$) in a shaking water bath. Reactions were terminated by heating to 100 °C for 3 min. Heat inactivated (100 °C for 10 min) extracts were routinely included as controls. Precipitated protein was removed by centrifugation and the pellet washed three times with 5 mL volumes of ice-cold methanol.

Extraction and Analysis of Products

The supernatant and combined washings were reduced to dryness, resuspended in 0.5 M potassium phosphate buffer (pH 8.5) and partitioned against diethyl ether (containing butylated hydroxytoluene, 20 mg L^{-1}) to remove neutral and basic impurities. The aqueous fraction was then adjusted to pH 2.5 and the acids partitioned into diethyl ether a further three times. The diethyl ether-soluble acids were purified on Sep-peak C_{18} cartridges and the distribution of radioactivity in the samples determined by chromatography either on thin layers of silica gel GF_{254} in the solvent system toluene/ethyl acetate (acetic acid (25:15:2, v/v) developed twice to 15 cm or, by reversed-phase HPLC on an ODS Spherisorb column (100 × 4.6 mm i.d.) using a linear gradient of 0–100% methanol (containing 0.5% acetic acid throughout) over 30 min at 2 mL min^{-1}.

Radioactive components co-chromatographing with authentic ABA, PA and 1′,4′-*trans*-ABA-diol were eluted from the silica gel with water-saturated ethyl acetate. Similar radioactive zones from HPLC eluates were pooled for further analysis. Individual components were methylated with ethereal diazomethane and their chromatographic behaviour monitored as described in «Results and Discussion». The methyl ester derivatives were further analyzed by treatment with either $NaBH_4$ or MnO_2 and rechromatographed as described by Milborrow and Noddle (1970). Capillary gas chromatography-mass spectrometry (GC-MS) was carried out using a Hewlett-Packard 5890 gas chromatograph fitted with a fused-silica capillary column (12 m × 0.32 mm i.d.) of HP-1 programmed from 120 °C at 5 °C min^{-1} with He as carrier gas (1.5–2.0 mL min^{-1}). Electron impact mass spectra were recorded at 70 eV and a source temperature of 250 °C using a Hewlett-Packard 5988A MS system and compounds identified from published mass spectra (Grey et al., 1974; Dörffling and Tietz, 1983).

Table 1: Incorporation of radioactivity into acidic products in cell-free extracts of *Citrus sinensis* flavedo supplied with *R*-[2-^{14}C]-MVA (370 kBq). Reaction conditions were as described in «Materials and Methods». Radioactive acids were analysed either by TLC (silica gel GF_{254} in toluene/ethyl acetate/acetic acid, 25:15:2 v/v) or by reversed-phase HPLC (linear gradient of 0–100% methanol containing 0.5% acetic acid throughout at 2 mL min^{-1}) and the distribution of radioactivity determined by liquid scintillation spectrometry as previously described (Cowan and Railton, 1987).

	Distribution of Radioactivity[a]		
	PA	1′,4′-*trans*-ABA-diol	ABA
	kBq/mg protein (% incorporation)		
Experiment 1	1.18 (0.32)	3.23 (0.87)	3.46 (0.94)
Experiment 2	1.41 (0.38)	2.92 (0.79)	4.22 (1.14)
Experiment 3	1.50 (0.41)	3.49 (0.94)	5.83 (1.58)

[a] Identification of metabolites based on co-chromatography with authentic standard.

Results and Discussion

Cell-free extracts were incubated in the presence of the squalene-2,3-oxide cyclase and kaurene synthetase inhibitor, AMO 1618, since a previous report had demonstrated that this compound stimulated ABA synthesis in cell-free systems (Cowan and Railton, 1987). The present study shows that cell-free enzyme preparations derived from *Citrus sinensis* flavedo incorporated label from *R*-[2-^{14}C]-MVA into acidic compounds that were chromatographically similar to ABA, 1′,4′-*trans*-ABA-diol and PA (Table 1). The identity of these acids was tentatively established by micro-chemical analysis of the methyl ester derivatives and by co-chromatography on thin layers of silica gel according to the criteria proposed for the identification of ABA and related compounds (Milborrow and Noddle, 1970) and the results are presented in Table 2.

In attempts to enhance incorporation of radioactivity into the acidic products, the contribution of developmental stage of *Citrus* fruits and the kinetics of the reaction were determined. Extracts prepared from chromoplast-containing flavedo, that is from fruits that had already undergone colour change, were better able to convert *R*-[2-^{14}C]-MVA into ABA and related acidic products (Fig. 1). Thus all further studies were carried out using fruits at this developmental stage. Kinetic studies revealed that production of putative ABA and PA was sustained for 2 h and thereafter declined, presumably due to product catabolism (Fig. 2 A and B). A decline in radioactivity associated with both ABA and PA

Table 2: Microchemical characterization and chromatographic behaviour of products formed from *R*-[12-^{14}C]-MVA by the crude enzyme preparation as described in Table 1.

Treatment	Putative		Authentic	
	ABA	1′,4′-*trans*-ABA-diol	ABA	1′,4′-*trans*-ABA-diol
	Rf values			
Untreated[1]	0.63	0.53	0.63	0.53
Diazomethane[2, 3]	0.63	0.83	0.63	0.83
$NaBH_4$[2, 5]	0.20, 0.40	NR[4]	0.20, 0.40	NR
MnO_2[2, 5]	NR	0.63	NR	0.63

[1] TLC on silica gel in toluene/ethyl acetate/acetic acid (50:30:4, v/v) developed 2 × to 15 cm.
[2] TLC on silica gel in *n*-hexane/ethyl acetate (1:1, v/v) developed 1 × to 15 cm.
[3] TLC on silica gel in *n*-hexane/ethyl acetate (1:1, v/v) developed 3 × to 15 cm.
[4] No reaction.
[5] Methyl ester derivatives treated.

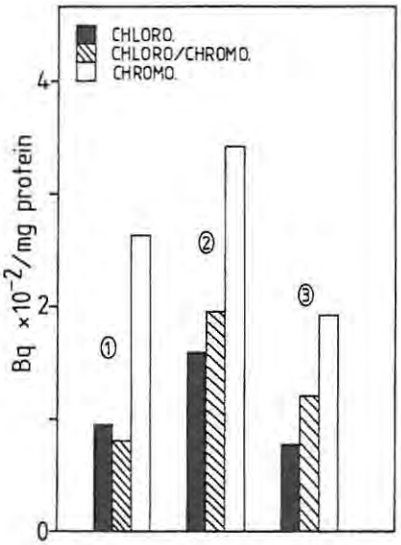

Fig. 1: The distribution of label between ABA (3), 1′,4′-*trans*-ABA-diol (2) and PA (1) obtained from cell-free enzyme systems of *Citrus sinensis* flavedo. Extracts were prepared from chloroplast-containing and chromoplast-containing flavedo and from exocarp of fruits during colour change. Enzyme preparations were incubated with *R*-[2-^{14}C]-MVA (50 kBq) as described in «Materials and Methods» and the products analysed as described in Table 1.

occurred concomitant with an increase in putative 1′,4′-*trans*-ABA-diol (Fig. 2 C). The accumulation of this metabolite facilitated its unequivocal identification by capillary GC-MS, as a product of MVA metabolism.

GC-MS of the methyl ester of putative 1′,4′-*trans*-ABA-diol, with helium as carrier gas, gave the spectrum presented in Fig. 3. The cracking pattern with a molecular ion (m/z) at 262 was identical to, and could be identified as 4′-desoxy-ABAMe, characterized previously by Tietz et al. (1979) and later shown to the product of dehydration of 1′,4′-*trans*-ABAMe-diol during GC analysis (Milborrow, 1983). Based on the positions at which label from *R*-[2-^{14}C]-MVA occurs in ABA (Milborrow, 1975), an apparent ion at m/z 268 (3.84% relative to b.p. m/z 125) may be attributable to 6 atomic mass units extra due to incorporation of [^{14}C].

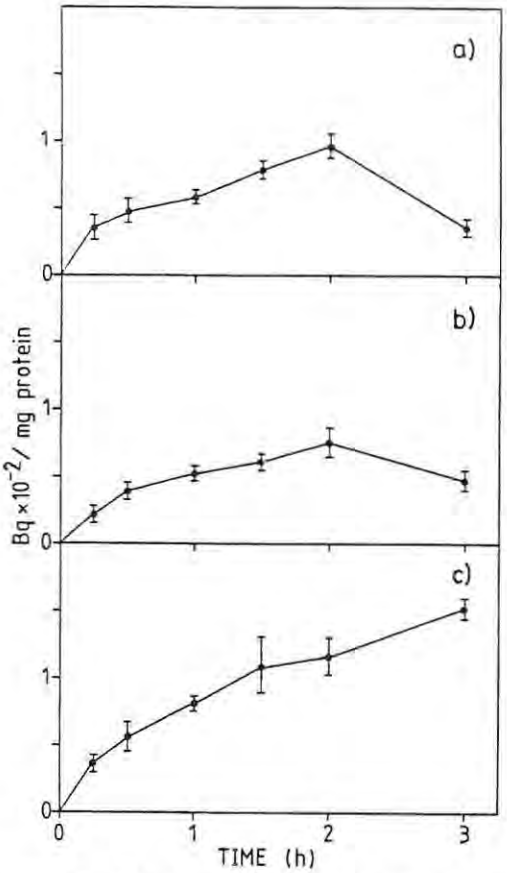

Fig. 2: Kinetics of the incorporation of label from *R*-[2-^{14}C]-MVA (50 kBq) into ABA (a), PA (b) and 1′,4′-*trans*-ABA-diol (c) by cell-free extracts of *Citrus sinensis* flavedo. Reaction conditions were as described in «Materials and Methods». Data represent the mean of three replicates ± S.E.

Table 3: The effect of AMO 1618 and (*R,S*)-ABA on the incorporation of label from *R*-[2-^{14}C]-MVA (50 kBq) into ABA, 1′,,4′-*trans*-ABA diol and PA by cell-free enzyme systems of *Citrus sinensis* flavedo. Reaction conditions were as described in «Materials and Methods».

Treatment	Distribution of Radioactivity			
	PA	1′,4′-*trans*-ABA-diol	ABA	Increase[1]
	Bq/mg protein (% incorporation)			
Control	123.1 (0.25)	209.7 (0.42)	123.9 (0.25)	0.0
+AMO 1618	218.5 (0.44)	323.6 (0.65)	203.8 (0.41)	163.3
+ABA	154.4 (0.31)	255.9 (0.51)	303.6 (0.61)	156.3
+AMO 1618 + ABA	235.5 (0.47)	408.0 (0.82)	718.5 (1.44)	300.2

[1] Total radioactivity in PA, 1′,4′-*trans*-ABA-diol and ABA relative to control.

The omission of AMO 1618 from cell-free extracts reduced incorporation of radioactivity into ABA, 1′,4′-*trans*-ABA-diol and PA (Table 3). A «cold-pool trap» of (*R,S*)-ABA increased the amount of radioacitivity associated with ABA but diluted the available radioactivity for incorporation into PA and 1′,4′-*trans*-ABA-diol. Several studies have

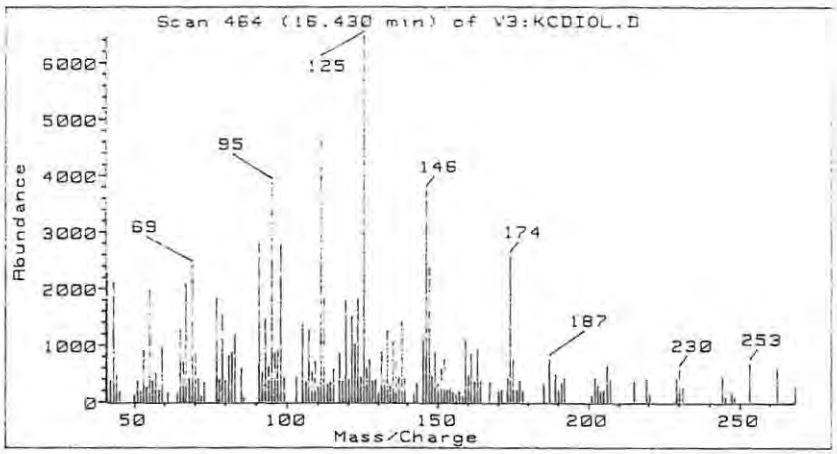

Fig. 3: Electron impact mass spectrum of the dehydration product of 1′,4′-*trans*-ABAMe-diol synthesized in a cell-free system from *Citrus sinensis* exocarp supplied with *R*-[2-^{14}C]-MVA (1.83 MBq, 120 µg). Conditions of incubation were as described in «Materials and Methods».

shown that 1′,4′-*trans*-ABA-diol can be converted to ABA in plant tissues (Okamoto et al., 1987 a; Parry et al., 1988) suggesting the possible origin of ABA *via* the «direct» pathway from FPP. This is particularly so given that ABA-producing fungi transform 1′,4′-*trans*-ABA-diol to ABA (Hirai et al., 1986; Okamoto et al., 1987 b), apparently in a «direct» pathway involving C_{15} intermediates derived from FPP. Milborrow (1972) showed that 1′,4′-*trans*-ABA-diol was converted to ABA and that the resultant high levels of ABA inhibited further synthesis by negative feedback. In fungi ABA is excreted into the culture medium and would therefore exert limited influence on the biosynthetic pathways, particularly as there is no information to suggest that these fungi catabolize exogenous ABA. In the present study excess cold ABA did not increase incorporation of label into 1′,4′-*trans*-ABA-diol at the expense of ABA. This finding strongly suggests that 1′,4′-*trans*-ABA-diol is not a precursor but rather a catabolite of ABA produced *in vitro* by the enzyme system prepared from *Citrus sinensis* exocarp, confirming a previous report (Rock and Zeevaart, 1990). We have recently demonstrated that similar cell-free extracts convert R-[2-^{14}C]-MVA into β-carotene and oxy-carotenoids in low yield (Richardson and Cowan, unpublished), an observation which suggests that ABA may be produced *via* the «indirect» pathway in extracts of *Citrus*. Unfortunately, we have thus far been unable to unequivocally characterize ABA as a product of MVA metabolism in this cell-free system. Nevertheless, the unequivocal identification of 1′,4′-*trans*-ABA-diol as a product in this cell-free system, coupled with the demonstration that it is produced as a catabolite of ABA, provide strong evidence for ABA biosynthesis *in vitro*.

Details of the above mentioned cell-free system will be published elsewhere and it is hoped that the further development of this system will assist studies aimed at elucidating the enzymology of ABA biogenesis.

Acknowledgements

The authors wish to acknowledge financial support from the Joint Research Committee (Rhodes University). Gaynor Richardson is supported, in part, by a grant from University of Fort Hare. Mr. Aubrey Sonneman is thanked for assistance with MS analysis.

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APPENDIX 2

A KEITH COWAN AND GAYNOR R RICHARDSON

The Biosynthesis of Abscisic Acid from *all-trans*-β-Carotene in a Cell-Free system from *Citrus sinensis* Exocarp.

Plant Cell Physiol 34(6): 969-972 (1993)

Plant Cell Physiol. 34(6): 969–972 (1993)

Rapid report

The Biosynthesis of Abscisic Acid from *all-trans*-β-Carotene in a Cell-Free System from *Citrus sinensis* Exocarp

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A cell-free system prepared from exocarp of *Citrus sinensis* fruits converted [^{14}C]-*all-trans*-β-carotene into zeaxanthin, antheraxanthin, violaxanthin and abscisic acid (ABA). ABA was unequivocally characterized by combined capillary GC-MS.

Key words: ABA biosynthesis — Cell free system — *Citrus sinensis*.

The biosynthetic route to the sesquiterpenoid growth regulator, ABA, in higher plants is now considered to occur predominantly via the metabolism of epoxy-carotenoids (Duckham et al. 1991, Li and Walton 1990, Parry et al. 1990, Parry and Horgan 1991, Rock et al. 1992, Rock and Zeevaart 1990, 1991). In addition, two recent reports have shown that increased ABA biosynthesis occurs coincident with changes in levels of β-carotene (Cowan and Rose 1991, Norman 1991). To assist in understanding more detailed aspects of ABA biogenesis and regulation, a cell-free system derived from exocarp of orange fruits has been developed. Here, we report the cell-free conversion of β-carotene to ABA via epoxy-carotenoids.

An acetone powder from the exocarp of ripe fruits of *Citrus sinensis* L. cv. Midknight was prepared as described by Pérez et al. (1980) and homogenized for 5 min at 0°C in 0.1 M Tris-HCl buffer (pH 7.4, 2 ml g^{-1} of powder). The homogenate was filtered through 2 layers of cheesecloth and centrifuged for 30 min at 23,500 × *g* at 2°C in a Sorvall RC-5 Superspeed refrigerated centrifuge. Aliquots of the supernatant, equivalent to 17 mg protein (Bradford 1976), were incubated by a procedure modified from that described by Cowan and Railton (1987). Briefly, incubations were carried out in 50 mM Tris-HCl buffer (pH 7.4) containing, 10 mM glutathione, 5 mM ATP, 1.25 mM $MgCl_2$, 1.25×10^{-2} mM of each of NAD^+, NADH and NADPH, 1 mM AMO 1618, 5 mM NaF, 1×10^{-3} mM Na_2MoO_4, and [^{14}C]-*all-trans*-β-carotene (0.88 MBq, 15 mg, Sp. act. 31 MBq mmol^{-1}) in a total volume of 25 ml.

Substrate [^{14}C]-*all-trans*-β-carotene was prepared by feeding 74 MBq NaH[^{14}C]O_3 (Sp. act. 1.92 GBq mmol^{-1}, obtained from Amersham International, U.K.) to an axenic culture of *Dunaliella salina* which was then exposed to high light intensity (1,000 μmol m^{-2} s^{-1}) for 5 days at 27°C, conditions which cause the alga to accumulate massive amounts of β-carotene (Lers et al. 1990). The radioactive β-carotene was extracted and purified as previously described (Cowan and Rose 1991) and the stereoisomers separated by reversed-phase HPLC, isocratically with methanol/acetonitrile (9 : 1 v/v) at 1 ml min^{-1} (Ben-Amotz et al. 1988).

Reaction mixtures containing [^{14}C]-*all-trans*-β-carotene and 'enzyme' were incubated for 2 h at 37°C in a shaking water bath under continuous illumination (42 μmol m^{-2} s^{-1}) and terminated by the addition of an equal volume of ice-cold methanol. Denatured protein was precipitated at −20°C and carotenoids and ABA extracted and analysed as described by Afitlhile et al. (1993).

Analysis of the carotenoid-containing fraction by reversed-phase HPLC revealed that [^{14}C]-*all-trans*-β-carotene had been metabolized into several oxygenated derivatives including zeaxanthin, antheraxanthin and violaxanthin (Fig. 1). The identity of the carotenoids produced from [^{14}C]-*all-trans*-β-carotene in the cell-free system from *Citrus sinensis* exocarp, was established by determining their absorption maxima in both hexane and benzene and by co-chromatography as previously described (Afitlhile et al. 1993). Briefly, the presence of 5,6-epoxide groups was confirmed by monitoring the spectral shift following the addition of 1.0-M HCl to ethanolic solutions of each purified carotenoid. The isomerization state

Abbreviations: GC-MS, gas chromatography-mass spectrometry; EI, electron impact.

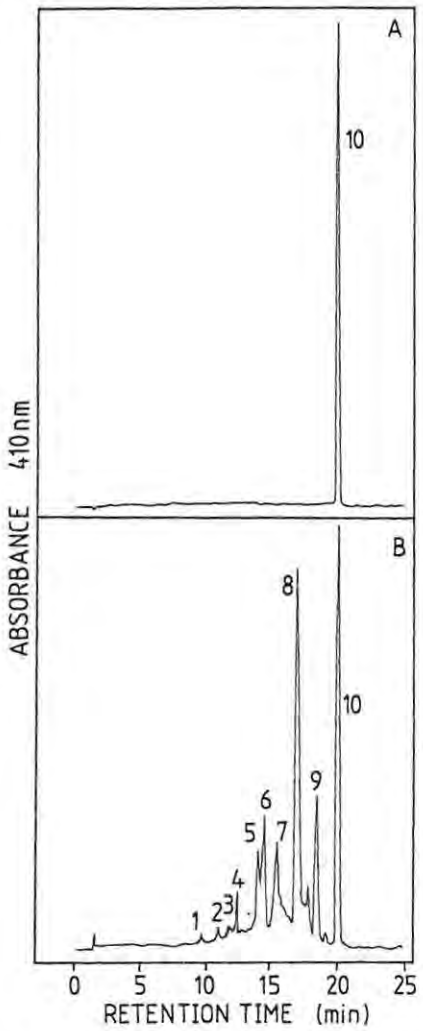

Fig. 1 HPLC chromatogram of the products of [^{14}C]-*all-trans*-β-carotene metabolism in extracts of *Citrus sinensis* exocarp. Carotenoids were separated on a 5 μm C_{18} Nucleosil column (250 × 4.6 mm i.d.) using a linear gradient of 0–100% ethyl acetate in acetonitrile/water (9 : 1 v/v) containing 0.1% triethylamine at 0.8 ml min^{-1} over 25 min. (A) Standard [^{14}C]-*all-trans*-β-carotene and (B) metabolites of β-carotene. Peaks: 1, *trans*-violaxanthin; 2, antheraxanthin; 3, unidentified; 4, zeaxanthin; 5, 9-*cis*-zeaxanthin; 6, 13-*cis*-zeaxanthin; 7, β-cryptoxanthin; 8, β-zea-carotene; 9, unidentified; 10, *all-trans*-β-carotene.

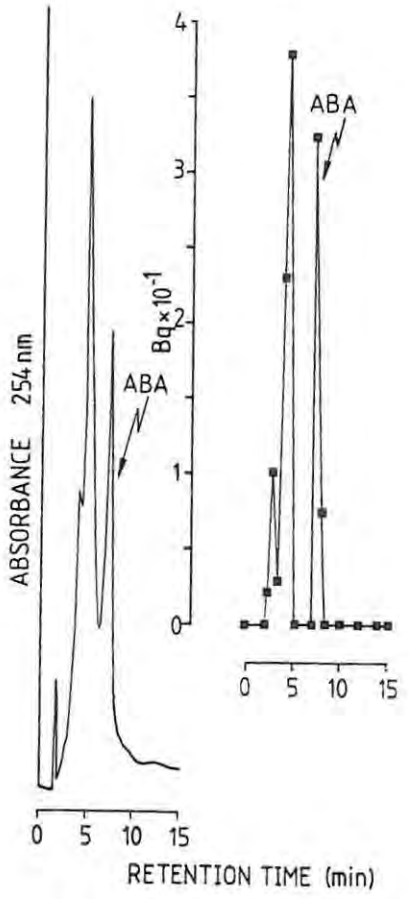

Fig. 2 Analysis by HPLC of the acidic products of incubation of [^{14}C]-*all-trans*-β-carotene with cell-free extracts of *Citrus sinensis* exocarp. Chromatography was performed on a Bondaclone 10 μm C_{18} column (150 × 3.9 mm i.d.). The column was eluted isocratically with methanol/water/acetic acid (40 : 60 : 1, v/v) at 1 ml min^{-1}.

was established by iodine-catalysed photomerization and subsequent analysis of the resultant components by reversed-phase HPLC using a linear gradient of 0–100% ethyl acetate in acetonitrile (9 : 1 v/v) containing 0.1% triethylamine. The diethyl ether-soluble acids, prepared from similar cell-free incubates were initially purified on thin layers of silica gel GF_{254} in the solvent system toluene/ethyl acetate/acetic acid (25 : 15 : 2, v/v) and the zone co-migrating with authentic ABA (Sigma), eluted from the silica gel with water-saturated ethyl acetate. Analysis of putative ABA by reversed-phase HPLC revealed a component of 277 μg which was chromatographically identical to authentic ABA at Rt = 7.5 min (Fig. 2).

After purification by HPLC, the component tentatively identified as ABA was derivatized in ethereal diazomethane and further analysed by combined capillary gas chromatography-mass spectrometry (GC-MS) and the electron impact (EI) mass spectrum is shown in Figure 3. This spectrum is almost identical to that of authentic ABA methyl ester (Gray et al. 1974) with characteristic ions at 222, 191, 190 (base peak), 162, 134, 125, 112 and 91. Under the conditions described (Fig. 3), authentic ABA methyl ester displayed a molecular ion at m/z 278.95. Since mevalonate and β-carotene are uniformly labelled via randomly labelled acetate, radiolabelled ABA produced from [^{14}C]-β-carotene should contain six atomic mass units extra

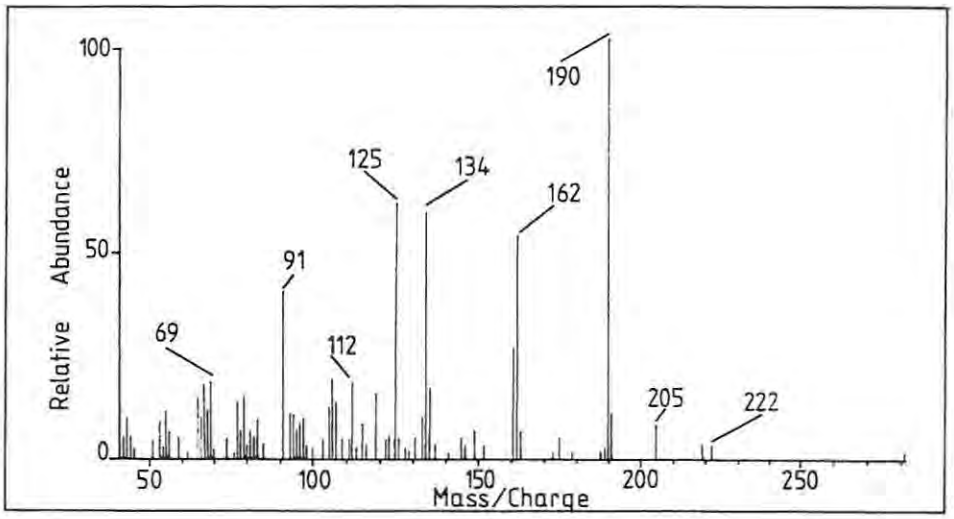

Fig. 3 Electron impact mass spectrum of the methyl ester derivative of ABA (277 μg; Sp. act. 29 Bq μmol^{-1}) prepared biosynthetically as described in Figure 2. Mass spectra were recorded at 70 eV and a source temperature of 250°C using a Hewlett-Packard 5988A mass spectrometer coupled to a Hewlett-Packard 5890 gas chromatograph, fitted with a fused-silica capillary column (12 m × 0.32 mm i.d.) of HP-1 programmed from 120° at 5°C min^{-1} with He as carrier gas (1.5–2.0 ml min^{-1}).

(Milborrow 1975). Unfortunately, no molecular ion at M^+ 284.95 was present in the EI spectrum (Fig. 3). Furthermore, none of the characteristic fragment ions of ABA methyl ester appeared to carry [^{14}C] which may reflect the relative insensitively of the analytical procedure used. Alternatively, the low level of incorporation (3.64%) coupled with the low specific activity of the product (29 Bq μmol^{-1}) may have contributed to the observed result. Nevertheless, the characterisation of ABA by combined capillary GC-MS as a major product in the cell-free system prepared from an acetone powder of *Citrus* exocarp, provides strong evidence for its origin from *all-trans*-β-carotene.

The cell-free system reported above augments similar cell-free studies using extracts from fruits of *Citrus sinensis* which have demonstrated mevalonate-activating enzymes (Potty and Bruemmer 1970a, b), prenyltransferase activity (De la Fuente et al. 1981) and sesquiterpene alcohol and aldehyde synthesis (Chayet et al. 1973). It is therefore envisaged that this cell-free system could easily be employed to elucidate the entire ABA biosynthetic pathway in vitro and to study more detailed aspects of the enzymology involved in this process.

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APPENDIX 3

GAYNOR R RICHARDSON AND A K COWAN

Abscisic acid content of *Citrus* flavedo in relation to colour development

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Journal of Horticultural Science (1995) **70** (5) 000–000

Abscisic acid content of *Citrus* flavedo in relation to colour development

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SUMMARY

The relationship between endogenous abscisic acid concentrations and the development of colour was examined in six cultivars of *Citrus sinensis*. Abscisic acid content increased throughout the course of colour development and reached a maximum at time of colour break. Late-maturing cultivars accumulated substantially more abscisic acid and development of colour occurred visibly later than in 'Navel' selections. A decline in abscisic acid concentration occurred concomitantly with full expression of colour. Analysis of the pigment composition of flavedo from *Citrus sinensis* cv. Midknight revealed that development of orange colour was associated with a decline in levels of β,β-carotenoids, an increase in violaxanthin monoesters and formation of xanthophyll acyl esters.

THERE is now substantial evidence to support the hypothesis that the plant growth substance, abscisic acid (ABA), is derived from metabolism of β,β-carotenoids (Parry, 1993). Thus the currently accepted biosynthetic sequence involves enzymatic cleavage of a 9-*cis* isomer of either neoxanthin or violaxanthin to yield xanthoxin, which is then successively oxidised to ABA-aldehyde and ABA. A similar, though less well-defined metabolic sequence has recently been established in cell-free extracts of *Citrus sinensis* flavedo where all-*trans*-β-carotene was used as substrate (Cowan and Richardson, 1993a). It is, therefore, not unreasonable to suggest that ABA would be, likewise, produced *in vivo* in *Citrus* fruit peel and that accumulation of ABA would occur coincident with development of orange colour.

Colour development in *Citrus* fruit peel involves temporal changes in chloroplast ultrastructure and the metabolism of both chlorophylls and carotenoids. For example, at colour break, thylakoid degeneration and the transition of chloroplasts to chromoplasts is accompanied by a decrease in carotenogenesis and enhanced chlorophyll breakdown, followed by an increase in carotenoid biosynthesis (Gross, 1981; Gross *et al.*, 1983). Early suggestions were that ABA may be involved in colour development in fruit, particularly as application of ABA enhances coloration in tomato (Khudairi and Arboleda, 1971) and grape (Coombe and Hale, 1973). However, all attempts to demonstrate an effect of ABA on coloration of *Citrus* fruit flavedo have thus far proved unsuccessful (Goldschmidt, 1988). Even so, the content of ABA in flavedo increases throughout the course of *Citrus* fruit development (Aung *et al.*, 1991; Hofman, 1990; Sagee and Erner, 1991) and concentrations in mature fruit peel are among the highest reported in the literature (Goldschmidt, 1976).

At present there is no published information on changes in carotenoid composition of *Citrus sinensis* flavedo during the course of fruit development. Furthermore it would be extremely difficult to assign changes in levels of individual carotenoids to an increase in ABA content, on a stoichiometric basis, due to

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the time-frame of the fruit developmental programme. This paper, therefore, illustrates the potential relationship between accumulation of ABA and development of colour in flavedo of *Citrus sinensis* and provides evidence to suggest that temporal changes in carotenoid composition could account for increases in ABA content of the tissue.

MATERIALS AND METHODS

Plant material

Fruits of *Citrus sinensis* cvs Midknight, Palmer Navel, Washington Navel, Moss Seedless, Lane Late Navel and Bahianinha were cultivated in orchards on the Baddaford farm in the Fort Beaufort district, Eastern Cape, South Africa. At two-week intervals throughout the 1991 and 1992 season, commencing mid-April, fruit from similarly aged trees on rough lemon rootstocks was harvested and the flavedo removed for determination of endogenous ABA content.

Extraction and analysis of abscisic acid and carotenoids

Harvested fruit were surface sterilized in a 1% sodium hypochlorite solution and then thoroughly rinsed with several changes of distilled water. The flavedo (30 g fresh weight) was grated from the fruit and homogenized at 0°C in ice-cold methanol/ethyl acetate (50:50, v/v), containing butylated hydroxytoluene (20 mg l^{-1}) and an aliquot of [^{2}H]-ABA (added to correct for losses during extraction), using an Ultra-Turrax top-drive homogenizer. The extract was filtered, reduced to dryness *in vacuo* and the residue resuspended in 0.5 M potassium phosphate buffer (pH 8.5). Aqueous fractions were partitioned against equal volumes of diethyl ether at pH 8.5 to remove neutral and basic impurities. To extract acids, the pH of the aqueous fraction was adjusted to 2.5 prior to a second partitioning against equal volumes of diethyl ether. Acidic diethyl ether soluble acid fractions were purified on thin layers of silica gel, developed in the solvent system toluene/ethyl acetate/acetic acid (25:15:2, v/v), and the UV-absorbing region co-chromatographing with authentic ABA eluted from the gel, derivatized with excess diazomethane and rechromatographed on silica gel in hexane/ethyl acetate (1:1, v/v). Putative ABA-methyl ester was recovered from the gel and quantified by gas chromatography electron capture (GC-EC) as described by Afitlhile *et al.*, (1993). Unequivocal identification of ABA was established by combined gas chromatography-mass spectrometry (Cowan and Richardson, 1993a).

Where specified, the composition of carotenoids in *Citrus* flavedo was determined by high performance liquid chromatography (HPLC) following extraction of tissue as described above. Methanol/ethyl acetate extracts were filtered, reduced to dryness and purified on C_{18} Sep-pak cartridges. Individual carotenoids were resolved on a 5 μm C_{18} Nucleosil column (Machery-Nagel, Düren, Germany) eluted with a linear gradient of 0–100% ethyl acetate in acetonitrile/water (9:1, v/v) at a flow rate of 0.8 ml min^{-1}. Peaks were detected at 410 nm using a variable wavelength detector and identified and quantified as described by Afitlhile *et al.* (1993). All manipulations were carried out in dim light to avoid photooxidation and isomerization of compounds of interest.

RESULTS AND DISCUSSION

The relationship between ABA accumulation and colour development in flavedo of the six cultivars examined is illustrated in Figure 1. It is apparent that ABA content of mature fruit increased throughout the duration of colour development (Figure 1). Only after development of colour was there an appreciable decline in ABA content, presumably due to enhanced catabolism and formation of ABA-conjugates (Harris and Dugger, 1986). Although the time taken to reach maximum ABA content was similar for all cultivars examined (37–39 weeks post anthesis), it is noteworthy that the late-maturing non-Navel oranges (viz. 'Midknight' and 'Moss Seedless') accumulated substantially more ABA, and colour development in these cultivars occurred visibly later (Figure 1). This observation may suggest that elevated endogenous ABA levels contribute to the apparent retardation of colour development in fruit of these cultivars. This is perhaps not surprising

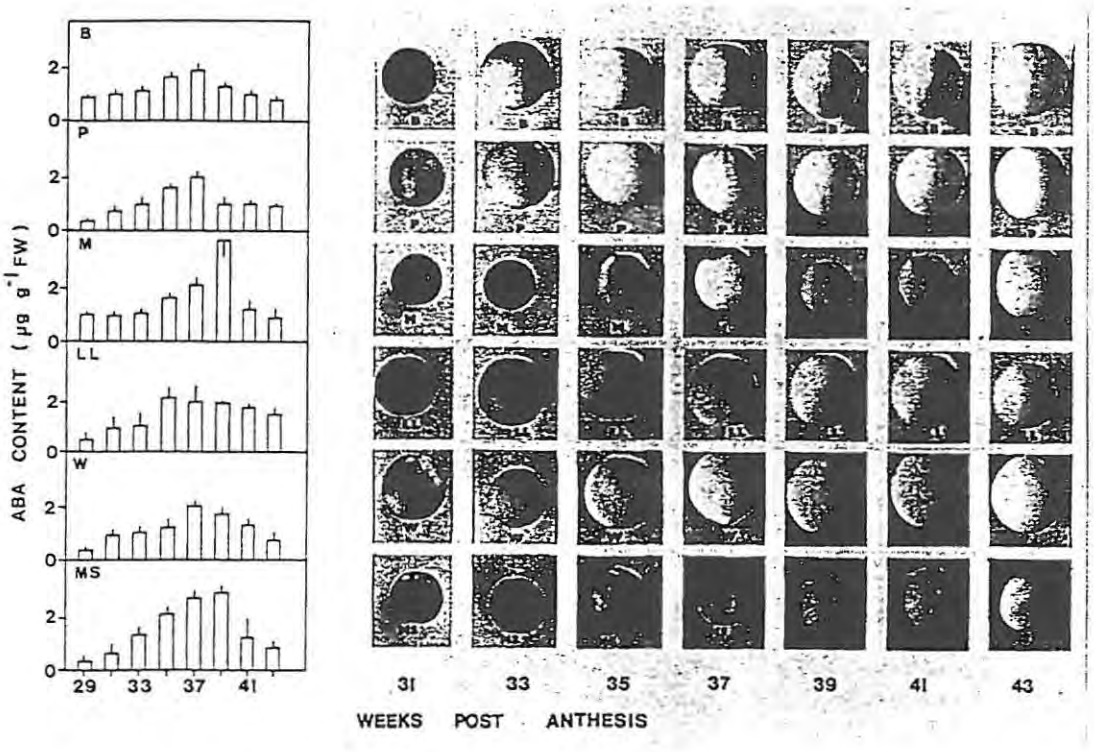

FIG. 1
The abscisic acid content in relation to colour development in flavedo from fruit of *Citrus sinensis* cvs Bahianinha (B), Palmer Navel (P), Midknight (M), Lane Late Navel (LL), Washington Navel (W) and Moss Seedless (MS). Changes in abscisic acid content and visual colour development of mature *Citrus* fruit. Abscisic acid data are expressed as the mean ± SE of quantifications made during the 1991 and 1992 seasons.

given that ABA participates in the regulation of terpenoid metabolism by feed-back inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA) reductase (Russell, 1985; Moore and Oishi, 1994), a key enzyme in the biosynthesis of isoprenoids and carotenoids (Bach and Lichtenthaler, 1983; Gray, 1987).

Analysis of the carotenoid composition of flavedo tissue at colour break (June) and after colour development revealed considerable differences and these are shown in the chromatograms presented in Figure 2. The pigment composition of flavedo tissue at colour break resembled closely that of photosynthetically active leaves and the major components were identified as neoxanthin, violaxanthin, antheraxanthin, lutein, zeaxanthin, β-cryptoxanthin, chlorophyll b, chlorophyll a and β-carotene (Figure 2A). Also present was β-citraurin, a characteristic component of extracts of *Citrus* flavedo (Gross, 1981). Following full development of colour, major differences in pigment composition included loss of chlorophyll and a substantial decline in levels of neoxanthin, violaxanthin, lutein and β-carotene, with an accompanying increase in antheraxanthin and the formation of violaxanthin monoesters and other unidentified xanthophyll acyl esters (Figure 2B). Quantification of these differences in pigment composition is presented in Table I. Although Goldschmidt (1976) concluded that there was little evidence to support a relationship between ABA and pigmentation in *Citrus* fruit peel (based on analysis of ABA levels in variegated lemon fruit), results from the

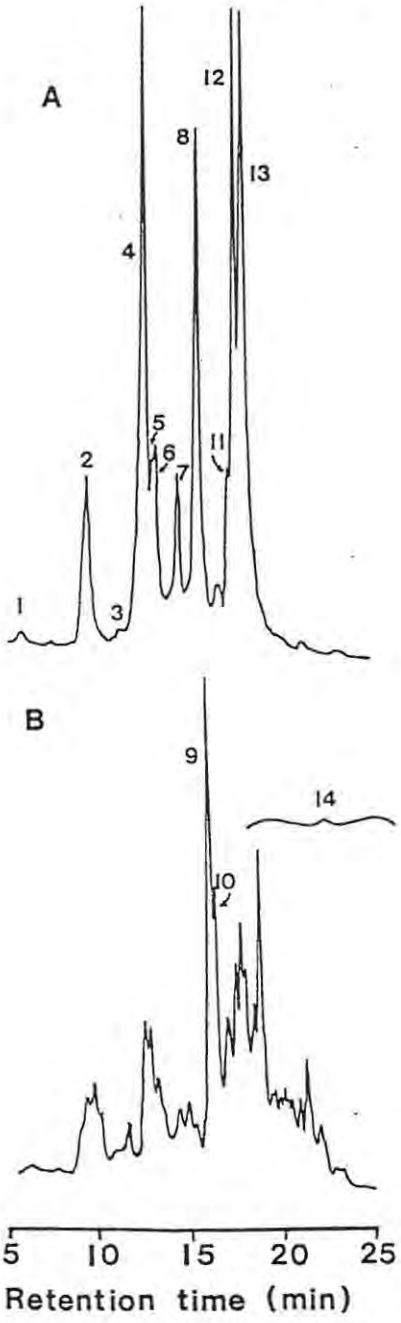

FIG. 2
Representative high performance liquid chromatographic profiles illustrating the pigment composition of flavedo from *Citrus sinensis* cv. Midknight fruit at colour break (A) and after development of full colour (B). Peaks: (1) neoxanthin, (2) violaxanthin, (3) antheraxanthin, (4) lutein, (5) zeaxanthin, (6) β-cryptoxanthin, (7) chlorophyll *b*, (8) chlorophyll *a*, (9) violaxanthin ME1, (10) violaxanthin ME2, (11) β-citraurin ME1, (12) β-citraurin ME2, (13) β-carotene and (14) unidentified xanthophyll acyl esters.

TABLE I
Pigment concentration of Citrus sinensis *cv. Midknight flavedo at colour break and after development of colour. Only data for identified pigments are presented*

Pigment	Concentration (μg g^{-1} fresh weight) Colour break	Full colour
Neoxanthin	0.50	tr.[a]
Violaxanthin	6.91	1.26
Antheraxanthin	0.10	1.78
Lutein	15.82	3.14
Zeaxanthin/ β-cryptoxanthin	6.73	6.46
Chlorophyll *b*	49.32	ND[b]
Chlorophyll *a*	164.91	ND
Violaxanthin ME	ND	17.99
β-citraurin ME	8.21	9.11
β-carotene	1.41	0.28
Total carotenoids	48.32	90.18

[a]trace amounts only.
[b]not detected.

present study indicate the contrary. For example: 1), a rise in endogenous ABA levels coincides with the onset of coloration but full development of colour occurs only after a decline in ABA levels (i.e. high endogenous ABA levels at colour break delay full development of colour), and 2), colour development is associated with an accumulation of xanthophyll acyl esters at the expense of free xanthophylls, the proposed progenitors of ABA. In the light of these observations we propose that a decline in free ABA due to increased capacity for ABA metabolism (Cowan and Richardson, 1993b) and conjugation (Harris and Dugger, 1986), coupled with a loss of unesterified xanthophylls would have the overall effect of reducing the pool of free ABA available for down regulation of HMGCoA-reductase activity and hence the synthesis of isoprenoids and carotenoids. Carotenoid concentration of *Citrus* flavedo has been shown to increase by as much as 462.5% during colour development (Gross, 1981).

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APPENDIX 4

GAYNOR R RICHARDSON AND A KEITH COWAN

Development of an abscisic acid biosynthesizing cell-free system from flavedo of *Citrus sinensis* fruit.

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Development of an abscisic acid biosynthesizing cell-free system from flavedo of *Citrus sinensis* fruit.

by

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Abstract

Cell-free extracts prepared from an acetone powder derived from the flavedo of mature, coloured fruits of *Citrus sinensis* L. cv. midknight transformed mevalonic acid, isopentenyl pyrophosphate, all-*trans*-β-carotene and 1',4'-*trans*-ABAdiol into ABA. This is the first report of a cell-free system capable of producing ABA from both a terpenyl pyrophosphate and carotenoid origin. ABA biosynthesizing activity was stimulated by reduced nicotinamide nucleotides, molybdate, AMO1618 and a cold-pool trap of (±)-ABA but was inhibited by FAD. Addition of FAD caused accumulation of label from isopentenyl pyrophophate in a compound with similar chromatographic and spectroscopic properties to those of β-carotene. 1',4'-*Trans*-ABAdiol, ABA and PA were unequivocally characterised as products of β-carotene metabolism in this cell-free system, a process that was markedly improved by extraction of enzyme in the presence of detergents. Stigmasterol, and to a lesser extent cholesterol reduced conversion of β-carotene to ABA but did not influence transformation of 1',4'-*trans*-ABAdiol to ABA. AMO1618 stimulated formation of ABA and appeared to exert its effect at the level of conversion of 1',4'-*trans*-ABAdiol to ABA. Ancymidol and zeatin reduced incorporation of label from all-*trans*-β-carotene into ABA suggesting involvement of a mixed function oxidase in this reaction sequence. Sodium dodecylsulphate polyacrylamide gel electrophoresis of the enzyme extract derived from *Citrus* flavedo revealed the presence of a 53 kD protein with peroxidase activity chacteristic of a cytochrome P-450.

Key words: Abscisic acid, biosynthesis, cell-free system, *Citrus sinensis*, flavedo, Rutaceae.

Introduction

The biosynthetic route to the sesquiterpenoid growth regulator, abscisic acid (ABA), in higher plants remains a contentious issue. ABA can be produced by either, a direct pathway involving a C-15 precursor derived from farnesyl pyrophosphate (FPP), or an indirect pathway involving cleavage of a C-40 carotenoid precursor. The arguments for and against each of these pathways have been extensively reviewed in recent publications (Zeevaart and Creelman, 1988; Creelman, 1989; Parry and Horgan, 1991; Taylor, 1991; Zeevaart *et al.*, 1991). Thus results from studies using stable isotopes, inhibitors of carotenogenesis and mutants deficient in both carotenoids and ABA tend to favour the production of ABA from a carotenoid origin (Parry, 1993). However, a recent investigation into the biosynthetic origin of ABA has revealed firstly, that there are two precursor pools involved in ABA synthesis and that neither consists of carotenoids (Willows *et al.*, 1994) and secondly, that the endogenous precursor of stress-induced ABA is an as yet unidentified compound (Netting and Milborrow, 1994). Furthermore in the absence of detailed information concerning the enzymology of ABA synthesis, the pathway of formation of this plant growth regulator from FPP, whether direct or indirect, must remain equivocal. The limited success of the aforementioned approaches to resolve the controversy surrounding the origin of ABA in higher plants suggests that an alternative be sought. One possibility is to develop cell-free systems, capable of synthesizing ABA from "simple" precursors, in which to study more detailed aspects of the biochemistry and enzymology of the post-FPP transformations. This approach has been successfully employed to study the biosynthesis (Graebe, 1987) and enzymology (Lange and Graebe, 1993) of gibberellins.

Surprisingly, only a few studies have attempted to demonstrate the cell-free biosynthesis of ABA and for the most part these have met with little success. A cell-free system of lysed chloroplasts was used to demonstrate ABA biosynthesis from mevalonic acid (MVA) but activity was low and no intermediates were reported (Milborrow, 1974). Unfortunately all subsequent attempts to demonstrate ABA biosynthesis in chloroplast isolates, whether intact or lysed, have proved unsuccessful (Hartung *et al.*, 1981; Cowan and Railton, 1986). More recently, a cell-free system derived from imbibed embryos of *Hordeum vulgare* was shown to synthesize a range of terpenyl pyrophosphates from labelled MVA and to transform MVA and isopentenyl pyrophosphate (IPP) into an acid with similar chromatographic properties to those of ABA (Cowan and Railton, 1987). Activity was however low and the identity of the acid as ABA was not unequivocally established. By comparison greater success has been achieved using putative post-FPP intermediates as substrates in ABA-biosynthesizing cell-free systems.

The *in vitro* transformations of xanthoxin (Xan), xanthoxin acid (Xan-acid), ABA-aldehyde and 1',4'-*trans*-ABAdiol into ABA have been demonstrated in extracts prepared from *Phaseolus vulgaris* leaves and turgid and water-stressed leaves of wild type *Lycopersicon esculentum* and the wilty mutants *sitiens*, *notabilis* and *flacca* (Sindhu and Walton, 1987; 1988; Sindhu *et al.*, 1990). Insignificant conversion was obtained when xanthoxin acid and 1',4'-*trans*-ABAdiol were used as substrates. However, xanthoxin was converted to ABA in good yield in extracts of *Phaseolus vulgaris* leaves, and both xanthoxin and ABA-aldehyde were transformed into ABA in extracts from leaves of wild type *Lycopersicon esculentum* and the wilty mutant, *notabilis*. While these results are consistent with a role for xanthoxin as an intermediate en route to ABA and suggest that ABA-aldehyde is the final ABA precursor, the biosynthetic origin of these compounds has yet to be unequivocally established.

Reactions in sesquiterpenoid synthesis occur slowly and the enzymes involved are usually at low intracellular concentrations (Alonso and Croteau, 1993). Thus it might be expected that it would be difficult to detect putative post-FPP intermediates such as xanthoxin, xanthoxin acid, ABA-aldehyde, 1',4'-*trans*-ABAdiol, and product ABA, in *in vitro* systems. We have attempted to resolve this problem by utilizing, as a source of enzyme, plant tissue with high prenyltransferase and cyclase activity. In addition, cell-free extracts of this tissue have been successfully used to study the *in vitro* synthesis of sesquiterpenoid alcohols and aldehydes (Chayet *et al.*, 1973; Perez *et al.*, 1980; De La Fuente *et al.*, 1981). We have thus demonstrated that similar cell-free extracts of *Citrus* flavedo incorporate label from MVA into 1',4'-*trans*-ABAdiol, ABA and phaseic acid (PA) and that extracts of an acetone powder of exocarp tissue convert all-*trans*-β-carotene to ABA (Cowan and Richardson, 1993a; 1993b). We now report details of the development of this cell-free system and present data to support a precursor role for 1',4'-*trans*-ABAdiol in ABA biosynthesis in this cell-free system.

Materials and methods

Labelled and non-labelled substrates

(*R,S*)-[2-^{14}C]-ABA (specific activity 833 MBq mol m^{-3}), 3*R*-[2-^{14}C]-MVA (specific activity 1.92 GBq mol m^{-3}) and [1-^{14}C]-IPP (specific activity 1.96 GBq mol m^{-3}) were purchased from Amersham International, Buckinghamshire, UK. [^{14}C]-All-*trans*-β-carotene (specific activity 31 MBq mol m^{-3}) was prepared biosynthetically from NaH[^{14}C]O_3 in axenic cultures of the unicellular green alga *Dunaliella bardawil* as previously described (Cowan and Richardson, 1993b) and stored in hexane at -20°C. Labelled and non-labelled 1',4'-*trans*-ABAdiol was prepared as described by Cowan and Railton (1986) and used immediately. Authentic all-*trans*-β-carotene and (*R,S*)-ABA were obtained from Sigma Chemical Co., St Loius, MO.

Preparation of cell-free extracts

Mature fruits of *Citrus sinensis* L. Osbeck cv. Midknight were kindly supplied by Riverside Enterprises, Fort Beaufort and Amanzi Estates, Uitenhage, South Africa. Fruit was harvested between June and August following development of colour, surface sterilized in 1% sodium hypochlorite and thoroughly rinsed with several changes of distilled water. Crude extracts were prepared as follows; the flavedo (exocarp) was grated off the whole fruit and the gratings homogenized at 0°C for 3 min, using an Ultra-Turrax top-drive homogenizer, in 0.1 M Tris-HCl buffer (pH 7.4) equivalent to 1.5 times the fresh weight. Acetone powder was prepared from freshly-grated exocarp tissue according to the method of Perez *et al.* (1980) and amounts of the dried powder, homogenized for 5 min at 0°C in

0.1 M Tris-HCl buffer (pH 7.4, 2 ml g^{-1} powder) followed by sonnication for 30 sec (3 by 10 sec bursts).

Homogenates were filtered through two layers of cheesecloth and centrifuged at 2°C for 30 min at 23 500 g in a Sorvall-RC5 refrigerated centrifuge. The supernatant was used as a source of enzyme activity following determination of the protein concentration (Bradford, 1976). Where specified, the 23 500 g supernatant from crude tissue homogenates was dialysed for 2 h agianst 20 mol m^{-3} Tris-HCl buffer (pH 7.4) at 0°C.

Incubation procedure

Incubations were carried out essentially as described by Cowan and Richardson (1993a). Routinely 2.5 ml of the 23 500 g supernatant (equivalent to 0.25-2.5 mg protein) was incubated in 50 mol m^{-3} Tris-HCl buffer (pH 7.4) containing glutathione (10 mol m^{-3}), sodium fluoride (5 mol m^{-3}), ATP (5 mol m^{-3}), $MgCl_2$ (12.5 mol m^{-6}), AMO 1618 (1 mol m^{-3}) and various combinations of NAD^+, NADH, NADPH, FAD and molybdate in a total volume of 5 ml. Where specified, non-labelled (*R,S*)-ABA (0.2 mol m^{-3}) was included as a cold-pool trap. Following addition of substrate, reactions were initiated by adding enzyme and allowed to proceed for 2 h (unless otherwise stated) at 37°C under continuous low-light illumination in a shaking water bath. Reactions were terminated by heating to 100°C for 3 min and protein precipitated by addition of an equal volume of ice-cold methanol, after incubates had been cooled to room temperature. Heat-inactivated (100°C for 10 min) were included as controls.

Extraction and analysis of products

Precipitated protein was removed by centrifugation and the pellet extracted three times with 5 ml volumes of ice-cold methanol/ethyl acetate containing butylated hydroxytoluene as an antioxidant. Where non-labelled products were to be analysed, a small amount of [^{3}H]-ABA was added to correct for losses during purification. Combined organic extracts were reduced to a small aqueous volume *in vacuo* and partitioned against diethyl ether to remove neutral and basic compounds. The pH of the aqueous fraction was then adjusted to 2.5 and the acids partitioned into diethyl ether. Diethyl ether-soluble acids were purified on Sep-pak C_{18} cartridges by sequential washing with 1% aqueous acetic acid followed by 20% methanol/1% aqueous acetic acid and ABA and related compounds eluted from the cartridge with 65% methanol/1% aqueous acetic acid. Purified acid fractions were analysed on thin layers of silica gel GF_{254} in toluene/ethyl acetate/acetic acid (25:15:2, v/v) and/or by reversed-phase HPLC on a 10 μm Bondaclone (Phenomenex, Torrance, CA) column (150 x 3.9 mm i.d.) eluted isocratically with methanol/water/acetic acid (40:60:1, v/v) at 1 ml min^{-1}, after recovery of compounds of interest from silica gel. Detection was at 254 nm using a Linear UV-Vis detector coupled to a Spectra-Physics SP4290 integrator. Distribution of radioactivity was determined by liquid scintillation spectrometry (Cowan and Richardson, 1993a).

Extracts containing non-labelled products were likewise separated either by thin layer chromatography or reversed-phase HPLC (see above) and zones co-chromatographing with authentic 1',4'-*trans*-ABAdiol, ABA and PA collected, methylated with excess diazomethane and rechromatographed on silica gel in *n*-hexane/ethyl acetate (1:1, v/v). The UV-absorbing bands co-eluting with authentic 1',4'-*trans*-ABAMediol, ABAMe and PAMe

were recovered into diethyl ether and quantified by gas chromatography-electron capture (GC-EC) as described by Afithlile *et al.* (1993). Unequivocal identification was established by combined capillary gas chromatography-mass spectrometry (GC-MS) as previously described (Cowan and Richardson, 1993a; 1993b).

Detection of cytochrome P-450 by SDS-PAGE

Proteins in the 23 500 g supernatant were separated electrophoretically on 12.5% polyacrylamide slab gels specifically under non-reducing conditions according to the method of Laemmli (1970). Gels were stained with Coomassie blue and cytochrome P-450 (P-420) detected by staining for the peroxidase activity with 3,3',5,5'-tetramethylbenzidine (TMBZ)-H_2O_2 as described by Thomas *et al.* (1976).

Results

Activity of cell-free preparations and co-factor requirements

Results in Table 1 provide a comparative assessment of the ABA-biosynthesizing activity of cell-free extracts prepared from crude, dialysed crude and acetone powder homogenates of *Citrus* flavedo supplied with 3*R*-[2-^{14}C]-MVA as substrate. The products were identified by microchemical analysis, co-chromatography and combined GC-MS (Cowan and Richardson, 1993a). It is evident that activity was enhanced almost 2-fold in extracts prepared from acetone powder homogenates. Heat-inactivated extracts showed no detectable enzyme activity. The influence of protein concentration, pH and the kinetics of incorporation of label from MVA into ABA, PA and 1',4'-*trans*-ABAdiol are shown in Fig. 1.

Reduced nicotinamide nucleotides enhanced ABA-biosynthesizing activity of crude extracts and acetone powder homogenates (Table 2). Addition of molybdate further increased enzyme activity. Although incorporation of label from both MVA and IPP into ABA and related acids was always greater in homogenates of acetone powder extracts, the effect of NADH, NADPH and molybdate on enzyme activity while stimulatory, was less marked in these preparations.

Several studies have implicated a molybdenum-requiring soluble oxidase as the terminal enzyme in ABA biosynthesis (Walker-Simmons *et al.*, 1989; Sindhu *et al.*, 1990). Likewise, the present work has shown that molybdate stimulates ABA-biosynthesizing activity in cell-free extracts prepared from *Citrus* flavedo. The functional regions in the amino acid sequence of molybdenum enzymes comprise both Mo-pterin and FAD binding domains (Wootton *et al.*, 1991). Since addition of molybdate enhanced ABA biosynthesis from MVA and IPP (Table 2), it was important to determine the effect of FAD on enzyme activity. Results in Table 3 show that addition of FAD inhibited incorporation of label from both MVA and IPP into ABA. Furthermore, the inhibitory effect of FAD was concentration dependent. FAD is an essential co-factor in the desaturation of phytoene to lycopene and for cyclization of the latter during formation of β-carotene (Bramley, 1985; Cunningham *et al.*, 1994). Analysis of the β-carotene-containing fraction by reversed phase HPLC and liquid scintillation spectrometry revealed accumulation of radioactivity in a 450 nm absorbing compound with identical chromatographic properties to those of authentic β-carotene. Unfortunately, insufficient mass precluded unequivocal identification of this pigment as β-carotene. Nevertheless, as shown in Table 4 incorporation of radioactivity into the β-carotene-like component was substantially enhanced in the presence of FAD. While this observation may account for the reduced incorporation of label into ABA in the presence of FAD, it suggested more importantly that β-carotene might be a precursor en

route to ABA in this cell-free system.

ABA biosynthesis from β-carotene

When [^{14}C]-β-carotene was used as substrate in acetone powder homogenates it was converted into radioactive acids that co-chromatographed with 1',4'-*trans*-ABAdiol, ABA and PA respectively (Table 5). ABA-biosynthesizing activity was increased nearly 7-fold with β-carotene as substrate (cf. Table 1). The radioactive acid tentatively assigned as ABA, was unequivocally identified by capillary GC-MS (Cowan and Richardson, 1993b). Although similar cell free extracts incorporated label from both MVA and IPP into ABA, the presence of non-labelled ABA in these extracts, as a cold-pool trap, made unequivocal identification of the radioactive acid an impossible task (data not shown). Since the acetone powder was essentially free of background ABA (< 1 ng 100 g^{-1} powder as determined by GC-EC) non-labelled all-*trans*-β-carotene was used as substrate to generate sufficient mass to enable unequivocal identification of the acidic products. Electron impact mass spectral analysis of the methyl ester derivatives confirmed the identity of 1',4'-*trans*-ABAdiol, ABA and PA as products of β-carotene metabolism in this cell free system.

The majority of carotenogenic enzymes are membrane proteins and solubilization is essential for catalytic activity. Although acetone powders routinely yield soluble, enzymatically active preparations (Bramley, 1985) attempts were nevertheless made to increase ABA-biosynthesizing activity using detergents. The results in Table 6 show that both Tween 20 and Triton X100 increased catalytic activity at 0.1% w/v. However, at a concentration of 0.5% w/v these detergents were much less effective.

1',4'-trans-*ABAdiol: precursor or product?*

In attempting to define the origin of 1',4'-*trans*-ABAdiol, the metabolism of both [2-^{14}C]-ABA and [2-^{14}C]-1',4'-*trans*-ABAdiol was investigated and the results are shown in Table 7. When [^{14}C]-ABAdiol was used as substrate it was readily converted into ABA in extracts of acetone powder homogenates, a process that was enhanced by AMO 1618. Results show that approximately 78% of the applied label was incorporated into ABA during the 2 h incubation procedure. The balance of radioactivity was distributed between substrate 1',4'-*trans*-ABAdiol and PA. Omission of non-labelled ABA decreased the yield of product [^{14}C]-ABA. In contrast, when [2-^{14}C]-ABA was used as substrate very little conversion to 1',4'-*trans*-ABAdiol was detected. Furthermore omission of either AMO 1618 or unlabelled ABA appeared to have no significant effect on this reaction. These results suggest that 1',4'-*trans*-ABAdiol is formed as an intermediate en route to ABA in extracts prepared from acetone powder homogenates.

The conversion of 1',4'-*trans*-ABAdiol to ABA involves oxidation whereas the reverse reaction requires reduction of the 4' keto function. Since biosynthesis of ABA is apparently dependent on molybdenum as a co-factor (Walker-Simmons *et al.*, 1989) and conversion of 1',4'-*trans*-ABAdiol to ABA would be expected to involve an oxidase enzyme, the co-factor requirements of this conversion were examined. The results shown in Table 8 indicate that conversion of 1',4'-*trans*-ABAdiol to ABA is stimulated by molybdate. Ommission of NADPH had little or no effect on enzyme activity. Likewise addition of FAD, a prosthetic group of some molybdenum-requiring enzymes, did not appear to influence enzyme activity. However FAD, in the absence of molybdate reduced enzyme activity appreciably.

Effect of ancymidol and zeatin on ABA biosynthesis

Several reports have indicated that cytokinins and the cytokinin analogue, ancymidol, inhibit ABA biosynthesis both *in vivo* and *in vitro* (Norman *et al.*, 1983a; 1983b; Cowan and Railton, 1987a; 1987b). Likewise, in the present study both ancymidol and zeatin reduced incorporation of label from β-carotene into ABA (Table 9). The formation of PA was not substantially influenced by the lower concentrations of both ancymidol and zeatin but was markedly inhibited by ancymidol at 0.5 mol m^{-3}. Interestingly, reduced incorporation of label into ABA, PA and 1',4'-*trans*-ABAdiol in extracts supplied with ancymidol and zeatin, occurred concomitant with increased incorporation of label into two neutral compounds that were detected following TLC analysis of the diethyl ether fraction obtained by partitioning the aqueous phase at pH 8.5. These products were tentatively identified as xanthoxin-alcohol (Xan-alc) and Xan by cochromatography, following feeds of non-labelled β-carotene, and by UV absorbance in methanol with maxima at 240 and 281 nm respectively (Taylor and Burden, 1972).

Effect of sterols and gibberellic acid on ABA biosynthesis

The ability of the kaurene synthetase and squalene oxide cyclase inhibitor, AMO 1618, to enhance incorporation of radioactivity from either MVA or IPP into terpenyl pyrophosphates and ABA, both *in vivo* and *in vitro* (Davies *et al.*, 1975; Milborrow, 1976; Cowan and Railton, 1987), coupled with the apparent requirement for this inhibitor during conversion of 1',4'-*trans*-ABAdiol to ABA suggested the possibility that either gibberellins or sterols, or intermediates en route to these compounds, interfere with ABA biosynthesis. In order to investigate this possibility the effect of stigmasterol, cholesterol and gibberellic acid (GA_3) on the conversion of all-*trans*-β-carotene and 1',4'-*trans*-ABAdiol to ABA was

examined. The results in Table 10 show that stigmasterol dramatically reduced incorporation of label from β-carotene into ABA. Furthermore, inhibition by stigmasterol was apparently concentration dependent. Cholesterol, a minor component of thylakoid membranes (Hartmann and Benveniste, 1987), was a much less effective inhibitor. GA_3 had no effect on conversion of β-carotene to ABA. By comparison, none of the compounds tested influenced conversion of 1',4'-*trans*-ABAdiol to ABA.

Detection of cytochrome P-450

Using a cell-free system derived from imbibed barley embryos we were able to demonstrate that incorporation of label from MVA into ABA was inhibited by both anaerobiosis and CO suggesting the participation of mixed function oxygenases in ABA biosynthesis (Cowan AND Railton, 1987a). This, coupled with the observations that ancymidol reduces incorporation of label into ABA in both the barley embryo and *Citrus* exocarp systems suggested the presence of a cytochrome P-450 enzyme. The results presented in Figure 2 illustrate the polypeptide pattern obtained following electrophoresis of the 23 500 g supernatant from homogenates of *Citrus* flavedo acetone powder. Treatment of unstained gels with TMBZ-H_2O_2 revealed the presence of a 53 kD protein with peroxidase activity characteristic of cytochrome P-450 (Thomas *et al.* 1976).

Discussion

This paper describes the development of a cell-free system prepared from *Citrus sinensis* flavedo that is capable of synthesizing ABA from MVA, IPP, all-*trans*-β-carotene and 1',4'-*trans*-ABAdiol. The results presented therefore support a carotenoid origin for ABA and suggest that the pathway proceeds from all-*trans*-β-carotene via 1',4'-*trans*-ABAdiol.

Although we concluded in a previous report that 1',4'-*trans*-ABAdiol was formed as a catabolite of ABA in similar cell-free preparations (Cowan and Richardson, 1993a), refeeding studies using both labelled ABA and 1',4'-*trans*-ABAdiol were not carried out to confirm this finding. The present study has revealed that 1',4'-*trans*-ABAdiol was readily converted to ABA and that this reaction was not the result of spontaneous oxidation in the *Citrus* cell-free system. 1',4'-*Trans*-ABAdiol may therefore be the immediate precursor to ABA in this cell-free system. Furthermore, a requirement for molybdate supports current conjecture that a molybdenum-containing oxidase enzyme catalyses the terminal step in ABA biosynthesis. The present study has shown that this enzyme is apparently unaffected by NADPH and FAD, inhibited by FAD in the absence of molybdate and dependent upon NAD^+. A requirement for NAD^+ or NADP (Sindhu and Walton, 1987) suggests that the enzyme concerned is a molybdenum-containing hydroxylase possibly of the $NAD(P)^+$-dependent dehydrogenase form (Wootton *et al.*, 1991).

While the identity and metabolic interrelationship of intermediates between β-carotene and 1',4'-*trans*-ABAdiol remain obscure, the following sequence is proposed based on analysis of products formed when non-labelled β-carotene is used as substrate; 9-*cis*-violaxanthin, Xan-alc, Xan, Xan-acid, 1',4'-*trans*-ABAdiol, ABA and PA. The routine appearance of Xan-alc and Xan as products of β-carotene metabolism in the *Citrus* cell-free system suggested involvement of a cytochrome P450-type mixed function oxygenase that catalyzes the successive oxidation of the side chain of the product of carotenoid cleavage. Coolbaugh (1984) has shown that both cytokinins and ancymidol can interact with cytochrome P450-mixed function oxidases in GA biosynthesis to inhibit kaurene oxidation in plants. Similarly, ancymidol and cytokinins, both inhibitors of ABA biosynthesis *in vivo* and *in vitro* (Cowan and Railton, 1987a; 1987b), significantly reduced incorporation of label from [^{14}C]-β-carotene into 1',4'-*trans*-ABAdiol and ABA with a concomitant rise in

Xan-alc and Xan. Further details of this proposed reaction sequence will be published elsewhere.

AMO 1618 has long been recognised as an inhibitor of *ent*-kaurene synthetase and squalene 2,3-oxidocyclase although it showed no activity against the squalene cyclase from pea microsomes (Duriatti *et al.*, 1985). Even so, the cyclizations of oxidosqualene and GGPP are mechanistically similar and cationic compounds such as AMO 1618 would be expected to inhibit these reactions (Hedden, 1990). The cyclization of lycopene to *β*-carotene might similarly be effected thereby reducing incorporation of labelled MVA and terpenyl pyrophosphates into ABA, particularly since ABA appears to be derived from a carotenoid origin. However, we have demonstrated that the *Citrus* cell-free system converts both FPP and GGPP to ABA in the presence of AMO 1618 (Richardson and Cowan, unpublished) and in the current study, AMO 1618 was shown to enhance ABA biosynthesis from all substrates used. Milborrow (1976) demonstrated that addition of AMO 1618 substantially increased the incorporation of MVA into ABA *in vivo* in avocado mesocarp. In addition, AMO 1618 stimulated production of ABA by *Botrytis cinerea*, an ABA-producing fungus from which 1',4'-*trans*-ABAdiol was isolated in mass amounts and shown to be converted into ABA (Hirai *et al.*, 1986). This suggests that the growth retarding property of AMO 1618 is related to its ability to facilitate enhanced biosynthesis of ABA.

It has been postulated that the inhibitory effect of AMO 1618 and related plant growth retardants is, in addition to interference with the A activity of kaurene synthetase (Graebe, 1987), due partly to inhibition of sterol biosynthesis (Douglas and Paleg, 1981). Also, plant sterols inhibit ABA-induced perturbations in phospholipid bilayers (Stillwell *et al.*, 1990) suggesting that ABA and sterols are antagonistic. Results from the present work

demonstrate that stigmasterol, an abundant plant membrane sterol (Hartmann and Benveniste, 1987), markedly reduced ABA biosynthesis from β-carotene but was ineffective when 1',4'-*trans*-ABAdiol was used as substrate. This is perhaps not surprising, given that the latter reaction would be expected to involve a soluble enzyme whereas the former interconversions are probably catalysed by membrane-bound enzymes. A possible point of interaction between AMO 1618, sterols and ABA could be cytochrome P-450. Both sterol and ABA metabolism involve at least one cytochrome P-450-catalysed reaction. For example, the enzyme catalyses the three oxygen-requiring stages during demethylation of 14α-methylsterols (Burden *et al.*, 1989) and the conversion of ABA to PA (Gillard and Walton, 1976). Furthermore, sterol cytochrome P-450-dependent enzyme inhibitors inhibited ABA biosynthesis in the fungus *Cercospora rosicola* whereas inducers of cytochrome P-450 (e.g. dithiothreitol) enhanced ABA synthesis in this organism (Norman *et al.* 1988). In fact, to obtain Xan oxidase activity from leaves of *Lycopersicon esculentum* it was necessary for Sindhu and Walton (1988) to include dithiothreitol in the extraction buffer which suggests that conversion of Xan to ABA involves a cytochrome P-450 catalysed reaction.

Evidence has been presented to suggest that activity of plant cytochrome P-450 is dependent on developmental stage of the tissue with high cytochrome P-450 activity in young and/or non-photosynthetic tissue such as fruit (Hendry, 1986). Mature, coloured *Citrus* flavedo would therefore be expected to be a rich source of cytochrome P-450. A minor protein detected following analysis of *Citrus* cell-free extracts by SDS-PAGE was shown to contain heam-associated peroxidase activity, characteristic of cytochrome P-450 (P-420). Whether or not this protein is intimately involved in the biosynthesis of ABA remains to be determined. Nevertheless, inhibition of ABA synthesis by ancymidol and stigmasterol coupled with detection of a cytochrome P-450 protein in *Citrus* cell-free

extracts suggests the involvement of a membrane-bound enzyme-catalysed reaction sequence, typical of mixed function oxygenases, in ABA biosynthesis.

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Figure Headings

Fig. 1. Effect of protein concentration (a) and pH (b) and the reaction kinetics (c) of incorporation of label from 3R-[2-^{14}C]-MVA into ABA and related acids (1′,4′-*trans*-ABAdiol + ABA + PA) in crude (○) and acetone powder (■) homogenates prepared from flavedo of *Citrus sinensis*. Conditions of incubation and analysis were as described for Table 1.

Fig. 2. SDS-PAGE analysis of proteins in extracts prepared from acetone powder homogenates of *Citrus sinensis* flavedo stained with Coomassie Brilliant Blue and detection of a cytochrome P-450 by staining for peroxidase activity with TMBZ-H_2O_2. Aliquots of the extract, equivalent to 5, 10 and 15 μg protein, were analysed by polyacrylamide gel electrophoresis as described in "Materials and Methods". Molecular weight markers are shown on the left side, while the arrow indicates the position of the protein band corresponding to cytochrome P-450.

Table 1. *ABA-biosynthesizing activity in cell-free preparations from* Citrus *flavedo.*

Incubations contained glutathione, NaF, ATP, $MgCl_2$, NAD, AMO 1618 and cold ABA, at the concentrations specified in "Materials and Methods", and were carried out at 37°C for 2 h. Incorporation of label from 3R-[2-^{14}C]-MVA (150 kBq) into 1',4'-*trans*-ABAdiol, ABA and PA was determined by liquid scintillation spectrometry following separation of the diethyl ether-soluble acids by TLC and/or reversed-phase HPLC. Enzyme activity is expressed as radioactivity incorporated into 1',4'-*trans*-ABAdiol + ABA + PA mg protein^{-1} h^{-1}.

Extract	Activity (Bq mg protein^{-1} h^{-1})
Undialysed Crude	158.48
Heat inactivated[a]	ND[b]
Dialysed Crude	211.91
Heat inactivated	ND
Acetone Powder	335.45
Heat inactivated	ND

[a] 100°C for 10 min.

[b] Not detected

Table 2. *Effeect of reduced nicotinamide nucleotides and molybdate on ABA biosynthesis in cell-free preparations from* Citrus *flavedo.*

Complete incubations were as described for Table 1. NADH (12.5 mol m^{-6}), NADPH (12.5 mol m^{-6}) and MoO_4^{-2} (1 mol m^{-6}), were included in the reaction mixture containing either 3R-[2-^{14}C]-MVA or [1-^{14}C]-IPP (50kBq) as substrates. Conditions of incubation and analysis were as described in "Materials and Methods". Data are expressed as [^{14}C] incorporated into ABA mg $protein^{-1}$ h^{-1}.

Extract	Assay Conditions	Substrate	Activity (Bq mg $protein^{-1}h^{-1}$)
Undialysed	Complete	MVA	55.60
Crude	Complete	IPP	204.59
	+NADH	MVA	90.24
	+NADPH	MVA	102.10
	+NADH+NADPH	MVA	123.65
	+NADH+NADPH +MoO_4^{-2}	MVA	157.76
	+NADH+NADPH +MoO_4^{-2}	IPP	404.29
Acetone	Complete	MVA	94.90
Powder	Complete	IPP	249.58
	+NADH	MVA	104.97
	+NADPH	MVA	111.96
	+NADH+NADPH	MVA	173.88
	+NADH+NADPH +MoO_4^{-2}	MVA	201.95
	+NADH+NADPH +MoO_4^{-2}	IPP	449.38

Table 3. *Effect of FAD on ABA biosynthesis in acetone powder homogenates prepared from* Citrus *flavedo.*

Complete acetone powder incubates contained glutathione, NaF, ATP, $MgCl_2$, AMO1618, cold ABA (all at the concentrations specified in "Materials and Methods") and NAD^+, NADH, NADPH and MoO_4^{-2} (as specified in Table 2) and either [2-^{14}C]-MVA (150 kBq) or [1-^{14}C]-IPP (150 kBq) as substrates. FAD was added at the concentrations indicated below. Reactions were for 2 h at 37°C. Data are expressed as [^{14}C] incorporated into ABA mg protein^{-1} h^{-1}.

Assay Conditions	Substrate	Activity (KBq mg protein^{-1} h^{-1})
Complete	MVA	0.16
+0.1 mol m^{-3} FAD	MVA	0.09
+1.0 mol m^{-3} FAD	MVA	0.02
Complete	IPP	10.70
+0.1 mol m^{-3} FAD	IPP	2.45
+1.0 mol m^{-3} FAD	IPP	0.08

Table 4. *Effect of FAD on incorporation of radioactivity from MVA and IPP into β-carotene.*

Assay conditions were as described for Table 3. The neutral diethyl ether fraction was analysed for β-carotene by reversed-phase HPLC using a 5 μm Nucleosil C_{18} column (250 x 4.0 mm i.d.) eluted isocratically with acetonitrile/methanol (9:1, v/v) at a flow rate of 0.8 mL min^{-1}. The eluate was monitored at both 287 and 450 nm and the distribution of radioactivity determined by liquid scintillation spectrometry.

Assay Conditions	Substrate	Radioactivity Incorporated (Bq)
Complete	[2-^{14}C]- MVA	19.0
+0.1 mol m^{-3} FAD	[2-^{14}C]- MVA	58.6
+1.0 mol m^{-3} FAD	[2-^{14}C]- MVA	56.0
Complete	[1-^{14}C]- IPP	1866.7
+0.1 mol m^{-3} FAD	[1-^{14}C]- IPP	4800.0
+1.0 mol m^{-3} FAD	[1-^{14}C]- IPP	3866.7

Table 5. *Conversion of [^{14}C]-all-*trans*-β-carotene to ABA in acetone powder homogenates of* Citrus *flavedo.*

Complete acetone powder incubates were prepared as described for Table 3 and reactions carried out at 37°C for 2 h with [^{14}C]-all-*trans*-β-carotene (10 kBq) as substrate. Incorporation of label into 1',4'-*trans*-ABAdiol, ABA and PA was determined after separation of the diethyl ether-soluble acids first by TLC and then reversed-phase HPLC analysis of the individual acids. Activity is the sum of labelled 1',4'-*trans*-ABAdiol, ABA and PA formed mg protein^{-1} h^{-1}.

	Distribution of radioactivity (kBq mg protein^{-1})			Activity (kBq mg protein^{-1} h^{-1})
	1',4'-*trans*-ABAdiol	ABA	PA	
Experiment 1	1.52	2.03	1.45	2.49
Experiment 2	1.21	1.81	0.82	1.92
Experiment 3	1.09	2.01	0.78	1.95

Table 6. *Effect of detergents on ABA-biosynthesizing activity of acetone powder homogenates of* Citrus *flavedo.*

Complete acetone powder incubates were supplied with synthetic all-*trans*-β-carotene (1 mg) and reactions carried out at 37°C for 2 h. ABA was extracted and quantified by GC-electron capture as described in "Materials and Methods".

Assay conditions	Activity (μg mg protein^{-1} h^{-1})
Complete	1.005
+0.1% w/v Tween 20	4.152
+0.05% w/v Tween 20	2.464
+0.1% w/v Triton x100	5.326
+0.5% w/v Triton x100	2.332

Table 7. *Metabolism of [^{14}C]-1',4'-*trans*-ABAdiol and 2-[^{14}C]-ABA in acetone powder homogenates of* Citrus *flavedo.*

Complete acetone powder incubates were supplied with either (R,S)-[2^{14}C]-ABA (333 Bq) or [^{14}C]-1',4'- *trans*-ABAdiol as substrate and reations allowed to proceed at 37°C for 2 h. The acids were partitioned into diethyl ether at pH 2.5 and the distribution of radioactivity determined by liquid scintillation spectrometry following separation on thin layers of silica gel GF_{254} in toluene/ethyl acetate/acetic acid (25:15:2, v/v).

Assay Conditions	Substrate	Radioactity in product [kBq mg protein^{-1} (%)]	
		1',4'-trans-ABA diol	ABA
Complete	ABA	0.06(0.3)	-
	ABA diol	-	15.68(78.4)
- cold ABA	ABA	0.05(0.2)	-
	ABA diol	-	7.26(36.3)
- AMO 1618	ABA	0.05(0.2)	-
	ABA diol	-	2.84(14.4)
- cold ABA,-AMO1618	ABA	0.05(0.2)	-
	ABA diol	-	1.11(5.5)

Table 8. *Cofactor requirements of conversion of 1',4'-*trans-*ABAdiol to ABA in acetone powder homogenates of* Citrus *flavedo.*

Complete acetone powder homogenates were supplied with [^{14}C]-1',4'-*trans*-ABAdiol (10 kBq) and reactions carried out at 37°C for 2 h and [^{14}C]-ABA extracted and analysed as described for Table 7.

Assay Conditions	Activity (kBq mg protein^{-1} h^{-1})	% Change[a]
Complete	3.58	0.00
-MoO_4^{-2}	1.51	-57.82
-MoO_4^{-2}, -NADPH	1.54	-56.98
+FAD[b]	3.59	0.28
+FAD, -MoO_4^{-2}	0.78	-78.21

[a] % change relative to complete assay conditions

[b] FAD included at 1 mol m^{-3}

Table 9. *Effect of ancymidol and zeatin on ABA biosynthesis in cell-free preparations from* Citrus *flavedo acetone powder homogenates.*

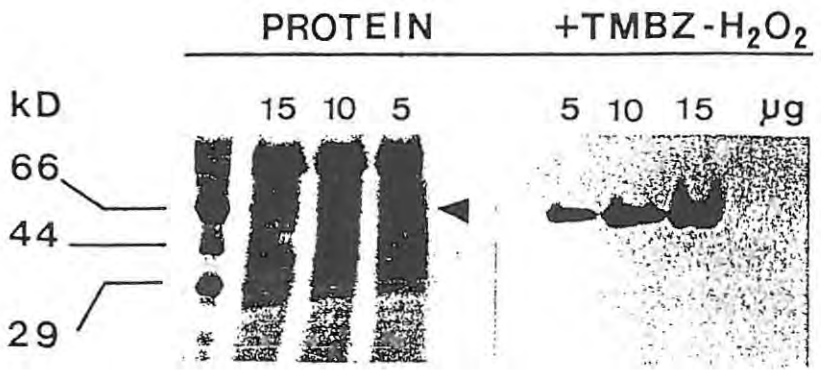
Complete acetone powder homogenates were supplied with [^{14}C]-all-*trans*-β-carotene in the presence or absence of ancymidol and zeatin. Reactions were carried out at 37°C for 2 h and [^{14}C]-ABA and related acids extracted and anlysed as described in "Materials and Methods". Xanthoxin-alcohol and xanthoxin were detected following TLC analysis of the neutral fraction, obtained by partitioning the aqueons phase against diethyl ether at pH 8,5, on silica gel GF_{254} in petroleum ether/acetone 3:1 (v/v) developed three times to 15 cm.

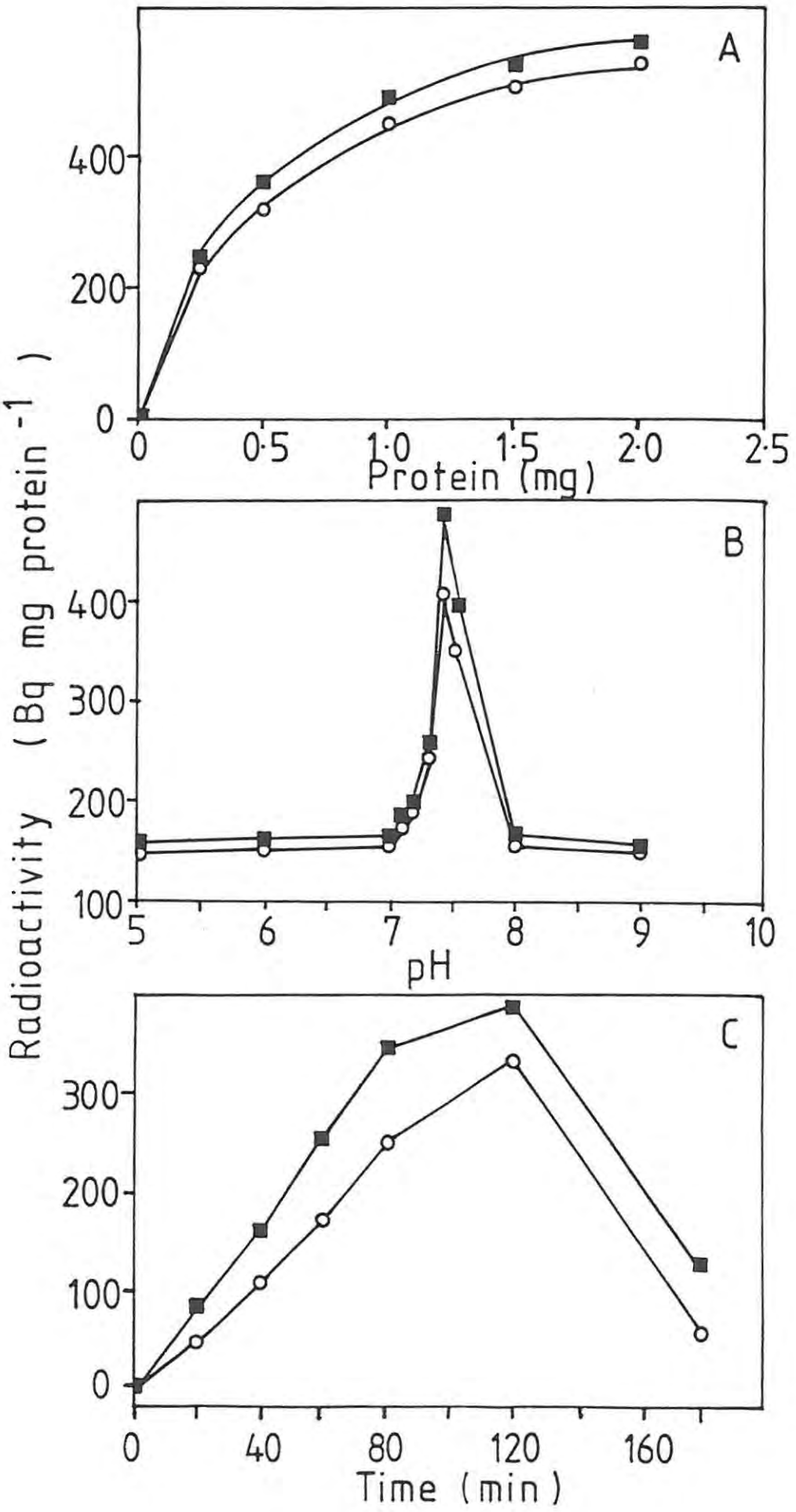
Treatment	Distribution of Radioactivity				
	Xan-Alc	Xan	1',4'-*trans* ABA diol	ABA	PA
	(Bq mg protein^{-1})				
Control	17.41	27.92	34.01	123.80	16.40
+0.1 mol m^{-3} ancymidol	45.86	68.17	10.60	34.53	12.46
+0.5 mol m^{-3} ancymidol	32.27	82.23	11.79	35.47	6.18
+0.1 mol m^{-3} zeatin	24.43	59.75	22.48	61.99	15.39

Table 10. *Effect of sterols and gibberellic acid on conversion of β-carotene and 1',4'-trans-ABAdiol to ABA in acetone powder homogenates of* Citrus *flavedo.*

Complete acetone powder homogenates were supplied with either [^{14}C]-β-carotene or [^{14}C]-1',4'-*trans*-ABAdiol in the presence or absence of stigmasterol, cholesterol and GA_3. Reactions were carried out at 37°C for 2 h and [^{14}C]-ABA extracted and analysed as described in "Materials and Methods".

Treatment	% Inhibition relative to control	
	[^{14}C]-β-carotene	[^{14}C]-1,4'-*trans*-ABAdiol
Control	0.00	0.00
+10 mol m^{-6} stigmasterol	-	0.00
+50 mol m^{-6} stigmasterol	76.35	0.00
+100 mol m^{-6} stigmasterol	84.22	0.00
+100 mol m^{-6} cholesterol	38.86	0.00
+100 mol m^{-6} GA_3	0.00	0.00


PROTEIN
+TMBZ-H2O2
kD
66
44
29
15 10 5
5 10 15 µg


A
400
200
0
0
0·5
1·0
1·5
2·0
2·5
Protein (mg)
B
400
300
200
100
5
6
7
8
9
10
pH
Radioactivity (Bq mg protein -1)
C
300
200
100
0
0
40
80
120
160
Time (min)