THE METABOLISM OF ABSCISIC ACID IN HIGHER PLANT TISSUES

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In memory of
Neil James Douglas Cowan 1932-1979

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ABSTRACT.

The biosynthesis of ABA from *R*-[2-^{14}C]-MVA was demonstrated in *Persea americana* cv. Fuerte mesocarp and in mature seeds of *Hordeum vulgare* cv. Dyan and cv. Himalaya. Radioactivity from *R*-[2-^{14}C]-MVA was also incorporated into the 1',4'-*trans* ABA diol in *Persea americana* mesocarp and a possible role for this metabolite as a precursor of ABA in plants is discussed. The biosynthesis of ABA from MVA could not be demonstrated in either turgid and water-stressed *Hordeum vulgare* cv. Dyan, *Pisum sativum* cv. Black-eyed Susan and *Phaseolus vulgaris* cv. Top-crop or in immature seeds of *Pisum sativum* and *Phaseolus vulgaris*.

(*R,S,*)-[2-^{14}C]-ABA was catabolised to PA, DPA and aqueous conjugates in leaves and mature seeds of *Hordeum vulgare* cv. Dyan, seedlings and immature seeds of *Pisum sativum* and *Phaseolus vulgaris* and in mesocarp from ripening fruits of *Persea americana*. PA and DPA were identified by either microchemical methods and/or capillary GC-MS. 7'-Hydroxy ABA was characterised as a novel ABA catabolite in light-grown and etiolated leaves of *Hordeum vulgare* by capillary GC-MS. Circular dichroism analysis revealed that it was derived predominantly from the (*R*)-enantiomer of ABA. This catabolite was absent in similar studies using the dicotyledons *Pisum sativum* and *Phaseolus vulgaris*. Refeeding studies with [^{14}C]-PA, [^{14}C]-DPA and [^{14}C]-7'-hydroxy ABA were used to confirm the metabolic interrelationship between ABA and its catabolites in both vegetative and non-vegetative tissues from monocotyledonous and dicotyledonous species. The methyl ester of (*R,S,*)-ABA was hydrolysed efficiently by light-grown leaves of *Hordeum vulgare*.

Older, vegetative tissues catabolised (*R,S,*)-ABA more efficiently than their younger counterparts. In contrast, small, immature seeds of *Pisum sativum* catabolised (*R,S,*)-ABA more effectively than larger, immature seeds of this species. Light did not appear to influence ABA biosynthesis but markedly enhanced ABA catabolism. Light stimulated the overall rate of ABA catabolism in both vegetative and non-vegetative tissue. Water stress reduced ABA catabolism in *Hordeum vulgare* leaves but had little effect on this process in *Phaseolus vulgaris* seedlings. Pretreatment of tissues with (*R,S,*)-ABA retarded the catabolism of (*R,S,*)-[2-^{14}C]-ABA, negating ABA-induced conversion to PA. Cycloheximide inhibited ABA biosynthesis and catabolism but did not affect ABA conjugation. Chloramphenicol and lincomycin had little or no effect on ABA metabolism suggesting that the enzymes involved were labile and cytoplasmic in origin. Ancymidol and cycocel

inhibited ABA biosynthesis while AMO1618 stimulated this process. The cytokinins, benzyladenine, kinetin, isopentenyl adenine and zeatin also inhibited ABA biosynthesis. These results are discussed in relation to the possible involvement of carotenoids in ABA biosynthesis. AMO1618, ancymidol and cycocel did not significantly influence the conversion of ABA to PA and DPA while cytokinins appeared to enhance this process only in vegetative tissue. The information derived from these studies was then used in attempts to develop a cell-free system from higher plants capable of metabolising ABA.

A cell-free system prepared from imbibed *Hordeum vulgare* cv. Dyan embryos biosynthesized and catabolised ABA. This is the first demonstration of a cell-free system from non-vegetative tissue capable of metabolising ABA and could prove useful for elucidating its biosynthetic route. This cell-free system generated the terpenyl pyrophosphates IPP, FPP and GGPP from MVA. ABA was produced from both MVA and IPP in the presence of O_2 and NADPH. The biosynthesis of ABA was stimulated by the addition of the squalene 2,3-oxide cyclase and kaurene synthetase inhibitor, AMO1618 and a "cold-pool trap" of (*R,S,*)-ABA. Ancymidol, cycocel and cytokinins reduced incorporation of label from MVA into ABA. Similar cell-free preparations, in the absence of AMO1618, converted (*R,S,*)-[2-^{14}C]-ABA into PA, 7'-hydroxy ABA and water-soluble conjugates. Although the methyl ester of (*R,S,*)-ABA was efficiently hydrolysed in this cell-free system no DPA was generated. The possible involvement of mixed function oxidase activity and soluble oxidases is discussed in relation to ABA metabolism.

While cell-free preparations from *Persea americana* cv. Fuerte mesocarp and immature seeds of *Pisum sativum* and *Phaseolus vulgaris* were unable to synthesize ABA from MVA, these tissue homogenates converted ABA into more polar acidic products. PA and DPA were identified as products of ABA catabolism in extracts from immature seeds of *Phaseolus vulgaris* and the 1',4'-*cis* diol of ABA in extracts from *Pisum sativum* immature seeds.

CHAPTER ONE

GENERAL INTRODUCTION

Abscisic acid (ABA; Figure 1.1) was orginally isolated from extracts of *Gossypium hirsutum* (Okhuma *et al*, 1965), *Acer pseudoplatanus* (Robinson and Wareing, 1964; Cornforth *et al*, 1965b; Cornforth *et al*, 1966a) and *Lupinus luteus* (Rothwell and Wain, 1964; Porter and van Steveninck, 1966; Koshimizu *et al*, 1966; Cornforth *et al*, 1966b).

The structure of ABA was confirmed by synthesis (Cornforth *et al*, 1965a), and shown to be dextrorotatory (Cornforth *et al*, 1966c) with the absolute configuration, (+)-(1'*S*,2Z,4E)-5- (1'-hydroxy-2',6',6'-trimethyl-4'-oxocyclohex-2'-enyl)-3-methyl penta-2,4-dienoic acid (Cornforth *et al*, 1967), by reference to Mills' rule. By a chemical correlation with malic acid, the absolute configuration of natural (+)-ABA was established as *S* (Ryback, 1972), according to the 1966 Cahn-Ingold-Prelog convention (Cahn *et al*, 1966).

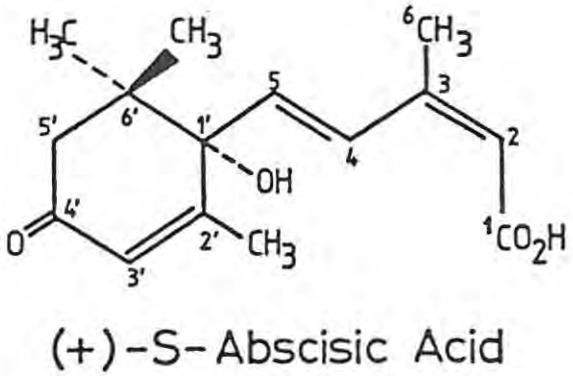

Figure 1.1. The structure of naturally-occurring abscisic acid.

The physical and chemical properties of ABA and related compounds, have been extensively reviewed by Addicott and Lyon, 1969; Dörffling, 1971; Bulard *et al*, 1972; Gross, 1972; Milborrow, 1974a; 1974c; 1978b; Zeevaart, 1979; Walton, 1983; Milborrow, 1984b and ABA has been reported to occur in a wide range of plants and plant tissues (Bearder, 1980), and has also been detected as a secondary metabolite in several fungi (Assante, 1977; Oritani *et al*, 1982; Marumo *et al*,1982).

Naturally-occurring (+)-*S*-ABA has been implicated in seed dormancy and germination (Wareing,

1965; Karssen, 1968; Wareing and Saunders, 1971; Wareing, 1978), bud dormancy (Eagles and Wareing, 1964; El-Antably *et al*, 1967; Rappaport and Wolf, 1969; During and Bachmann, 1975; Harrison and Saunders, 1975; Wright, 1975, Wareing and Phillips, 1983), apical dominance (Tamas *et al* 1979; Tucker, 1980; Knox and Wareing, 1984), tuberization (El-Antably *et al* 1967; Biran *et al* 1972), abscission (Addicott *et al* 1964; Addicott, 1970; Davis and Addicott, 1972; Addicott, 1983), senescence and fruit ripening (Rudnicki *et al*, 1968; Paranjothy and Wareing, 1971; Coombe and Hale, 1973; Even-Chen and Itai, 1975; Beevers, 1976; Thomas and Stoddart, 1980; Sacher, 1983; Palejwala *et al*, 1985) and gravitropism (Juniper, 1976; Feldman, 1981; Chanson and Pilet, 1982; Wilkins, 1984; Feldman, 1985). Of greater importance however, is the role of ABA in stomatal closure in response to water-stress (Wright, 1969; Wright and Hiron, 1969; Tucker and Mansfield, 1971; Wareing, 1978; Davies *et al*, 1979; Milborrow, 1981; Davies and Mansfield, 1983). Despite the aforementioned physiological implications, relatively little is known concerning the biosynthetic pathway to ABA in higher plants.

1.1. Abscisic acid biosynthesis.

All terpenoids are derived from mevalonic acid (MVA) by a well characterised pathway (Popjak and Cornforth, 1966; Goodwin, 1971; Goodwin and Mercer, 1983) and thus it would be expected that the sesquiterpenoid, ABA, should be similarly produced in higher plants (Figure 1.2). The biosynthesis of sesquiterpenes from labelled sodium acetate and MVA has been widely demonstrated in fungal preparations (Evans and Hanson, 1975; Baker *et al*, 1975; Arigoni, 1975; Bradshaw *et al*, 1978; Cane *et al*, 1981; Cane, 1983) and farnesyl pyrophosphate (FPP) has been implicated as the key intermediate in the biosynthesis of these compounds (Loomis and Croteau, 1980; Banthorpe and Charlwood, 1980). In addition, studies employing MVA as the substrate have demonstrated the synthesis of numerous sesquiterpenoids in higher plants (Croteau *et al*, 1972; Croteau and Loomis, 1972; Loomis and Croteau, 1980; Gleizes *et al*, 1984; Banthorpe *et al*, 1985; Coolbear and Threlfall, 1985) and the synthesis of pyrophosphorylated intermediates, between MVA and FPP, has been shown to occur in several plant extracts (Potty and Bruemmer, 1970; Chayet *et al*, 1973; Davies *et al*, 1975; Stieger *et al*, 1985). Furthermore, partial purification of prenyltransferase (EC 2.5.1.1: assayed as farnesyl pyrophosphate synthetase) has been achieved in extracts from *Pisum sativum* seedlings (Allen and Banthorpe, 1981). It is not unreasonable to suggest that ABA should therefore be derived in a manner consistent with that, for the biosynthesis, of other sesquiterpenoids. 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 tritium at C-2 and C-5' in the ABA molecule, when [(4R)-4-^{3}H]-mevalonate was used as the substrate.

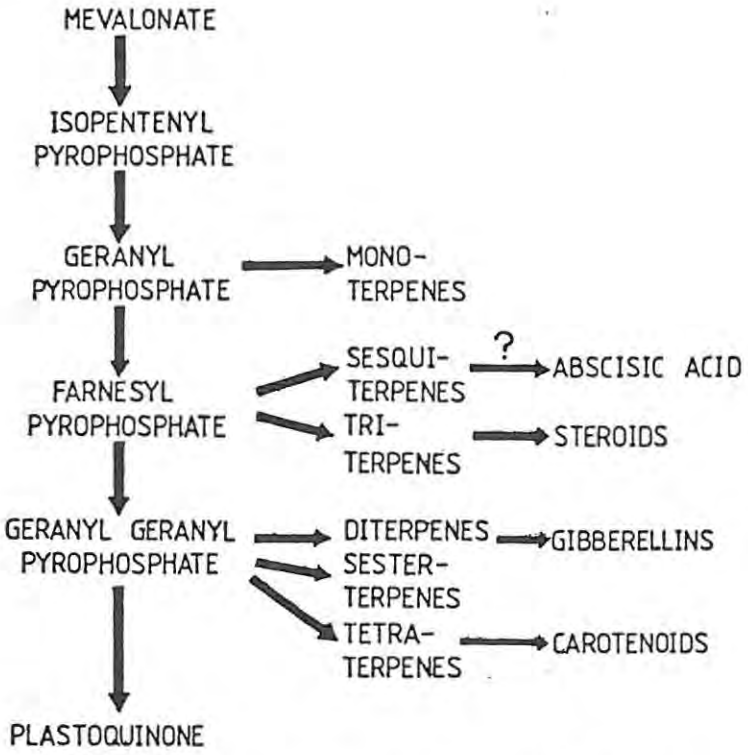

Figure 1.2. Schematic representation of the terpenoid biosynthetic pathway.

Initially, Noddle and Robinson (1969) reported that label from MVA was incorporated into ABA in fruits of *Lycopersicon esculentum* and *Persea gratissima*[1]. 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 gratissima* (Milborrow and Robinson, 1973), lysed chloroplasts from *Persea americana* and *Phaseolus vulgaris* (Milborrow, 1974b) and in sterile liquid suspension cultures prepared from *Vitis vinifera* pericarp tissue (Loveys *et al*, 1975).

Contrary to the above findings, it was suggested that ABA might be biosynthesized by the photolytic cleavage (Burden and Taylor, 1970) or enzymatic cleavage (Firn and Friend, 1972), with

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[1]*Persea gratissima* Gaertn. has been reclassified as *Persea americana* Mill. The latter name is used in this thesis to specify where studies have been carried out using mesocarp tissue from ripening fruits of this species.

either lipoxygenase or linoleate, of the C-40 carotenoid violaxanthin (see Figure 1.3; structure 1). One of the resultant products being a C-15 intermediate, *cis*-xanthoxin (Figure 1.3; structure 2), which was converted to ABA when supplied to shoots of *Phaseolus vulgaris* and *Lycopersicon esculentum* (Taylor and Burden, 1972; ' 1973; Burden and Taylor, 1976). Thus, this series of investigations suggested the synthesis of ABA from MVA *via* the carotenoid biosynthetic pathway. However, 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, even though attempts to distinguish between the two, using stereochemical considerations (Milborrow, 1978b), proved inconclusive. An earlier attempt was made to distinguish between the two possible pathways by feeding [^{14}C]-phytoene, the precursor of carotenoids, and [^{3}H]-MVA to fruits of *Persea americana* (Robinson, cited in Milborrow, 1974c). The results demonstrated [^{14}C]-label associated with carotene only, whereas [^{3}H]-label was found in both carotene and ABA. However, these data remain inconclusive since, [^{14}C]-phytoene may not have reached the subcellular sites of ABA biosynthesis, which might include chloroplasts, although it is known to cross the envelope membranes of this organelle (Benz *et al*, 1984), and the presence of [^{14}C]-label in xanthophylls was not specifically demonstrated.

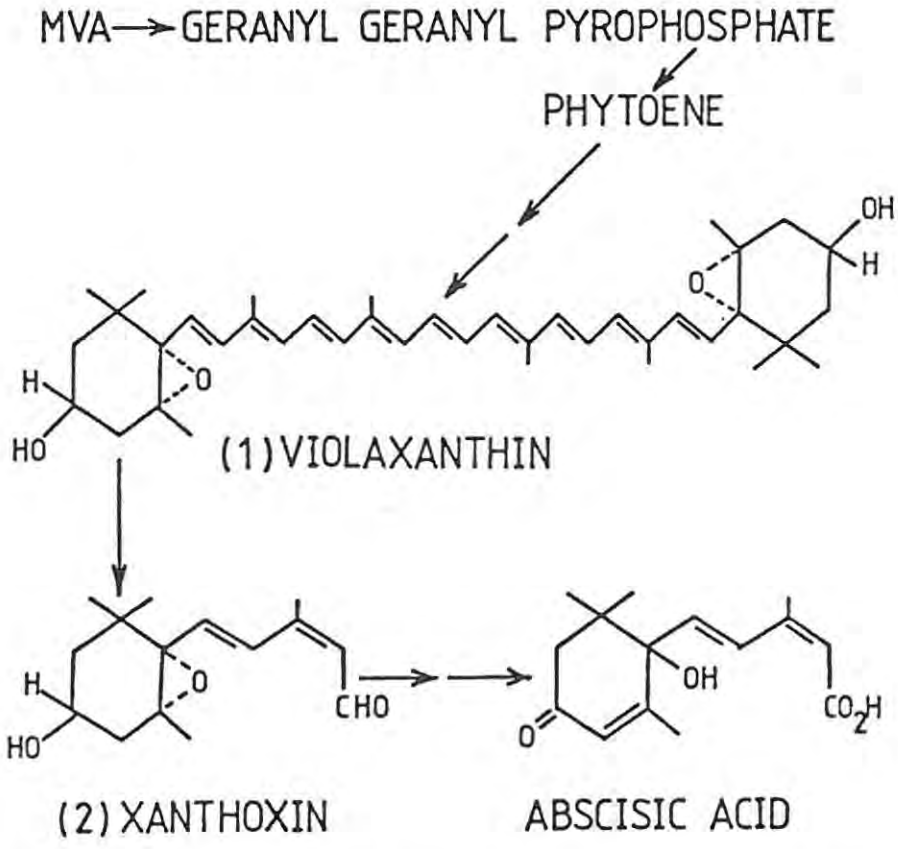


Figure 1.3. The hypothetical carotenoid ("C-40") pathway for abscisic acid biosynthesis.

The discovery of the ABA-producing fungi, *Cercospora rosicola* (Assante *et al*, 1977), *Cercospora cruenta* (Oritani *et al*, 1982) and *Botrytis cenerea* (Marumo *et al*, 1982) has provided researchers with a powerful means whereby the biosynthetic pathway to ABA may be elucidated. Although the pathway by which these fungi produce ABA need not be identical to that used by higher plants (Walton, 1980), the absence of violaxanthin in these fungi implies that only the "direct" pathway would be operating. To date, it has been demonstrated that mycelial suspensions of *Cercospora rosicola* 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 (Norman *et al*, 1982; Bennett *et al*, 1984; Norman *et al*, 1986), ^{2}H-α-ionylidene ethanol and ^{2}H-α-ionylidene acetic acid (Neill *et al*, 1982b; Neill and Horgan, 1983) into ABA, and the possible routes for the later stages of ABA biosynthesis, in this fungus, are depicted in Figure 1.4. Together, these data clearly demonstrate the merit of using hypothetical intermediates in attempts to elucidate the ABA biosynthetic pathway.

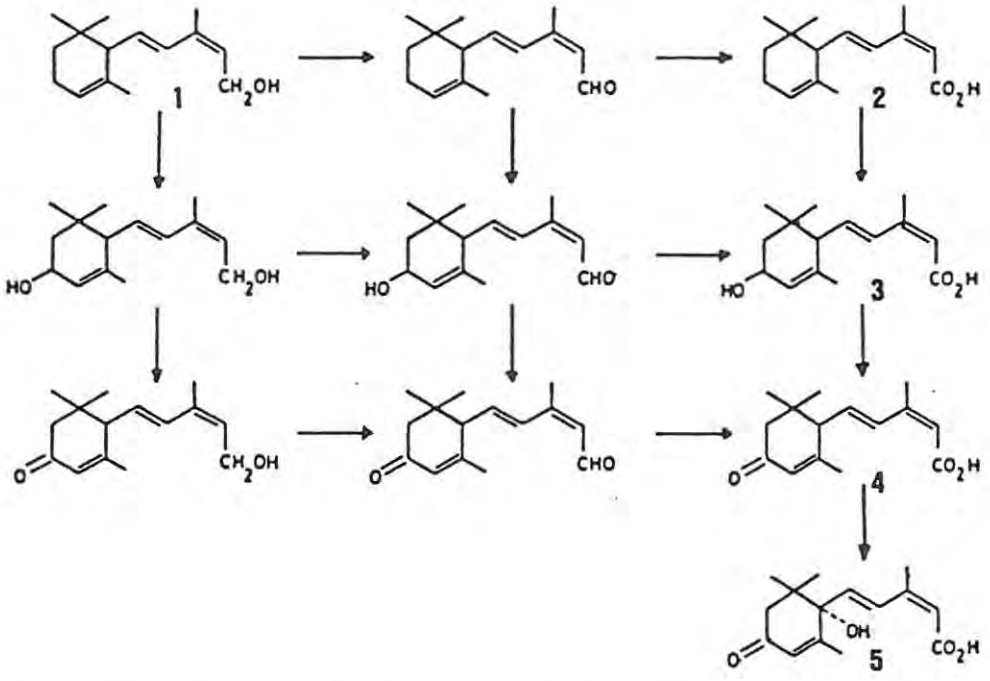


Figure 1.4. Hypothetical routes for the latter stages of abscisic acid biosynthesis in *Cercospora rosicola* (after Neill *et al*, 1984). 1, α-ionylidene ethanol; 2, 4'-deoxy-α-ionylidene acetic acid; 3, 1'-deoxy ABA; 4, 4'-hydroxy-α-ionylidene acetic acid; 5, ABA.

In addition to the aforementioned studies in higher plants, various hypothetical precursors to ABA have been fed to plant tissues. Mallaby and Milborrow (cited in Milborrow, 1974c) 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. Similarly, several compounds with pre-formed ABA skeletons have been examined as possible precursors. The 1',4'-*cis* and 1',4'-*trans* diols (Figure 1.5; structures 8 and 9) of ABA are transformed to ABA in high yield in excised axes of *Phaseolus vulgaris* (Walton and Sondheimer, 1972b) and in wilted leaves of *Triticum aestivum* (Milborrow and Noddle, 1970), although some of this oxidation is reported to occur spontaneously (Milborrow and Garmston, 1973). The 1',4'-*trans* diol of ABA appears to be a catabolite of ABA in stressed seedlings of *Pisum sativum* (Milborrow, 1983a). Thus, since naturally-occurring 1',4'-*cis* diol of ABA has been detected in seeds of *Vicia faba* (Dathe and Sembdner, 1982), it has been suggested (Neill *et al*, 1984) that the 1',4'-*cis* diol could be natural precursor of ABA in plants.

However, Hirai *et al*, (1986) showed that the 1',4'-*trans* diol of ABA is a precursor of ABA in the fungus *Botrytis cinerea* and Neill *et al* (1987) have demonstrated that both the 1',4'-*cis* and 1',4'-*trans* diols are converted to ABA in *Cercospora rosicola*. Recently, the 1',4'-*trans* diol of ABA was characterised as an endogenous compound in *Persea americana* fruit and *Pisum sativum* shoots (Vaughan and Milborrow, 1987; Okamoto *et al*, 1987). In addition, Okamoto *et al* (1987) demonstrated the conversion of [^{2}H]-1',4'-*trans* diol into ABA in *Pisum sativum* and *Persea americana* but were unable to show the transformation of ABA into the 1',4'-*trans* diol using these tissues. However, Vaughan and Milborrow (1987) presented data to suggest that the 1',4'-*trans* diol of ABA was produced as a catabolite of applied (*R,S,*)-ABA in *Persea americana* fruits and shoots of *Vicia faba*. Thus a certain amount of contorversy still surrounds the role of both the 1',4'-*cis* and 1',4'-*trans* diols as precursors of ABA in the biosynthetic route.

In other studies, 1',2'-epoxyionylidene acetic acid was converted to ABA by fruits of *Lycopersicon esculentum* (Milborrow and Noddle, 1970). This was further substantiated in studies using ^{18}O-labelling which demonstrated that the oxygen atom in the tertiary hydroxyl group of ABA was derived from the oxygen atom in the epoxy group. However, it is unlikely that the epoxy acid is a natural intermediate since it has not been identified in plant extracts and did not accumulate label when added as a "cold-pool trap". When (*R,S,*)-epoxyionylidene acetic acid was fed to shoots of *Lycopersicon esculentum* and *Persea americana* mesocarp, it was metabolised into (*S*)-ABA and

(*R*)-*cis*-xanthoxin acid. (*R*)-*cis* xanthoxin acid was not converted into ABA whereas (*S*)-*cis* xanthoxin acid, synthesized from natural violaxanthin, was converted into ABA. Similar findings have been reported in studies using the fungus *Cercospora cruenta*, where (*R,S,*)-epoxyionylidene acetic acid was metabolised to (*R,S,*)-xanthoxin acid, (2Z,4E)-5'-hydroxy-1',2'-epoxy-1',2'-dihydro-β-ionylidene acetic acid, (*R,S,*)-1',2'-epoxy-1',2'-dihydro-β-ionone and trace amounts of ABA (Oritani and Yamashita, 1985).

Neill *et al*, (1984) suggested that xanthoxin acid could be a possible intermediate between xanthoxin and ABA and between epoxyionylidene acetic acid and ABA in higher plants, but there is no direct evidence to support this suggestion. Xanthoxin acid has not been identified as a natural compound and it did not accumulate radioactivity when fed as a "cold-pool trap" to *Persea americana* mesocarp synthesizing ABA from [^{14}C]-MVA. However, xanthoxin, the postulated intermediate in the "C-40" cartenoid pathway of ABA biosynthesis, has been detected in higher plants. Both xanthoxin isomers were identified in extracts of *Phaseolus vulgaris* and *Lycopersicon esculentum* (Taylor and Burden, 1970) but only *cis*-xanthoxin was converted to ABA when supplied to shoots of *Phaseolus vulgaris* and *Lycopersicon esculentum* (Taylor and Burden, 1972; 1973; Burden and Taylor, 1976). Its detection in a wide range of plant species (Firn *et al*, 1972; Zeevaart, 1974; Anstis *et al*, 1975; King *et al*, 1977; Böttger, 1978; Dörffling *et al*, 1978) coupled with its characterisation in roots of *Zea mays* (Feldman *et al*, 1985) and shoots of *Lycopersicon esculentum* (Vaughan and Milborrow, 1987; Parry *et al*, 1988) implies that it could be an intermediate *en route* to ABA. However, recent studies by Nonhebel and Milborrow (1987) on the contrasting incorporation of ^{2}H from $^{2}H_2O$ into ABA, xanthoxin and carotenoids in *Lycopersicon esculentum* suggest otherwise. Nevertheless, a role for xanthoxin in ABA biosynthesis cannot be discounted.

1'-deoxy ABA (Figure 1.4; structure 3) has been characterised as an intermediate in the biosynthesis of ABA in *Cercospora rosicola* (Neill *et al*, 1982a). It was further demonstrated that label from MVA and either α-ionylidene ethanol or α-ionylidene acetic acid was incorporated into 1'-deoxy ABA which was then converted to ABA (Neill *et al*, 1982a; 1982b; Horgan *et al*, 1983; Neill and Horgan, 1983). These authors have suggested that 1'-deoxy ABA is the immediate precursor of ABA in *Cercospora rosicola* (Figure 1.4).

Studies with vegetative higher plant tissues have shown that α-ionylidene acetic acid was converted to 1'-deoxy ABA (4-oxo-α-ionylidene acetic acid) but not to ABA in *Hordeum vulgare* seedlings (Lehmann and Schütte, 1976). Similar results were obtained for *Persea americana* and

Phaseolus vulgaris supplied with α-ionylidene acetic acid, however in cuttings of *Vicia faba*, α-ionylidene acetic acid and 1'-deoxy ABA were converted to ABA (Neill *et al*, 1982a). These results would appear to suggest the possibility that 1'-deoxy ABA may be an intermediate on route to ABA in plant tissue although it has yet to be identified as an endogenous compound.

More recently, studies by Oritani *et al* (1985) have demonstrated that α-ionylidene acetic acid is converted to 1'-deoxy ABA and not to ABA in seedlings of *Lycopersicon esculentum*, *Glycine max*, *Avena sativa* and *Oryza sativa*. This is in contrast to studies in the fungi *Cercospora rosicola* (Norman *et al*, 1985a) and *Cercospora cruenta* (Oritani *et al*, 1982; Ichimura *et al*, 1983) where α-ionylidene acetic acid is enantioselectively oxidized to (*R*)-4'-hydroxy-α-ionylidene acetic acid, 1'-deoxy ABA and (*S*)-ABA.

Contrary to results obtained in studies on *Cercospora rosicola* (Neill and Horgan, 1983), Oritani and Yamashita (1979) demonstrated the conversion of (*R,S,*)-(2Z,4E)-4'-hydroxy-β-ionylidene acetic acid into (*S*)-ABA by *Oryza sativa* seedlings and recently, the conversion of (*R,S,*)-(2Z,4E)-1'-hydroxy-α-ionylidene acetic acid into (2Z,4E)-dehydro-β-ionylidene acetic acid and (*S*)-ABA in *Lycopersicon esculentum* seedlings (Oritani *et al*, 1985). These findings have recently been supported by Norman *et al*, (1985a), in studies using *Cercospora rosicola*, who suggested that ABA may be formed through a (2Z,4E)-1'-hydroxy-β-ionylidene-type intermediate in addition to the previously proposed route through 1'-deoxy ABA.

In the main these results show that with the exception of xanthoxin, and recently the 1',4'-*cis* and 1',4'-*trans* diols of ABA, no other possible intermediates beyond FPP have been identified in plants. Nevertheless, Nonhebel and Milborrow (1986) have provided evidence for the existence of a large pool of precursors to ABA, based on studies on the incorporation of ^{2}H from $^{2}H_2O$ into ABA in *Lycopersicon esculentum* seedlings, however these have yet to be characterised.

On the other hand, evidence is accumulating which favours the biosynthesis of ABA from a carotenoid origin. Firstly, when seedlings are grown in the presence of fluridone or norflurazon, which inhibit the desaturation of phytoene to phytofluene (Bartels and Watson, 1978), and in high light intensities, their endogenous ABA levels are greatly reduced (Henson, 1984; Moore and Smith, 1984; Quarrie and Lister, 1984b; Gamble and Mullet, 1986). Similarly, work on carotenoid-deficient mutants of *Zea mays* has shown that such mutants contain no detectable levels of ABA, *albeit* within the constraints of the technique used (Moore and Smith, 1985). Contrary to these

results, Feldman and Sun (1986) have demonstrated that when roots of *Zea mays* were treated with norflurazon the levels of xanthoxin increased while the levels of ABA were markedly reduced, suggesting that xanthoxin and ABA, at least in part, were derived *via* different biosynthetic routes. Secondly, a reappraisal of the conformation of ABA by NMR analysis (Milborrow, 1984a) demonstrated that the mechanism of cyclisation occurring during its biosynthesis was identical to that originally proposed for the β- and ε-rings of higher plant carotenoids (Britton *et al*, 1979). Thirdly, attempts to demonstrate the origin of the four oxygen atoms in ABA, where wilted leaves of *Xanthium strumarium* and *Phaseolus vulgaris* were incubated in an 80% $^{18}O_2$ containing atmosphere, were undertaken (Creelman and Zeevaart, 1984). Mass spectral analysis showed that one ^{18}O atom was incorporated into the carboxyl group in the side chain of ABA. Based on the structural similarities between ABA and carotenoids, it was expected that the 4'-keto and 1'-hydroxyl groups would also contain ^{18}O. Since this was not the case, the authors proposed that the oxygen atoms in the 1'- and 4'- positions either arose from water, or were already present in the form of a stored precursor such as xanthoxin, which was then converted to ABA under conditions of stress. Similar results were obtained using stressed roots of *Xanthium strumarium* (Creelman *et al*, 1987). When violaxanthin, in which the epoxide oxygen was labeled *in situ* with ^{18}O, was fed to *Phaseolus vulgaris* seedlings and the tissue subjected to stress, the ABA isolated contained ^{18}O in the 1'-hydroxyl group (Li and Walton, 1985; 1987), thus, suggesting a role for the positive involvement of carotenoids in the biosynthesis of ABA.

While Creelman and Zeevaart (1984) demonstrated the incorporation of a single ^{18}O atom into ABA, similar studies, conducted by Horgan and Walton (pers comm in: Creelman and Zeevaart, 1984) in the ABA producing fungus *Cercospora rosicola*, demonstrated that four atoms of ^{18}O were present in ABA. These results suggest that the pathway by which ABA is produced in the fungus differs from that for the stress-induced synthesis of ABA in higher plants. Furthermore, the existence of two separate, independent biosynthetic pathways cannot be ruled out, one operating in turgid tissue and another in stressed tissue.

1.2. Abscisic acid catabolism.

In contrast to studies on ABA biosynthesis, the catabolism of ABA in plants has been extensively investigated. However, the unequivocal spectroscopic identification of the products has only been achieved in a few cases (Milborrow, 1970; Zeevaart and Milborrow, 1976; Teitz *et al*, 1979). Initially, Milborrow (1968) described the existence of three catabolites of exogenously applied (*R,S,*)-[2-

^{14}C]-ABA one of which was identified as a 6'-hydroxymethyl derivative of ABA (6'-HM-ABA; Figure 1.5, structure 1), which was spontaneously rearranged to phaseic acid (PA) (Milborrow, 1969). The original structure proposed for PA (Figure 1.5, structure 2), isolated from *Phaseolus vulgaris* (MacMillan and Pryce, 1969), was incompatible with isomerisation from a hydroxylated form of ABA, and an alternative structure (Figure 1.5 , structure 3) for PA was proposed (Milborrow, 1969). Milborrow (1970) further demonstrated the conversion of ABA into a water-soluble neutral product, abscisic acid-β-D-glucose ester (ABAGE; Figure 1.5, structure 11), originally characterised as an endogenous compound from seed of *Lupinus luteus* (Koshimizu *et al*, 1968). Studies by Walton and Sondheimer (1972a) demonstrated that excised embryonic axes of *Phaseolus vulgaris* cv. White Marrowfat, rapidly catabolised [^{14}C]-ABA into two acidic compounds subsequently identified as PA (Walton and Sondheimer, 1972a) and 4'-dihydrophaseic acid (4'-DPA), (Figure 1.5, structure 4), (Tinelli *et al*, 1973). The absolute configurations of PA and 4'-DPA were later determined by Milborrow (1975b) as being, (-)-3-methyl-5[8{1(R), dimethyl-8(5)-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, 2, 1)-octane}] 2-cis-4-trans-pentadienoic acid, respectively.

PA and DPA have regularly been detected by co-chromatography and by comparrison with published Rf values, as catabolites of ABA in plant tissues. These include fruits of *Lactuca sativa* (McWha and Hillman, 1973; Robertson and Berrie, 1977; Orlandini *et al*, 1984), *Hordeum vulgare* (Cummins, 1973; Gross and Schütte, 1974; Railton and Cowan, 1985b) leaf tissue, seeds of *Fraxinus americana* (Sondheimer *et al*, 1974), *Betula lutea* seedlings (Loveys *et al*, 1974), wilted light-grown seedlings of *Pisum sativum* (Dörffling *et al*, 1974; Milborrow, 1983a; Tietz, 1985), excised water-stressed and turgid leaves of *Phaseolus vulgaris* (Harrison and Walton, 1975), the liquid endosperm from *Echinocystis lobata* (Gillard and Walton, 1976), immature seeds of *Pyrus communis* (Martin *et al*, 1977; Martin *et al*, 1982), *Triticum aestivum* ears (Dewdney and McWha, 1978; King, 1979), seeds of *Pyrus malus* (Powell and Seeley, 1974; Rudnicki and Czapski, 1974; Barthe and Bulard, 1981; Milborrow and Vaughan, 1979), leaf epidermal tissue from *Tulipa gesneriana* L. and *Commelina communis* L. (Singh *et al*, 1979), light-grown seedlings of *Lycopersicon esculentum* (Loveys, 1979; Vaughan and Milborrow, 1984), *Hodeum vulgare* aleurone layers (Dashek *et al*, 1979; Ho and Uknes, 1982; Uknes and Ho, 1984), leaves of *Spinacia oleracea* (Hartung *et al*, 1980; Railton and Symon, 1983), leaves and pod tissue from *Glycine max* (Setter *et al*, 1981), cell suspension cultures from various higher plant species (Lehmann *et al*, 1983b; Lehmann and Glund, 1986) and in excised stressed leaves of *Triticum aestivum* (Murphy, 1984; Lehmann and Schütte, 1984).

Other plant tissues in which studies on ABA catabolism have been conducted include, ripening grains of *Hordeum vulgare* (Naumann and Dörffling, 1982), stressed leaves of *Phaseolus vulgaris* (Pierce and Rashke, 1981) and *Xanthium strumarium* (Zeevaart and Boyer, 1982; Zeevaart, 1983; Cornish and Zeevaart, 1984, Bray and Zeevaart, 1985; Boyer and Zeevaart, 1986; Zeevaart *et al*, 1986), seedlings and guard cells of *Vicia faba* (Everat-Bourborloux, 1982; Grantz *et al*, 1985), *Vitis vinifera* (Loveys, 1984), *Beta vulgaris* (Daie *et al*, 1984), in *Ricinus communis* and *Xanthium* (Zeevaart and Boyer, 1982; Zeevaart and Boyer, 1984) and in stressed *Populus robustus*, *Euphorbia lathyrus* (Sivakumaran *et al*, 1980) and *Gossypium hirsutum* seedlings (Radin and Hendrix, 1986).

During such catabolic studies, the presence of 6'-hydroxymethyl ABA has only been reported once (Milborrow, 1969). However, its inferred presence as a catabolite of ABA in a cell-free system prepared from *Echinocystis lobata* liquid endosperm (Gillard and Walton, 1976; Walton, 1980), its tentative identification in fruits of *Vigna unguiculata* (Adesomoju *et al*, 1980) coupled with the recent characterisation of (*R*)-3-hydroxy-3-methyl-glutaryl-6'-hydroxy ABA (Figure 5, structure 6) from seeds of *Robinia pseudoaccacia* (Hirai *et al*, 1978; Hirai and Koshimizu, 1981) suggests that it is the initial acidic product of ABA catabolism.

The conversion of ABA to PA in the *Echinocystis lobata* cell-free system was shown to require oxygen and NADPH. Furthermore, this reaction was positively inhibited by carbon monoxide (CO), suggesting that the enzyme responsible was a Cytochrome P450 mixed function oxidase (Gillard and Walton, 1976; Walton, 1980). Creelman and Zeevaart (1984) have demonstrated that when stressed and subsequently rehydrated *Xanthium* leaves are incubated in an atmosphere containing labelled oxygen ($^{18}O_2$), one atom of ^{18}O is incorporated into the 6'-hydroxy group of PA further suggesting that the ABA hydroxylating enzyme is a mono-oxygenase and implicating 6'-hydroxymethyl ABA as the initial product of ABA catabolism in plants.

Although DPA appears to be the major product of PA reduction, its epimer, 4'-*epi*-DPA (Figure 1.5, structure 5), has been isolated from *Phaseolus vulgaris* seedlings in considerable amounts (± 18% of DPA) following the application of [^{14}C]-ABA (Zeevaart and Milborrow, 1976).

The possible routes for the catabolism of ABA are presented in Figure 1.6. In addition to the aforementioned catabolites there is now considerable evidence to suggest that other acidic

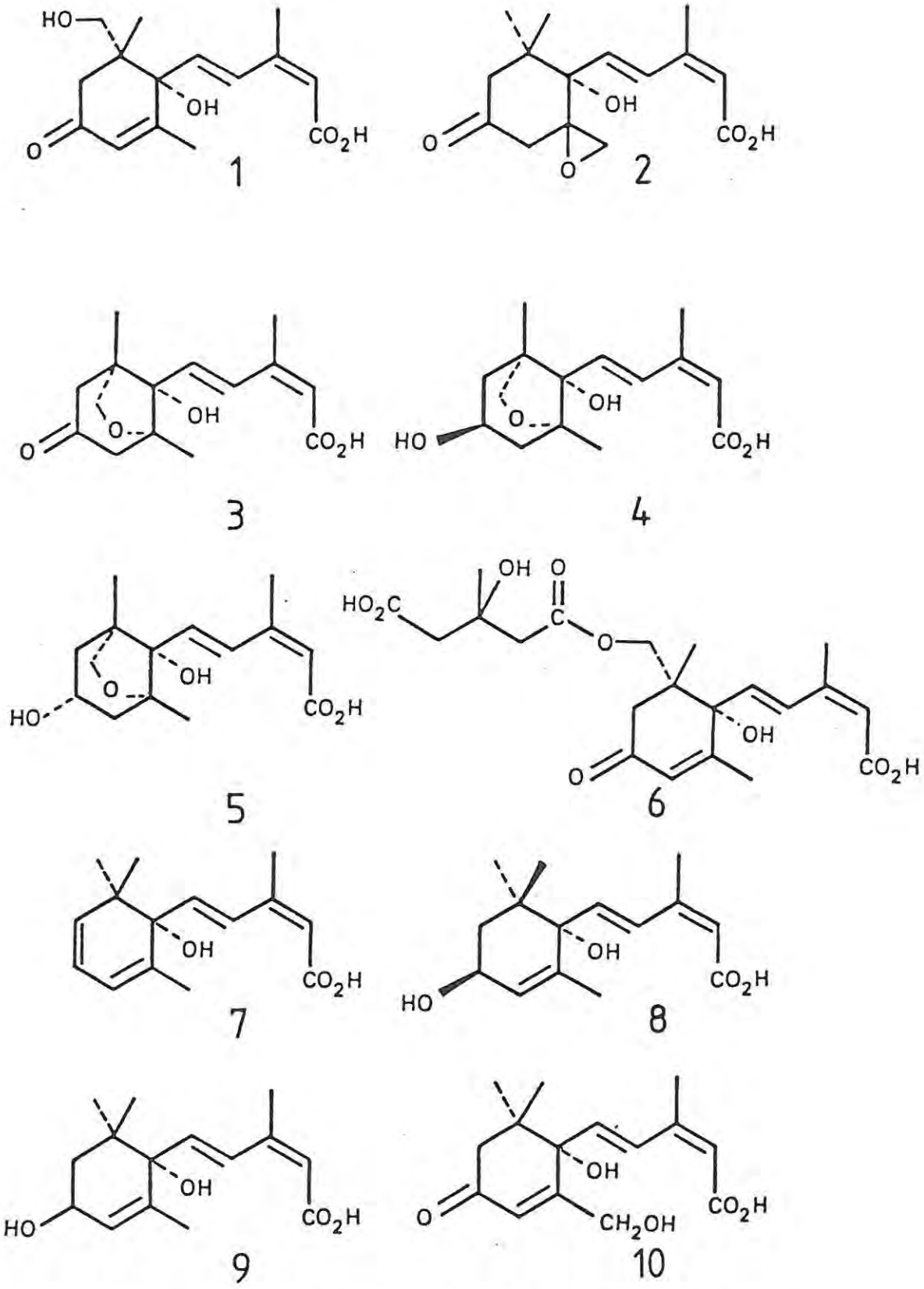
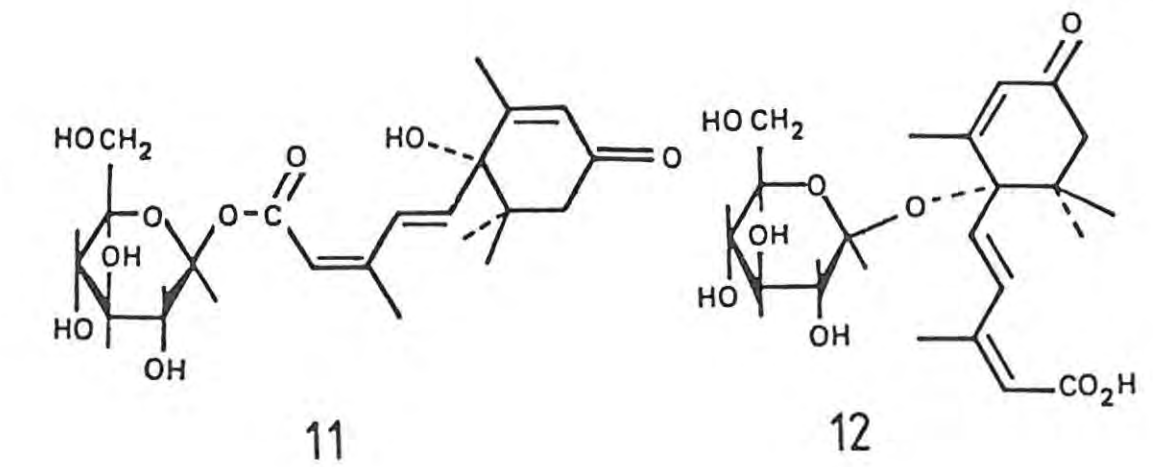
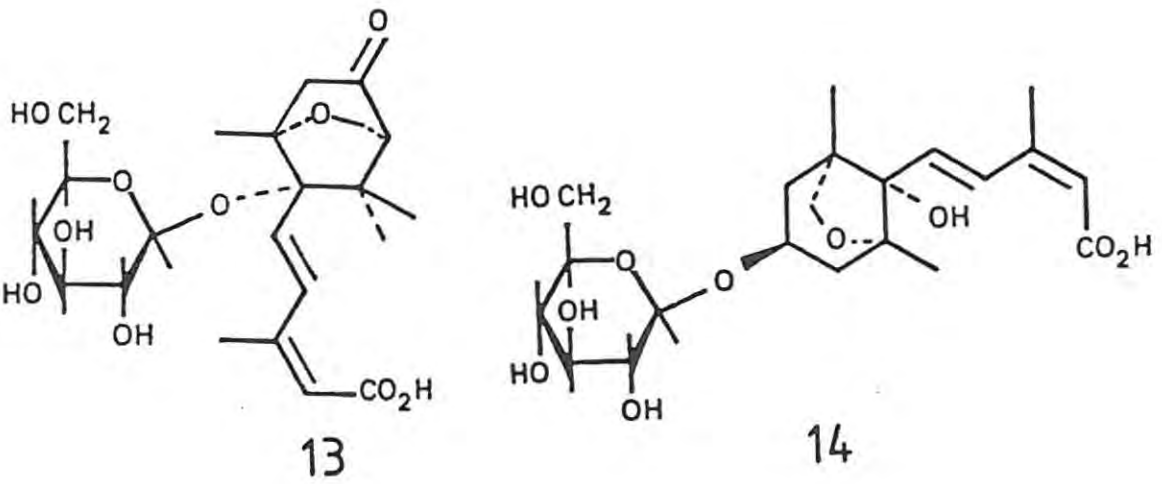


Figure 1.5. Structural formulae of the proposed catabolites of abscisic acid in plant tissues.





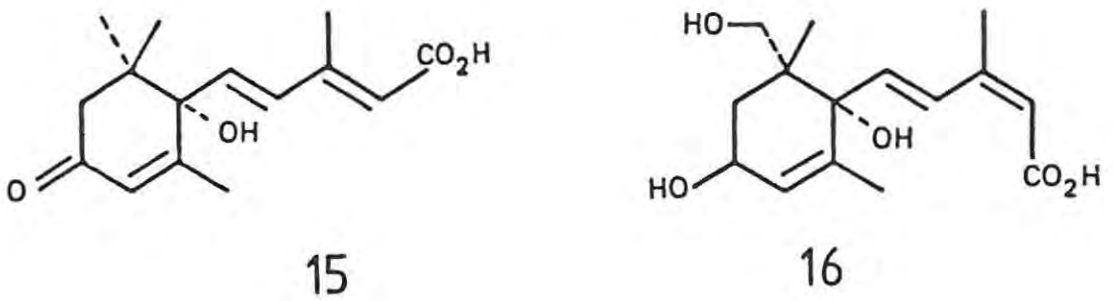


Figure 1.5. Continued.

products of ABA are formed in higher plant tissues. Martin *et al*, (1977) provided evidence to suggest that immature seeds of *Pyrus communis* contain several novel catabolites of ABA including, *trans*-DPA, a hydroxylated derivative of DPA and either a keto derivative of DPA or a hydroxylated derivative of PA. Similar catabolic products have been tentatively identified in phloem exudates from the monocots *Yucca flaccida* and *Cocos nucifera* (Hoad and Gaskin, 1980). Two compounds, possibly derived from ABA, provided mass spectra consistent with DPA and either hydroxyphaseic acid or oxo-dihydrophaseic acid.

Dewdney and McWha (1978) presented evidence for the formation of a variety of ABA catabolites when *Triticum aestivum* plants were incubated with [^{14}C]-ABA. An unknown catabolite has also been detected in stressed leaves of *Triticum aestivum* (Murphy, 1984) which has yet to be unequivocally characterised. Likewise, Railton and Symon (1983) detected a novel acidic catabolite of [^{14}C]-PA in leaves of *Spinacia oleracea* which has still to be identified.

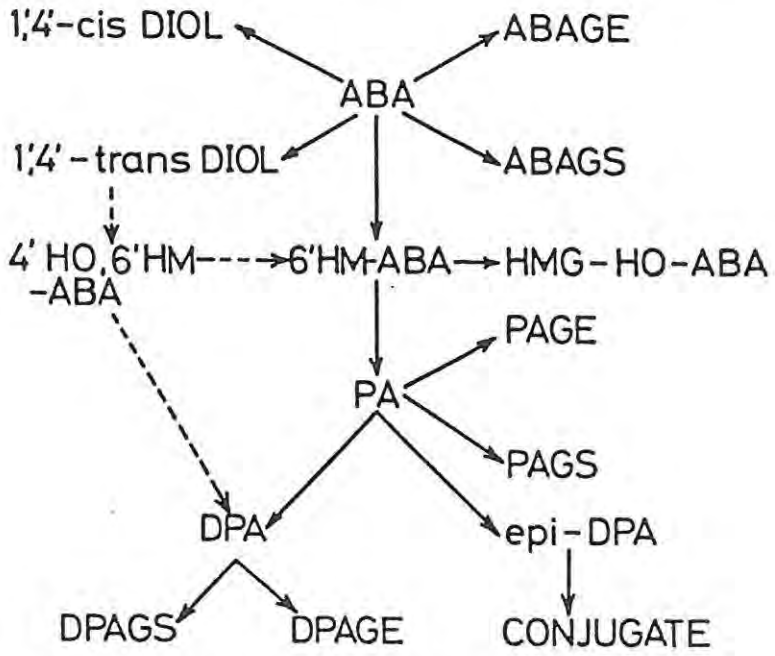


Figure 1.6. Possible routes for the catabolism of abscisic acid in higher plant tissues.

In water-stressed *Pisum sativum* seedlings, 4'-deoxy ABA (Figure 1.5, structure 7) was unequivocally characterised by combined gas chromatography-mass spectrometry (GC-MS) as a major catabolite of ABA (Tietz *et al*, 1979). The identity of this compound has been questioned by Milborrow (1983a) who subsequently demonstrated that it was produced as an artefact from the 1',4'-*trans* diol of ABA (Figure 1.5, structure 9) by dehydration during GLC analysis, although this has

not been substantiated in recent studies by Norman *et al* (1985a) and Hirai *et al* (1986).

4'-dihydro-6'-hydroxy ABA (pisumic acid, PISA) (Figure 1.5, structure 17) has also been isolated and characterised as a catabolite of ABA in stressed seedlings of *Pisum sativum* (Tietz, 1985). It was suggested, based on earlier proposals (Walton *et al*, 1973), that PISA is derived from ABA *via* the 1',4'-*trans* diol and is then subsequently converted to DPA. This represents the first positive support for the suggested (Walton *et al*, 1973) hypothetical alternative for DPA production from ABA in higher plants, and this pathway is depicted in Figure 1.6. In addition, Milborrow (1986) was able to show that when (*S,S,*)-[2-^{14}C]-1',4'-*trans* ABA diol was fed to *Pisum sativum* seedlings, almost seven times more radioactivity was incorporated into DPA-4'-O-β-D-glucopyranoside and other oxidized catabolites, than was formed from the (*R,R,*)-[2-^{14}C]-1',4'-*trans* ABA diol, which was either glucosylated or converted to ABA and its conjugates. Furthermore, when the [2-^{14}C]-1',4'-*trans* diol of ABA, with its 4'-protium atom replaced by deuterium, was fed to *Lycopersicon esculentum* plants, DPA-4'-O-β-D-glucopyranoside was rapidly formed and chemical ionization mass spectral analysis showed that no deuterium had been retained. Hence the hydroxyl group of 1',4'-*trans* diol had been oxidized to a ketone, implying that either the 1',4'-*trans* diol was hydroxylated to 4'-dihydro-6'-hydroxy ABA and then oxidized to 6'-hydroxymethyl ABA, or first oxidized to ABA before undergoing 6'-hydroxylation. Nevertheless, it would appear from the results obtained by Tietz (1985) that the 1',4'-*trans* diol is hydroxylated to 4'-dihydro-6'-hydroxy ABA, in which the hydroxyl group at 4' in the diol is retained, suggesting that an enzyme catalysed reaction would be required to effect the subsequent cyclisation to DPA. However, the significance of this pathway and the mechanism whereby this series of reactions takes place, has yet to be fully elucidated.

A novel catabolite of supplied (*R,S,*)-[^{14}C]-ABA, (*S*)-3-methyl-5-(1'-hydroxy-2'-hydroxymethyl-6'-dimethyl-4'-oxo-cyclohex-2'-enyl)-penta-2Z,4E dienoic acid (Figure 1.5, structure 10) has been characterised in cell suspension cultures of *Nigella damascena* (Lehmann *et al*, 1983a; 1983b; Lehmann and Schütte, 1984) and the suggested name, nigellic acid, assigned to this compound.

Further aspects of ABA catabolism include investigations into the degradation of the (+)-(*S*)-enantiomer and (-)-(*R*)-enantiomer of ABA[2]. Milborrow (1979) fed [^{14}C]-ABA to shoots of *Lycopersicon esculentum* and *Beta vulgaris* and was able to demonstrate that while oxidation *via* the PA pathway occurred involving the (+)-(*S*)-enantiomer, both the (+)-(*S*)- and (-)-(*R*)- enantiomers of ABA were conjugated. Since conjugates of (+)-(*S*)- and (-)-(*R*)-ABA, *trans*-ABA (Figure 1.5, structure 16), PA and DPA are formed, hydroxylation would appear more specific, (+)-(*S*)-ABA

being accepted and (-)-(*R*)-ABA not or more slowly (Sondheimer *et al*, 1971; Harrison and Walton, 1975; Zeevaart, 1980; Pierce and Rashke, 1981; Mertens *et al*, 1982; Milborrow, 1983a; Vaughan and Milborrow, 1984; Murphy, 1984; Boyer and Zeevaart, 1986). Thus the formation of PA seems to be a specific inactivation mechanism whereas conjugation appears to be a general catabolic mechanism for unwanted acids, at least in *Lycopersicon* and *Beta* (Milborrow, 1983b), although this might not be the case in other plant tissues.

Treatment of aqueous plants extracts with base, to cleave ester linkages between ABA and sugars, has shown that ± 10 % of the ABA in leaf tissue is present as sugar conjugates (Milborrow, 1981). Thus, in addition to the PA-DPA catabolic sequence, ABA is converted to its water-soluble β-D-glucopyranosyl ester, ABAGE (Figure 1.5, structure 11), first isolated from immature seeds of *Lupinus luteus* (Koshimizu *et al*, 1968) and shown to be naturally occurring in the psuedocarp of *Rosa averensis* (Milborrow, 1970). ABAGE was also characterised as a derivative of [^{14}C]-ABA in shoots of *Lycopersicon esculentum* (Milborrow, 1970) and has since been isolated from leaves of *Xanthium* (Zeevaart, 1980), *Spinacia oleracea* (Boyer and Zeevaart, 1982) and in receptacles of *Pyrus communis* (Martin *et al*, 1982). ABAGE has also been detected in extracts from *Citrus sinensis*, *Persea americana* and *Phaseolus vulgaris* (Neill *et al*, 1983). In addition, Sembdner *et al*, (1979) detected a further conjugate of ABA, linked *via* the carboxyl group seemingly to a disaccharide containing at least one glucose unit, occurring both in the wood and bark of *Acer pseudoplatanus*. Another conjugate of ABA, 1'-O-abscisic acid-β-D-glucopyranoside given the acronym, ABAGS (Figure 1.5, structure 12), has recently been characterised from shoots of *Lycopersicon esculentum* (Loveys and Milborrow, 1981) and is said to occur naturally. Evidence suggests that its epimer, α-ABAGS is also present in higher plant tissues (Milborrow, 1979).

Earlier suggestions (Milborrow, 1975a; Zeevaart and Milborrow, 1976) that conjugates of PA and DPA could arise from the exogenous application of ABA have recently been confirmed. Milborrow (1979) isolated and characterised 1'-O-phaseic acid-β-D-glucopyranoside (PAGS) from leaf tissue of *Lycopersicon esculentum* (Figure 1.5, structure 13). Recently, Zeevaart and Boyer (1982) demonstrated the presence of β-D-glucopyranosyl phaseate, given the acronym PAGE (Figure 1.5, structure 14), as a major product of [^{14}C]-PA catabolism in leaves of *Xanthium strumarium*. PAGE has also been isolated and unequivocally characterised as a catabolite of applied (*R,S,*)-ABA in shoots of *Lycopersicon esculentum* (Carrington *et al*, 1988). Furthermore,

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[2]Although (+)-, (±)- and (-)- are acceptable, in the present investigation (*S*)-, (*R,S,*)- and (*R*)- will be used to specify labelled and non-labelled substrates.

Setter *et al*, (1981) characterised dihydrophaseic acid aldopyranoside as a catabolite of ABA in pod tissue from *Glycine max*. Subsequent investigations (Milborrow and Vaughan, 1982), on ABA catabolism in vegetative shoots of *Lycopersicon esculentum* have resulted in the recharacterisation of this conjugate as dihydrophaseic acid-4'-O-β-D-glucopyranoside, given the acronym DPAGS (Figure 1.5, structure 15). In addition, DPAGS has been isolated and characterised from fruits of *Persea americana* (Hirai and Koshimizu, 1983). It is now envisaged that conjugates of ABA, PA and DPA may represent the stable end-products of ABA catabolism (Powell and Seeley, 1974; Zeevaart and Boyer, 1982; Neill *et al*, 1983), which are the compartmentalised within the leaf tissue (Bray and Zeevaart, 1985).

1.3. Cell-free studies.

Although Milborrow demonstrated the cell-free biosynthesis of ABA in lysed chloroplast preparations obtained from ripened *Persea americana* mesocarp (Milborrow, 1974b; 1976), current evidence suggests that chloroplasts are not the major site of ABA biosynthesis and catabolism (Hartung *et al*, 1980; 1981; Cowan and Railton, 1986).

Only one report has been published on the cell-free catabolism of ABA. In a cell-free system prepared from the liquid endosperm of *Echinocystis lobata*, Gillard and Walton (1976) were able to demonstrate the conversion of ABA to PA and DPA. However, this work has never been substantiated and further reports on functional ABA cell-free catabolising systems have not been forthcoming. Even so, accounts of cell-free systems, prepared from plant tissue, which actively synthesize and catabolise terpenoids are numerous and include, the synthesis of carotenoids and polyterpenoids (Goodwin, 1971; Davies and Taylor, 1976; Britton, 1976a; 1976b; Spurgeon and Porter, 1980; Goodwin, 1983; Britton, 1984), the synthesis of mono- and sesquiterpenoids (Cori, 1983; Cane, 1983), the synthesis of diterpenoids (Hanson, 1983) and in particular the phytoallexin casbene (Robinson and West, 1970a; 1970b; Sitton and West, 1975; Dudley *et al*, 1986a; 1986b) and the gibberellins (GAs) and intermediates in the GA biosynthetic pathway (Railton, 1976; Graebe and Ropers, 1978; Hedden *et al*, 1978; Sembdner *et al*, 1980; Railton, 1982; Bearder, 1983; Coolbaugh, 1983; Hedden, 1983; MacMillan, 1983; 1984; Graebe, 1986; 1987).

These reports, coupled with the published attempts to develop ABA-metabolising cell-free systems and the recent advances on ABA biosynthesis in the fungus, *Cercospora rosicola* (Horgan *et al*, 1983; Neill *et al*, 1984) should be used as a basis for further attempts to develop a suitable

cell-free system in which to study the synthesis and catabolism of ABA.

1.4. Objectives.

In the current research programme, experiments were carried out to assess the potential of various plant tissue as sources of enzymes for possible use in later studies on the cell-free metabolism of ABA. It was hoped that the detailed experiments leading up to cell-free studies would provide information regarding the best conditions under which an ABA-metabolising cell-free system might be developed. Thus,

1.) the effect of environmental and chemical factors on the biosynthesis of ABA from MVA and the catabolism of applied, radiolabelled ABA were examined in various plant tissues in order to establish whether such factors could alter ABA metabolism, possibly by influencing the levels and/or activities of the ABA-metabolising enzymes.

2.) attempts were made to develop an ABA-metabolising cell-free system from the selected tissues investigated in 1).

CHAPTER TWO

MATERIALS AND METHODS.

2.1. CHEMICALS.

2.1.1. Radioactive substrates.

(*R,S,*)-[2-^{14}C]-Abscisic acid (ABA) marketed as DL-*cis*, *trans*-[2-^{14}C]-ABA (sp.act. 947MBq/mmol), DL-[2-^{14}C]-mevalonic acid lactone (MVAL) (sp.act. 1.89GBq/mmol), *R*-[2-^{14}C]-MVAL (sp.act. 1.89GBq/mmol and [1-^{14}C]-isopentenyl pyrophosphate (IPP), ammonium salt (sp.act. 2.1GBq/mmol) were purchased from Amersham International, Buckinghamshire, England.

2.1.2. Plant growth regulators and growth retardants.

Abscisic acid methyl ester (ABAMe), sold as the (*R,S,*)-*cis*, *trans* isomer, (*R,S,*)-ABA, kinetin, 6-(furfurylamino)purine; benzyladenine, 6-(benzylamino)purine (BA); isopentenyl adenine, 6-(γ,γ-dimethylallyl-amino)purine (IAP) were purchased from Sigma Chemical Co., St Louis, MO, USA. AMO 1618 (2'-isopropyl-4'-(trimethylammonium chloride)-5'-methyl phenyl piperidine-1'-carboxylate) was obtained from Calbiochem, Behring Corp., La Jolla, Ca., USA. Cycocel (2-chloro-ethyl)-trimethylammonium chloride (CCC) was purchased from SA Cyanamid (Pty) Ltd., Johannesburg, SA. Ancymidol (α-cyclopropyl-α-(p-methoxy phenyl)-5-pyrimidine methyl alcohol), was a gift from Dr. R. Coolbaugh, Botany Department, Iowa State University, Ames, Iowa, USA.

2.1.3. Inhibitors.

Leupeptin (acetyl-L-leucyl-L-leucyl-L-argininal), trypsin inhibitor, pepstatin, lincomycin (α-methyl-6, 8-dideoxy-6- [1-methyl-4-propyl-2-pyrrolidine carboxamido] -1-thio-D-erythro-D-galactooctopyranoside) and chloramphenicol (chloromycetin; D (-)-threo-2,2-dichloro-N[β-hydroxy-α-(hydromethyl)-p-nitro-phenethyl] acetamide) were purchased from Sigma Chemical Co., St Louis, MO, USA.

Phenylmethylsulfonylfluoride (PMSF) and cycloheximide (3-[2 (3, 5-dimethyl-2-oxocyclohexyl)-2-hydroxyethyl]glutarimide (CHI) were purchased from Boehringer Mannheim, West Germany.

2.1.4. Cofactors.

Adenosine-5'-triphosphate (ATP) the disodium salt ($C_{10}H_{14}N_5O_{13}P_3Na_2$); the monosodium salts of adenosine-5'-diphosphate (ADP) and adenosine-5'-monophosphate (AMP); 2-oxoglutaric acid; L-ascorbic acid and glutathione were purchased from Sigma Chemical Co., St Louis, MO, USA.

β-Nicotinamide-adenine dinucleotide (NAD), the free acid ($C_{21}H_{21}N_7O_{14}P_2$); β-nicotinamide-adenine dinucleotide reduced (NADH) the disodium salt ($C_{21}H_{27}N_7O_{14}P_2Na_2$); β-nicotinamide-adenine dinucleotide phosphate reduced (NADPH), the tetrasodium salt ($C_{21}H_{26}N_7O_{17}P_3Na_4$); flavine adenine dinucleotide (FAD), the disodium salt ($C_{27}H_{31}N_9O_{15}P_2Na_2$) and flavine mononucleotide (FMN), the monosodium salt ($C_{17}H_{20}N_4O_9PNa$) were purchased from Boehringer Mannheim, West Germany. 2-Mercaptoethanol (C_2H_6OS) was obtained from Merck Chemicals, Darmstadt, West Germany.

2.1.5. Chromatographic standards.

(*R,S,*)-farnesol (3, 7, 11-trimethyl-2, 6, 10-dodecatrien-1-ol), isoamyl alcohol (isopentyl alcohol; 3-methyl-1-butanol), (*R,S,*)-linalool (D, L-3, 7-dimethyl-3-hydroxy-1, 6-octadiene), (*R,S,*)-phytol (3, 7, 11, 15-tetramethyl-2-hexadecen-1-ol), (*R,S,*)-nerolidol (3-hydroxy-3, 7, 11-trimethyl-1, 6, 10-dodecatriene) and (*R,S,*)-geraniol were purchased from Sigma Chemical Co., St Louis, MO, USA.

2.1.6. Chromatographic media.

Sep-pak silica and C_{18} cartridges were obtained from Waters Associates, Milford, MA, USA. DEAE cellulose (diethylamino ethyl cellulose), a microgranular anion exchanger and polyvinylpyrrolidone (PVP) were purchased from Sigma Chemical Co., St Louis, MO, USA.

For thin layer chromatography (TLC), Kieselgel 60 GF_{254} and Kieselgel G (Typ 60) were obtained from Merck Chemicals, Darmstadt, West Germany. The gas liquid chromatographic phases 2% OV-1 and 2% GC SE-30 on Gas Chrom Q (80-100 mesh) were purchased from Applied Science Inc., State College, Penn, USA and 1% XE-60 on Gas Chrom Q (80-100 mesh) was obtained from Alltech Associates, Deerfield, ILL, USA.

2.2. GENERAL TECHNIQUES.

2.2.1. Preparation of N-nitroso-N'-methyl urea.

The preparation of N-nitroso-N'-methyl urea was esentially the same as described by Arndt (1944). To a tarred 1l flask containing 200ml of a 24% aqueous methylamine (1.5M) solution, concentrated HCl was added until the solution was acid to methyl red. The resultant mixture was diluted to a final volume of 500ml with distilled water.

300g (5M) urea was then added and the solution boiled under reflux for 3h with vigorous boiling during the last 15min. The solution was cooled to room temperature and 110g of 95% sodium nitrite added and further cooled to $0^{o}C$. N-nitroso-N'-methyl urea, the crystalline foamy precipitate, was then filtered under vacuum, washed with 50ml distilled water and dried in a vacuum desiccator to a constant weight with a 60-70% yield.

2.2.2. Preparation of ethereal diazomethane.

Ethereal diazomethane was generated at room temperature without co-distillation, by the hydrolysis of N-nitroso-N'-methyl urea 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). 133mg N-nitroso-N'-methyl urea and 0.5ml distilled water, to dissipate any heat generated, were placed in the inside tube. 3.0ml dried 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 0.6ml 5N NaOH injected through the teflon rubber septum and the reaction allowed to proceed for ±45min or until the ether developed a deep yellow colour.

2.2.3. Preparation of JONES' reagent.

The reagent comprised a solution of chromic acid and sulphuric acid in water (Bowden *et al*, 1946) and oxidations were carried out by titrating a stirred solution of the alcohol, in acetone at 15-$20^{o}C$, with Jones' reagent. Jones' reagent was prepared using the method described by Fieser and Fieser (1967). Briefly 267mg of powdered chromium trioxide (Cr_2O_3) was dissolved in a solution of H_2O/98% H_2SO_4 (3.45:1, v/v) with constant stirring until a dark brown colour developed. The

solution was then allowed to cool to room temperature. Jones' reagent was freshly prepared each time before use.

2.2.4. Protein determination.

The levels of protein in plant tissue cell-free extracts were determined colorimetrically using the Bradford dye-binding assay (Bradford, 1976).

To 0.1ml of the protein solution, or any dilution thereof, 5.0ml of Bradfords Reagent (100mg Coomassie Brilliant Blue G-250 (Sigma Chemical Co., USA) dissolved in 50ml 95% ethanol to which was added 100ml 85% (w/v) phosphoric acid and the solution diluted to a volume of 1l with distilled water) was added, the samples throughly mixed and the absorbance at 595nm determined using a MSE Spectroplus spectrophotometer, after 2min and before 1h.

2.3. ENZYME ASSAYS.

2.3.1. Protease assays.

Proteolytic activity associated with plant tissue cell-free preparations was assayed as described for chloroplast preparations by Drivdahl and Thimann (1977) and Hampp and De-Filippis (1980). Acid-denatured haemaglobin (Sigma Chemical Co.) was used as substrate since it is readily soluble over a wide range of pH.

The reaction mixture for acid protease determination contained sodium acetate buffer (0.2M sodium acetate; 0.2M acetic acid, pH4.2), 0.25 ml 4% haemaglobin (in distilled water) and 'enzyme' in a total volume of 0.75ml. For neutral protease activity the reaction mixture contained haemaglobin, 'enzyme' and phosphate citrate buffer (0.1M citric acid; 0.2M K_2HPO_4, pH6.8 containing 10mM 2-mercaptoethanol) in a total volume of 0.75ml.

Reactions were initiated by the addition of 'enzyme', incubated at 50^{o}C in a water-bath for 90min and terminated by the addition of 0.25ml 40% trichloroacetic acid. Precipitated protein was removed by centrifugation and the levels of aromatic amino acids released by proteolytic activity, in solution, were determined at 280nm (Chow and Cassell, 1968; Chang and Takahashi, 1973; Cavadore *et al*, 1979) and confirmed by measuring the amount of α-amino nitrogen in the supernatant

by the method of Moore and Stein (1954). Protease activity was expressed as μg-L-tryptophan released per ml, determined from a standard curve for L-tryptophan (MERCK).

2.3.2. Phosphatase assay.

Alkaline phosphatase activity in cell-free extracts was determined by a method modified from those descibed by Lowry *et al* (1951), Garen and Levinthal (1960) and Godeh *et al*, (1981).

The reaction mixture comprised 1.0M diethanolamine (MERCK), 0.5mM $MgCl_2$ and 15mM p-nitrophenyl phosphate (Koch-light Laboratories, England), at pH10.4, and enzyme in a total volume of 1.5ml. Reactions were initiated by the addition of 'enzyme' and incubated at 37°C for 30min in a water-bath. The reactions were then subsequently terminated by the addition of 1.0ml 0.3M NaOH and centrifuged at 10 000xg for 10min to remove insoluble protein. The amount of free nitrophenyl in the supernatant, released by hydrolysis of the phosphate, was determined at 400nm using a MSE-Spectroplus spectrophotometer. Absolute amounts of alkaline phosphatase activity were determined from a standard curve prepared by incubating dilutions of stock (10mg/ml) alkaline phosphatase (sp.act. 35u/mg), from calf intestine obtained from Boehringer Mannheim, West Germany; in a manner identical to that described above.

2.4. PLANT MATERIAL.

Seeds of *Phaseolus vulgaris* L. cv. Top-Crop and *Pisum sativum* L. cv. Black-eyed Susan and an unkown variety of *Helianthus annuus* were purchased from Phoenix Roller Mills, Grahamstown, S.A. Seeds of *Hordeum vulgare* L. cv. Dyan were kindly supplied by the W.P. Cooperative, Malmesbury, S.A. Seeds of *Hordeum vulgare* L. cv. Himalaya (1980 harvest) were purchased from Washington Sate University, Pullman, WA, USA and seeds of *Pisum sativum* L. cv. Progress No9 were obtained from Sutton Seeds, Reading, England.

Immature fruits of *Phaseolus vulgaris* and *Pisum sativum*, of unknown variety, and ripening fruits of *Persea americana* Mill. cv. Fuerte were purchased from a local fresh-produce dealer.

2.4.1. Growth of plants.

Seeds of *P. vulgaris, P. sativum, H. annuus* and *H. vulgare* were imbibed in aerated tap-water for 6-8h,

sown in flats of moist vermiculite and germinated in constant environment chambers under conditions of continuous illumination (66μmol m^{-2} s^{-1}) at 25°C and 60% relative humidity. Eliolated seedlings were grown in total darkness under the same conditions as described for their light-grown counterparts. Following germination, a single treatment with the fungicide PROTEKTA KOPPERSPREY (Supra Horticultural Products, Johannesburg, S.A.), in distilled water (2g/l) was given and thereafter the plants watered once a day with tap-water until harvested. 10d old seedlings of *P. sativum* were transplanted into potting soil/vermiculite (4:1) and grown under constant illumination (66μmol m^{-2} s^{-1}) at 25°C and the immature fruits harvested 10 to 21 days following anthesis.

2.5. PREPARATION OF PLANT TISSUES.

2.5.1. Preparation of embryo and endosperm halves from mature seeds of *Hordeum vulgare*.

Embryo and endosperm portions were dissected from the dry seeds of *Hordeum vulgare* L. cv. Dyan and cv. Himalaya, using a sterile razor blade. These were then surface sterilised in a 1% sodium hypochlorite solution for 20min. The embryo and endosperm portions were then washed in 5x100ml aliquots of distilled water under filtration. All manipulations were carried out under aseptic conditions. Embryos were imbibed in aerated distilled-water for 2 to 12h, excess water removed under filtration and the wet weight of the tissue determined.

2.5.2. Preparation of aleurone layers from mature seeds of *Hordeum vulgare*.

Aleurone layers were prepared by the methods of Dashek *et al* (1979) and Uknes and Ho (1984), modified from Chrispeels and Varner (1967). Non-embryonic half seeds of *Hordeum vulgare* L. cv. Dyan and cv. Himalaya were surface sterilised as described above. Half seeds were incubated on acid washed sand (50g in a 10cm i.d. glass petri dish) with 13ml of 20mM sodium succinate buffer (pH5.0) containing 20mM $CaCl_2$, for 4-5 days at room temperature in the dark. At the end of the incubation period, aleurone layers were easily removed from the endosperm using two sets of sterile dissecting forceps. All manipulations were carried out under aseptic conditions.

2.6. APPLICATION OF CHEMICALS TO PLANT TISSUES.

2.6.1. Cytokinins.

Foliar applications of cytokinins to intact *Phaseolus vulgaris* and *Pisum sativum* seedlings were carried out in the following way. 50mg of either zeatin, kinetin, benzyl adenine, isopentenyl adenine or adenine (as a control) were dissolved in a small volume of 0.5M K_2HPO_4/KH_2PO_4 buffer (pH8.5) with gentle heating where necessary, and diluted to a volume of 1l in Tween 80/water (0.01%). To each tissue batch, 200ml of the respective cytokinin solution was applied as a foliar spray and 200ml was applied as a soil drench. Treatment was carried out each day for a period of 5 days, under continuous illumination (66μmol m^{-2} s^{-1}) at 25°C, prior to harvesting. In addition, solutions of these cytokinins at a concentration of 500μM (Norman *et al*, 1982), prepared in 0.5ml 10mmol KPi buffer (pH7.5) as above, were applied to excised *Hordeum vulgare* leaves and *Phaseolus vulgaris* seedlings *via* the transpiration stream and incubated under constant illumination (66μmol m^{-2} s^{-1}) at 25°C over a 12h period, prior to the addition of radiolabelled substrates. Similarly, concentration ranges (0-1.0mM) of kinetin and isopentenyl adenine were fed to excised, light-grown *Hordeum vulgare* leaves in exactly the same way. Where required, cytokinins (0.2-1.0mM) were solubilised in 0.5ml of KPi buffer (pH7.5)/ethanol (1:1,v/v) before application to the tissue.

In addition, cytokinins (500μM) in 200μl Tween 80/acetone/water (1:1:8, v/v) were applied to the sliced surface of ripened *Persea americana* mesocarp tissue, incubated over a 24h period in a water-saturated environment under continuous illumination (66μmol m^{-2} s^{-1}) at 25°C (Milborrow and Robinson, 1973), prior to the addition of radiolabelled substrates.

Applications of cytokinins to excised immature seed of *Pisum sativum* and *Phaseolus vulgaris in vitro*, were made as follows. Excised immature seed, thoroughly washed in distilled water was placed into Erlenmyer flasks containing 20ml of nutrient medium (Nitsch, 1951) prepared in the following way; the mineral salt solution contained $Ca(NO_3)_24.H_2O$ (500mg/l), KNO_3 (125mg/l), $MgSO_4.7H_2O$ (125mg/l) in 1l KH_2PO_4 (125mg/l); the trace element solution contained H_2SO_4 (0.5ml/l), $MnSO_4.4H_2O$ (3g/l), $ZnSO_4.7H_2O$ (500mg/l), H_3Bo_3 (500mg/l), $CuSO_4.5H_2O$ (25mg/l) and $Na_2MoO_4.2H_2O$ (25mg/l) in distilled water; anhydrous ferric chloride (10g/l), as per Frydman and MacMillan (1975), and 50g sucrose. One litre of the basic nutrient medium contained 50g

sucrose, 1ml of the anhydrous ferric chloride solution and 1ml of the trace element solution in the mineral salt solution. Cytokinins (500μM), dissolved in a small volume (0.5ml) of the nutrient medium were added to the immature seeds *in vitro* and uptake facilitated by placing the incubates under vacuum for 1min. Incubations were carried out over a 12h period in a Gallenkamp orbital shaker, under continuous illumination (42μmol m^{-2} s^{-1}) at 28°C prior to the addition of the radiolabelled substrates. Where specified, cytokinins (500μM) were included in cell-free incubation media.

2.6.2. Inhibitors of gibberellin biosynthesis.

Seedlings of *Phaseolus vulgaris* growing under conditions of continuous illumination (66μmol m^{-2} s^{-1}) at 25°C were pretreated with 200ml of aqueous solutions of AMO1618 (500μM) and CCC (500μM) and 100ml ancymidol (500μM) as a soil drench on 4 alternate days prior to harvesting and application of radiolabelled substrate. In addition, AMO1618 (500μM); CCC (500μM) and ancymidol (500μM) in 0.5ml 10mM KPi buffer (pH7.5) were applied to excised leaves of *Hordeum vulgare via* the transpiration stream, prior to the addition of radiolabelled substrate.

Applications of aqueous solutions of AMO1618 (500μM), ancymidol (500μM) and/or CCC (500μM) to ripened *Persea americana* mesocarp, immature seeds of *Phaseolus vulgaris* and *Pisum sativum* and cell-free homogenates, were made in an identical manner to that described for the application of cytokinins.

2.6.3. Inhibitors of protein biosynthesis.

The inhibitors of protein synthesis, chloramphenicol (CAP) (1mg/ml), lincomycin (LIN) (100μg/ml), and cycloheximide (CHI) (1μg-1mg/ml) each dissolved in 0.5ml K_2HPO_4/KH_2PO_4 buffer (10mM, pH7.5) were supplied to excised, light grown leaves of *Hordeum vulgare* and/or seedlings of *Pisum sativum* and/or *Phaseolus vulgaris via* the transpiration stream under identical conditions to those already described for cytokinins and inhibitors of gibberellin biosynthesis. Once all solutions had been absorbed by the seedlings, further buffer, without inhibitor, was added and the tissue incubated over a period of 6h prior to the addition of radiolabelled substrates.

The protein synthesis inhibitors were supplied to ripened *Persea americana* mesocarp tissue and immature seeds of *Phaseolus vulgaris* and *Pisum sativum* in an identical manner to that already

described for cytokinins and inhibitors of gibberellin biosynthesis.

2.7. APPLICATION OF RADIOCHEMICALS TO PLANT TISSUES.

2.7.1. Vegetative tissues.

2.7.1.1. Biosynthesis of ABA from [2-^{14}C]-MVA.

Leaves from *Hordeum vulgare* (10g.f.w.) and seedlings of *Pisum sativum* and *Phaseolus vulgaris* (20g f.w.) were excised and placed with their proximal ends into glass beakers, each containing 5.0ml of 10mM K_2HPO_4/KH_2PO_4 buffer (pH7.5)/ethanol (50:1, v/v) as described by Noddle and Milborrow (1970) and either *R*-[2-^{14}C]-MVAL or *R*-[2-^{14}C]-MVA (hydrolysed from the lactone in 5mM NaOH at 30°C for 30min to yield the corresponding Na salt, Britton and Goodwin (1971)) which was taken up *via* the transpiration stream under conditions of constant illumination (66μmol m^{-2} s^{-1}) at 25°C. Once all the solution had been absorbed the tissue samples were split into equal batches and one batch was transferred to a stream of warm air from a hair-dryer until it had lost 12-20% of its original fresh weight. Leaves were then sealed in transparent polythene bags and allowed to metabolise the substrate under constant illumination (66μmol m^{-2} s^{-1}) at 25°C for 24h. Turgid leaves were sealed in polythene bags containing paper towels soaked with water (Milborrow and Noddle, 1970). Incubations were terminated by freezing the tissue samples at -20°C.

2.7.1.2. Catabolism of (*R,S,*)-[2-^{14}C]-ABA.

Light-grown or etiolated shoots of *Phaseolus vulgaris* and *Pisum sativum* were excised under water and placed with their proximal ends into small glass vials, each containing 0.5ml of 10mM K_2HPO_4/KH_2PO_4 buffer (pH7.5) and (*R,S,*)-[2-^{14}C]-ABA. Similarly, light-grown or etiolated excised leaf tissue from *Hordeum vulgare* was incubated with (*R,S,*)-[2-^{14}C]-ABA. Uptake of the substrate was achieved *via* the transpiration stream under constant illumination (66μmol m^{-2} s^{-1}) or, in the case of eliolated seedlings, in darkness at 25°C. Where excised seedlings had been pretreated with chemicals (see Section 2.6), (*R,S,*)-[2-^{14}C]-ABA was added directly to the incubation medium. Once all the solution had been absorbed by the tissue, a further 0.5ml of the same 10mM K_2HPO_4/KH_2PO_4 buffer (pH7.5) was added, to ensure thorough uptake of substrate, after which a further 5.0ml of 10mM K_2HPO_4/KH_2PO_4 buffer (pH7.5) was added and catabolism allowed to proceed for varying lengths of time as specified in the Results (Chapters 5 and 6). A

similar procedure was adopted to study the catabolism of (*R,S,*)-[2-^{14}C]-ABA methyl ester (ABAMe) (prepared by esterifying (*R,S,*)-[2-^{14}C]-ABA with CH_2N_2) in light-grown leaves of *Hordeum vulgare*, using a 30h incubation period with continuous illumination (66μmol m^{-2} s^{-1}) at 25^{o}C.

Applications of radioactive PA, DPA and unknown catabolites of (*R,S,*)-[2-^{14}C]-ABA, generated biosynthetically in Section 2.16.1 were carried out in a similar manner. For studies on the effect of N_2 on (*R,S,*)-[2-^{14}C]-ABA catabolism in *Hordeum vulgare* leaves, uptake of radiolabelled substrates was performed in a N_2 atmosphere in a glass chromatography tank in which the lid was sealed with vacuum grease and a stream of N_2 entrained over the excised leaves throughout the duration of catabolism.

For wilting studies, leaves of *Phaseolus vulgaris* and *Hordeum vulgare* were treated with (*R,S,*)-[2-^{14}C]-ABA as described above and once the initial 0.5ml of 10mM K_2HPO_4/KH_2PO_4 buffer (pH7.5) had been absorbed, they were transferred to a stream of warm air from a hair-dryer, until they had lost between 0 and 15% of their orginal fresh weight. They were then sealed in plastic bags and maintained under constant illumination (66μmol m^{-2} s^{-1}) at 25^{o}C for varying lengths of time as specified in the Results (see Chapter 6). Turgid leaves were sealed in plastic bags containing paper towels soaked with water. Uptake of radiolabelled substrates was determined by measuring the residual radioactivity in the incubation vessels following the incubation periods using liquid scintillation spectrometry (Section 2.13), and was routinely found to be greater than 90 %.

2.7.2. Immature seed and imbibed, mature seed.

Studies on the *in vitro* metabolism of ABA in immature seed were carried out in a similar way to that described for the metabolism of gibberellins (Frydman and MacMillan, 1975). 5-10g excised immature seed from *Pisum sativum* cv. Black-eyed Susan, *Pisum sativum* cv. Progress No 9 and *Phaseolus vulgaris* were thoroughly washed in distilled water and placed into Erlenmyer flasks containing 20ml of nutrient medium (Nitsch, 1951). To the contents of each flask an aliquot of either (*R,S,*)-[2-^{14}C]-ABA of *R*-[2-^{14}C]-MVAL was added either directly or following pretreatment with chemicals (see section 2.6). Uptake of radiolabelled substrates was facilitated by placing the incubates under vacuum for a period of 1min.

Flasks, containing immature seed were incubated under white light (42μmol m^{-2} s^{-1}) at 28^{o}C in a

Gallenkamp orbital shaker for varying lengths of time as specified in the Results (Chapter 5). At the end of their respective incubation periods, the nutrient medium was removed by filtration and the tissue washed with distilled water. Residual radioactivity remaining in the nutrient medium was determined by scintillation spectrometry, and the tissue and nutrient media were frozen at -20°C until analysed.

2.7.3. *Persea americana* fruits.

Routinely, blocks (20g fresh weight) of ripened mesocarp from *Persea americana*, excised from skinned fruits, were sliced with a razor-blade, and either *R*-[2-^{14}C]-MVAL, *R*-[2-^{14}C]-MVA, or (*R,S,*)-[2-^{14}C]-ABA in 200μl Tween 80/acetone/H_2O (1:1:8, v/v) was infiltrated into the cuts, which were then closed, and metabolism allowed to proceed for varying lengths of time, as specified in the Results (Chapters 4 and 5), under continuous illumination (66μmol m^{-2} s^{-1}) at 25°C in a H_2O-saturated environment (Milborrow and Robinson, 1973). Where tissue had been pretreated with chemicals (see Section 2.6), following the initial incubation period, radiolabelled substrates were applied as described above.

2.7.4. Aleurone layers, excised embryo and endosperm halves from mature seeds of *Hordeum vulgare*.

2.7.4.1. Biosynthesis of ABA from *R*-[2-^{14}C]-MVAL.

20 Aleurone layers, dissected from *Hordeum vulgare* cv. Dyan endosperm halves under aseptic conditions (see Section 2.4.3), were placed into 25 ml Erlenmyer flasks containing 2.0ml of 20mM sodium succinate buffer (pH5.0) and 20mM $CaCl_2$. Likewise, 50-100 excised, dry embryos (1g dry weight) or endosperm halves (3g dry weight) prepared from either *Hordeum vulgare* cv. Dyan or *Hordeum vulgare* cv. Himalaya, were placed into 25ml Erlenmyer flasks containing 2.0ml of 10mM K_2HPO_4/KH_2PO_4 buffer (pH7.5). *R*-[2-^{14}C]-MVAL was added to each flask and uptake was facilitated by placing the flasks under vacuum for 1min. Aleurone layers were incubated in darkness while imbibing embryo and endosperm halves were incubated under laboratory light at 26°C for varying lengths of time as specified in the Results (see Chapter 5). Following incubation, the liquid medium was separated from the tissue under filtration and the tissue washed with succesive rinses using the respective buffers. The tissue and incubation media (containing the buffer washings) were stored at -20°C until analysed.

2.7.4.2. Catabolism of (*R,S,*)-[2-^{14}C]-ABA.

10-20 Aleurone layers and 30 excised embryos (1g dry weight) and endosperm (3g dry weight) halves, prepared from *Hordeum vulgare* seeds as previously described, were placed into 25ml Erlenmyer flasks containing 2.0ml of either 20mM sodium succinate buffer (pH5.0) and 20mM $CaCl_2$, or 10mM K_2HPO_4/KH_2PO_4 buffer (pH7.5) and (*R,S,*)-[2-^{14}C]-ABA. Uptake of the radiolabelled substrates was facilitated by placing the incubates under vacuum for 1min. Aleurone layers were incubated in a metabolic shaker at 26^{o}C in the dark for periods up to 24h. Imbibing embryo and endosperm halves were incubated in a metabolic shaker at 26^{o}C in either darkness or under laboratory light (14.4μmol m^{-2} s^{-1}) for periods up to 24h.

In studies on the effect of (*R,S,*)-ABA on the catabolism of (*R,S,*)-[2-^{14}C]-ABA in aleurone layers, the tissue was pretreated with (*R,S,*)-ABA at 10^{-3}M for 24h prior to the addition of labelled substrate, using identical conditions to those described above. At the end of this period the tissue was thoroughly rinsed (to remove excess (*R,S,*)-ABA) and fresh buffer containing (*R,S,*)-[2-^{14}C]-ABA was added and the tissue incubated for a further 24h period. Following termination of the incubations the tissue was washed in a solution of 10^{-4}M (*R,S,*)-ABA to remove non-specifically bound [^{14}C]-ABA and the tissue, incubation buffers and washings stored at -20^{o}C until analysed.

2.8. CELL-FREE STUDIES.

2.8.1. PREPARATION OF CELL-FREE HOMOGENATES.

2.8.1.1. Leaf tissue of *Hordeum vulgare*, immature seed of *Pisum sativum* and *Phaseolus vulgaris* and *Persea americana* mesocarp cell-free homogenates.

Cell-free homogenates were prepared from leaf tissue of etiolated and light-grown *Hordeum vulgare* seedlings, immature seed from *Phaseolus vulgaris* and *Pisum sativum* and ripened mesocarp tissue from *Persea americana* fruits by a modification of the methods described by Milborrow (1974b), Gillard and Walton (1976), Ropers *et al*, (1978), Hedden and Phinney (1979), Kamiya and Graebe (1983) and Railton *et al*, (1984). Preweighed quantities of tissue and in the case of *Persea americana*. the outer green layer of the ripened mesocarp, were homogenised on ice in grinding medium (2ml/g fresh weight), containing PVP (1g/10g fresh weight of tissue), added to bind phenolics, using an IKA-WERK Ultra-Turrax top-drive homogeniser (2x90sec bursts).

Preweighed immature seed was homogenised on ice for 5min in grinding medium (2ml/g fresh weight), using a mortar and pestle with acid washed sand and PVP (1g/10g fresh weight of tissue).

For ABA biosynthetic studies with *R,S*-[2-^{14}C]-MVA and *R*-[2-^{14}C]-MVA, plant tissue was homogenised in 20mM K_2HPO_4/KH_2PO_4 buffer, pH7.5, containing ATP (2.0mM) and $MgCl_2$ (2.0mM) and for ABA catabolic studies, in 0.1M K_2HPO_4/KH_2PO_4 buffer (pH7.2) containing $MgCl_2$ (2.0mM). In subsequent experiments 2-mercaptoethanol (20mM) and the inhibitors of protease activity leupeptin (2.0mM), pepstatin (10mM), trypsin inhibitor (1.742mg/5ml) and PMSF (10mM) were added to the grinding medium during tissue homogenisation (Pike and Biggs, 1972; Alpi and Beevers, 1981).

The tissue homogenates were routinely filtered through 8 layers of muslin and the filtrates centrifuged at 27 000xg for 30min in a Du Pont RC-5 Sorvall Superspeed Refrigerated Centrifuge, between 0-4^{o}C, and the supernatants retained as a source of enzyme. All manipulations were carried out at 0^{o}C. Protein in the supernatants was determined using the dye-binding assay (Bradford, 1976) previously described.

2.8.1.2. *Hordeum vulgare* embryo cell-free homogenates.

Cell-free homogenates from embryo tissue of *Hordeum vulgare* cv. Himalaya and cv. Dyan, were prepared essentially in the same way as that described by Davies *et al*, (1975). However, in their case, embryos were dissected following imbibition and the time taken to effect this preparation may alter the levels of enzyme present in the tissue extract. Thus, for accuracy, the embryos were dissected prior to imbibition in aerated distilled-water after which, the wet weight of the tissue was determined.

The embryos were homogenised on ice, using a mortar and pestle, in ice-cold 0.1M K_2HPO_4/KH_2PO_4 buffer, pH7.5 (1.5ml/g fresh weight) for 5min. The homogenate was centrifuged at 10 000x g for 20min in Du Pont RC-5 Sorvall Superspeed Refrigerated Centrifuge at 0-4^{o}C and protein in the resulting supernatant determined as previously described.

2.8.2. CELL-FREE INCUBATION PROCEDURES.

2.8.2.1. Leaf tissue of *Hordeum vulgare*, immature seed of *Pisum sativum* and *Phaseolus vulgaris* and *Persea americana* mesocarp homogenates.

2.8.2.1.1. Biosynthesis of ABA from *R*-[2-^{14}C]-MVA.

Following the hydrolysis of either *R,S*-[2-^{14}C]-MVAL or *R*-[2-^{14}C]-MVAL, [2-^{14}C]-MVA and NADPH (0.5μM) were added to 9.0ml of the cell-free homogenates (2mg/ml protein), prepared as previously described from light-grown and etiolated *Hordeum vulgare* leaf tissue, immature seed of *Phaseolus vulgaris* and *Pisum sativum*.

In a separate experiment, cell-free homogenates from *Persea americana* were incubated with aqueous solutions of ATP (0.1ml, 0.02M). $MgCl_2$ (0.02ml,0.05M), 2-mercaptoethanol (0.05ml, 0.2M) and a mixture of FAD, FMN, NAD, NADH, NADP, NADPH (each 0.02M in the same 0.1ml 20mM K_2HPO_4/KH_2PO_4 buffer, pH7.5) together with either [2-^{14}C]-MVA or (*R,S,*)-[2-^{14}C]-ABA and distilled water, to a final volume of 2.0ml (Milborrow, 1974b). The reactions were initiated by the addition of radiolabelled substrate and incubated at 28^{o}C in a metabolic shaker under constant illumination (42μmol m^{-2} s^{-1}) for 20h periods, unless otherwise stated. Where specified $FeSO_4$ (0.5mM), 2-oxoglutarate (0.5mM) and ascorbate (5.0mM) were added to the incubation media, as has been described for the cell-free biosynthesis of gibberellins (Hedden and Graebe, 1982: Kamiya and Graebe, 1983; Kamiya *et al*, 1984; Hedden *et al*, 1984; Turnbull *et al*, 1985). The reactions were terminated by the addition of an equal volume of ice-cold methanol and insoluble protein precipitated at -20^{o}C. Where necessary heated controls (100^{o}C x 10min) were included.

2.8.2.1.2. Catabolism of (*R,S,*)-[2-^{14}C]-ABA.

To each cell-free homogenate (9.0ml; 2mg/ml protein), prepared as previously described from either *Hordeum vulgare* leaf tissue, immature seed of *Pisum sativum* and *Phaseolus vulgaris* or ripened *Persea americana* mesocarp, (*R,S,*)-[2-^{14}C]-ABA and NADPH (0.5μM) were added in 1.0ml grinding medium. Heated controls (100^{o}C x 10min) were included where specified.

Reactions were initiated by the addition of radiolabelled substrate and incubations carried out in a

metabolic shaker at 28°C under white light (42μmol m^{-2} s^{-1}) for 24h periods, unless otherwise stated. Reactions were terminated by the addition of an equal volume of ice-cold methanol and insoluble protein precipitated at -20°C.

2.8.2.2. *Hordeum vulgare* embryo homogenates.

2.8.2.2.1. Biosynthesis of terpenyl pyrophosphates from *R*-[2-^{14}C]-MVA and [1-^{14}C]-IPP.

Either *R*-[2-^{14}C]-MVA or [1-^{14}C]-IPP was added to 25ml Erlenmyer flasks containing 2.5ml of the cofactor solution in 0.1M K_2HPO_4/KH_2PO_4 buffer, pH7.5. The final volume was 5.0ml and the concentrations of cofactors and inhibitors were: ATP(10mM), GSH(10mM), $MgCl_2$(6mM), AMO1618 (1mM) and NaF (5mM). The flasks, containing the co-factor solutions were equilibrated at 24°C for 5min. The reactions were initiated by the addition of 2.5ml (±20mg protein) of the *Hordeum vulgare* embryo supernatant. Incubations were carried out under laboratory light (14.4μmol m^{-2} s^{-1}) at 24°C in a metabolic shaker under an atmoshpere of nitrogen and were terminated after 2h by heating the mixture at 80°C in a water-bath for 2min or until a protein precipitate formed. The mixture was centrifuged at 5000 rpm in a MSE bench centrifuge, and the precipitate washed 2x with 2.0ml volumes of distilled H_2O. Washings were combined with the supernatant and the radioactive pyrophosphate products separated by DEAE-column chromatography (see Section 2.12.1).

2.8.2.2.2. Biosynthesis of ABA from *R*-[2-^{14}C]-MVA and [1-^{14}C]-IPP.

Reaction mixtures for the biosynthesis of ABA from *R*-[2-^{14}C]-MVA and [1-^{14}C]-IPP, in homogenates of either *Hordeum vulgare* cv. Dyan or *Hordeum vulgare* cv. Himalaya embryo tissue, were prepared as described above. 25ml Erlenmyer flasks containing radiolabelled substrate, NADPH (0.5μM) and (*R,S,*)-ABA (75μg) as a "cold-pool trap", all in 2.0ml of the cofactor solution were equilibrated at 28°C. Reactions were initiated by the addition of 2.0ml (±20mg protein) of the *Hordeum vulgare* embryo supernatant. Incubations were carried out under laboratory light (14.4μmol m^{-2} s^{-1}) at 28°C in a metabolic shaker for varying lenghts of time as specified in the Results (Chapter 7). Reactions were terminated by the addition of an equal volume of ice-cold methanol and the insoluble protein precipitated at -20°C. Heated controls (100°C x 10min) were routinely included during incubation procedures. Where required solutions of trypsin inhibitor (0.6mg/ml), PMSF (10mM), pepstatin (10mM), leupeptin (2.0mM), trypsin (1.0mg/ml), BSA (1.0

mg/ml), BA (500μM), kinetin (500μM), IPA (500μM), adenine (500μM), ancymidol (500μM), CCC (500μM) and (*R,S,*)-ABAMe (100μg) all in cofactor-containing incubation medium were added. Where specified the hydroxylating system, comprising $FeSO_4$ (0.5mM), 2-oxoglutarate (0.5mM) and ascorbate (5.0mM), was included in the incubation system as has been described for the biosynthesis of gibberellins (Hedden and Graebe, 1982).

2.8.2.2.3. Catabolism of (*R,S,*)-[2-^{14}C]-ABA.

Hordeum vulgare cv. Dyan embryo supernatants, were prepared as previously described. 25ml Erlenmyer flasks containing labelled substrate, NADPH (0.5μM) and ATP, GSH, NaF and $MgCl_2$ in 2.0ml of 0.1M K_2HPO_4/KH_2PO_4 buffer, pH7.5 (final concentrations in 4.0ml; ATP, 10mM; GSH, 10mM; NaF, 5mM; $MgCl_2$, 6mM) were equilibrated at 28°C. Reactions were initiated by the addition of 2.0ml of embryo supernatant (±20mg protein). Reactions were carried out under laboratory light (14.4μmol m^{-2} s^{-1}) at 28°C in a metabolic shaker for varying lengths of time as specified in the Results (Chapter 7). Reactions were terminated by the addition of an equal volume of ice-cold methanol and insoluble protein precipitated at -20°C. Heated controls (100°Cx10min) were included where necessary. Where specified $FeSO_4$ (0.5mM), 2-oxoglutarate (0.5mM) and ascorbate (5.0mM) were included in the incubation mixtures.

2.9. EXTRACTION OF ABSCISIC ACID AND ITS CATABOLITES.

An outline of the extraction and partial purification procedures adopted for ABA and its catabolites is depicted in Figure 2.1. In order to obtain information regarding the efficiency of these extraction procedures and solvent partitioning processes, (*R,S,*)-[2-^{14}]-ABA was added to crude plant extracts and the recovery of radioactivity monitored throughout the procedure. The data are presented in the flow-diagram depicted in Figure 2.1. This procedure clearly demonstrates that 95.79±1.82% (n = 3) of the applied (*R,S,*)-[2-^{14}]-ABA (4.2 kBq) was recovered in the ethyl acetate soluble acid fraction while 93.14±1.55% (n = 3) in the diethyl ether fraction.

2.9.1. Effect of the extraction solvents on abscisic acid recovery and sample dry weight.

Although 80% aqueous methanol and acetone are the most commonly selected extraction solvents for ABA (Saunders, 1978; Yokota *et al*, 1980; Brenner, 1981), there are numerous reports where other extraction solvents have been used (Hillman *et al*, 1974; Loveys, 1979; Hubick and Reid, 1980;

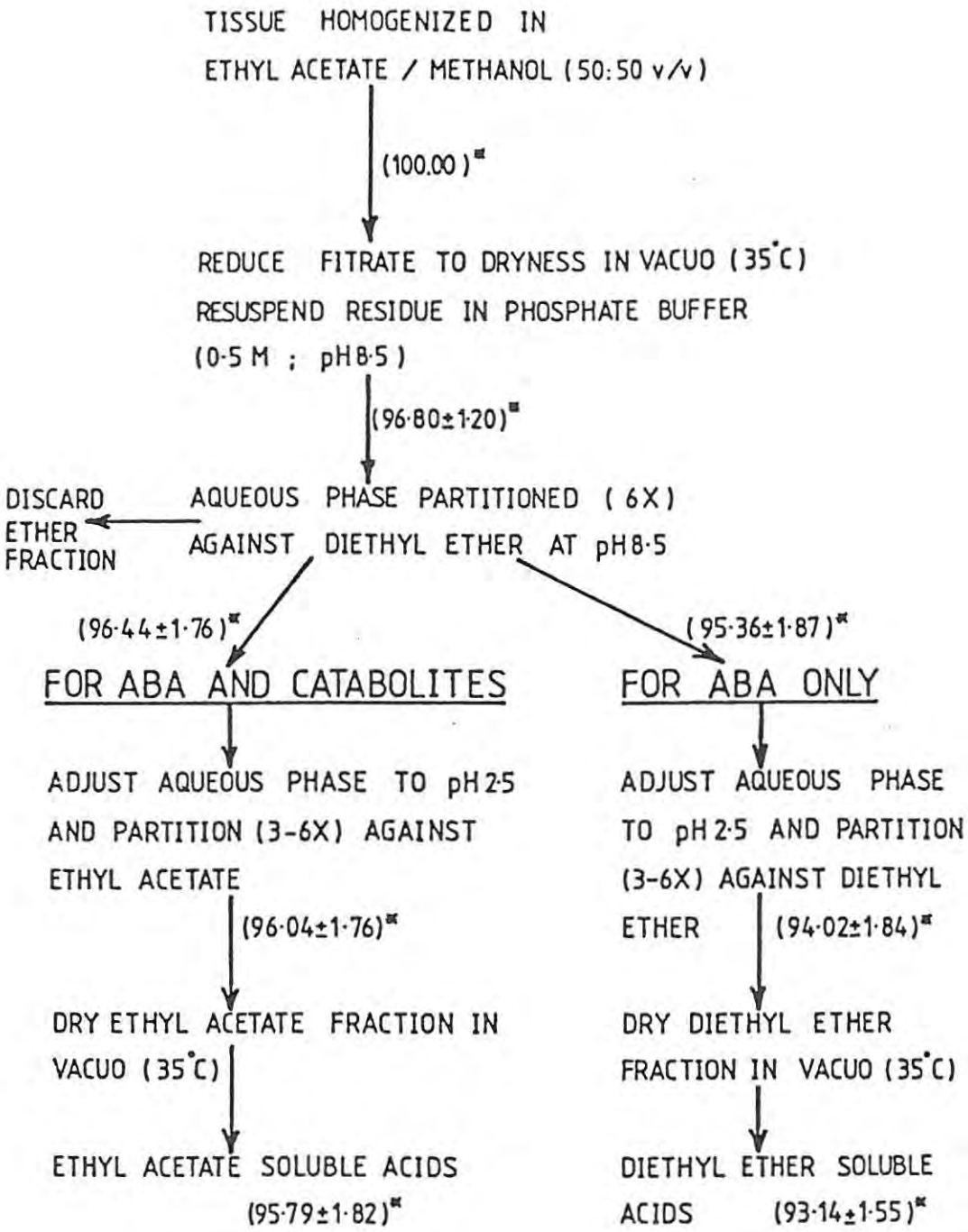

Figure 2.1. Flow diagram of the procedure for the extraction and partial purification of abscisic acid and its catabolites from plant tissues. ( )* Percentage (*R,S,*)-[2-^{14}C]-ABA (4.2kBq), added as an internal standard to the crude tissue homogenate, recovered at each stage during the procedure (n=3).

Railton and Symon, 1983). Loveys (1977) suggested that methanol/ethyl acetate/acetic acid (50:50:1, v/v) was a superior solvent for extracting ABA from leaf tissue. Thus, it seemed pertinent to examine the contributions of various extraction solvents to the final dry weight of the soluble acid fractions obtained.

Three samplesof leaf tissue, each 10g fresh weight, from *Hordeum vulgare* seedlings were extracted in either 80% aqueous methanol, ethyl acetate/methanol (50:50, v/v) or ethyl acetate/methanol/acetic acid (50:50:1, v/v) and partially purified as depicted in Figure 2.1, to generate ethyl acetate- and diethyl ether-soluble fractions, and the final dry weight determined.

The data presented in Table 2.1 demonstrates the contribution of the extraction solvents to the final dry weights of the respective soluble acid fractions generated. Although the recovery of (*R,S,*)-[2-^{14}]-ABA (added as an internal standard) was unaffected by the extraction solvent used, it is evident that tissue homogenised and extracted with ethyl acetate/methanol (50:50, v/v) produced soluble acid fractions of lowest dry weight. Thus, all subsequent tissue extractions were undertaken using this solvent mixture.

2.9.2. Extraction of excised plant tissues.

Frozen plant material was homogenised, using either a mortar and pestle, with acid washed sand (BDH Chemicals Ltd. Poole, England) or using an IKA-WERK Ultra Turrax top-drive homogeniser, in ice-cold ethyl acetate/methanol (50:50, v/v) (10ml/g fresh weight) unless otherwise stated. The tissue homogenates were then filtered through Whatman No. 1 filter paper in a Buchner funnel, under vacuum, and the residue washed with further ethyl acetate/methanol solution until all the radioactivity had been removed or until the tissue was rendered colourless. Where *R*-[2-^{14}]-MVA or *R*-[2-^{14}]-MVAL had been supplied to excised, intact plant tissues, ABA (2.0mg/l) and butylated hydroxytoluene (BHT; 2,6-di-*tert*- butyl-p-cresol, 20mg/l), BDH Chemicals Ltd., Poole, England were added to the extraction solvent prior to tissue homogenisation as described by Milborrow and Robinson (1973). It should be noted that BHT was not included in the extraction and partial purification of tissues fed with (*R,S,*)-[2-^{14}]-ABA, in catabolic studies, since no discernable differences were observed in the percentage of radioactivity recovered in ABA and its catabolites (Table 2.2) generated *in vivo*. The filtrates were then reduced to dryness *in vacuo* at 35^{o}C on a rotary evaporator (Buchi Rotavapor-RE).

TABLE 2.1: The effect of the extraction solvent on the dry weight of the soluble acid fractions.

A comparison of the dry weight associated with ethyl acetate and diethyl ether-soluble acid fractions (prepared as outlined in Figure 2.1) and the recovery of added (R,S)-[2-^{14}C]-ABA (1 kBq) in extracts prepared from 10 d old Hordeum vulgare seedlings (10g fresh weight).

	EXTRACTION SOLVENT		
Soluble-Acid Fraction	Ethyl Acetate/ Methanol (50:50, v/v)	Ethyl Acetate/ Methanol/Acetic Acid (50:50:1, v/v)	80% Aqueous Methanol
	mg dry weight (% radioactivity recovered)		
Ethyl Acetate	16.0 (97.89)	32.7 (98.92)	43.5 (96.20)
Diethyl Ether	4.6 (98.93)	16.8 (97.53)	23.2 (94.01)

TABLE 2.2: The effect of butylated hydroxytoluene (BHT; 2,6-Di-tert-butyl-p-cresol) on the recovery of radioactivity in ABA and its catabolites.

Leaves of 10 d old Hordeum vulgare (2g f.w.) were fed with (R,S)-[2-^{14}C]-ABA (0.5 kBq) for 30 h. ABA and its catabolites were extracted as described in Section 2.9 with or without BHT and the ethyl acetate soluble acids were separated by TLC on silica gel GF_{254} in toluene/ethyl acetate/acetic acid (50:30:4, v/v) developed 2 x to 15 cm. Where specified BHT (20 mg/l) was included in the organic solvents.

	Radioactivity in ABA and its catabolites					
Treatment	Origin (Rf 0-0.1)	DPA (Rf 0.16-0.23)	X (Rf 0.33-0.36)	PA (Rf 0.5-0.56)	ABA (Rf 0.66-0.73)	Conjugates
	Bq (%)					
-BHT	52.1 (10.42)	126.9 (25.38)	67.5 (13.49)	66.1 (13.22)	85.6 (17.11)	101.9 (20.38)
+BHT	58.8 (11.75)	147.4 (29.47)	60.2 (12.03)	66.0 (13.19)	86.3 (17.25)	81.6 (16.31)

2.9.3. Extraction of *Hordeum vulgare* aleurone layers.

Frozen aleurone layers were homogenised using a mortar and pestle with a small amount of acid washed sand and 1.5ml ice-cold 90% methanol. The homogenate was centrifuged at 1500xg for 10min and the pellet re-extracted in 1.0ml 90% aqueous methanol and centrifuged as above. The second pellet was further extracted by heating at 60°C in 90% aqueous ethanol for 1.0min with vortexing and centrifugation. The resultant three supernatants were pooled and reduced to dryness *in vacuo* at 35°C on a rotary evaporator.

2.9.4. Extraction of cell-free incubates.

Methanol-denatured, cell-free incubates were centrifuged at 5000rpm for 10min in a MSE bench-top centrifuge to remove precipitated protein and cellular debris. The protein pellet was washed three times (vortexing and centrifugation) using 4.0ml ice-cold ethyl acetate/methanol (50:50, v/v). In biosynthetic studies, where either *R*-[2-^{14}]-MVA or [1-^{14}C]-IPP had been used as substrates, BHT (20mg/l) was added as an antioxidant to the ethyl acetate/methanol extraction solvent. The combined supernatants were then reduced to a small, aqueous volume *in vacuo* at 35°C on a rotary evaporator.

Reduced aqueous filtrates were routinely mixed with an equal volume of 0.5M K_2HPO_4/KH_2PO_4 buffer (pH8.5) and partitioned (3-6X) against equal volumes of diethyl ether to remove pigments and neutral/basic impurities. The aqueous phases were then adjusted to pH2.5 with concentrated HCl and partitioned (3-6X) against equal volumes of either, ethyl acetate (for the extraction of ABA and its catabolites) or diethyl ether (for the extraction of ABA only) to extract both radioactive and non-radioactive acids. The ethyl acetate and diethyl ether fractions were dried by freezing-out the H_2O at -20°C (removed under filtration) and were then reduced to dryness *in vacuo* at 35°C to yield the ethyl acetate- and diethyl ether-soluble acid fractions. In biosynthetic studies all organic solvents used routinely contained BHT (20mg/l) as an antioxidant.

In the case of biosynthetic and catabolic cell-free studies, aqueous incubates were resuspended in 5.0ml 0.5M K_2HPO_4/KH_2PO_4 buffer (pH8.5) and were transferred to graduated test-tubes for partitioning. Aqueous samples were then partitioned as described above and the acids extracted into the organic phases by vortexing and centrifugation. Likewise, water was removed (by freezing-out at

-20°C) under filtration and the soluble acid fractions reduced to dryness *in vacuo* at 35°C. Again, in studies on the cell-free biosynthesis of ABA from either *R*-[2-^{14}C]-MVA or [1-^{14}C]-IPP, BHT (20mg/l) was included in all organic solvents used.

All manipulations were carried out under conditions of dim light to minimise the isomerisation of ABA and its catabolites, and where necessary ethyl acetate- and diethyl ether-soluble acids were further dried in a vacuum desiccator for periods up to 12h.

2.10. PURIFICATION OF EXTRACTS USING SEP-PAK CARTRIDGES.

2.10.1. Sep-pak silica cartridge purification of ABA and its catabolites.

Purification of plant extracts using Sep-pak silica cartridges was carried out essentially as described by Hubick and Reid (1980), with minor modifications.

For the analysis of ABA alone, known weights of either the initial crude organic filtrates and ethyl acetate, or the diethyl ether soluble acids, containing a known quantity of (*R,S,*)-[2-^{14}]-ABA, were loaded onto cartridges in 1.0ml of methylene chloride or in varying amounts of methanol. Where methanol was used, this was subsequently evaporated from the silica with N_2 prior to elution. Both [^{14}C]-PA and [^{14}C]-DPA, which were insoluble in methylene chloride, were routinely applied to the cartridges in methanol.

Step-wise elution was carried out using the solvent mixtures described by Hubick and Reid (1980). Six aliquots of methylene chloride were followed sequentially by 5% (v/v) ether in methylene chloride, 5% ethyl acetate in methylene chloride, 5% acetone in methylene chloride, 4% methanol in methylene chloride, and 10, 20 and 60% methanol in methylene chloride. Where specified, a 1-10% methanol in methylene chloride gradient was included in the eluotropic series. The dry weight of each fraction was determined using a Cahn TA-4100 analytical balance and levels of radioactivity were determined by liquid scintillation spectrometry (Section 2.13).

2.10.2. Sep-pak C_{18} cartridge purification of ABA and its catabolites.

Either the diethyl ether- or ethyl acetate- soluble acids from partially purified plant extracts were redissolved in 200-500µl of 32% methanol in 20mM K_2HPO_4/KH_2PO_4 buffer, pH8.0. The samples

were then applied to prewashed (2ml absolute methanol followed by 5ml distilled water) Sep-pak C_{18} cartridges (Waters Associates, Milford, MA, USA) using a glass syringe with a Luer end-fitting. ABA and its catabolites were eluted from the Sep-pak C_{18} cartridge with a single 6.0ml aliquot of 32% methanol in 20mM K_2HPO_4/KH_2PO_4 buffer, pH8.0 at a flow rate of 1-2ml/min. The eluates were reduced to dryness *in vacuo* at 35°C and the purified acids resuspended in a small volume of ethyl acetate/methanol (50:50, v/v) for further analysis. This procedure routinely resulted in greater than 80% reduction in dry weight and greater than 95% recovery of ABA and its catabolites (see Chapter 3).

Where necessary, plant extracts were also purified on Sep-pak C_{18} cartidges using the procedures outlined by Pierce and Raschke (1981) and Lewis and Visscher (1982). In the Lewis and Visscher (1982) method extracts were applied to Sep-pak C_{18} cartridges in 50µl of methanol. The cartridge was then washed with 7ml aliquots of 20% and 32% aqueous methanol pH2.8, followed by 6ml of 32% aqueous methanol pH8.0 and 5ml of 100% methanol at a flow rate of 2-3ml/min.

In the procedure described by Pierce and Raschke (1981), aqueous plant extracts were loaded onto Sep-pak C_{18} cartidges which were then washed with 5ml of 1% aqueous acetic acid and 7ml of ethanol/acetic acid (40:60, v/v). In both procedures organic solvents were removed *in vacuo* at 35°C and the recovery of labelled ABA and the reductions in dry weight determined.

2.11. MICRO-CHEMICAL ANALYSES.

2.11.1. Identification of terpenyl pyrophosphates.

Prior to micro-chemical analyses, the terpenyl pyrophosphates, produced in cell-free preparations from *Hordeum vulgare* embryo tissue were tentatively identified by DEAE-cellulose column chromatography by comparison with authentic standards and published elution properties. They were further identified using descending paper chromatography (see Section 2.12.2) in the solvent system, *iso*-propanol/*iso*-butanol/NH_3/H_2O (40:20:1:39, v/v). Tentative identifications were made by co-chromatography with authentic [1-^{14}C]-IPP and by comparison to the Rf values obtained for these compounds in studies using the identical solvent system (Anderson *et al*, 1960; Dugan *et al*, 1968).

Putative terpenyl pyrophosphates were hydrolysed using either enzymic or acid procedures as

described by Davies *et al*, (1975). For enzymic hydrolysis, putative IPP (generated from [^{14}C]-MVA) and FPP (generated from [^{14}C]-MVA and [^{14}C]-IPP), redissolved in a small volume of 0.2M $(NH_4)_2CO_3$ buffer, were added to incubation mixtures consisting of 1.0ml 2M Tris-HCl buffer, pH8.0 which contained 12M $MgCl_2$ and calf-intestine alkaline phosphatase (Grade II-Boehringer Mannheim) equivalent to 2mg protein. The mixture was incubated for 4h at 37^{o}C in a water-bath and the reaction terminated by the addition of 2.0ml ethanol. The incubation mixtures were extracted (3x) with 5.0ml aliquots of diethyl ether and the combined extracts reduced to dryness *in vacuo* at 35^{o}C and the residue analysed by TLC (see Section 2.12.3) in the solvent system, *n*-propanol/NH_3/H_2O (6:3:1, v/v) developed once to 10.0cm (Shechter, 1973). The data depicted in Figure 2.2 show [^{14}C]-isopentenol was released when [1-^{14}C]-IPP was treated with alkaline phosphatase.

For acid hydrolysis, 1N HCl was added to putative [^{14}C]-IPP and [^{14}C]-FPP in 1.0ml of 0.1M K_2HPO_4/KH_2PO_4 buffer (pH7.5) and the pH adjusted to 1.0. The acidic solution was heated in a stoppered test tube to 60^{o}C for 60min in a water bath. At the end of the incubation periods the solutions were diluted to 5.0ml with distilled water and extracted (3x) with 5.0ml aliquots of diethyl ether. The diethyl ether extracts were reduced to dryness *in vacuo* at 35^{o}C and the residue analysed by TLC as described above. The data depicted in Figure 2.2C and D show that acid hydrolysis of authentic [^{14}C]-IPP (Figure 2.2C) produced the free alcohol, [^{14}C]-isopentenol (Figure 2.2D).

2.11.2. The tentative identification of biosynthetically-produced abscisic acid and the catabolites of applied abscisic acid.

2.11.2.1. Identification of biosynthetically-produced ABA.

Putative ABA, generated from either *R*-[2-^{14}]-MVA or [1-^{14}C]-IPP during biosynthetic studies in intact tissue and cell-free homogenates, was dissolved in a small volume of absolute methanol and methylated (at room temperature) by adding excess ethereal diazomethane (prepared as described in Section 2.2.2). Tentative identification of ABA was achieved by comparing its chromatographic properties as the methyl ester on TLC (silica gel GF_{254}) with authentic (*R,S,*)-ABAMe using the solvent system, *n*-hexane/ethyl acetate (1:1, v/v) developed once to 15cm (Noddle and Robinson, 1969; Milborrow and Noddle, 1970; Loveys *et al*, 1975).

The identity of ABA was further determined by reduction of its methyl ester with $NaBH_4$ in ice-cold

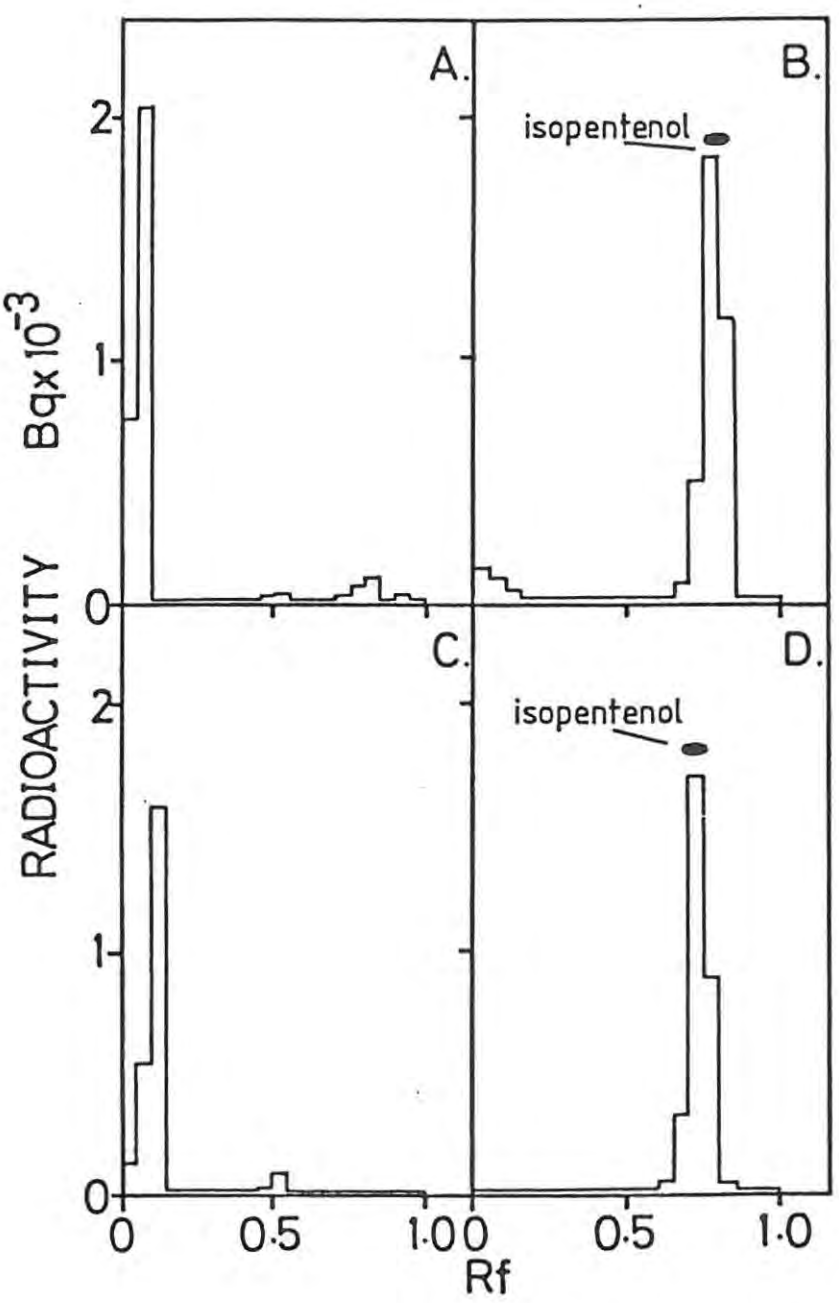

Figure 2.2. Acid and enzymic hydrolysis of standard isopentenol pyrophosphate. Authentic [1-^{14}C]-IPP (A and C) was hydrolyzed with either acid (B) or alkaline phosphatase (D) as described in Section 2.10.1 to produce the free terpenol, isopentenol. Samples were chromatographed on silica gel G(Type 60) in *n*-propanol/NH_3/H_2O (6:3:1, v/v), developed once to 10cm.

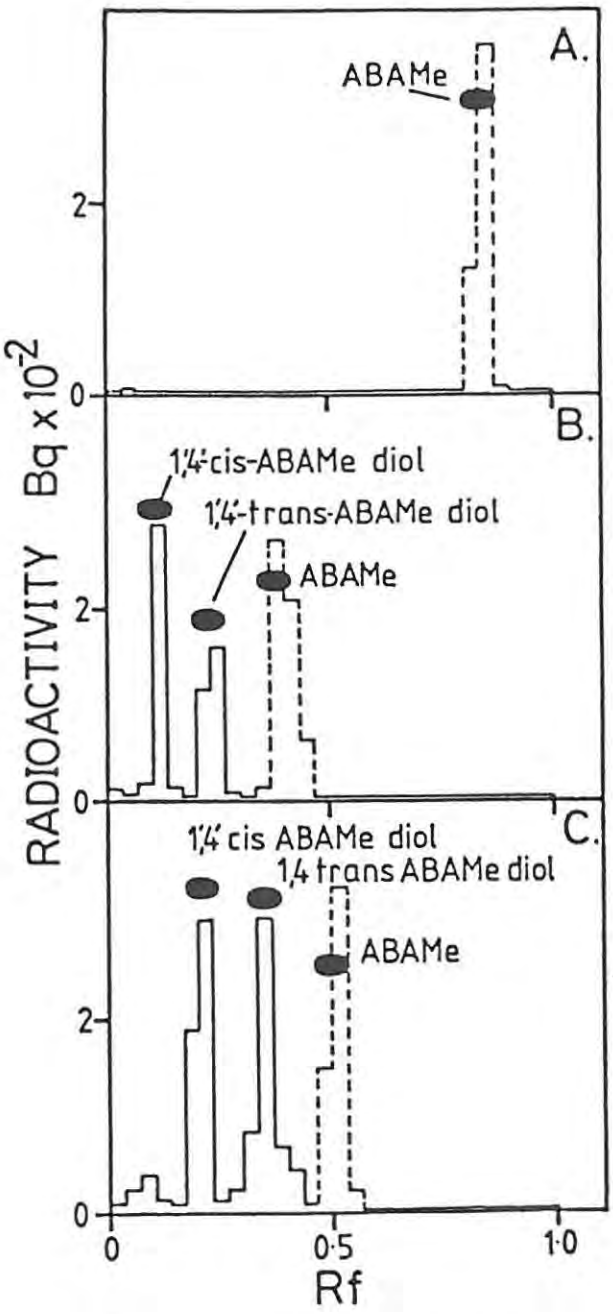

Figure 2.3. TLC separation of ABAMe and its products of $NaBH_4$ reduction. A), TLC of the methyl ester of authentic (*R,S,*)-[2-^{14}C]-ABA on silica gel GF_{254} in *n*-hexane/ethyl acetate (1:1, v/v) developed (1x) to 15cm; B), products of $NaBH_4$ reduction of ABAMe separated by TLC on silica gel GF_{254} in benzene/ethyl acetate/acetic acid (25:3:4, v/v) developed (1x) to 15cm; and C), reduction products of ABAMe separated on TLC in benzene/ethyl acetate/acetic acid (15:3:1, v/v) developed (1x) to 15cm.

methanol/H_2O (2:1, v/v) at 0°C for 15min to give an equal mixture of the 1',4'-*cis* and 1',4'-*trans* diols of ABAMe (see Section 2.16.3). These were separated by TLC (silica gel GF_{254}) using the solvent systems, benzene/ethyl acetate/acetic acid (15:3:1, v/v): 1',4'-*cis* diol, Rf 0.3; 1',4'-*trans* diol, Rf 0.43 and benzene/ethyl acetate/acetic acid (25:3:4, v/v): 1',4'-*cis* diol, Rf 0.4; 1',4'-*trans* diol, Rf 0.56 (Milborrow and Noddle, 1970; Noddle and Robinson, 1969; Loveys *et al*, 1975). The distributions of radioactivity, following micro-chemical manipulations and analysis by TLC in the solvent systems described above, for authentic (*R,S,*)-[2-^{14}C]-ABAMe are presented in Figure 2.3. A typical separation of (*R,S,*)-[2-^{14}]-ABAMe in *n*-hexane/ethyl acetate (1:1, v/v) is depicted in Figure 2.3A, while the distribution of radioactivity associated with the products of $NaBH_4$ reduction are depicted in Figure 2.3C and D following TLC on silica gel GF_{254} in two different solvent systems.

2.11.2.2. Identification of ABA catabolites.

Putative PA, DPA and unidentified acidic catabolites, generated from (*R,S,*)-[2-^{14}]-ABA, were tentatively identified by comparing their chromatographic properties as the methyl esters on TLC (silica gel GF_{254}) with methylated standards using the solvent system, *n*-hexane/ethyl acetate (1:1, v/v) developed (3x) to 15cm. The separation of PA and DPA isolated from *Phaseolus vulgaris* seedlings, fed with (*R,S,*)-[2-^{14}]-ABA (100kBq), as their methyl esters on TLC (silica gel GF_{254}) in *n*-hexane/ethyl acetate (1:1, v/v) is depicted in Figure 2.4A.

The identity of PA was further determined by reduction of its methyl ester with $NaBH_4$ (as described above) to yield 4'-DPAMe and its 4'-epimeric ester (Milborrow, 1975b). Following removal of the methanol under N_2 and extraction of the compounds into ethyl acetate, DPAMe and its epimeric ester were separated on TLC (silical gel GF_{254}) using the solvent system, *n*-hexane/ethyl acetate (1:1, v/v) developed (3x) to 15 cm. Separation of the reduction products of PAMe, orginally prepared from *Phaseolus vulgaris* seedlings, fed with (*R,S,*)-[2-^{14}]-ABA (100kBq), on TLC is depicted in Figure 2.4B. Similarly, the identity of DPA was determined following oxidation with Jones' reagent (prepared as described in Section 2.2.3) to produce PAMe (Zeevaart and Milborrow, 1976).

Jones' reagent was added to a well-stirred mixture of the alcohol (DPAMe) in 0.5ml acetone at 5-10°C. The solution was stirred for a further 30min, diluted with water (3.5ml) and the products extracted (3x) into equal volumes of ethyl acetate. Ethyl acetate samples, containing DPAMe and its oxidation product were separated on TLC (silica gel GF_{254}) using the solvent system, *n*-

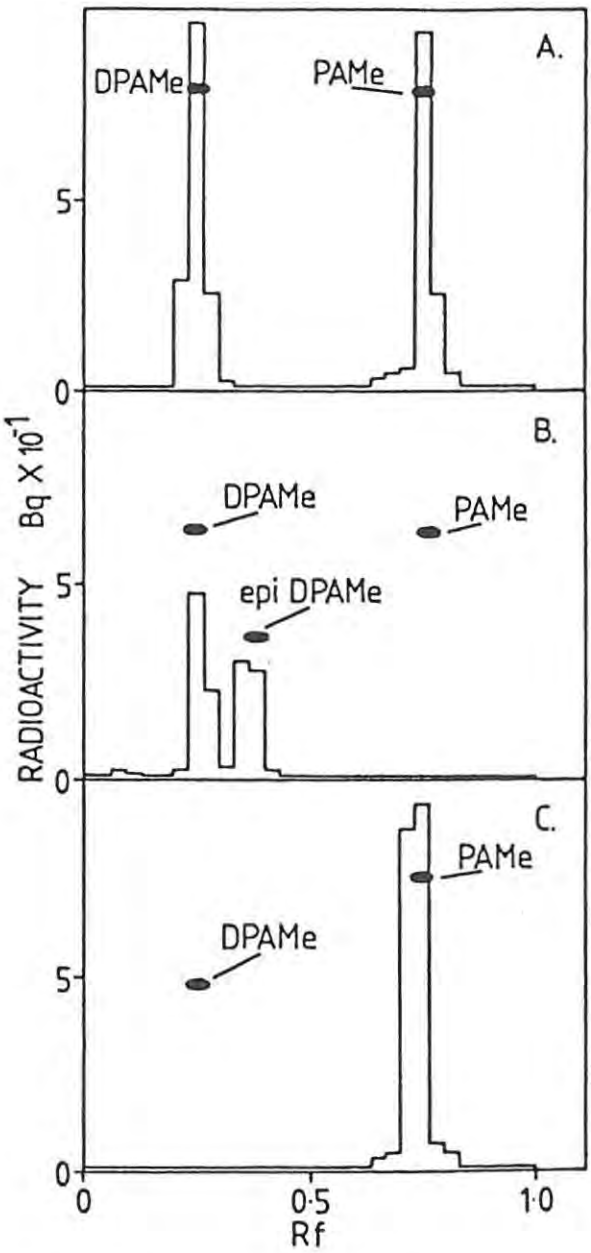

Figure 2.4. Micro-chemical characterization of the acidic catabolites of (*R,S,*)-[2-^{14}C]-ABA. A), TLC of the methyl esters of the acidic catabolites of (*R,S,*)-[2-^{14}C]-ABA from extracts of light-grown *Phaseolus vulgaris* seedlings; B), the reduction products of PAMe following treatment with $NaBH_4$; and C), the oxidation product of DPAMe following treatment with Jones' reagent. Samples were separated on silica gel GF_{254} in *n*-hexane/ethyl acetate (1:1, v/v) developed (3x) to 15cm.

hexane/ethyl acetate (1:1, v/v) developed (3x) to 15cm. The oxidation products of DPAMe, isolated from *Phaseolus vulgaris* seedlings fed with (*R,S,*)-[2-^{14}]-ABA (100kBq), separated on TLC as described above are depicted in Figure 2.4C. Identical procedures were conducted on additional, previously undetected, radioactive acidic catabolites of applied (*R,S,*)-[2-^{14}]-ABA.

2.11.2.3. Alkaline hydrolysis of conjugates.

The aqueous fractions remaining after solvent partitioning, containing putative glucosyl ethers and esters of ABA and its acidic catabolites, were detected by treating one half of the aqueous phase with base (5N NaOH), pH11.0 at 60^{o}C for 1h (Milborrow, 1970; Loveys and Milborrow, 1981; Hirai and Koshimizu, 1983). The free acids released by hydrolysis were extracted (4x) into ethyl acetate after the incubations had been cooled and adjusted to pH2.5 with conc HCl. Control incubations were held at pH7.0 and 60^{o}C for 1h and were processed similarly.

In order to demonstrate that this procedure had no adverse effect on ABA *per se*, incubations with authentic (*R,S,*)-[2-^{14}]-ABA were carried out in the same way as described above. The data depicted in Figure 2.5 demonstrate that ABA is unaffected by both temperature (Figure 2.5A) and treatment with base (Figure 2.5B). These findings suggest that any radioactive ABA released by hydrolysis from a conjugated form will not be transformed into acidic breakdown products by treatment with base at 60^{o}C for 1h.

Hydrolysis of highly polar, radioactive compounds from the origin of TLC plates following chromatography of the orginal ethyl acetate-soluble acid fraction in toluene/ethyl acetate/acetic acid (50:30:4, v/v), was carried out as above after eluting the compounds from the silica gel with methanol.

2.12. CHROMATOGRAPHIC PROCEDURES.

2.12.1. DEAE-cellulose column chromatography.

The separation of terpenyl pyrophosphates was essentially the same as that described by Davies *et al*, (1975). Incubation mixtures, supernatants and washings, generated from *R*-[2-^{14}]-MVA or [1-^{14}C]-IPP feeds to *Hordeum vulgare* embryo cell-free preparations, were immediately applied to a 1.8x13.5cm Pierce Chromato Flo^{TM} column (Pierce, Rockford, ILL, USA) of DEAE cellulose No.

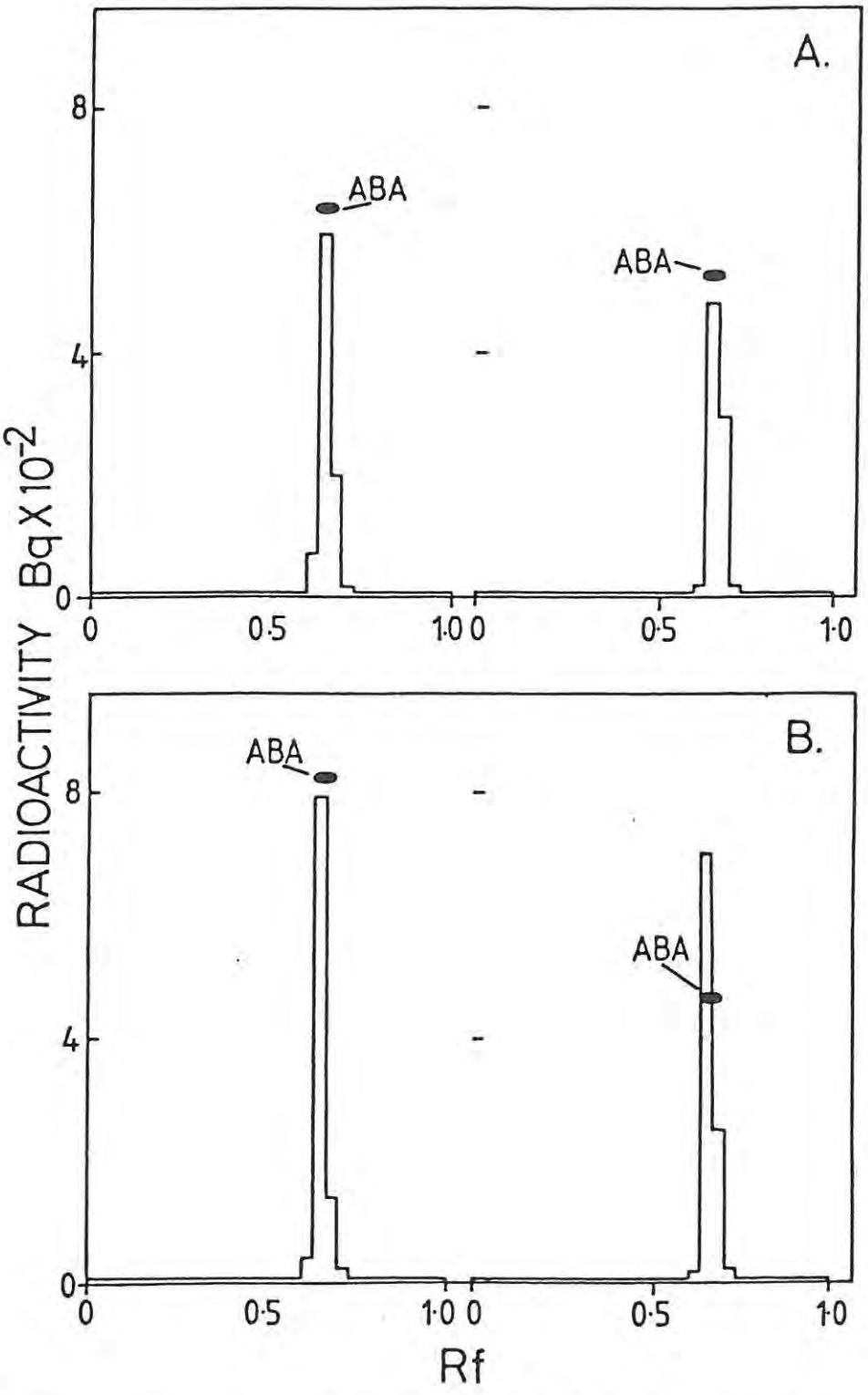

Figure 2.5. The effect of base and temperature on (*R,S,*)-ABA. (*R,S,*)-[2-^{14}C]-ABA (1kBq) in 0.5M K_2HPO_4/KH_2PO_4 buffer was incubated at A), room temperature and 60°C at pH7.0 for 1h and B), at room temperature and 60°C at pH11.0 for 1h. Samples were processed as described in Section 2.10.2.3 and chromatographed on silica gel GF_{254} in toluene/ethyl acetate/acetic acid (50:30:4, v/v) developed (2x) to 15cm.

D-3764 which had been washed, packed and pre-equilibrated at 4°C with distilled water (Millipore, Milli-Q Reagent Water System). After application of the sample, the column was eluted at 4°C with a 600ml concave gradient (k=2) of 0-0.2M $(NH_4)_2CO_3$ (Merck Chemicals, Darmstadt, Germany) at a flow rate of 40ml/h, and 5.0ml fractions were collected using an ISCO Model 328 automatic fraction collector. Column fractions were examined for absorbance at 265nm in a MSE-Spectro Plus spectrophotometer and then the distribution of radioactivity was determined using liquid scintillation spectrometry (see Section 2.13).

To determine the elution properties of the radiolabelled substrates used in the preparation of terpenyl pyrophosphates and the adenosine derivatives, resulting from the enzymic hydrolysis of ATP, a 5ml aliquot of 0.1M K_2HPO_4/KH_2PO_4 buffer (pH7.5) containing ATP, ADP, AMP, (all 1mg/100ml) and [2-^{14}]-MVA, [2-^{14}]-MVAL, [1-^{14}C]-IPP (all 90kBq) was chromatographed on a DEAE-cellulose column as described above. The data depicted in Figure 2.6 show a typical chromatographic separation on DEAE-cellulose of the standard adenosine derivatives (AMP, ADP and ATP), adenine and the radiolabelled subtrates MVA, MVAL and IPP. This elution profile was then used to locate the various components derived during the cell-free preparation of terpenyl pyrophosphates.

In addition, conductivity measurements were taken every 10 fractions using a dds 200 temperature/pH/conductivity meter.

2.12.2. Paper chromatography.

This was carried out only for studies on the biosynthesis of terpenyl pyrophosphates. Aliquots from the pooled column fractions containing the putative terpenyl pyrophosphates were spot loaded onto Whatman No1 paper chromatography strips (6x45cm) and dried using a hair-dryer. Authentic [1-^{14}C]-IPP was used as a standard for comparison with published Rf values of this compound (Dugan *et al*, 1968; Davies *et al*, 1975) in the solvent system used. Once the spots had been dried the chromatograms were developed using the solvent system, *iso*-porpanol/*iso*-butanol/NH_3 (sp.gr. 0.91)/H_2O (40:20:1:39, v/v) and the solvent front allowed to migrate 35cm from the origin in a decending manner. The radioactive components were subsequently detected by scintillation spectrometry (Section 2.13).

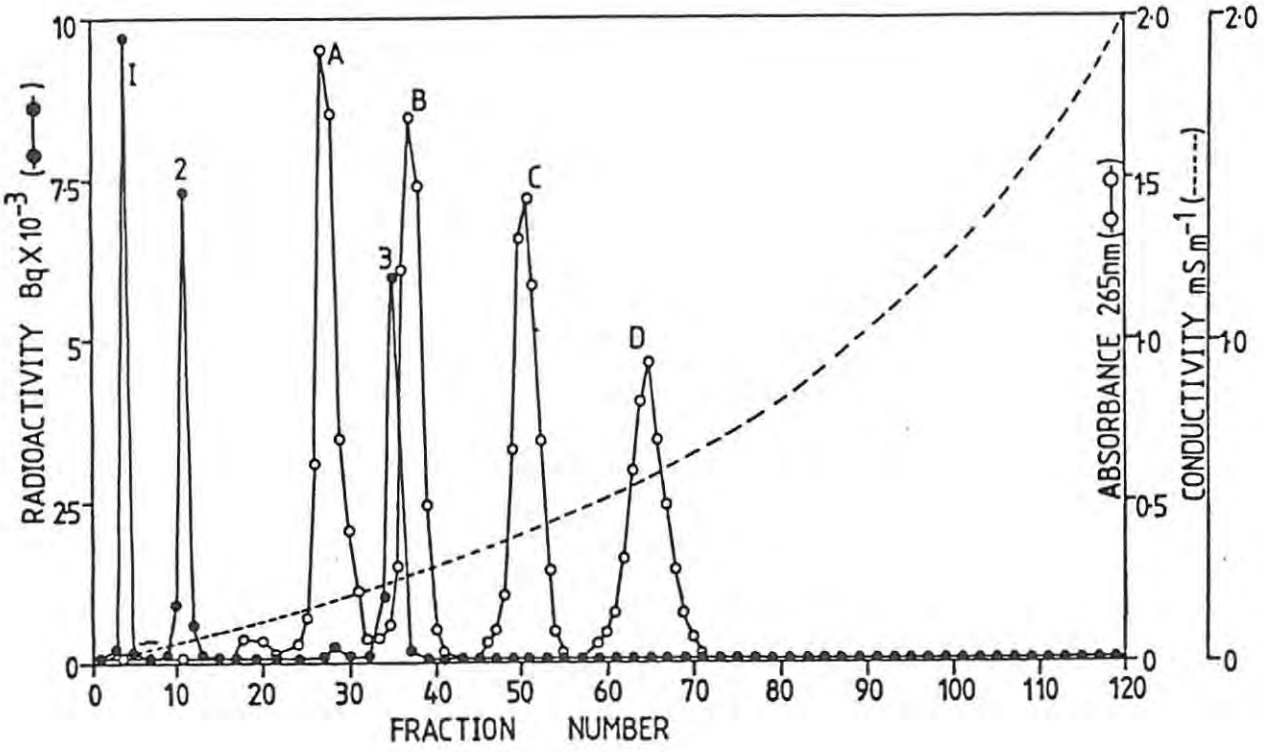

Figure 2.6. Elution profile of standard adenosine derivatives, adenine and radiolabelled substrates separated by DEAE-cellulose column chromatography (Section 2.11.1) developed with 0-0.2M $(NH_4)_2CO_3$ and 5ml fractions collected. Column effluent was monitored for radioactivity (●) and absorbance at 265nm (o). Alternate baseline points have been omitted. Conductivity was determined in every 10 fractions eluted from a DEAE-cellulose column developed with 0-0.2M $(NH_4)_2CO_3$ buffer only, in order to demonstrate the gradient (k = 2) generated. 1, MVA; 2, MVAL; 3, IPP; A, adenine; B, AMP; C, ADP; D, ATP.

2.12.3. Thin layer chromatography.

Thin layer plates (0.25mm thickness) of silica gel GF_{254} (MERCK) and silica gel G (Type 60) were prepared by adding 60ml of distilled H_2O to 30g of silica gel which was then vigorously shaken for 2min and 5, 20x 20cm thin layer plates prepared using a DESAGA TLC spreader and the plates allowed to air dry overnight.

Aqueous samples of terpenyl pyrophosphates, ether fractions containing terpenols and soluble acid fractions containing ABA and its catabolites and the methyl esters thereof were applied to TLC plates 2.0cm from the bottom and the plates developed in an acending manner in glass chromatography tanks using the following solvent systems.

Terpenyl pyrophosphates, their free alcohols and standard terpenols were separated on TLC (silica gel G Type 60) using the solvent system, *n*-propanol/NH_3 (sp.gr. 0.91)/H_2O (6:3:1, v/v) developed once to 10cm (Shechter, 1973). Their relative mobilities in this solvent system are presented in Table 2.3. Following chromatography, standard terpenols were detected by exposing the TLC plates to iodine vapours in a chromatography tank for 45min. The distribution of radioactivity was determined by liquid scintillation spectrometry (see Section 2.13).

ABA and its catabolites were separated on TLC (silica gel GF_{254}) using the solvent systems; toluene/ethyl acetate/acetic acid (50:30:4, v/v) developed (2x) to 15cm (Zeevaart and Milborrow, 1976), toluene/ethyl acetate/methanol/acetic acid (50:30:7:4, v/v) developed (1x) to 15cm (Lehmann *et al*, 1983b); chloroform/methanol/water (75:22:3, v/v) developed once to 11cm and then to 16cm, after briefly drying the plate in air (Murhpy, 1984) and in benzene/butanol/acetic acid (70:15:25, v/v) developed (1x) to 15cm (Dashek *et al*, 1979; Uknes and Ho, 1984). Polar catabolites were separated on TLC (silica gel GF_{254}) using the solvent system, chloroform/methanol/acetic acid/water (45:15:3:2, v/v) developed (1x) to 15cm (Dashek *et al*, 1979). The relative mobilities of authentic ABA and its biosynthetically-prepared catabolites and related compounds in these solvent systems are presented in Table 2.4. In biosynthetic studies, diethyl ether fractions containing [^{14}C]-ABA were chromatographed on TLC (silica gel GF_{254}) using the solvent system, toluene/ethyl acetate/acetic acid (50:30:4, v/v) containing BHT (10mg/l) as an antioxidant and developed 2x to 15cm (Milborrow and Robinson, 1973), as described above.

TABLE 2.3: Chromatographic properties of standard terpenols and 1-[^{14}C]-IPP on thin layers of silica gel.

Relative mobilities of standard terpenols and authentic 1-[^{14}C]-IPP on thin layers (0.25 mm) of silica gel (Type 60) using the solvent system, n-propanol/NH_3 (sp. gr. 0.91)/H_2O (6:3:1, v/v) developed once to 10 cm. Terpenols were analysed by exposure of the developed TLC plate to iodine vapours (x 45 min) in a glass chromatography tank. 1-[^{14}C]-IPP was detected by liquid scintillation spectrometry.

COMPOUND	RELATIVE MOBILITY ON TLC	Rf x 100 ON TLC
(±) - Farnesol	0.90	90
(±) - Linalool	0.94	94
(±) - Nerolidol	0.80	80
(±) - Geraniol	0.94	94
(±) - Phytol	0.57	57
1-[^{14}C]-IPP	0.12	12

TABLE 2.4: Thin layer chromatography of ABA and its catabolites on silica gel GF_{254}.

COMPOUND	Rf x 100 in Solvent System*				
	(1)	(2)	(3)	(4)	(5)
ABA	66	68	76	83	-
t- ABA	76	-	-	-	-
1',4'-cis diol	40	-	-	-	-
1',4'-trans diol	60	-	66	-	-
PA	50	53	60	70	-
DPA	16	30	60	56	73
epi-DPA	16	-	60	56	-

*Solvent systems: (1) Toluene/ethyl acetate/acetic acid (50:30:4, v/v); (2) toluene/ethyl acetate/methanol/acetic acid (50:30:7:4, v/v); (3) chloroform/methanol/water (75:22:3, v/v); (4) Benzene/n-butanol/acetic acid (70:15:25, v/v); (5) chloroform/methanol/acetic acid/water (45:15:3:2, v/v).

Radioactive zones, detected by liquid scintillation spectrometry (see Section 2.13), and non-radioactive zones, detected under UV light, with Rf values corresponding to those of authentic (*R,S,*)-ABA (Sigma) and biosynthetically generated PA and DPA (see Section 2.16.1) were immediately scraped from the TLC plates into short glass columns plugged with glass wool which was covered with a 5mm layer of glass beads. Compounds were then eluted from the silica gel using H_2O-saturated ethyl acetate. Likewise, previously unidentified catabolites of ABA, detected as either UV-quenching bands or radioactive zones, were similarly eluted from the silica gel.

In order to determine the efficiency of recovery of compounds from silica gel, radioactive ABA, PA and DPA were chromatographed on TLC as described above and the zones containing these compounds eluted in the same way as previously described. The data depicted in Figure 2.7 demonstrate that 20ml of H_2O-saturated ethyl acetate, used to elute the silica gel, resulted in 100% recovery of labelled ABA, PA and DPA.

ABAMe was routinely separated on TLC (silica gel GF_{254}) using the solvent system, *n*-hexane/ethyl acetate (1:1, v/v) developed (1x) to 15 cm (Noddle and Robinson, 1969; Milborrow and Noddle, 1970; Loveys *et al*, 1975). In addition to PAMe and DPAMe, their respective reduction and oxidation products were separated on TLC (silica gel GF_{254}) using *n*-hexane/ethyl acetate (1:1, v/v) developed (3x) to 15cm, while the 1',4'-*cis* and 1',4'-*trans* diols of ABAMe were separated in either benzene/ethyl acetate/acetic acid (15:3:1, v/v) or *n*-hexane/ethyl acetate (1:1, v/v) developed (1x) to 15cm. The relative mobilities of the standard methyl esters are presented in Table 2.5. 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 12h.

It has been reported that silica-coated TLC plates decrease the recovery of indole-3-acetic acid (IAA) when compared to cellulose plates (Sagi, 1969), resulting from oxidation on the silica gel (Mann and Jaworski, 1970). For this reason it was decided to examine the effect of drying time on the recovery of (*R,S,*)-[2-^{14}]-ABA, chromatographed on silica gel GF_{254} as described above. The data presented in Table 2.6 indicate that with an increase in drying time there is a concomitant decrease in the percentage radioactivity recovered from the silica gel. Thus, TLC plates were never dried for periods exceeding 12h and in all cases an aliquot of the radioactive sample was removed, prior to chromatography, and the absolute amounts of radioactivity determined using liquid scintillation

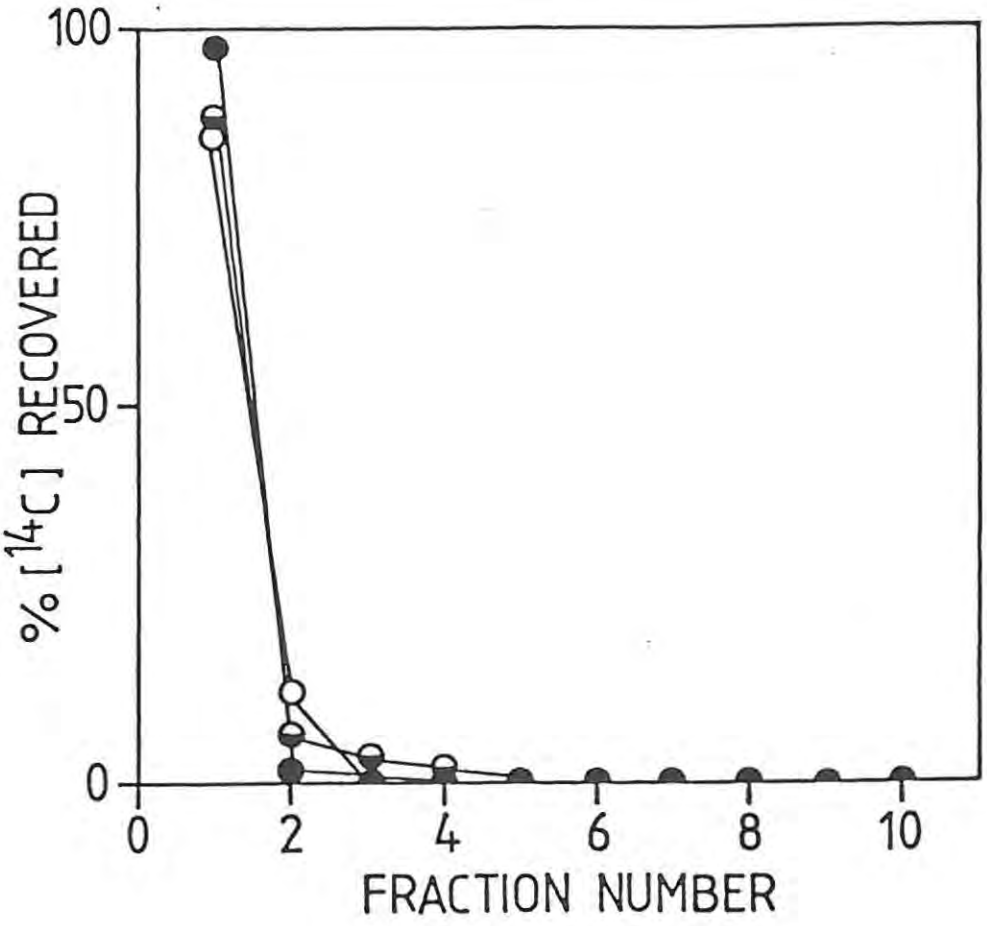

Figure 2.7. The recovery of labelled ABA, PA and DPA from silica gel GF_{254} using H_2O-saturated ethyl acetate. (*R,S,*)-[2-^{14}C]-ABA, [^{14}C]-PA and [^{14}C]-DPA were chromatographed on thin layers of silica gel GF_{254} in toluene/ethyl acetate/acetic acid (50:30:4, v/v) developed (2x) to 15cm. The zones of silica gel containing the radioactive compounds were scraped from the TLC plates and each was eluted through a glass column plugged with glass wool using H_2O-saturated ethyl acetate. Fractions (10ml) were collected and the recovery of radioactive ABA (●), PA (o) and DPA (⊖) in each fraction was determined by liquid scintillation spectrometry.

TABLE 2.5 Thin layer chromatography of the methyl esters of ABA and its catabolites on silica gel GF_{254} (0.25 mm thickness).

Compound	Rf x 100 in Solvent System*			
	(1)	(2)	(3)	(4)
ABAMe	60	80	-	70
PAMe	-	56	73	-
DPAMe	-	-	26	-
epi-DPAMe	-	-	40	-
1', 4' -cis diol of ABAMe	23	60	-	40
1', 4' -trans diol of ABAMe	36	70	-	56

*Solvent systems: (1) Benzene/ethyl acetate/acetic acid (15:3:1, v/v); (2) n-hexane/ethyl acetate (1:1, v/v), developed once; (3) n-hexane/ethyl acetate (1:1, v/v), developed three times; (4) Benzene/ethyl acetate/acetic acid (25:3:4, v/v).

TABLE 2.6 Kinetics of the recovery of (R,S)-[2- ^{14}C]-ABA during air drying of thin layers of silica gel GF_{254} following chromatography.

Five aliquots of (R,S) - [2- ^{14}C]-ABA (1.0 kBq) were chromatographed on silica gel GF_{254} in toluene/ethyl acetate/acetic acid (50:30:4, v/v) developed (2X) to 15 cm. The recovery of radioactivity from the TLC plate was determied each day for a 5 day period using liquid scintillation spectrometry.

Drying Time (h)	% (R,S)- [2-^{14}C]-ABA Recovered
12	100.0
24	96.49 ± 0.98
48	92.33 ± 0.59
72	84.48 ± 3.41
96	72.80 ± 1.61

spectrometry to allow for correction of elution efficiency.

2.12.4. Gas liquid chromatography.

Methylated samples for analysis by gas liquid chromatography (GLC) were routinely dissolved in a small volume of redistilled acetone. GLC was performed on a dual column, Perkin-Elmer 990 instrument using silanized glass columns (1.8mx2mm i.d.) packed with either 2% SE-30 or 1% XE-60 on Gaschrom Q (80-100 mesh) with Ar as the carrier gas (43ml/min) at an oven temperature of 190°C. The retention times of ABAMe and its catabolites on the stationary phases 2% SE-30 and 1% XE-60 are presented in Table 2.7.

Capillary GC was carried out on a Varian 4600 instrument coupled to a Varian CDS 401 data system using a wall-coated open tubular (WCOT) capillary column (50mx0.31mm fused-silica) coated with OV-1 (Hewlett-Packard, Wendywood, Johannesburg) and a He flow rate of 1ml/min and temperature programming (start temp 180°C, to 190°C at 2°C min^{-1}).

2.12.5. Radio-gas liquid chromatography.

Radioactive zones on TLC plates, detected by scintillation spectrometry, were eluted with H_2O-saturated ethyl acetate and reduced to dryness *in vacuo* for further analysis by radio-gas liquid chromatography (radio-GLC). Residues were methylated with ethereal diazomethane as previously described.

Radio-GLC was performed on a dual column Perkin-Elmer 990 gas chromatograph interfaced with a Panax Radiogas System (Panax Equipment Ltd., Redhill, Surrey, England) equipped with a gas flow proportional counter, scaler timer, automatic print-out facilities and twin-pen flat bed recorder. Samples were analysed on silanized glass columns (1.8mx 2mm i.d.) using the liquid stationary phases 1% XE-60 and 2% SE-30 on Gaschrome Q (80-100 mesh) with argon as carrier gas at a flow rate of 42 ml min^{-1} in a similar manner to that used for gibberellin analysis (Durley *et al*, 1973; Railton *et al*, 1973; 1974a, b, c; Railton, 1976; Railton, 1980). The effluent carrier gas was split between the flame ionization detector and the gas flow proportional counter in a ratio of 1:9 and the [^{14}C] labelled compounds were oxidised to $^{14}CO_2$ over copper oxide at 650°C in a silica combustion tube connected to a freshly packed furnace tube containing magnesium perchlorate. Argon "make-up" gas was added at the entrance to the combustion tube to minimise "tailing" of the radioactive peaks

TABLE 2.7: Gas chromatography of the methyl esters of ABA and its catabolites.

Compound	Stationary phases*	
	2% SE-30	1% XE-60
	Retention time (min)	
ABAMe	3.8	3.4
t-ABAMe	5.2	4.3
PAMe	4.6	5.2
t-PAMe	5.8	6.8
DPAMe	6.0	4.2
t-DPAMe	ND[a]	ND
epi-DPAMe	-	3.8

*Packed glass columns (1.8m x 2mm i.d.) of 2% SE-30 or 1% XE-60 on Gas-chrom Q (80 - 100 mesh), oven temp. 180°C; Ar carried gas at a flow rate of 43 ml/min.

[a] not determined.

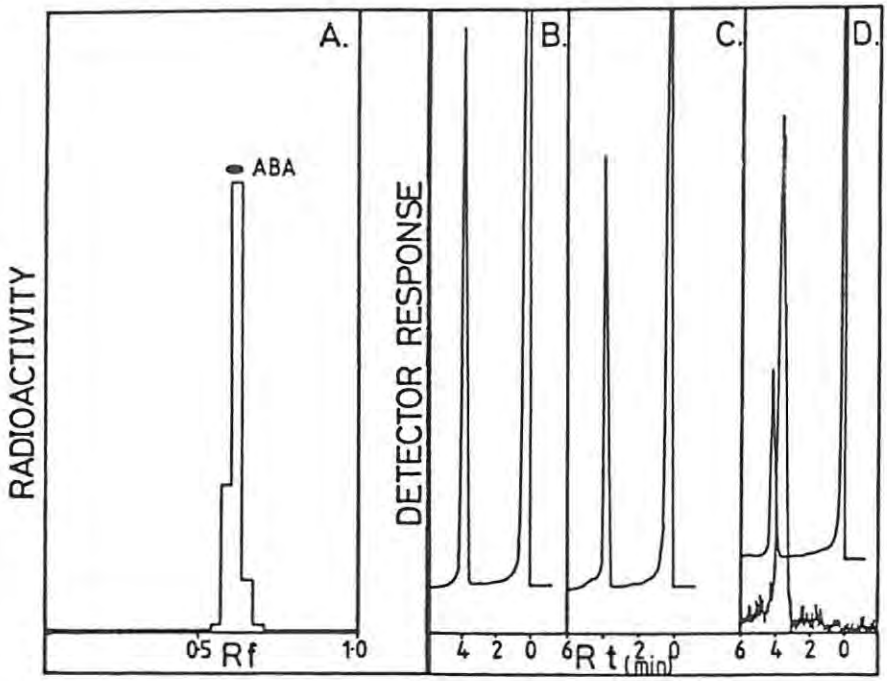

Figure 2.8. Purity of authentic (*R,S,*)-[2-^{14}C]-ABA, (*R,S,*)-ABA and (*R,S,*)-ABAMe analysed by TLC, GLC and radio-GLC. A), separation of (*R,S,*)-[2-^{14}C]-ABA by TLC on silica gel GF_{254} in toluene/ethyl acetate/acetic acid (50:30:4, v/v) developed (2x) to 15cm; B) and D), esterified, authentic (*R,S,*)-ABA analysed on packed GLC columns of 1% XE-60 and 2% SE-30; C), GLC analysis of authentic (*R,S,*)-ABAMe on a packed column of 1% XE-60; and D), radio-GLC of esterified, authentic (*R,S,*)-[2-^{14}C]-ABA on a packed GLC column of 2% SE-30. GLC and radio-GLC conditions were as described in Sections 2.12.4 and 2.12.5.

before carrier gas entry to the proportional counter.

The purity of (*R,S,*)-[2-^{14}]-ABA stock solutions was determined by both TLC (as previously described) and by radio-GLC (as above) (Figure 2.8A and D). Similarly, the purity of non-radiolabelled (*R,S,*)-ABA (Sigma) was determined by GLC using both 1% XE-60 and 2% SE-30 as stationary phases (Figure 2.8B and D). The purity of authentic (*R,S,*)-ABAMe (Sigma), used in some studies, was also determined (Figure 2.8C).

2.13. LIQUID SCINTILLATION SPECTROMETRY.

TLC plates, used for the separation of radioactive ABA and its catabolites, were each divided into 30 equal strips which were then scraped into scintillation vials and eluted with methanol (0.5ml). Similarly, TLC plates used for the separation of radioactive terpenols and terpenyl pyrophosphates were each divided into 20 equal strips and scraped into scintillation vials. In addition, paper chromatograms, used for the separation of radioactive terpenyl pyrophosphates, were divided into equal strips which were then cut into small pieces and placed into scintillation vials. The level of radioactivity in the vials was determined using either a Beckman LS 3150T scintillation spectrometer, with a counting efficiency of 92.73% for [^{14}C], or a Beckman LS 5801 scintillation spectrometer, programmed for automatic quench correction, following the addition of 10ml of cocktail (2,5-dipenyloxazole pyrophosphate (PPO) in toluene, 5g/l).

Radioactivity in aqueous phases following partitioning, aqueous column eluates, residual incubation media and phosphate buffer remaining after uptake of labelled substrate was counted in Bray's scintillant prepared by dissolving naphthalene (60g) and PPO (4g) in 100ml methanol and diluting the solution to 1l in 1,4-dioxan (Bray, 1960). Results were corrected for uptake of radiolabelled substrates into tissues, elution efficiency of radioactivity from silica gel and where necessary, for total recovery of radioactivity.

2.14. COMBINED CAPILLARY GAS CHROMATOGRAPHY-MASS SPECTROMETRY.

Capillary gas chromatography-mass spectrometry (GC-MS) was carried out on a Varian Aerograph series 2700 GC coupled to a Varian MAT 311A mass spectrometer and Varian SS-100 MS data system. A fused-silica column (50mx0.3mm i.d.) coated with OV-1 was employed, which was temperature programmed (start temp. 180^{o}C, final temp. 190^{o}C, rising at 2^{o}C/min), using He as the

carrier gas (flow rate = 1ml/1min 10sec). Mass spectra were obtained at 70eV with a source temperature of 175°C and were recorded at 11.5sec per mass decade.

Identification of ABAMe was achieved by reference to the published fragmentation pattern of ABAMe (Gray *et al*, 1974). The mass spectrum of methyl abscisate and its fragmentation pathway are depicted in Figure 2.9.

2.15. CIRCULAR DICHROISM MEASUREMENTS.

Esterified samples were measured in absolute methanol, in a Model J-20 ORD-CD instrument (Japan Spectroscopic Co.), between 200 and 400nm (Cornforth *et al*, 1966b; Saunders, 1978; Dörffling and Tietz, 1983).

2.16. PREPARATION OF STANDARD COMPOUNDS.

2.16.1. Biosynthetic preparation of phaseic acid and dihydrophaseic acid.

Radioactive and non-radioactive standards of PA and DPA were prepared biosynthetically using shoots of *Phaseolus vulgaris* (Zeevaart and Milborrow, 1976) and/or etiolated and light-grown leaf tissue from *Hordeum vulgare*. (*R,S,*)-[2-^{14}]-ABA (100kBq) or (*R,S,*)-ABA (5-20mg) was fed to excised, intact tissue (10-30g fresh weight) in KPi buffer as previously described (see Section 2.7 for details). Following a 30h incubation period, under constant illumination (66μmol m^{-2} s^{-1}) at 25°C or in the dark at 25°C for etiolated tissue, the plant material was homogenised in ice-cold ethyl acetate/methanol (50:50, v/v) and ABA and its catabolites extracted and purified as described in Sections 2.9 and 2.10. Compounds were further purified by TLC as described in Section 2.12.3. Identification of radiolabelled compounds was achieved by micro-chemical derivatisation (see Section 2.11) and radio-GLC (Section 2.12.5) while non-radioactive compounds were characterised by combined capillary GC-MS as described in Section 2.14.

The GLC of biosynthetically-prepared PA as its methyl ester on either a packed column of 2% SE-30 (Figure 2.10A) or on a capillary column coated with OV-1 (Figure 2.10B) demonstrated the presence of a single mass peak which was identified by capillary GC-MS as PAMe. The mass spectrum (Figure 2.11) generated from PAMe was found to be identical to the published mass spectrum (Dörffling and Tietz, 1983) of this compound.

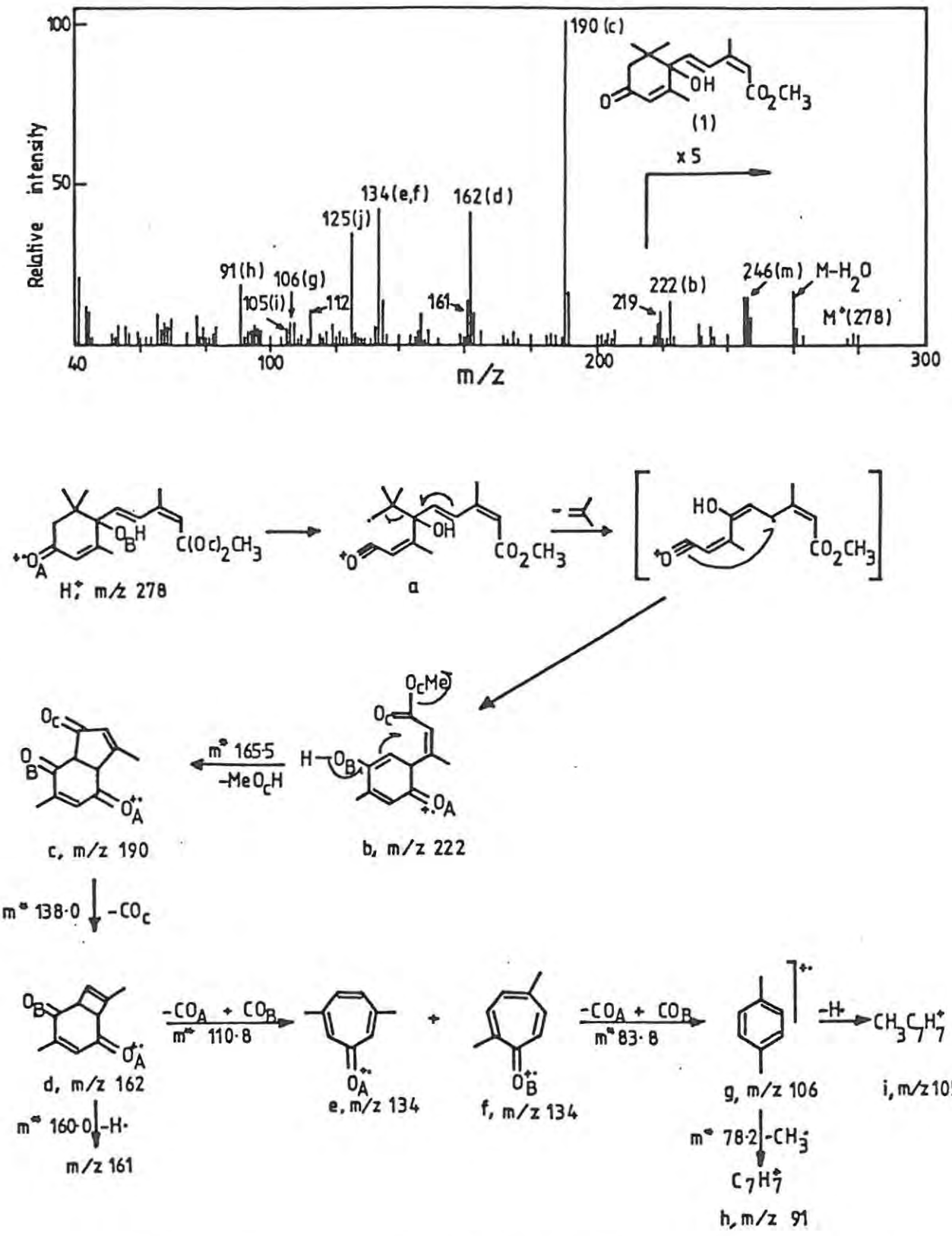

Figure 2.9. Electron impact mass spectrum of ABAMe and its major fragmentation pathway (after Gray *et al*, 1974).

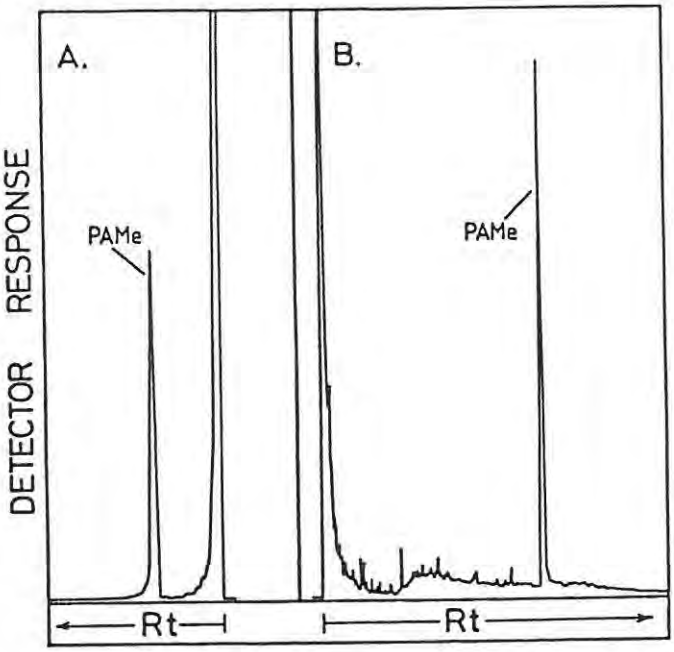

Figure 2.10. Gas chromatograms of esterified, biosynthetically-produced PA. PA was generated in light-grown *Phaseolus vulgaris* seedlings following a high-dose feed of (*R,S,*)-ABA (20mg) to 60g fresh weight of tissue. PA was extracted and purified as described in Sections 2.8 and 2.9. A), GLC of esterified PA on a packed column of 2% SE-30 and B), capillary GC of esterified PA on a capillary column coated with OV-1. GLC was carried out using a silanized glass column (1.8m x 2mm i.d.) packed with 2% SE-30 on Gaschrom Q (80-100 mesh) with Ar as carrier gas (43ml/min) at an oven temperature of 190°C. Capillary GC was carried out using a wall-coated open tubular (WCOT) capillary column (50m x 0.31mm fused-silica coated with OV-1) with a He flow rate of 1 ml/min and temperature programming (start 180°C to 190°C at 2°C min^{-1}).

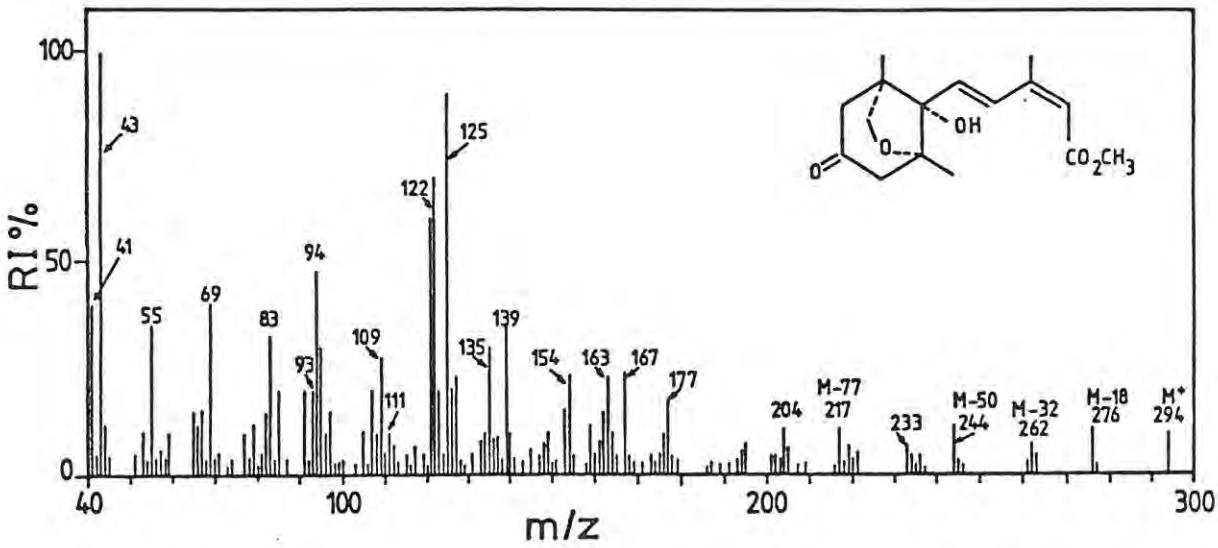

Figure 2.11. Electron impact mass spectrum of PAMe, separated by capillary GC on OV-1 as described in Figure 2.10. The mass spectrum was obtained at 70eV with a source temperature of 175°C and was recorded at 11.5 sec per mass decade.

Similarly, chromatography of biosynthetically-prepared DPAMe on both packed and capillary columns is depicted in Figure 2.12A and B and its mass spectrum is shown in Figure 2.13. This was identical to the published spectrum for DPAMe (Dörffling and Tietz, 1983).

2.16.2. Preparation of *epi*-dihydrophaseic acid.

Standard *epi*-DPAMe was prepared by the reduction of PAMe with $NaBH_4$ as described for the diols (Section 2.16.3). *epi*-DPAMe was separated from DPAMe using the solvent system, *n*-hexane/ethyl acetate (1:1, v/v) developed (3x) to 15cm. Further analysis of the PAMe reduction products was carried out by GLC on a packed column using the stationary phase 1% XE-60 (Figure 2.14).

2.16.3. Preparation of 1',4'-*cis* and 1',4'-*trans* diol of abscisic acid.

Standards of the 1',4'-*cis* and 1',4'-*trans* diols of ABA were prepared by reducing ABAMe (4mg) with $NaBH_4$ (4mg) in 2.0ml of H_2O-methanol (1:2, v/v) at 0^oC. Kinetic studies (Figure 2.15) demonstrated that the reaction went to completion within 15min.

The reaction mixture was then extracted (3x) with equal volumes of ethyl acetate after removal of the methanol under N_2. The products were separated on TLC (silica gel GF_{254}) in benzene/ethyl acetate/acetic acid (25:3:4, v/v) and the 1',4'-*cis* diol of ABAMe (Rf 0.40) and 1',4'-*trans* diol of ABAMe (Rf 0.56) were eluted from the silica gel with H_2O-saturated ethyl acetate as described in Section 2.12.3. Analysis of these products was performed by capillary GC-MS using the conditions described in Section 2.14. The 1',4'-*cis* diol of ABAMe gave a single, sharp peak (see Figure 2.16A) with a molecular ion at M^+280 and the spectrum, depicted in Figure 2.17, was consistent with the structure of this diol (Dathe and Sembdner, 1982). The 1',4'-*trans* diol of ABAMe dehydrated on capillary GC (see Figure 2.16B) as described before (Milborrow, 1983a) to yield a compound, X, with a molecular ion at M^+262 which was identified as 4'-desoxy ABAMe (Milborrow, 1983a) and the spectrum is depicted in Figure 2.18. Thus, the identity of the 1',4'-*trans* diol was sought by direct probe analysis where a weak molecular ion at M^+280 was detected using a Varian MAT 212 mass spectrometer. The fragmentation pattern of this compound was consistent with the structure of the 1',4'-*trans* diol of ABAMe and the mass spectrum is depicted in Figure 2.19.

The free acid of the 1',4'-*cis* diol of ABA and its 1',4'-*trans* diol isomer were produced by hydrolysis

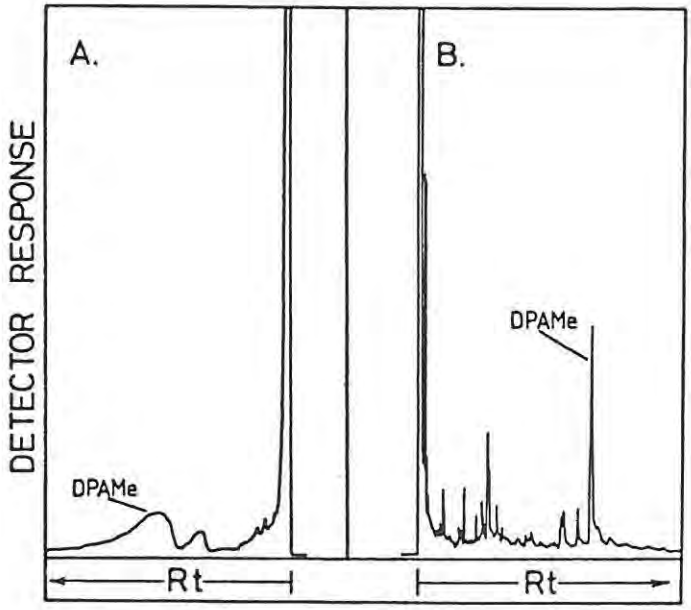

Figure 2.12. Gas chromatograms of esterified, biosynthetically-produced DPA. DPA was generated in light-grown *Phaseolus vulgaris* seedlings following a high-dose feed of (*R,S,*)-ABA (20mg) to 60g fresh weight of tissue. DPA was extracted and purified as described in Sections 2.8 and 2.9. A), GLC of esterified DPA on a packed column of 2% SE-30 and B), capillary GC of esterified DPA on a capillary column coated with OV-1. GLC was carried out using a silanized glass column (1.8m x 2mm i.d.) packed with 2% SE-30 on Gaschrom Q (80-100 mesh) with Ar as carrier gas (43ml/min) at an oven temperature of 190°C. Capillary GC was carried out using a wall-coated open tubular (WCOT) capillary column (50m x 0.31mm fused-silica coated with OV-1) with a He flow rate of 1 ml/min and temperature programming (start 180°C to 190°C at 2°C min^{-1}).

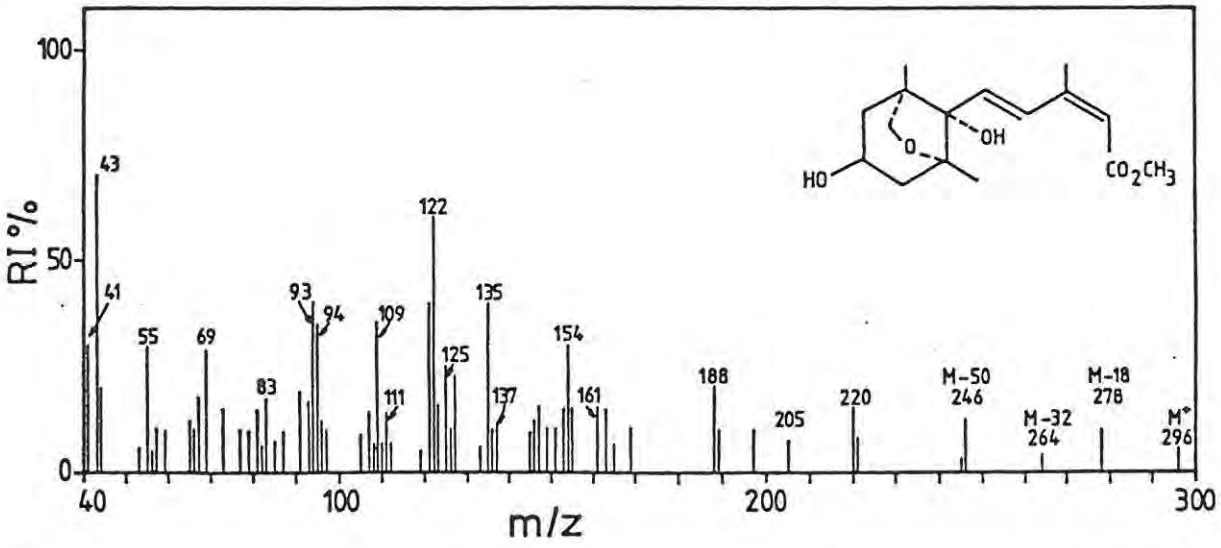

Figure 2.13. Electron impact mass spectrum of DPAMe, separated by capillary GC on OV-1 as described in Figure 2.12. The mass spectrum was obtained at 70eV with a source temperature of 175°C and was recorded at 11.5 sec per mass decade.

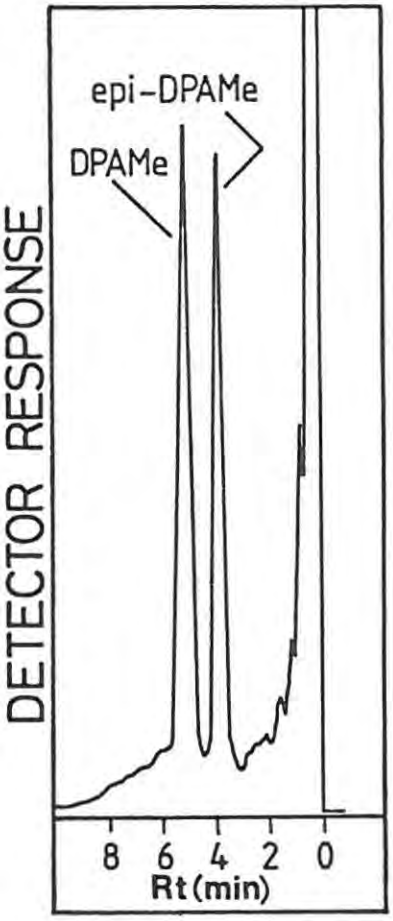

Figure 2.14. Gas chromatogram of DPAMe and *epi*-DPAMe separated on a packed column of 1% XE-60. Standard DPAMe and *epi*-DPAMe were generated by treatment of PA (biosynthetically prepared in light-grown leaves of *Hordeum vulgare*), following esterification with CH_2N_2, by reduction with $NaBH_4$. GLC was carried out using silanized glass columns (1.8m x 2mm i.d.) packed with 2% SE-30 on Gaschrom Q (80-100 mesh) with Ar as carrier gas (43ml/min) at an oven temperature of 190°C.

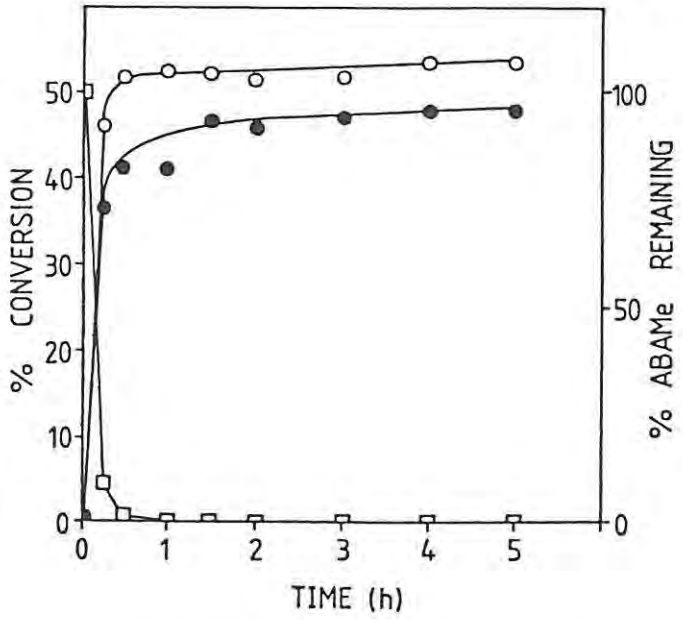

Figure 2.15. Kinetics of the reduction of (*R,S,*)-[2-^{14}C]-ABAMe to the 1',4'-*cis* and 1',4'-*trans* diols of ABAMe using $NaBH_4$. Authentic, radiolabelled ABAMe (□) was reduced to a mixture of the 1',4'-*cis* (●) and 1',4'-*trans* (o) diols of ABAMe by treatment with $NaBH_4$ (4mg) in ice-cold methanol/H_2O (1:2, v/v). At intervals aliquots were removed and partitioned into ethyl acetate (3x) after removal of excess methanol under N_2. The products were separated by TLC on silica gel GF_{254} in *n*-hexane/ethyl acetate (1:1, v/v), developed once to 15cm.

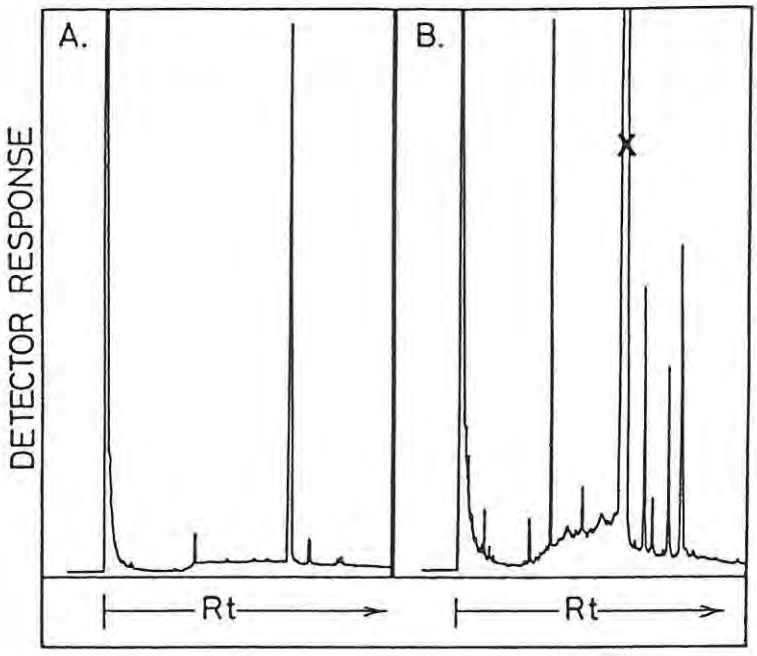

Figure 2.16. Capillary GC of the 1',4'-*cis* (A) and 1',4'-*trans* (B) diols of ABAMe. The diols were prepared by treating authentic (*R,S,*)-ABAMe (4mg) with $NaBH_4$ (4mg) and were processed as described in Figure 2.15. Capillary GC was carried out using a wall-coated open tubular (WCOT) capillary column (50m x 0.31mm fused-silica coated with OV-1) with a He flow rate of 1 ml/min and temperature programming (start 180°C to 190°C at 2°C min^{-1}).

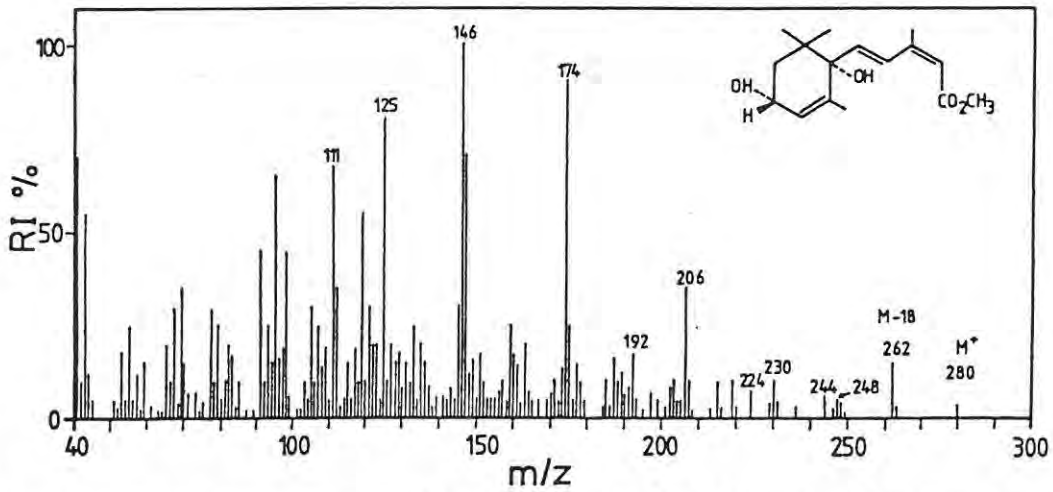

Figure 2.17. Electron impact mass spectrum of the 1',4'-*cis* diol of ABAMe, separated by capillary GC on OV-1. The mass spectrum was obtained at 70eV with a source temperature of 175°C and was recorded at 11.5 sec per mass decade.

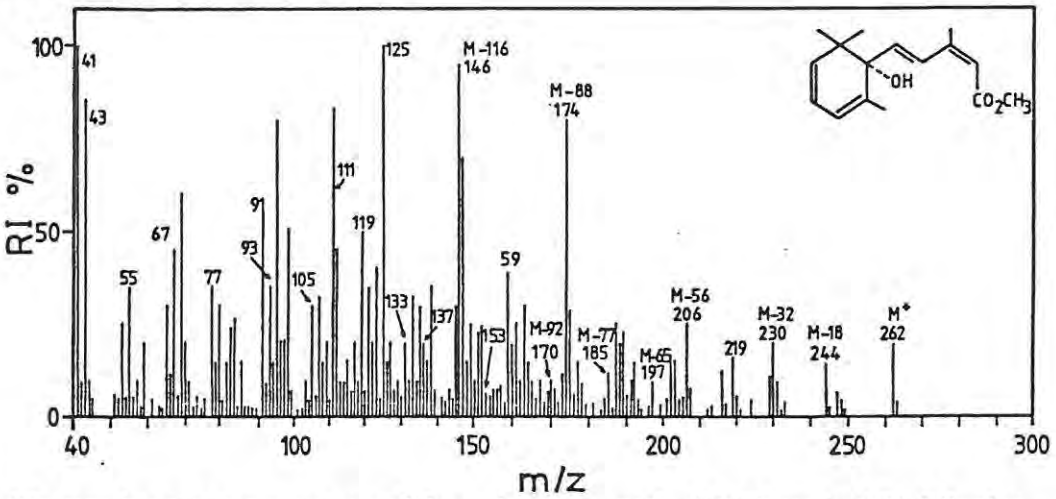

Figure 2.18. Electron impact mass spectrum of the major mass peak (X; Figure 2.16 B) following injection of the 1',4'-*trans* diol of ABAMe onto a capillary column coated with OV-1 and identification of this compound as 4'-deoxy-ABAMe. The mass spectra was obtained at 70eV with a source temperature of 175°C and was recorded at 11.5 sec per mass decade.

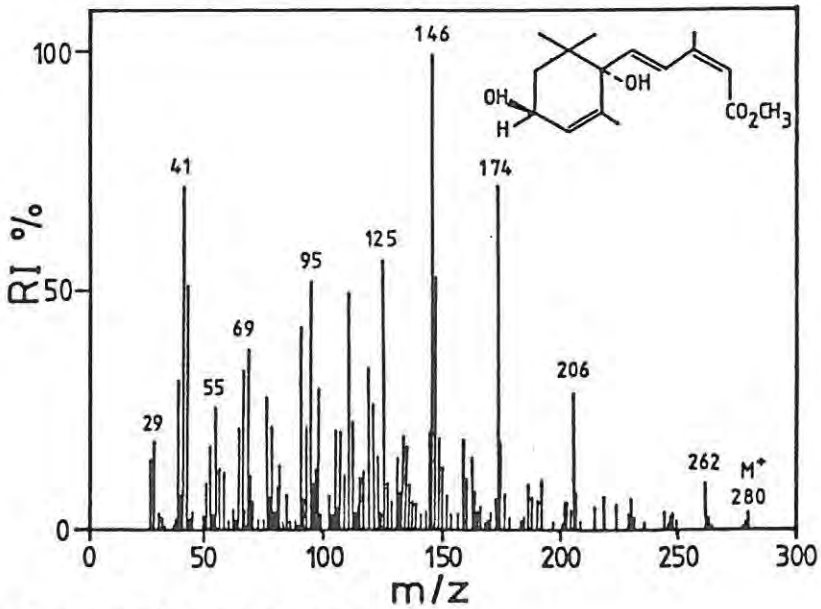

Figure 2.19. Direct probe mass spectrum of the 1',4'-*trans* diol of ABAMe.

of the corresponding ester with 2N KOH/ethanol (1:2, v/v) at 22°C for 45min. The reaction mixture was carefully adjusted to pH2.5 with 2N HCl and the products extracted into ethyl acetate.

2.17. ISOMERISATION OF ABSCISIC ACID AND PHASEIC ACID.

The tentative identification of ABA in plant extracts can be achieved by UV irradiation of putative ABA samples dissolved in acetone or methanol when isomerisation of ABA occurs to produce a mixture of ABA and its *trans* isomer, which can then be separated by GLC (Lenton *et al*, 1971; Zeevaart and Milborrow, 1976; Saunders, 1978; Dörffling and Tietz, 1983). Although PA and DPA could be similarly identified, no attempts have been made to employ the technique for this purpose (Dörffling and Tietz, 1983).

(*R,S,*)-ABAMe (1mg/ml) and esterified, biosynthetically-prepared PA (1 mg/ml), in acetone, were irradiated with UV light in quartz cuvettes. At intervals, aliquots were removed and the rate of appearance of the *trans* isomers determined by GLC analysis on packed columns of 2% SE-30. Typical GLC traces obtained for ABAMe, PAMe and their respective *trans* isomers are depicted in Figure 2.20A and B. A plot of percentage isomerisation versus time for ABAMe and PAMe is depicted in Figure 2.21. These data indicate that an equal mixture of ABAMe and *trans*-ABAMe is obtained within 4h and that the rate of isomerisation of ABAMe appears similar to that obtained in a study by Lenton *et al* (1971). However, during the same time period less than half of the *trans*-PAMe isomer had been produced suggesting that the absence of the 2',3' double bond in PAMe reduces its rate of isomerisation. This implies that the 2',3' double bond in the cyclohexene ring of ABA is necessary for energy transfer during the isomerization process.

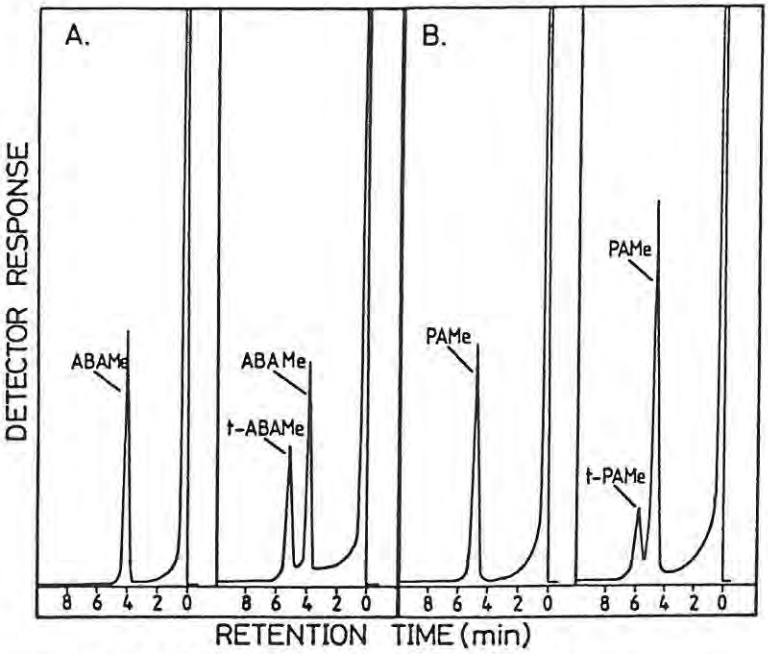

Figure 2.20. Gas chromatographic analysis of ABAMe, PAMe and their respective *trans* isomers. Acetone solutions of authentic ABAMe and esterified PA (biosynthetically-prepared in excised, light-grown leaves of *Hordeum vulgare*) in quartz cuvettes were irradiated with UV light for a period of 2h after which time aliquots were analysed by GLC on a packed column of 2% SE-30. A), GLC analysis of ABAMe before and after irradiation with UV light and B), PAMe before and after UV irradiation. GLC was carried out using a silanized glass column (1.8m x 2mm i.d.) packed with 2% SE-30 on Gaschrom Q (80-100 mesh) with Ar as carrier gas (43ml/min) at an oven temperature of 190°C.

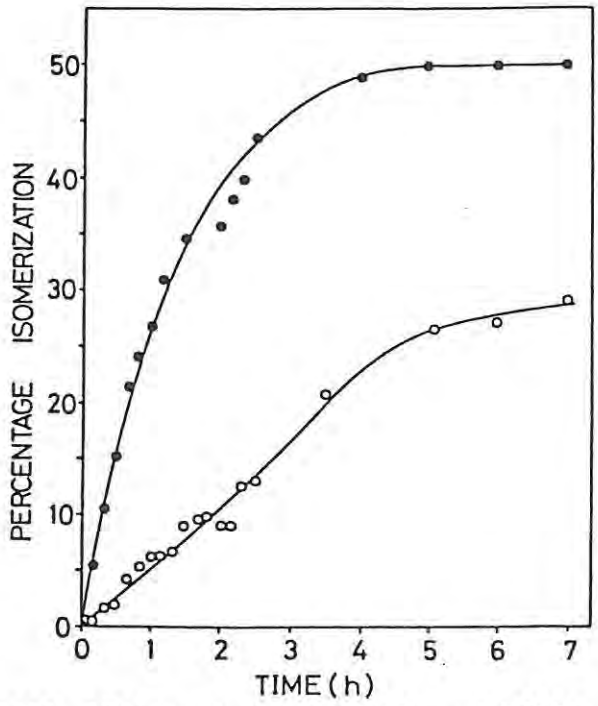

Figure 2.21. Isomerization curves for ABAMe and PAMe. UV isomerization of ABAMe and PAMe was achieved as described in Figure 2.20. At intervals aliquots were removed and the rate of production of the *trans* isomers determined by GLC analysis on a packed column of 2% SE-30 as described in Figure 2.20.

CHAPTER THREE

PURIFICATION OF ABSCISIC ACID AND SOME OF ITS CATABOLITES IN PLANT EXTRACTS USING SEP-PAK CARTRIDGES.

3.1. INTRODUCTION.

Routine purification of ABA and its catabolites from plant extracts requires a method for reducing the associated high levels of impurities prior to characterisation and quantification by GLC and/or combined GC-MS. Numerous methods for the extraction and purification of ABA and its catabolites have been described (Milborrow, 1974a; Milborrow, 1978b; Saunders, 1978; Walton, 1980; Yokoto *et al*, 1980; Brenner, 1981; Dörffling and Tietz, 1983; Loveys and Milborrow, 1984). However, at the start of the present work (1983) efforts to improve the available methods seemed to be necessary, particularly the development of more rapid purification techniques for subsequent analysis of ABA and its catabolites.

In the past, techniques such as charcoal-celite column chromatography (Walton *et al*, 1973; Zeevaart, 1974; Adesomoju *et al*, 1980), silica-gel or silica-gel celite column chromatography (Koshimizu *et al*, 1966) and partition column chromatography (Isogai *et al*, 1967; Hashimoto *et al*, 1968) had been used to purify ABA and its catabolites in plant tissue extracts.

Lenton *et al* (1971) used poly-N-vinyl pyrrolidone (PVP) column chromatography to purify diethyl ether fractions, giving 95% reduction in dry weight in the ABA-containing fractions, 47% recovery and negligible interconversion of the isomers. Furthermore, Mousedale and Knee (1979) described PVP column chromatography of ABA using methanol as the eluent, thus facilitating more rapid purification. Sweetster and Vatvars (1976) prepared ABA fractions using Sephadex G-25 column chromatography. The resultant ABA fraction was of such purity that it could be analysed directly using high performance liquid chromatography (HPLC).

Nevertheless, the above purification techniques are time consuming and appear inadequate for the rapid manipulations required in investigations on the metabolism of ABA in higher plants where a large number of samples need to be processed. The advent of Sep-pak cartridges has provided a means to circumvent these problems. At the time the present investigation was initiated, only limited use had been made of Sep-pak cartridges containing either silica (Hubick and Reid, 1980) or C_{18}

(Knegt *et al*, 1981; Pierce and Raschke, 1981; Lewis and Visscher, 1982; Milborrow and Vaughan, 1982; Corradi, 1983; Zeevaart, 1983; Koorneef *et al*, 1984; Oden and Danberg, 1984) as a "clean-up" procedure, and no meaningful, quantitative information is currently available on the contribution of Sep-pak cartridges to reductions in the weights of plant extracts during the isolation of ABA and/or its catabolites.

Two studies (Hubick and Reid, 1980; Lewis and Visscher, 1982) which have attempted to investigate this aspect, presented either insufficient (Hubick and Reid, 1980) or no (Lewis and Visscher, 1982) quantitative data on the purification procedures *per se*. The primary aim of these investigations, and others (Pierce and Raschke, 1981; Corradi, 1983; Dumbroff *et al*, 1983; Zeevaart, 1983), was to use Sep-pak cartridges as a preparative purification procedure for ABA prior to either GC-ECD or HPLC analyses and consequently, only very low masses of tissue were extracted. In addition, the use of selective detectors may mask the true contribution of Sep-pak cartridges to these purification procedures. Furthermore, no attempts have been made to investigate the suitability of Sep-pak cartridges for the purification PA and DPA in plant tissue extracts.

In studies on ABA metabolism in plants it is sometimes necessary to extract large amounts of tissue (ca. 80-100g fresh weight) during, for example, the biosynthetic production of radiolabelled and non-radiolabelled catabolites of ABA, for refeeding studies and for use as authentic chromatographic standards (Zeevaart and Milborrow, 1976; Tietz *et al*, 1979; Railton and Symon, 1983; Railton and Cowan, 1985a). Similarly, studies on the biosynthesis of ABA from labelled mevalonate (MVA) in intact plant tissues have, in the past, been undertaken using large tissue samples (Milborrow and Noddle, 1970; Milborrow and Robinson, 1973), emphasizing the need for an efficient method of purifying any ABA produced prior to chromatographic analyses. Therefore, the suitability of both silica and C_{18} Sep-pak cartridges for purifying ABA and its major catabolites, PA and DPA, was examined in tissue samples of high dry weight.

3.2. RESULTS.

3.2.1. SEP-PAK SILICA CARTRIDGE PURIFICATION.

3.2.1.1. Purification of ABA.

Initially, the experiment conducted by Hubick and Reid (1980) was repeated in order to obtain the

essential quantitative data which was absent from their study.

Leaf tissue from *Helianthus annuus* seedlings (equivalent to 2g fresh weight) was homogenised in methanol/ethyl acetate/acetic acid (50:50:1, v/v) and the filtrates reduced to dryness. The crude plant extract was then solubilised in 1.0ml of methylene chloride, applied to a silica cartridge which had been pre-washed with 0.5ml methylene chloride and the sample eluted in exactly the same way as described by Hubick and Reid (1980) (see Chapter 2, Section 2.10). The distribution of radioactivity and dry weight in each fraction was determined and the results are depicted in Figure 3.1. These data demonstrated that the bulk of the dry weight was associated with the ABA-containing fractions. The procedure afforded a 50.72% reduction in dry weight of the CH_2Cl_2 solubilised material with 74.76% of the orginal [^{14}C]-ABA being recovered, following back-extraction into ethyl acetate.

The major loss of [^{14}C]-ABA appeared to be due largely to the inability of methylene chloride to completely solubilise the material present in the crude plant extract (Table 3.1). Thus, increases in the dry weight of such extracts resulted in a marked reduction in the recovery of labelled ABA. Furthermore, increasing the dry weight of the crude extracts resulted in a marked decline in the efficiency of Sep-pak silica cartridges to reduce the ABA-associated dry weight, suggesting that the cartridge was beig overloaded. Consequently, it was decided to firstly, fractionate the aqueous extracts by partitioning them against organic solvents in order to allow larger tissue samples to be processed without the subsequent need for back-extraction.

It had previously been demonstrated that tissue which had been homogenised and extracted in methanol/ethyl acetate (50:50, v/v), generated soluble acid fractions of lower dry weight than tissue extracted in methanol/ethyl acetate/acetic acid (50:50:1, v/v), with no discernible difference in the recovery of [^{14}C]-ABA (see Chapter 2, Section 2.9), and for this reason all subsequent tissue homogenisations were undertaken in this solvent mixture.

Ethyl acetate-soluble acids, which would contain ABA and its more polar acidic catabolites, PA and DPA, and diethyl ether-soluble acids, which would contain ABA only, were prepared from several plant species and purified on Sep-pak silica cartridges as described previously. The data depicted in Figure 3.2A and B show that a significant reduction in dry weight was achieved with the bulk of the added [^{14}C]-ABA being recovered. In addition, it is evident that the distribution of dry weight is "soluble acid fraction" dependent, since diethyl ether-soluble acids contain more non-polar

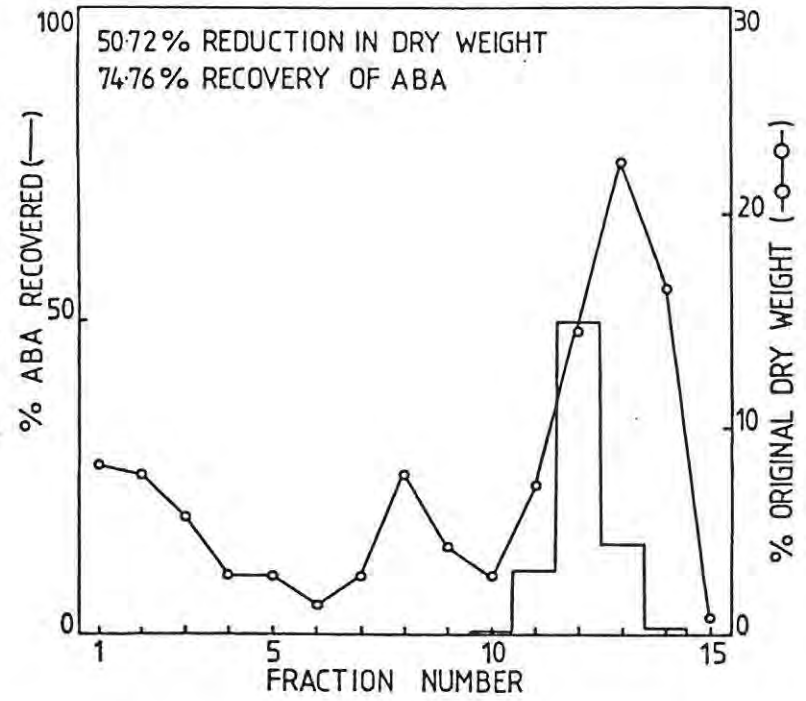

Figure 3.1. Purification of a crude plant extract (56.2mg dry weight) prepared from *Helianthus annuus* seedlings (2g fresh weight) by homogenization in methanol/ethyl acetate/acetic acid (50:50:1, v/v), internally standardized with (*R,S,*)-[2-^{14}C]-ABA (1.5kBq) and applied to a pre-washed Sep-pak silica cartridge in 1.0ml methylene chloride and eluted using the solvent mixtures described in Section 2.9.1. Fractions were monitored for radioactivity (—) and the dry weights (o) associated with each fraction were quantitatively determined.

TABLE 3.1 The effect of increasing tissue fresh weight on the recovery of (R,S)-[2-^{14}C]-ABA (1.5kBq added as an internal standard) and the reduction in dry weight in the ABA containing fractions following purification on silica Sep-pak cartridges and back-extraction into ethyl acetate. Crude plant extracts were prepared from Helianthus annuus seedlings and purified as described by Hubick and Reid (1980).

Tissue Sample (g f.w.)	1.0	2.0	5.0	10.0	15.0	20.0
Crude extract (mg dry wt.)	27.6	56.2	134.4	234.9	414.7	524.7
CH_2Cl_2 soluble material (mg dry wt.)	5.9	13.8	27.5	43.5	95.9	96.3
ABA fraction (mg dry wt.)	2.6	6.8	13.7	20.8	30.6	31.5
Dry weight after back-extraction (mg)	2.6	6.5	12.8	20.3	30.2	31.1
% Reduction in dry weight*	55.93	52.89	53.45	53.33	68.51	67.71
% [^{14}C]-ABA recovered	78.15	74.76	39.00	26.10	19.21	15.83

* = % Reduction in dry weight calculated from methylene chloride soluble material

material than do ethyl acetate-soluble acids. Evenso, the bulk of the associated dry weight co-eluted with ABA. In an attempt to separate ABA from this associated dry weight, a 1-10% methanol in methylene chloride gradient was included in the eluotropic series.

When ethyl acetate-soluble acids, from *Phaseolus vulgaris* seedlings were purified using this modification of the usual method (Figure 3.3B), a significant displacement and reduction in the dry weight of the ABA-containing fractions was achieved. The inclusion of a 1-10% methanol in methylene chloride gradient provided 97.2% reduction in original dry weight which was 25% more efficient than the result obtained from a similar purification when the method described by Hubick and Reid (1980) was used (Figure 3.3A). Given this result, the extended gradient was similarly examined, for its ability to elute and separate ABA and its major catabolites, PA and DPA.

3.2.1.2. Purification of ABA, PA and DPA.

PA and DPA were only partially soluble in methylene chloride, and since high losses in radioactivity were incurred as a result of the inability of methylene chloride to solubilise all the dry weight, methanol was used to apply PA and DPA to silica Sep-pak cartridges. Following application of these compounds to the cartridge, the excess methanol was removed by evaporation under a stream of N_2. The elution profiles obtained from individual samples of [^{14}C]-ABA, and biosynthetically-prepared [^{14}C]-PA and [^{14}C]-DPA and a mixture of all three compounds are depicted in Figure 3.4A, B, C and D. The data depicted in Figure 3.4D demonstrate that ABA, PA and DPA were not adequately separated using the extended gradient, and co-eluted from the silica cartridge with the bulk of the associated dry weight (see Figure 3.3B).

Thus, for convenience, extracts were finally eluted from silica cartridges with 60% methanol in methylene chloride, following initial purification steps using the low polarity solvent mixtures described by Hubick and Reid (1980). Ethyl acetate-soluble acids, prepared from seedlings of *Phaseolus vulgaris*, containing known amounts of (*R,S,*)-[2-^{14}C]-ABA and biosynthetically prepared [^{14}C]-PA and [^{14}C]-DPA were purified in this manner. This procedure resulted in 85.25% reduction in dry weight and 98.23% recovery of the radiolabelled compounds and the data are depicted in Figure 3.5.

Clearly, this modified procedure is more efficient than the one described by Hubick and Reid

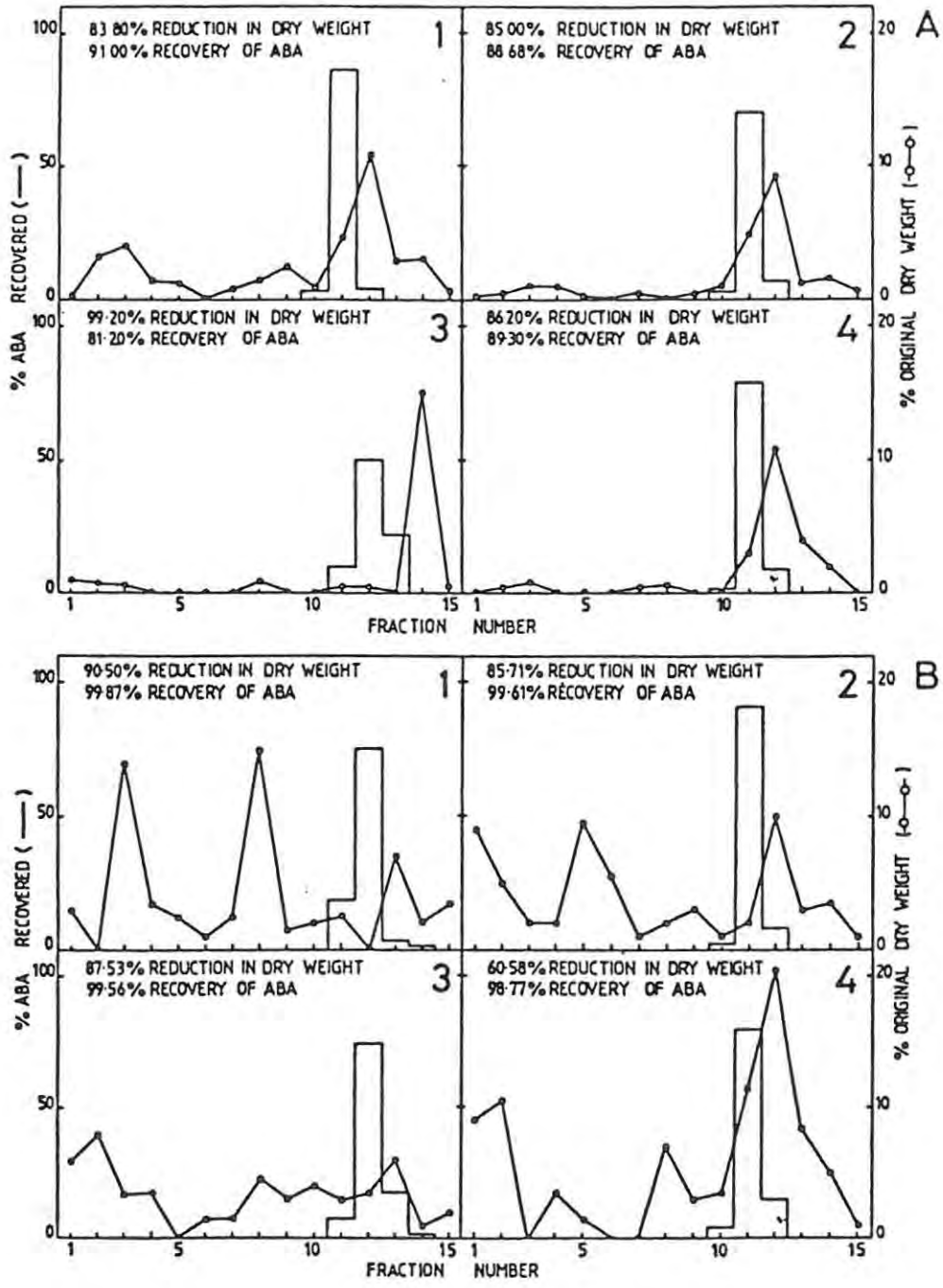

Figure 3.2. Purification of A), ethyl acetate- (50mg dry weight) and B), diethyl ether- (20mg dry weight) soluble acid fractions prepared from 4 plant species as described in Chapter 2, Sections 2.8 and 2.9. Soluble acid fractions, containing (*R,S,*)-[2-^{14}C]-ABA (1.5kBq) were loaded and eluted from Sep-pak cartridges as described in Figure 3.1. 1, *Phaseolus vulgaris*; 2, *Pisum sativum*; 3, *Helianthus annuus*; 4, *Persea americana*.

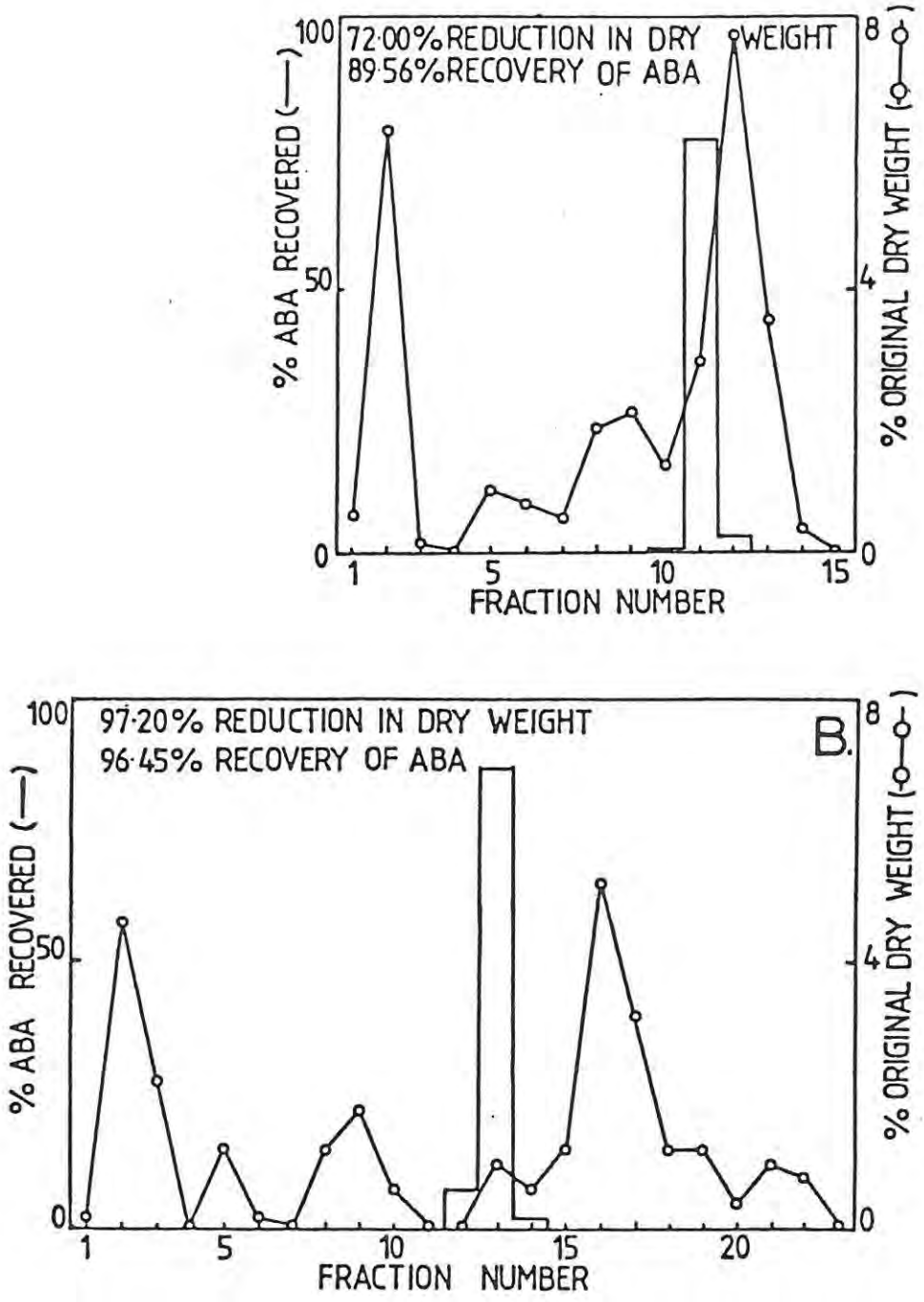

Figure 3.3. Purification of ethyl acetate-soluble acids (50mg dry weight) prepared from *Phaseolus vulgaris* seedlings, containing (*R,S,*)-[2-^{14}C]-ABA (1.5kBq), on Sep-pak silica cartridges using an extended gradient of 1-10% methanol in methylene chloride. A), control; B), extended gradient. Fractions were monitored for radioactivity (—) and the dry weight (o) associated with each fraction was expressed as % original dry weight applied to the cartridge.

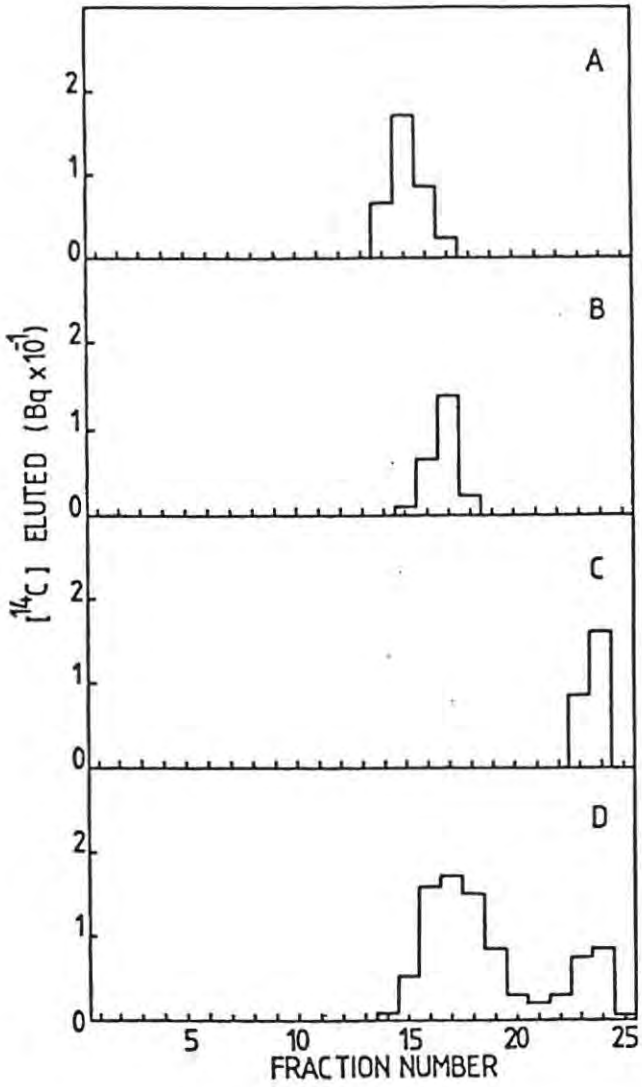

Figure 3.4. The elution of A), (*R,S,*)-[2-^{14}C]-ABA; B), [^{14}C]-PA and C), [^{14}C]-DPA from Sep-pak silica cartridges using the extended gradient described in Figure 3.3B. Samples were loaded in methanol which was removed from the cartridge by evaporation under a stream of N_2 (x 45min). D), elution of a sample containing radioactive ABA, PA and DPA from a Sep-pak silica cartridge as described above.

(1980) and had the advantage that increasing the dry weight of the soluble-acid fractions, up to 50mg (equivalent to 50g fresh weight) routinely provided more than 70% reduction in the original dry weight and greater than 80% recovery of ABA, PA and DPA (Figure 3.6). These data also showed that the efficiency of the cartridge decreased significantly for dry weights greater than 50mg. This implied that where it is necessary to extract large tissue samples ($\pm$100g fresh weight) during the biosynthetic preparation of standard compounds (Zeevaart and Milborrow, 1976; Tietz *et al*, 1979), purification of these extracts using Sep-pak silica cartridges is limited. This prompted an investigation into the suitability of Sep-pak C_{18} cartridges for this purpose.

3.2.2. SEP-PAK C_{18} CARTRIDGE PURIFICATION.

When the present investigation began in 1983, there were only two detailed reports on the use of Sep-pak C_{18} cartridges for the purification of ABA in plant tissue (Pierce and Raschke, 1981; Lewis and Visscher, 1982) and these concentrated on the efficiency of recovery of ABA rather than on the reductions in levels of associated impurities. Thus, experiments were carried out to examine the latter aspect.

3.2.2.1. Purification of ABA.

Initially, the method employed by Lewis and Visscher (1982) was studied using both the ethyl acetate and diethyl ether-soluble acids, containing (*R,S,*)-[2-^{14}C]-ABA, from tissue homogenates of several plant species.

The levels of radioactivity and the dry weights in each fraction were determined and the results are presented in Table 3.2. Unlike the procedure described by Lewis and Visscher (1982), ABA-containing fractions were not back-extracted into ethyl acetate since the investigation was primarily aimed at determining the role of the Sep-pak C_{18} cartridge in the purification procedure *per se*.

Although a significant reduction in dry weight was observed, the recovery of [^{14}C]-ABA in "fraction 3" (32% aqueous methanol, pH8.0) did not compare favourably with the data presented orginally by Lewis and Visscher (1982). The bulk of the residual radioactivity was found to be associated with "fraction 2" (32% aqueous methanol, pH2.8), which might have been due to either the methanol concentration of the second eluting buffer (fraction 2) or the volume of methanol used to

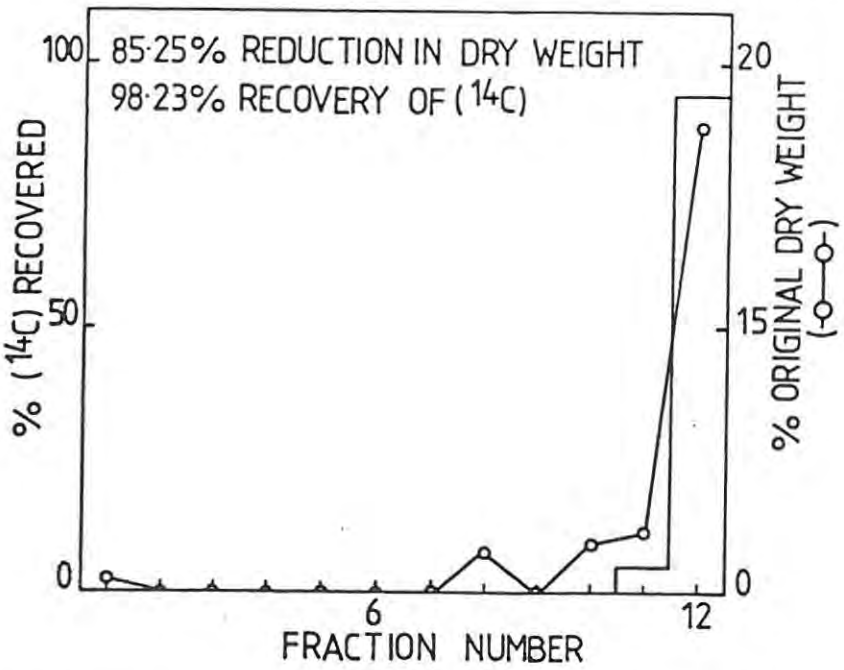

Figure 3.5. Elution profile of an ethyl acetate-soluble acid fraction (20mg dry weight) prepared from *Phaseolus vulgaris* seedlings, containing (*R,S,*)-[2-^{14}C]-ABA, [^{14}C]-PA and [^{14}C]-DPA (all 0.83kBq) applied to a Sep-pak silica cartridge in 50μl methanol which was then evaporated under N_2 and eluted from the cartridge using firstly, the non-polar solvent mixtures and finally 60% methanol in methylene chloride. The distribution of radioactivity (—) and dry weight (o) was determined as previously described.

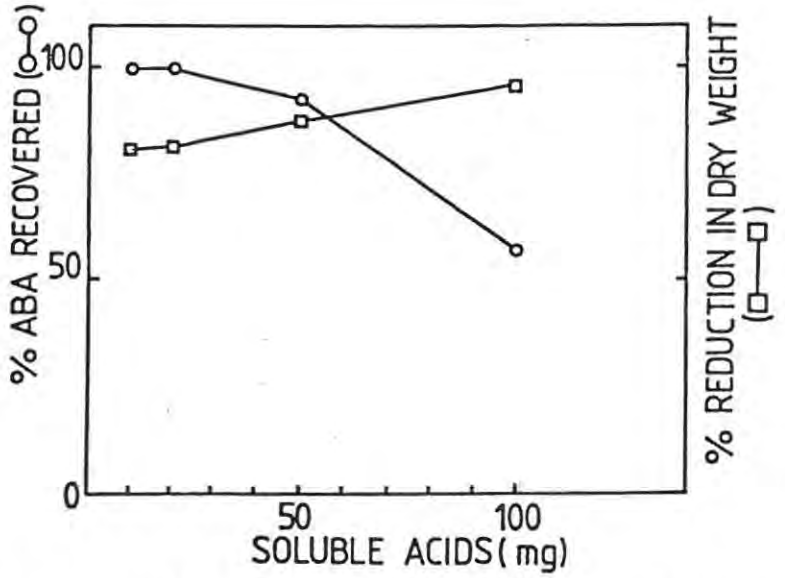

Figure 3.6. The effect of increasing the dry weight of the soluble acid fraction on the capacity of the Sep-pak silica cartridge to reduce ABA associated dry weight and on the recovery of ABA. Soluble acid fractions of increasing dry weight, prepared from *Pisum sativum* seedlings and containing (*R,S,*)-[2-^{14}C]-ABA (0.83kBq), were purified as described in Figure 3.5. The % ABA recovered (o) and the reductions in associated dry weight (□) were determined as previously described.

apply the extract to the cartridge. The detailed data presented in Figure 3.7 support the suggestion (Lewis and Visscher, 1982) that the volume of methanol used to apply the extract to the cartridge is critical. It is evident that volumes of methanol exceeding 50μl result in a marked decline in the efficiency of this purification procedure.

In addition, substitution of the second eluting buffer (32% aqueous methanol, pH2.8), used by Lewis and Visscher (1982), with 26% aqueous methanol, pH2.8 resulted in a marked increase in the recovery of [^{14}C]-ABA in "fraction 3" with little dry weight eluting in the first three fractions.

Ethyl acetate-soluble acids, prepared from *Pisum sativum* seedlings and containing a known amount of (*R,S,*)-[2-^{14}C]-ABA dissolved in 50μl of methanol, were applied to C_{18} cartridges, purified using the above procedures, and the four eluates fractionated into 1.0ml aliquots. The distribution of radioactivity and dry weight was quantitatively determined and these data are depicted in Figure 3.8A and B. Since little dry weight eluted in the first two fractions (7ml 20% aqueous methanol, pH2.8 followed by 7.0ml 26% aqueous methanol, pH2.8) it seemed reasonable to elute the Sep-pak cartridges with a single 6.0ml aliquot of 32% aqueous methanol, pH8.0. However, since the pH of the eluting buffers, prepared as described by Lewis and Visscher (1982), was generally unstable, 20mM K_2HPO_4/KH_2PO_4 buffer (pH8.0) was substituted for water in the eluting buffers. Furthermore, ethyl acetate and diethyl ether-soluble acids (up to 100mg dry weight) were soluble in 32% aqueous methanol prepared in 20mM phosphate buffer (pH8.0), and volumes of this buffer up to 500μl had no effect on the elution properties and recovery of [^{14}C]-ABA from the Sep-pak C_{18} cartridge (Figure 3.9).

Nevertheless, a further aspect which needed to be determined was the optimal methanol concentration for the eluting buffer. Thus, ethyl acetate-soluble acids (20mg dry weight), containing a standard amount of (*R,S,*)-[2-^{14}C]-ABA, were purified on Sep-pak C_{18} cartridges using increasing concentrations of methanol in 20mM KPi buffer (pH 8.0). The result depicted in Figure 3.10 indicated that the optimal methanol concentration was between 30 and 40%. Higher concentrations of methanol reduced the capacity of the cartridge to remove associated dry weight.

3.2.2.2. Purification of PA and DPA.

PA and DPA were very soluble in 32% methanol in 20mM K_2HPO_4/KH_2PO_4 buffer (pH8.0) and the data presented in Figure 3.11 demonstrated that on fractionation of the eluates (6.0ml, 32%

TABLE 3.2: Purification on ethyl acetate (50 mg dry wt.) and diethyl ether (20 mg dry wt.) soluble acids, containing (R,S)-[2-^{14}C]-ABA (1.6kBq), from four different plant species using C_{18} Sep-pak cartridges by the method of Lewis and Visscher (1982).

Plant Tissue	Acids	% [^{14}C]-ABA recovered	% Reduction of dry weight in ABA zone*
Phaseolus vulgaris	EtoAc	87.60	97.80
	Et_2O	84.14	90.50
Pisum sativum	EtoAc	82.44	90.78
	Et_2O	81.80	86.02
Helianthus annuus	EtoAc	88.33	93.60
	Et_2O	78.00	92.60
Persea americana	EtoAc	89.60	88.80
	Et_2O	86.99	92.40

* ABA eluted in 32% aqueous methanol, pH 8.0

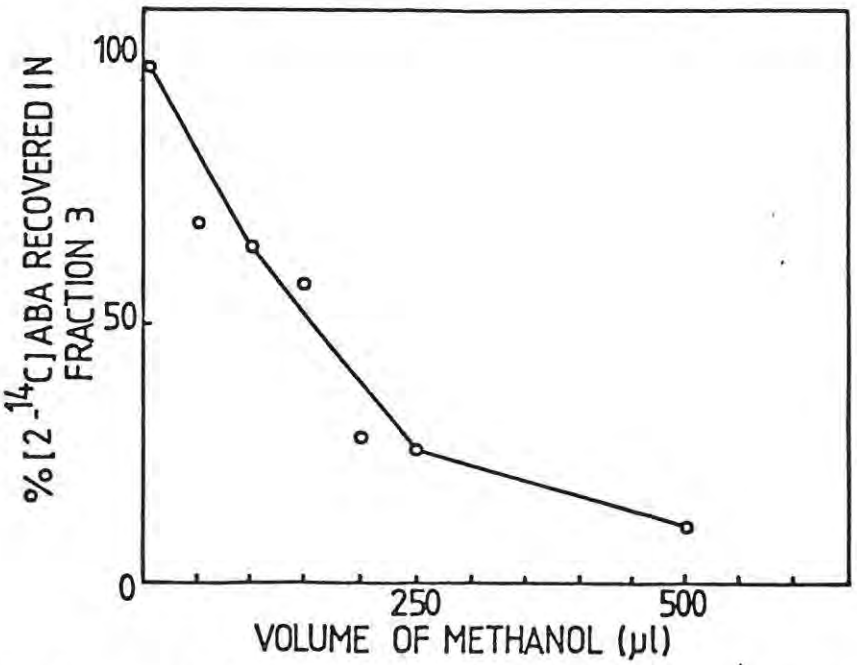

Figure 3.7. The effect of varying the amounts of methanol during sample loading on the recovery of (*R,S,*)-[2-^{14}C]-ABA (1.5kBq) in "fraction 3" (32% aqueous methanol, pH8.0). Samples were eluted as described by Lewis and Visscher (1982), see Chapter 2, Section 2.9.2.

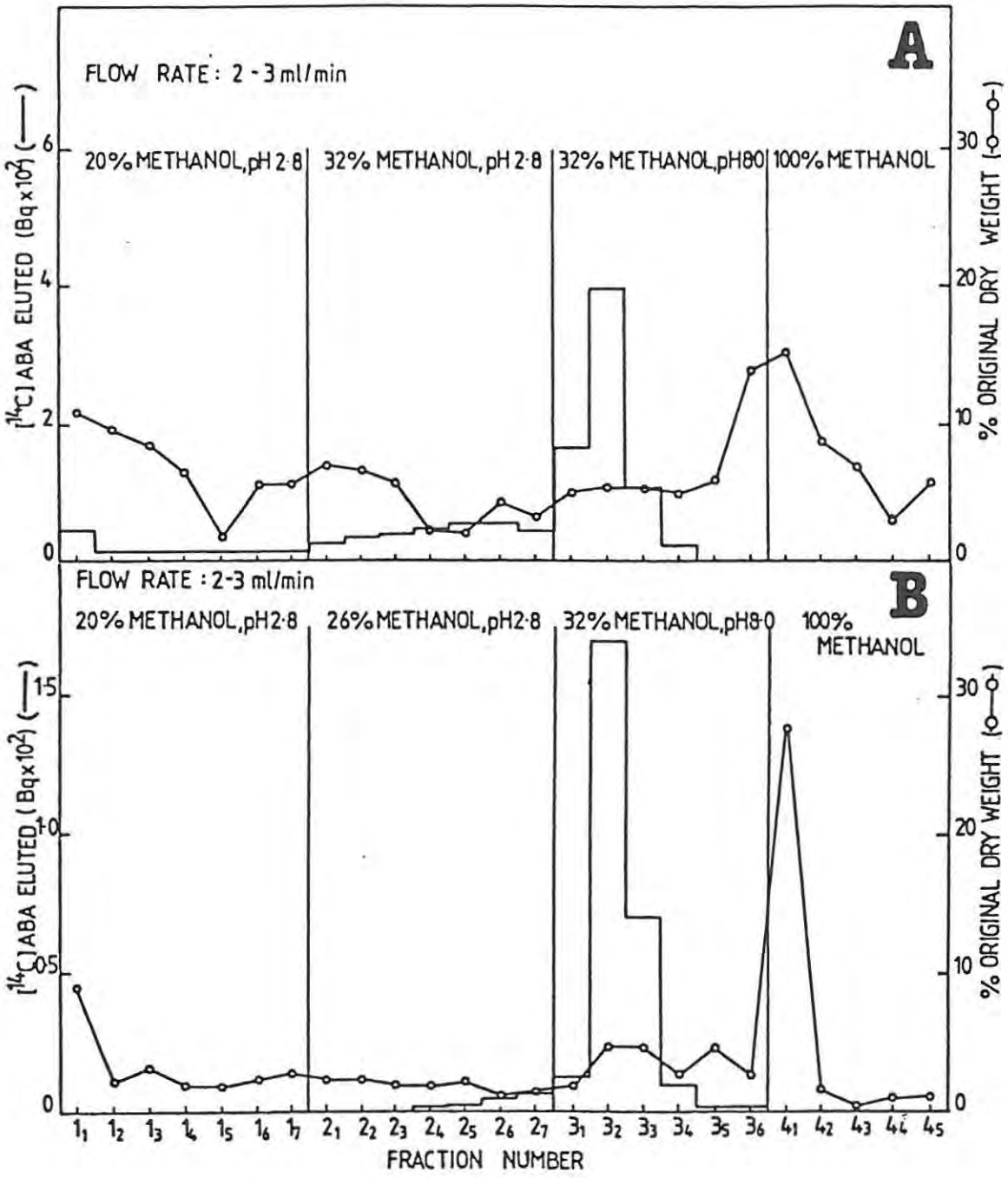

Figure 3.8. The distribution of radioactivity (—) and dry weight (o) following the purification of ethyl acetate-soluble acids (20mg dry weight), containing (*R,S,*)-[2-^{14}C]-ABA (0.33kBq), prepared from *Helianthus annuus* seedlings on Sep-pak C_{18} cartridges. A), aqueous methanolic solutions prepared as described by Lewis and Visscher (1982) and B), 26% aqueous methanol (pH2.8) replaced 32% aqueous methanol (pH2.8) as the second eluting buffer.

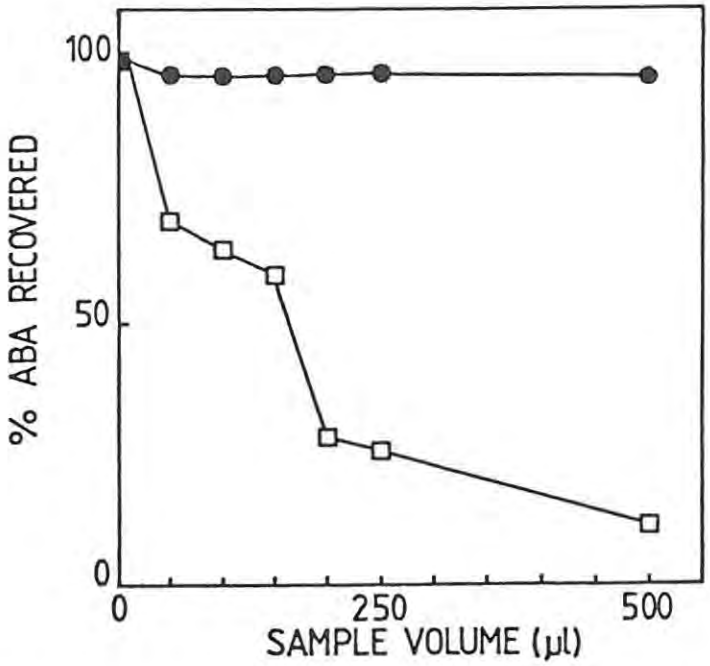

Figure 3.9. A comparison between increasing volumes of absolute methanol (□) and 32% methanol in 20mM K_2HPO_4/KH_2PO_4 buffer, pH8.0 (●), used to apply the sample to a Sep-pak C_{18} cartridge, on the recovery of radioactivity in the ABA containing fraction.

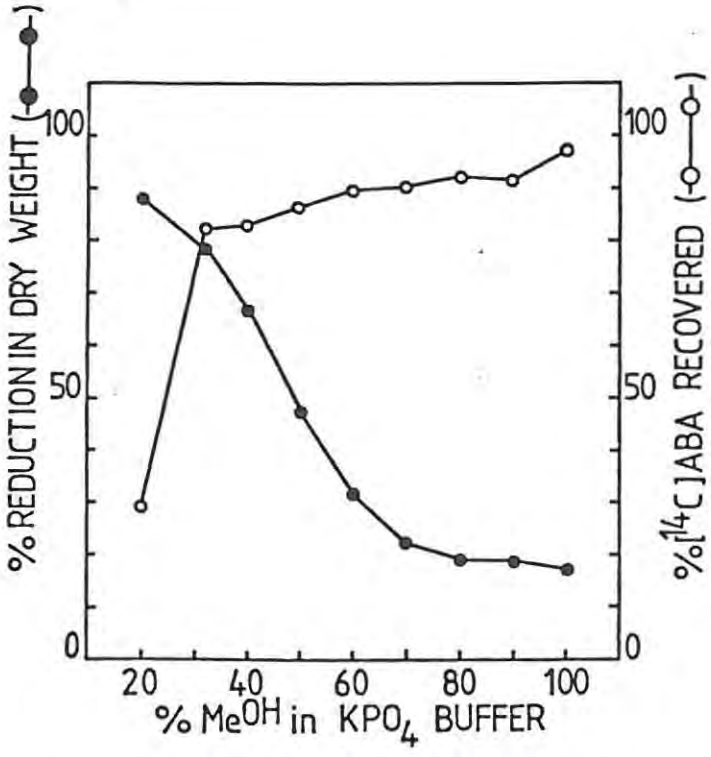

Figure 3.10. The effect of increasing methanol concentration in the eluting buffer (20mM K_2HPO_4/KH_2PO_4 buffer, pH 8.0) on the reduction in dry weight (●) and the recovery of ABA (o) from ethyl acetate soluble- acids (20mg dry weight) prepared from *Pisum sativum* seedlings, containing (*R,S,*)-[2-^{14}C]-ABA (1.5kBq). Samples were applied to pre-washed Sep-pak C_{18} cartridges in 250µl 32% methanol in 20mM K_2HPO_4/KH_2PO_4 buffer (pH8.0) and eluted with 6.0ml of the same buffer.

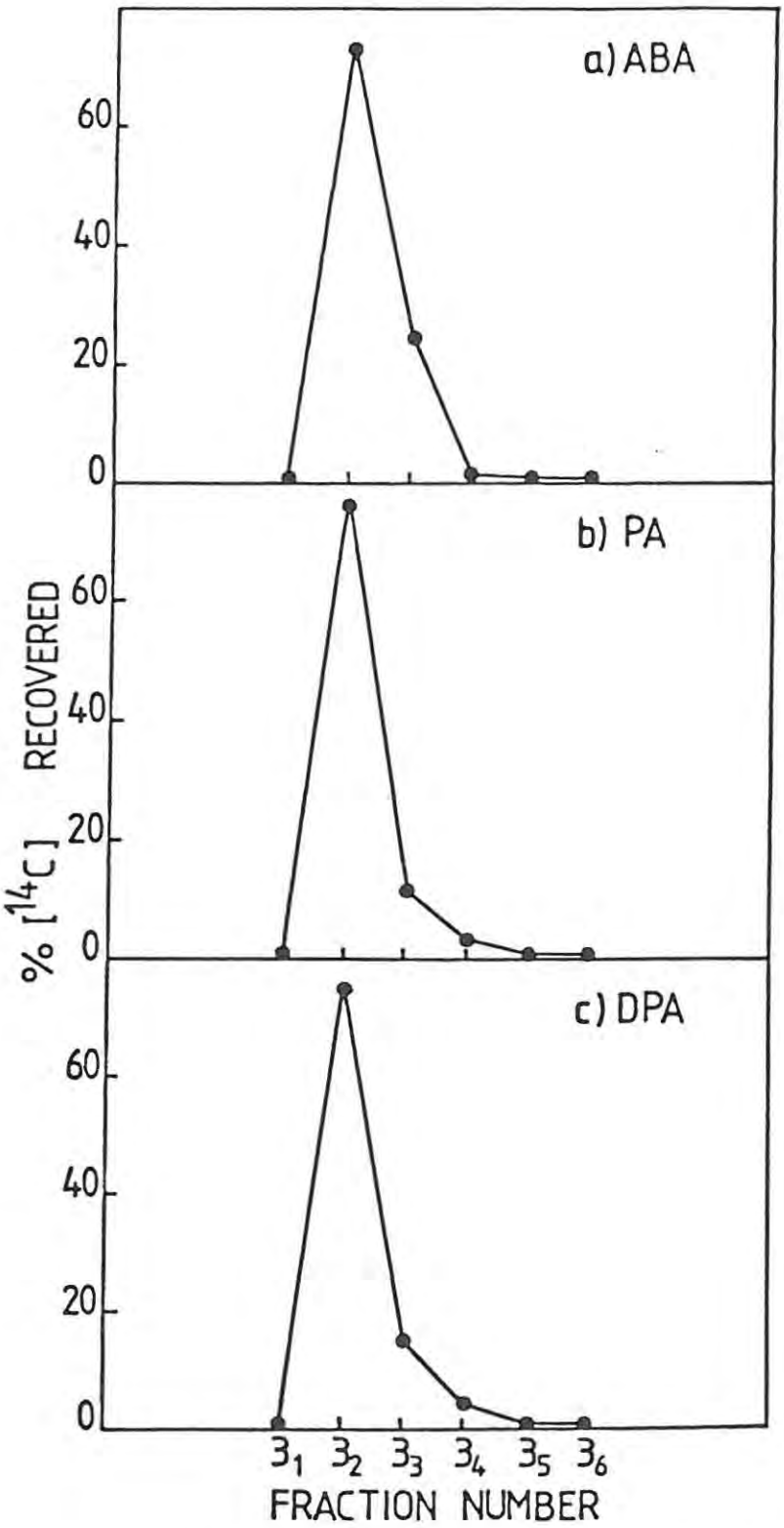

Figure 3.11. The distribution of radioactivity following the elution of A), (*R,S,*)-[2-^{14}C]-ABA; B), [^{14}C]-PA and C), [^{14}C]DPA from Sep-pak C_{18} cartridges. Samples were loaded and eluted with 32% methanol in 20mM K_2HPO_4/KH_2PO_4 buffer (pH8.0) and the eluates fractionated into 1.0ml aliquots.

methanol in 20mM phosphate buffer, pH8.0) into 1.0ml aliquots, the bulk of the applied [^{14}C]-ABA, [^{14}C]-PA and [^{14}C]-DPA eluted in the first 4 fractions.

The application of ethyl acetate-soluble acids, containing ABA and its catabolites, and diethyl ether-soluble acids, containing ABA only, to Sep-pak C_{18} cartridges, followed by elution of the cartridge with a single 6.0ml aliquot of 32% methanol in 20mM KPi buffer (pH8.0) gave the elution profiles depicted in Figure 3.12. This procedure routinely resulted in greater than 80% reduction in orginal dry weight with greater than 90% recovery of the radioactivity associated with ABA, PA and DPA. Furthermore, the data depicted in Figure 3.13 also demonstrated that up to 100mg dry weight could be purified without any loss in efficiency of dry weight reduction or recovery of ABA.

3.2.2.3. GLC analysis of Sep-pak C_{18} purified extracts.

Both Hubick and Reid (1980) and Lewis and Visscher (1982) used GLC with an electron capture detector to determine the purity of endogenous ABA in plant extracts following Sep-pak treatment, which would have under estimated the level of residual impurities.

In order to further assess the efficiency of Sep-pak C_{18} cartridges to reduce the dry weight of plant extracts, Sep-pak purified, ethyl acetate-soluble acids were methylated and compared with a similar non-purified, methylated sample by GLC on a packed column of 2% SE-30 using a flame ionisation detector. The results (Figure 3.14A and B) showed that an overall, marked reduction in extraneous compounds of widely differing polarities had been achieved.

3.2.2.4. Sep-pak silica versus Sep-pak C_{18} cartridge purification.

The second method employing Sep-pak C_{18} cartridges which was also available when the present study was initiated was described by Pierce and Raschke (1981). This procedure had been developed as a preparative clean-up method prior to the analysis of endogenous ABA and its catabolites by HPLC. It differed from the method described by Lewis and Visscher (1982) in that acidified aqueous samples were applied to the cartridge ensuring retention of ABA and other lipophilic substances in the C_{18} layer. As with other methods (Hubick and Reid, 1980; Lewis and Visscher, 1982) no quantitative data was given regarding the role of the Sep-pak cartridge in this purification procedure and only small quantities of tissue were processed.

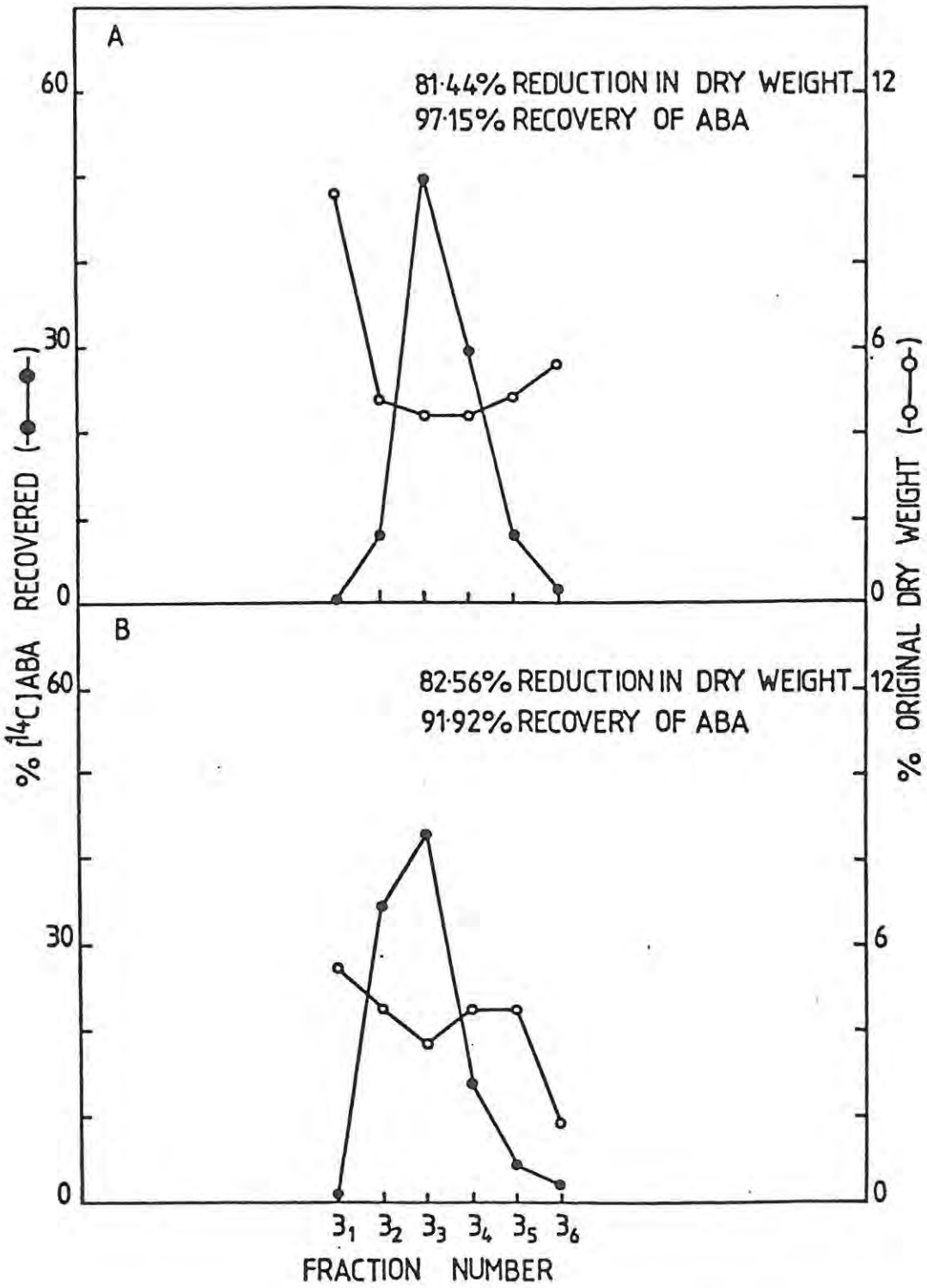

Figure 3.12. The distribution of dry weight (o) and radioactivity (●) from A), ethyl acetate-soluble acids (25mg dry weight), containing radioactive ABA, PA or DPA and B), diethyl ether-soluble acids (10mg dry weight) containing ABA only. Soluble acids, containing a known amount of radioactivity were purified as described in Figure 3.10.

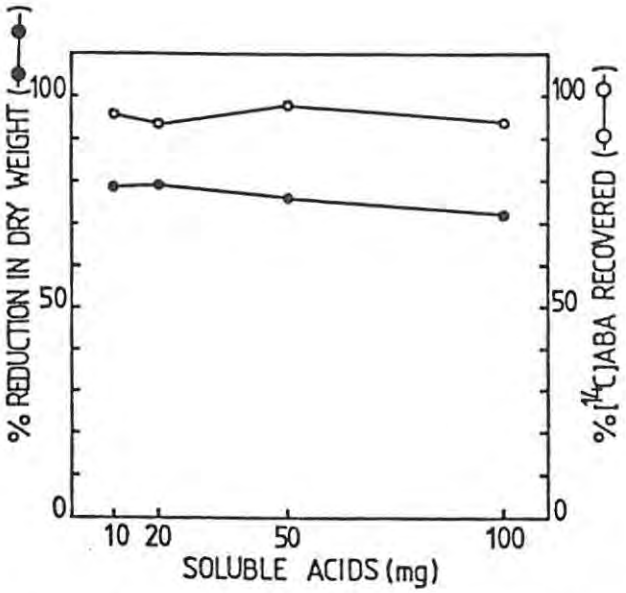

Figure 3.13. The effect of increasing dry weights of ethyl acetate-soluble acids, prepared from *Pisum sativum* seedlings containing (*R,S,*)-[2-^{14}C]-ABA (1.5kBq), on the recovery of ABA (o) and the reduction in associated dry weight (●) following purification on Sep-pak C_{18} cartridges. Samples were purified as described for Figure 3.11.

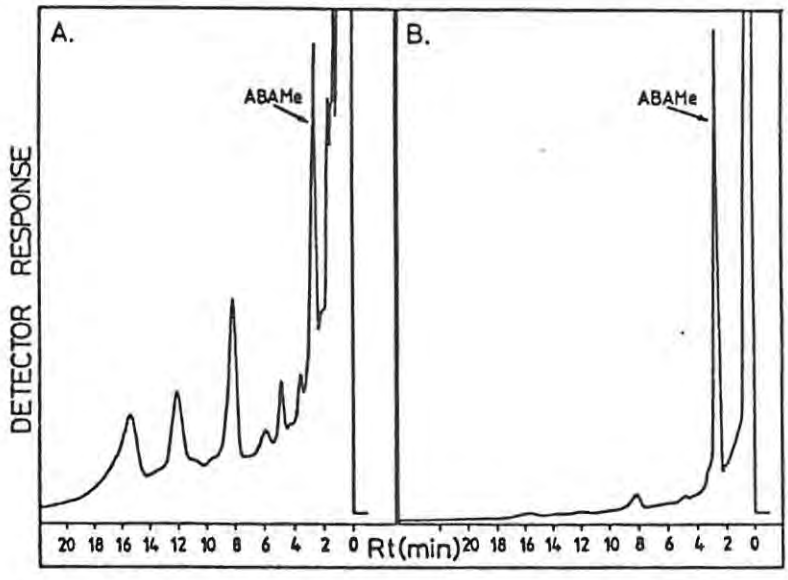

Figure 3.14. Gas chromatograms of A), esterified crude ethyl acetate-soluble acids and B), esterified Sep-pak C_{18} purified ethyl acetate-soluble acids, co-injected with authentic (*R,S,*)-ABAMe. Extracts were prepared from *Phaseolus vulgaris* seedlings (10g fresh weight) and chromatographed on a packed column of 2% SE-30. GLC was carried out using a silanized glass column (1.8m x 2mm i.d.) packed with 2% SE-30 on Gaschrom Q (80-100 mesh) with Ar as carrier gas (43ml/min) at an oven temperature of 190^{o}C.

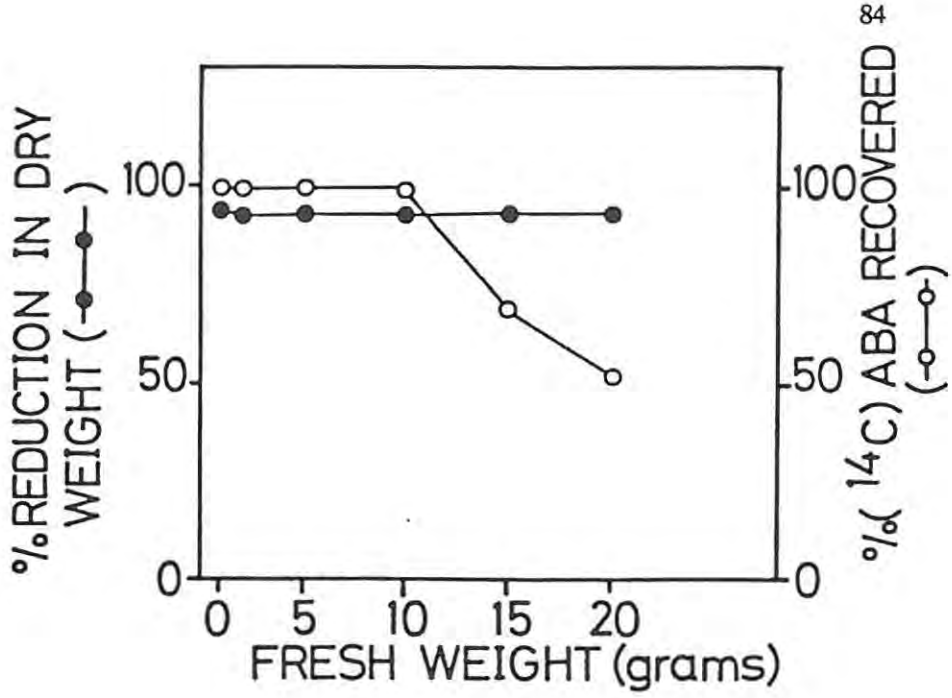

Figure 3.15. The effect of increasing tissue sample size (fresh weight equivalent) on the reduction in associated dry weight (●) and the recovery of (*R,S,*)-[2-^{14}C]-ABA (o) in plant extracts of light-grown *Helianthus annuus* seedlings purified on C_{18} cartridges using the method described by Pierce and Raschke (1981).

TABLE 3.3: A comparison between the available methods and those described in this thesis for the purification of abscisic acid and its catabolites in plant extracts using silica and C_{18} Sep-pak cartridges.

	Silica 1	Silica 2	C_{18} 1	C_{18} 2	C_{18} 3
Tissue sample (fr. wt. in g)	10.00	10.00	10.00	10.00	10.00
Crude extract (dry wt. in mg)	234.90*	14.67	48.00	261.10	11.63
ABA fraction (dry wt. in mg)	20.80	4.14	6.60	16.90	2.20
% Reduction in dry weight	53.18	71.78	86.25	93.53	81.08
% reduction in dry weight following back-extraction	53.33	-	90.73	-	-
% [^{14}C]-ABA recovered	26.10	98.73	90.86	99.57	99.49

SILICA 1 = Hubick and Reid (1980).

C_{18} 1 = Lewis and Visscher (1982)

C_{18} 2 = Pierce and Raschke (1981)

* 43.5 mg constituted the methylene chloride soluble material

Therefore, crude extracts were prepared from *Helianthus annuus* seedlings, of increasing dry weights (containing (*R,S,*)-[2-^{14}C]-ABA as an internal standard), and were purified on Sep-pak C_{18} cartridges in the same way as described by Pierce and Raschke (1981). The results are depicted in Figure 3.15 and show that with increasing dry weights of the crude plant extracts there was a decline in the recovery of added radiolabelled ABA. This was particularly evident in plant extracts prepared from tissue samples in excess of 10g fresh weight. Thus, this procedure appeared unsuitable for use in metabolic studies where large tissue samples are required for the biosynthetic preparation of PA and DPA. However, since smaller tissue samples are generally used in studies on the catabolism of (*R,S,*)-[2-^{14}C]-ABA in plants, the efficiency of both Sep-pak silica and C_{18} cartridges for purifying plant extracts was compared.

The data presented in Table 3.3 using extracts from light-grown seedlings of *Pisum sativum* showed that C_{18} cartridges were more efficient than silica cartridges. In addition, Sep-pak C_{18} cartridge purification was more rapid and better suited for use in this study on ABA metabolism since a large number of tissue samples of high dry weight were to be processed. Although the method described by Pierce and Raschke (1981) was as efficient as the method modified from that described by Lewis and Visscher (1982), it remained time consuming and generally favoured the purification of small tissue samples. Therefore, the Lewis and Visscher method was employed routinely for purifying extracts of high dry weight from plants which had been fed mg amounts of ABA to generate non-radiolabelled PA and DPA for use as chromatographic standards.

CHAPTER FOUR

STUDIES ON THE BIOSYNTHESIS OF ABSCISIC ACID IN VARIOUS PLANT TISSUES AND ITS MODIFICATION BY CHEMICAL AND ENVIRONMENTAL FACTORS.

4.1. INTRODUCTION.

The studies described in this chapter were initiated in order to assess the usefulness of various higher plant tissues as possible sources of enzymes for later studies on the cell-free biosynthesis of ABA. Thus, selected tissues were examined for their efficiency to incorporate label from MVA into ABA. A greater understanding of how ABA biosynthesis is regulated in plants can only be obtained by investigating factors which might modify this process. Therefore, the effects of various environmental and chemical factors on ABA biosynthesis in plants were investigated. Such studies were carried out using plant tissues in which ABA biosynthesis could be repeatedly demonstrated and where it might be possible to observe any effect of these factors.

4.1.1. Abscisic acid biosynthesis in plants.

ABA is a sesquiterpenoid and therefore would be expected to be derived from MVA (see Figure 1.2). However, evidence suggests that ABA may arise by either of two possible routes. One route involves the synthesis of ABA from the C-15 sesquterpenoid precursor, FPP. The other, an indirect route, involves enzymatic cleavage of a putative C-40 carotenoid intermediate such as the xanthophyll, violaxanthin. These alternative pathways for ABA biosynthesis are depicted in Figure 4.1.

The incorporation of radioactivity from labelled MVA into ABA has only been demonstrated in a limited number of higher plant tissues (for reviews see: Milborrow, 1976; 1978b) and the intermediates are largely unknown (see Chapter 1). Although ripening mesocarp from fruit of *Persea americana* has been used in most of the studies on ABA biosynthesis from MVA in higher plants, other plant organs were also examined for their ability to incorporate label from MVA into ABA in order to determine which tissues might be suitable for the development of an ABA-metabolising cell-free system.

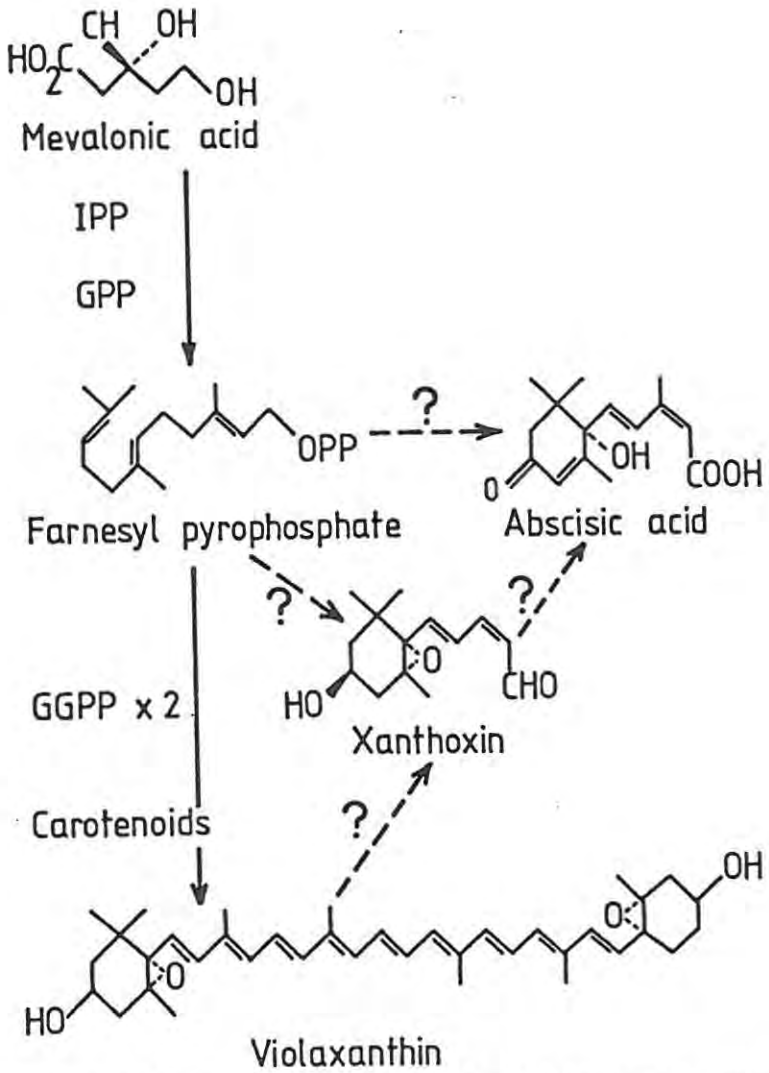

Figure 4.1. Possible routes for the biosynthesis of abscisic acid in higher plants: C-15 vs C-40 (after Neill *et al*, 1984).

ABA has been unequivocally characterised as a naturally-occurring compound in immature seeds of *Pisum sativum* (Isogai *et al*, 1967; Frydman *et al*, 1974) and *Phaseolus vulgaris* (Hiraga *et al*, 1974) where the levels of this compound increase dramatically during seed maturation (Hsu, 1979; Browning, 1980), possibly as a result of ABA biosynthesis. Similarily, a close relationship between changes in extractable amounts of ABA and changes in growth rates of both pod and seed (Eewens and Schwabe, 1975) implies that growth of the pod depends largely on hormones supplied by the seed, further suggesting that immature seed could be a site of ABA biosynthesis. Terpenoid biosynthesis in these organs is most clearly seen in studies on the biosynthesis of GAs where the whole GA pathway has been delineated (Graebe, 1987). Thus, the information suggests that immature seed are potentially, a rich source of enzymes involved in ABA biosynthesis. Likewise, the synthesis and metabolism of GAs has been demonstrated in cereal grains, including *Triticum*

aestivum and *Hordeum vulgare* (Pharis and King, 1985). While ABA has been characterised in seeds of *Triticum aestivum* and *Hordeum vulgare* (Morris *et al*, 1988), its synthesis from MVA has only been demonstrated in *Triticum aestivum* seeds (Milborrow and Robinson, 1973). Thus, seeds of *Hordeum vulgare* were also examined for their ability to synthesize ABA from MVA.

4.1.2. Modification of ABA biosynthesis by environmental and chemical factors.

4.1.2.1. Water-stress.

The increase in ABA levels in plants in response to water stress is well documented (Wright, 1969; Zeevaart, 1980; Henson and Quarrie, 1981) and it has been suggested (Zeevaart, 1980; Pierce and Raschke, 1981) that this increase is produced by *de novo* biosynthesis (Wright, 1969) rather than by release from a storage form. Although recent evidence suggests that stress-induced ABA accumulation requires rapid enzyme production in the cytoplasm (Quarrie and Lister, 1984b) and nuclear gene transcription (Guerrero and Mullet, 1986) only in leaves of *Triticum aestivum* (Milborrow and Noddle, 1970) and *Persea americana* tissues (Milborrow and Robinson, 1973) has an increase in incorporation of label from MVA into ABA been demonstrated in response to water stress. Thus, this aspect was examined further using excised, light-grown leaves from other vegetative higher plants.

4.1.2.2. Light.

Studies on the effect of light on ABA metabolism in plants have concentrated on changes in endogenous levels of this hormone and have produced controversial results. It was shown that light-grown *Pisum sativum* plants contained higher levels of ABA than did their dark-grown counterparts (Barnes and Light, 1969). However, attempts to repeat these experiments did not prove wholly successful (Kende and Kays, 1971; Burden *et al*, 1971; Barnes, 1972; Dörffling, 1973; Henson, 1983) although Simpson and Saunders (1972) showed that there was an interaction between the effects of light on the growth of *Pisum sativum* plants and their ability to accumulate ABA in response to water stress. Their results showed that the levels of ABA in light-grown seedlings were considerably higher than in dark-grown plants, and that this effect was even more dramatic following the imposition of stress. It has also been shown that increases in ABA levels in *Phaseolus vulgaris* seedlings (Tillberg, 1974) and *Pisum sativum* roots (Tietz, 1974; 1975) can be brought about by exposure to light. Likewise, illumination of cultures of *Cercospora rosicola* have

been shown to double the amount of ABA produced (Norman *et al*, 1981).

Several studies have also indicated that the length of the photoperiod and the wavelength of the radiation used are important factors in the effect of light on ABA levels in plants (Zeevaart, 1971; 1974; Tucker and Mansfield, 1972; Tanada, 1973; Loveys *et al*, 1974; Even-Chen and Itai, 1975; Tucker, 1976; Wellburn, 1978), implying a role for phytochrome in this process. However, these data give no information about the way in which such changes could occur.

Although attempts have been made to explain the effect of light on ABA levels in terms of its synthesis from a carotenoid origin, the results of such studies have been inconclusive (Taylor and Smith, 1967; Taylor and Burden, 1970; 1973; Firn *et al*, 1972; Firn and Friend, 1972; Burden *et al*, 1973; Malaby, 1974; Zeevaart, 1974; Anstis *et al*, 1975; Dörffling, 1978; De Greef and Frederick, 1983; Milborrow, 1983b) and Neill *et al* (1984) concluded that light is not normally required for ABA biosynthesis in plants.

4.1.2.3. Chemical factors.

Inhibitors of GA and carotenoid biosynthesis such as phosphon D, 2-chloroethyl trimethyl ammonium chloride (CCC; Figure 4.2; structure VIII), diphenylamine and 3-amino-1,2,4-triazole have been tested on ABA biosynthesis in ripening fruits of *Persea americana*, but without any effect on the incorporation of label from MVA into ABA (Milborrow, 1976). The effects of 2-isopropyl-4-dimethyl amino-5 methylphenyl-1-piperidine carboxylate methyl chloride (AMO1618; Figure 4.2; structure VII) and sandoz 6706, claimed to inhibit the desaturation of cartenoid precursors (Bartels and McCullough, 1972), have also been examined. At 10^{-3}M, AMO1618 enhanced the incoporation of label from MVA into ABA, whereas sandoz 6706 at 10^{-3}M strongly inhibited ABA synthesis but enhanced the incorporation of label from MVA into ABA at 10^{-5}M (Milborrow, 1976). Thus, no specific inhibitor of ABA biosynthesis in higher plants has been found, and consequently investigations into the role of ABA *in vivo*, by prevention of both its synthesis and catabolism have been unattainable.

Cytokinins have been reported to inhibit the biosynthesis of ABA in cultures of *Cercospora rosicola* (Norman *et al*, 1982) and *Botrytis cinerea* (Hirai *et al*, 1986). This inhibition was found to be concentration dependent, and in studies using farnesyl-[1-^{14}C]-pyrophosphate it was suggested to be specific rather than general for terpenoid biosynthesis. Likewise, the oxidation of *ent*-kaurene in

cell-free extracts prepared from *Marah oreganus* fruits was inhibited by several cytokinins and cytokinin analogues. Based on the structural similarities between cytokinins and α-cyclopropyl-α-[p-methoxyphenyl]-5-pyrimidine methyl alcohol (ancymidol; Figure 4.2; structure VI), Coolbaugh (1984) suggested that the inhibition of *ent*-kaurene oxidation by cytokininsand ancymidol occurred both *via* an interaction with cytochrome P450. However, clarification of this aspect requires the purification of the cytochrome P450, kinetic studies and cytokinin binding assays. In addition, ancymidol, AMO1618 and CCC inhibit the synthesis of ABA in cultures of *Cercospora rosicola* (Norman *et al*, 1983). In contrast to these findings, Hirai *et al* (1986) showed that AMO1618 enhanced the biosynthesis of ABA in mycelial cultures of *Botrytis cinerea* while CCC inhibited this process.

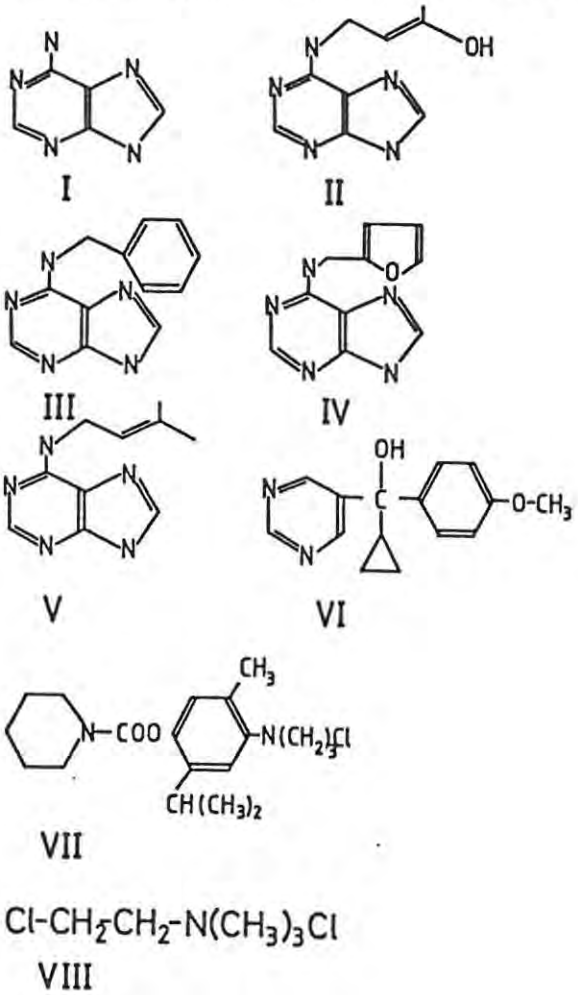

Figure 4.2. Structural formulae of adenine, several cytokinins, plant growth retardants and inhibitors of gibberellin biosynthesis. I, adenine; II, zeatin; III, benzyladenine; IV, kinetin; V, isopentenyl adenine; VI, ancymidol; VII, AMO1618; VIII, cycocel.

It is well known that ancymidol, a substituted pyrimidine, exhibits potent growth regulatory activity

in higher plants (Shive and Sisler, 1976; Coolbaugh and Hamilton, 1976) and is an inhibitor of *ent*-kaurene oxidation in microsomal preparations from liquid endosperm of immature *Marah macrocarpus* seeds (Coolbaugh *et al*, 1978) and to a lesser extent inhibits the incorporation of label from MVA into *ent*-kaurene (Coolbaugh and Hamilton, 1976). Recent information, obtained in studies on *Pisum sativum* and cell-free extracts of *Marah macrocarpus*, suggests that at low concentrations ancymidol, and its analogues, act to inhibit *ent*-kaurene oxidation and GA biosynthesis (Coolbaugh *et al*, 1982) although other modes of inhibition may operate at higher concentrations.

Similarly, AMO1816 and CCC inhibit GA biosynthesis in plant tissue (Zeevaart, 1966; Jones and Phillips, 1967; Dale and Felippe, 1968; Reid *et al*, 1968; Cleland and Zeevaart, 1970; Zeevaart, 1971; Kuo and Pharis, 1975). AMO1618 has been shown to inhibit the production of *ent*-kaurene (Dennis *et al*, 1965; Anderson and Moore, 1967) and the A activity of kaurene synthetase, responsible for the conversion of geranylgeranyl pyrophosphate (GGPP) to copalyl pyrophosphate (CPP) both intermediates in the production of *ent*-kaurene, in cell-free homogenates from *Gibberella fujikuroi* and *Ricinus communis* (Schechter and West, 1969; Robinson and West, 1970a). Similarly, CCC inhibits the A-activity of kaurene synthetase (Frost and West, 1977) and thus the cyclisation of GGPP to CPP. In addition, Douglas *et al*, (1972, 1974, 1978) have demonstrated that AMO1618 is an active inhibitor of sterol biosynthesis in *Nicotinia tobaccum* which may account for suggestions (Crozier *et al*, 1973) that some effects of AMO1618 are mediated by factors other than the inhibition of GA biosynthesis. The knowledge that inhibitors of GA biosynthesis and cytokinins have the ability to inhibit ABA biosynthesis in the fungus *Cercospora rosicola*, clearly indicates the need to investigate the effects of these compounds on ABA biosynthesis in higher plants. This in turn, could provide a means whereby the levels and/or activities of the enzymes involved in ABA biosynthesis could be modified in tissues which might be used for cell-free systems in which to investigate ABA biosynthesis.

Studies on ABA biosynthesis from MVA have intimated that the enzymes required for this process are present in plant tissues. However, nothing is known about their stability or their subcellular site of synthesis. In order to explore this aspect several well-known inhibitors of protein biosynthesis were examined for their effect on ABA biosynthesis. Thus, cycloheximide (CHI; Figure 4.3; structure 1), an inhibitor of protein biosynthesis on 80s ribosomes (Galling, 1982) was compared with chloramphenicol (CAP; Figure 4.3, structure 2) and lincomycin (LINC; Figure 4.3, structure 3), two inhibitors of 70s ribosomal protein biosynthesis (Brock, 1961; Armstrong *et al*, 1971; Ellis and

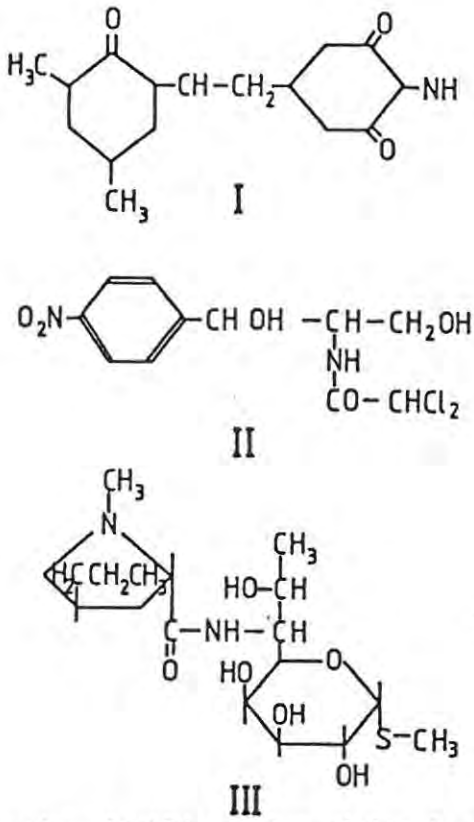

Figure 4.3. Structural formulae of inhibitors of translation. I, cycloheximide; II, chloramphenicol; III, lincomycin.

Hartley, 1971; Neumann and Parthier, 1973; Ellis, 1975; Yang and Scandalios, 1977).

4.2. RESULTS.

4.2.1. STUDIES ON ABSCISIC ACID BIOSYNTHESIS IN VARIOUS PLANT TISSUES.

4.2.1.1. ABA biosynthesis in ripening fruits of *Persea americana* Mill. cv. Fuerte.

Most of the studies on ABA biosynthesis from MVA have been carried out using ripening *Persea americana* mesocarp tissue (for reviews see: Milborrow, 1974a; 1976; 1978). In order to establish the efficiency of both MVA and MVAL as precursors of ABA under the present conditions *R,S*-[2-^{14}C]-MVAL (111kBq), *R,S*-[2-^{14}C]-MVA (111kBq) and *R*-[2-^{14}C]-MVAL (111kBq) were supplied to blocks (20g fresh weight) of ripened *Persea americana* mesocarp tissue and incubated under constant illumination for 24h in a water-saturated environment. Following incubation, the

tissue was extracted and the acidic fraction separated on thin layers of silica gel (GF_{254}) in toluene/ethyl acetate/acetic acid (50:30:4, v/v), as described in Chapter 2. The identity of any labelled ABA produced was confirmed by determining the retention of label in ABAMe, following methylation of the radioactive zone cochromatographing with authentic (*R,S,*)-ABA, and by reduction of ABAMe into the 1',4'-*cis* and 1',4'-*trans* diols of ABAMe. The results obtained are presented in Table 4.1. In addition, the distribution of radioactivity associated with ABAMe and the 1',4'-*cis* and 1',4'-*trans* diols of ABAMe, following derivitisation and chromatography, are depicted in Figure 4.4.

These data showed that illuminated *Persea americana* mesocarp synthesized ABA from [2-^{14}C]-MVAL as reported before (Milborrow and Robinson, 1973). However, this represents the first time these results have been corroborated in another study and confirms that applied [2-^{14}C]-MVA was a less efficient precursor of ABA than [2-^{14}C]-MVAL in this system, supporting earlier observations (Milborrow, 1974c).

The identity of ABA, synthesized from [2^{14}C]-MVAL, was further established by radio-GLC and UV-isomerisation (Lenton *et al*, 1971). One half of a destoned ripening *Persea americana* fruit (168g fresh weight) was supplied with *R*-[2-^{14}C]-MVAL (0.943MBq) to the cut surface and incubated for 24h as described before. The purified radioactive zone, cochromatographing with authentic (*R,S,*)-ABA on TLC, was methylated and an aliquot analysed by radio-GLC. Analysis of the putative ABAMe on a packed column of 1% XE-60 (Table 4.2) gave a single peak with an identical retention time (Rt=3.2min) to that of authentic ABAMe (Rt=3.2min).

The identity of the radioactive acid as ABA was further substantiated by UV-isomerisation where analysis of the products by radio-GLC on a packed column of 1% XE-60 gave radio peaks at Rt=3.2min and Rt=4.4min which displayed identical retention times to those of authentic (*R,S,*)-[2-^{14}C]-ABAMe and (*R,S,*)-[2-^{14}C]-*trans*-ABAMe (Table 4.2).

4.2.1.1.1. Kinetics of ABA biosynthesis in ripening fruits of *Persea americana* cv. Fuerte.

Kinetic studies on the biosynthesis of ABA from MVA were undertaken in order to determine the time of maximum incorporation of label from supplied MVAL into ABA, for later studies on the effect of applied chemicals on this pathway. *R*-[2-^{14}C]-MVAL (91.7kBq) was supplied to each of a 20g fresh weight block of *Persea americana* mesocarp tissue, which were then incubated in a H_2O-

TABLE 4.1: Biosynthesis of ABA in *Persea americana* cv. Fuerte mesocarp.

Blocks of softening mesocarp (20g fresh weight) were supplied with 111kBq of either R,S-[2-^{14}C]-MVAL, R,S-[2-^{14}C]-MVA or R-[2-^{14}C]-MVAL in Tween 80/acetone/H_2O (1:1:8, v/v) to the cut surface and incubated at 25°C under constant illumination (66µmol m^{-2} s^{-1}) for 24 hr in a H_2O-saturated environment. Tissue was extracted with methanol/ethyl acetate (50:50 v/v) containing BHT (20 mg/l) and (R,S)-ABA (2 mg/l). The diethyl ether-soluble acids were separated on TLC (silica gel GF_{254}) in toluene/ethyl acetate/acetic acid (50:30:4, v/v). Putative [^{14}C]-ABAMe was separated in n-hexane/ethyl acetate (1:1,v/v) while the products of $NaBH_4$ reduction were separated in benzene/ethyl acetate/acetic acid (15:3:1, v/v).

	Incorporation of Radioactivity			
Substrate	ABA	ABAMe	1',4'-cis diol of ABAMe	1',4'-trans diol of ABAMe
	Bq (%)			
R,S-[2-^{14}C]-MVAL	62.0 (0.057)	62.0 (0.057)	12.0 (0.011)	16.5 (0.015)
R,S-[2-^{14}C]-MVA	17.6 (0.016)	-	-	-
R-[2-^{14}C]-MVAL	236.4 (0.213)	206.5 (0.186)	104.3 (0.094)	107.7 (0.097)

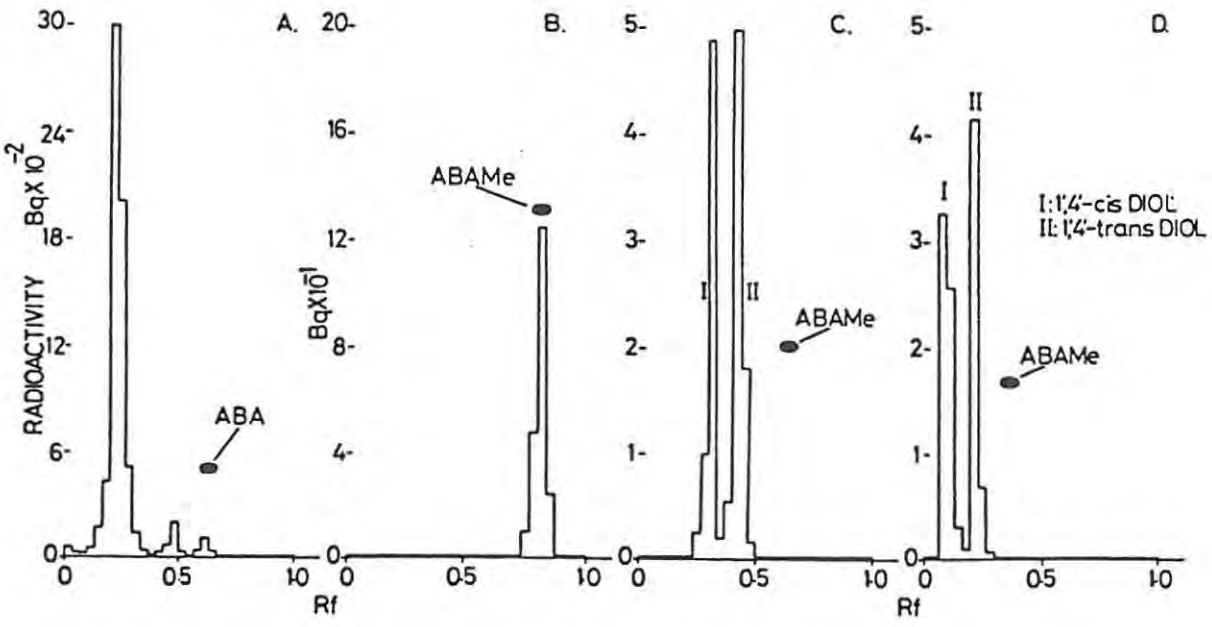

TABLE 4.2: Radio-gas liquid chromatography of the methyl ester of putative [^{14}C]-ABA generated from R-[2-^{14}C]-MVAL feeds to Persea americana cv. Fuerte mesocarp.

Persea americana tissue (16Bg f.w.) was fed with R-[2-^{14}C]-MVAL (0.943 MBq) and allowed to metabolize the substrate for 24h as previously described (Chapter 2: Section 2.7.3). Putative [^{14}C]-ABA was esterified with CH_2N_2 and analysed by radio-GLC on a packed glass column (1.8 m x 2 mm i.d.) of 1% XE-60 on gaschrom Q (80 - 100 mesh) with Ar as carrier gas at a flow rate of 42ml min^{-1}. In addition, putative [^{14}C]-ABAMe was isomerized under UV light for 2h and the products analysed as described above.

	Retention time on 1% XE-60 (min)	
Compound	Authentic	Putative
[^{14}C]-ABAMe	3.2	3.2
[^{14}C]-t-ABAMe	4.4	4.4

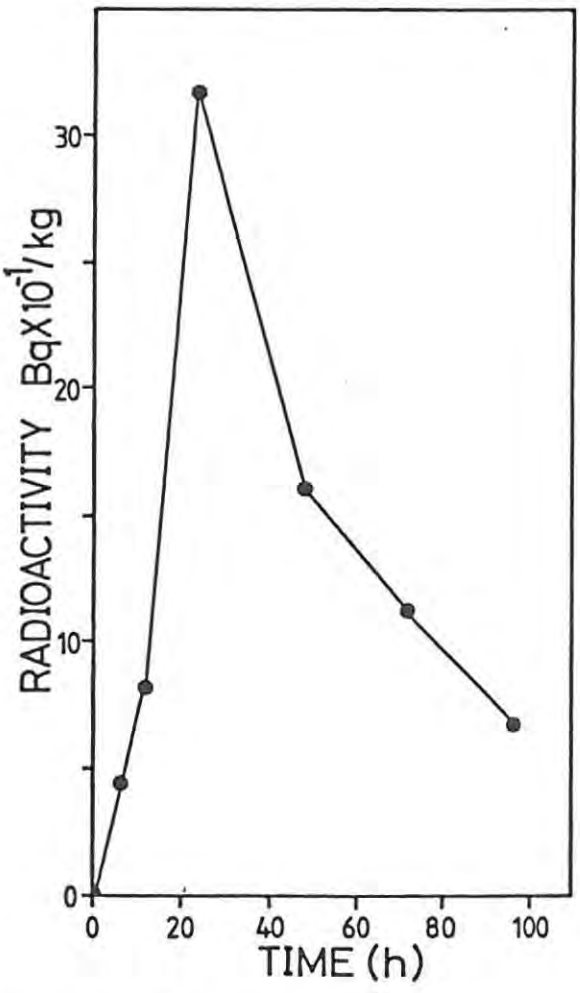

Figure 4.5. The kinetics of ABA biosynthesis in *Persea americana* cv. Fuerte mesocarp tissue.

saturated environment, under constant illumination for periods up to 96h and the data are depicted in Figure 4.5. It is evident that highest incorporation into ABA occurred at 24h and thereafter declined rapidly, probably as a result of product catabolism.

4.2.1.1.2. Tentative identification of PA and the 1',4'-*trans* diol of ABA produced from *R*-[2-^{14}C]-MVAL by mesocarp tissue of *Persea americana* cv. Fuerte.

Further analysis of the purified diethyl ether-fraction from *Persea americana* fruit supplied with *R*-[2-^{14}C]-MVAL by TLC in toluene/ethyl acetate/acetic acid (50:30:4, v/v) (Figure 4.6A), revealed in addition to ABA, a second radioactive zone, metabolite zone 1 (Rf 0.5) which displayed similar chromatographic properties to those of authentic PA in this solvent system.

Metabolite zone 1 was methylated and an aliquot chromatographed in *n*-hexane/ethyl acetate (1:1, v/v). The results depicted in Figure 4.6B demonstrated the existence of two radioactive compounds which displayed identical chromatographic properties to those of PAMe (Rf 0.63) and the 1',4'-*trans* diol of ABAMe (Rf 0.73) in this solvent system. The identity of the putative PAMe was established by microchemical methods (Figure 4.6C), as described in Chapter 2. Putative 1',4'-*trans* diol of ABAMe was identified by cochromatography using the solvent systems, benzene/ethyl acetate/acetic acid (15:3:1, v/v) and *n*-hexane/ethyl acetate (1:1, v/v) (Figure 4.6D and E). Thus, good circumstantial evidence was obtained to suggest that label from supplied *R*-[2-^{14}C]-MVAL is incorporated into ABA, its 1',4'-*trans* diol and PA in fruits of *Persea americana*, and the absolute amounts of radioactivity incorporated into these compounds is presented in Table 4.3.

TABLE 4.3: Incorporation of radioactivity from supplied R-[2-^{14}C]-MVAL (0.943 MBq) into ABA and related compounds, applied to one half of a Persea americana fruit (168g fresh weight) and incubated for 25h under constant illumination (66μmol $m^{-2}s^{-1}$) in a H_2O-saturated environment. ABA and related compounds were extracted and analysed as described in Chapter 2.

	Radioactivity incorporated (Bq) / kg fruit		
Substrate	ABA	PA	1',4'-trans diol of ABA
R-[2-^{14}C]MVAL	2440	4532	19882

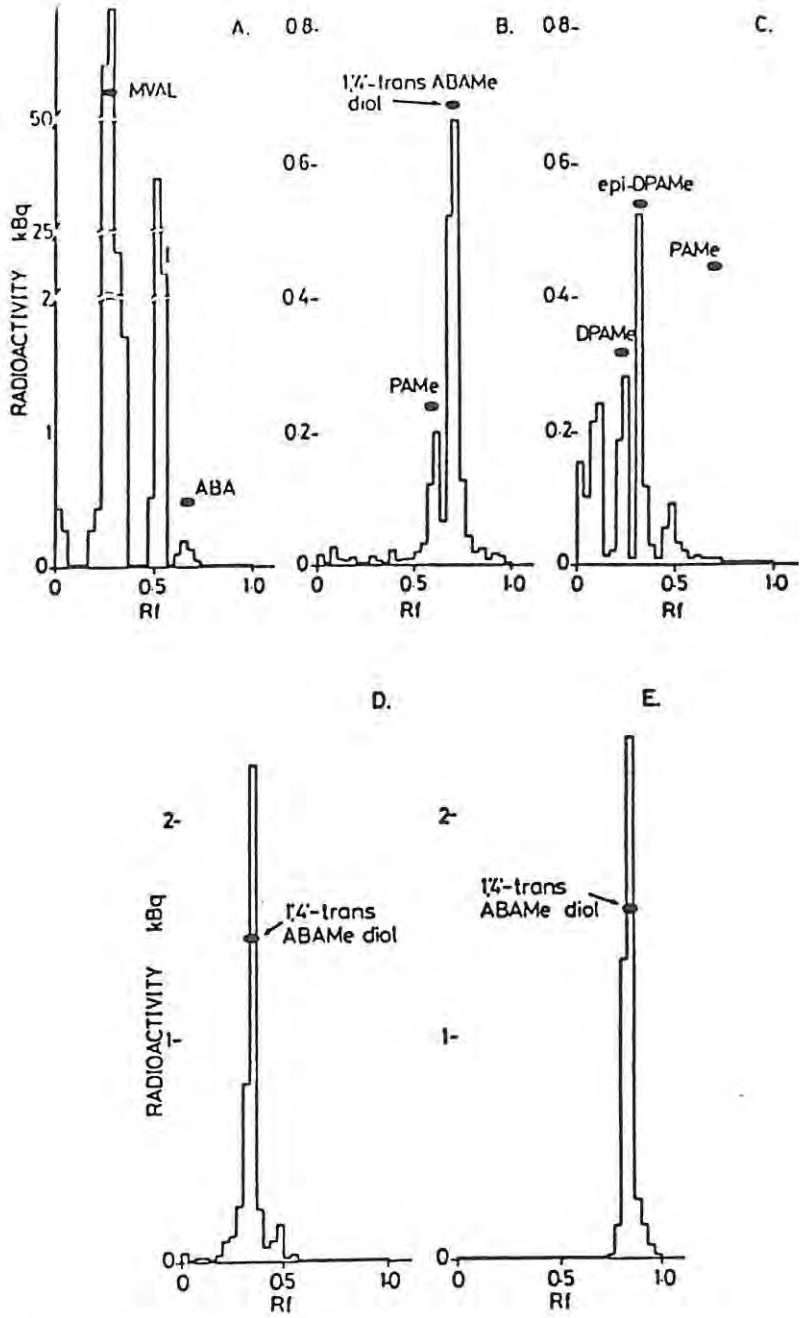

Figure 4.6. The metabolism of mevalonate by *Persea americana* cv. Fuerte.

A) Separation of the diethyl ether-soluble acids generated as described in Table 4.2; B), TLC separation of esterified metabolite zone 1 on silica gel GF_{254} in *n*-hexane/ethyl acetate (1:1, v/v) developed (1x) to 15cm; C), TLC separation of the $NaBH_4$ reduction products of the radioactive zone cochromatographing with authentic PAMe in B, separated by TLC on silica gel GF_{254} in *n*-hexane/ethyl acetate (1:1, v/v) developed (3x) to 15cm; D), TLC of the radioactive zone co-chromatographing with the 1',4'-*trans* diol of ABAMe in B, on silica gel GF_{254} in benzene/ethyl acetate/acetic acid (15:3:1, v/v) developed (1x) to 15cm and E), in *n*-hexane/ethyl acetate (1:1, v/v) developed (3x) to 15cm.

4.2.1.2. Studies on ABA biosynthesis in mature seeds of *Hordeum vulgare*.

ABA has been detected by bioassay, UV-spectrometry and GLC in developing grains of various cereals including *Triticum aestivum* (McWha and Hillman, 1974; McWha, 1975) and in maturing *Hordeum vulgare* seeds (Goldbach and Michael, 1976; Naumann and Dörffling, 1982). ABA has also been detected by immunoassay and characterised by GC-MS in embryos of *Triticum aestivum* and *Hordeum vulgare* (Morris *et al*, 1988).

Milborrow and Robinson (1973) showed that label from *R,S*-[2-^{14}C]-MVAL became incorporated into ABA in embryo and endosperm halves of *Triticum aestivum* seeds. No similar studies have been conducted on mature seeds of *Hordeum vulgare*. Therefore, a comparison was made between the biosynthetic competence of embryo and endosperm halves and aleurone layers for the synthesis of ABA from MVAL. 100 Embryos and endosperm halves were each imbibed in 2.0ml 0.1M KPi buffer (pH7.5), while 20 aleurone layers were imbibed in 2.0ml of 20mM sodium succinate buffer (pH5.0) containing 20mM $CaCl_2$ and *R*-[2-^{14}C]-MVAL for 24h under laboratory light (42μmol m^{-2} s^{-1}) in a metabolic shaker at 28^{o}C. Following incubation, the tissue was extracted and analysed as described in Chapter 2. The radioactive zone cochromatographing with authentic (*R,S,*)-ABA on thin layers of silica gel in toluene/ethyl acetate/acetic acid (50:30:4, v/v) was identified by microchemical means as [^{14}C]-ABA and the results are presented in Table 4.4. In contrast, isolated *Hordeum vulgare* aleurone layers were unable to synthesize ABA from applied, radioactive MVAL (Table 4.4).

These data demonstrate that both embryo and endosperm halves from seeds of *Hordeum vulgare* cv. Himalaya and cv. Dyan could synthesize ABA from applied *R*-[2-^{14}C]-MVAL (Table 4.5).

4.2.1.3. Studies on ABA biosynthesis in immature seed of *Pisum sativum* L. and *Phaseolus vulgaris* L.

Unlike studies on GA metabolism (Sponsel, 1983; 1985; Graebe, 1986), nothing is known about the ability of immature seeds to biosynthesize ABA. ABA has been characterised as an endogenous compound in immature seeds of *Pisum sativum* (Isogai *et al*, 1967; Frydman *et al*, 1974) and *Phaseolus vulgaris* (Hiraga *et al*, 1974), two species in which GA biosynthesis has been demonstrated (Graebe, 1987). In addition, increases in the endogenous levels of ABA during seed maturation in both species (Hsu, 1979; Browning, 1980) suggests that this increase might be achieved

TABLE 4.4: The biosynthesis of ABA in aleurone layers, embryo and endosperm halves of Hordeum vulgare cv. Dyan seeds.

20 Aleurone layers, embryo and endosperm portions were embibed in 2.0ml K_2HPO_4/KH_2PO_4 (0.1M; pH 7.5) containing R-[2-^{14}C]-MVAL (94kBq) for 24h under constant illumination (42μmol $m^{-2}s^{-1}$) in a metabolic shaker at 28°C. Following incubation, the tissue was extracted and the amounts of radioactivity associated with ABA and its derivatives determined as described in Chapter 2.

	Radioactivity incorporated / g dry weight				
		Bq (%)			
Tissue	Dry Weight (g)	ABA	ABAMe	1',4'-cis diol ABAMe	1',4'-trans diol of ABAMe
Embryos	0.82	58.44(0.0623)	51.83(0.0550)	23.61(0.0250)	24.66(0.0263)
Endosperms	3.00	3.46(0.0037)	3.42(0.0036)	1.54(0.0017)	1.77(0.0020)
Aleurone layers	-	4.16(0.0044)	-	-	-

TABLE 4.5: The biosynthesis of ABA from R-[2-^{14}C]-MVAL in Hordeum vulgare cv. Dyan and cv. Himalaya embryo tissue. (For incubation, extraction and identification procedures, see Table 4.4).

	Radioactivity incorporated / g dry weight				
		Bq (%)			
Cultivars	Dry weight (g)	ABA	ABAMe	1',4'-cis diol of ABAMe	1',4'-trans diol of ABAMe
cv. Dyan	0.92	63.94(0.0697)	57.62(0.0613)	27.55(0.0293)	21.60(0.0229)
cv. Himalaya	0.70	53.99(0.0574)	51.63(0.0549)	19.39(0.0206)	24.60(0.0262)

by biosynthesis.

Immature seeds of *Pisum sativum* and *Phaseolus vulgaris* were incubated in nutrient medium (Nitsch, 1951) containing *R*-[2-^{14}C]-MVAL for 48h after which any ABA produced was partitioned into diethyl ether at pH2.5. The diethyl ether-soluble acid fractions, from 48h feeds of *R*-[2-^{14}C]-MVAL to immature seed from *Pisum sativum* and *Phaseolus vulgaris*, were separated on thin layers of silica gel (GF_{254}) in toluene/ethyl acetate/acetic acid (50:30:4, v/v). Only small amounts of radioactivity were associated with authentic (*R,S,*)-ABA (Rf 0.66-0.7) and the data are presented in Table 4.6.

Attempts to determine the identity of this radio-peak as ABA, by micro chemical methods, proved unsuccessful and no radioactivity was found associated with authentic (*R,S,*)-ABAMe, following derivatisation and re-chromatography on thin layers of silica gel in *n*-hexane/ethyl acetate (1:1, v/v). One explanation for the inability to demonstrate the incorporation of label from MVAL into ABA in immature seeds in these studies might have been permeability problems which prevented MVAL from reaching the subcellular sites of ABA biosynthesis.

4.2.2. THE EFFECT OF WATER STRESS AND LIGHT ON ABA BIOSYNTHESIS AND THE MODIFICATION OF ABA BIOSYNTHESIS BY CHEMICAL FACTORS.

4.2.2.1. Studies on ABA biosynthesis in water stressed leaves of *Hordeum vulgare* cv. Dyan, *Pisum sativum* cv. Black-eyed Susan and *Phaseolus vulgaris* cv. Top-crop.

Only in one instance has the stress-induced synthesis of ABA from radiolabelled precursors been demonstrated in vegetative tissue (Milborrow and Noddle, 1970). ABA has been identified in *Hordeum vulgare* leaves and its levels increase in response to stress (Quarrie and Lister, 1984a). Likewise, ABA has been characterised in seedlings of *Pisum sativum* and *Phaseolus vulgaris* (Komoto *et al*, 1972; Zeevaart and Milborrow, 1976). Thus, in view of the results obtained by Milborrow and Noddle (1970) using leaves of *Triticum aestivum*, the stress-induced increase in ABA biosynthesis from applied, radiolabelled mevalonate was investigated in light-grown leaves of *Hordeum vulgare* and excised seedlings of *Pisum sativum* and *Phaseolus vulgaris*.

Initially, leaf tissue from 10d old *Hordeum vulgare* seedlings was supplied with *R*-[2-^{14}C]-MVAL

via the transpiration stream. Following uptake, half of the tissue was transferred to a stream of warm air until it lost 20% of its original fresh weight. Turgid and wilted tissue samples were then incubated as described in Chapter 2. Following incubation, the diethyl ether fractions, prepared from the non-wilted (control) and wilted tissue were separated by TLC in toluene/ethyl acetate/acetic acid (50:30:4, v/v) to yield the distribution of radioactivity depicted in Figure 4.7. These data suggested the presence of a compound with similar chromatographic properties to those of ABA which was significantly higher in the wilted tissue (Figure 4.7B).

To establish the identity of the radioactive zone cochromatographing with authentic ABA, nine identical experiments were carried out and the results are presented in Table 4.7. In only one case was this radioactive acid tentatively identified as ABA following cochromatography and microchemical analyses. Thus, the significance of this result appears minimal. Furthermore, in an experiment to determine whether leaf age contributed to the capacity of this tissue to synthesize ABA, attempts were made to demonstrate the effect of water- stress on the synthesis of ABA, from applied *R*-[2-^{14}C]-MVAL, in excised leaves of different ages. The data presented in Table 4.7 (experiments 8, 9 and 10) indicate that leaf age had no apparent effect on the ability of this tissue to incorporate label from MVAL into ABA in response to water stress.

Studies were also carried out to investigate the stress-induced synthesis of ABA from applied, radiolabelled mevalonate using excised, light-grown seedlings of *Pisum sativum* and *Phaseolus vulgaris*. In these experiments both turgid and wilted seedlings from these species were unable to incorporate label from applied mevalonate into ABA (data not shown).

The inability to demonstrate the biosynthesis of ABA from MVAL in response to stress in light-grown leaves of *Hordeum vulgare* and seedlings of *Pisum sativum* and *Phaseolus vulgaris*, might be due to competition with other pathways (carotenoid and chlorophyll biosynthesis) for substrate MVA. Thus, whether ABA increases in response to water-stress occur by *de novo* synthesis in these tissues remains to be shown.

4.2.2.2. Studies on the effect of light on the biosynthesis of ABA in ripening fruits of *Persea americana* cv. Fuerte.

The effect of light on the incorporation of label from [^{14}C]-MVAL into ABA was investigated. *R*-[2-^{14}C]-MVAL was supplied to *Persea americana* mesocarp tissue which was then incubated for 24h

TABLE 4.6: The metabolism of R-[2-^{14}C]-MVAL in immature seed from Pisum sativum and Phaseolus vulgaris.

Immature seed was incubated in 20ml nutrient medium (Nitsch, 1951) containing R-[2-^{14}C]-MVAL (105kBq) in an orbital shaker at 28°C under constant illumination (42μmol $m^{-2}s^{-1}$) for 24h. The diethyl ether soluble acids were separated by TLC in silica gel GF_{254} in toluene/ethyl acetate/acetic acid (50:30:4, v/v) while the putative methyl ester was separated in n-hexane/ethyl acetate (1:1, v/v).

	Radioactivity incorporated Bq (%)		% [^{14}C] incorporated /g fresh weight
Tissue	ABA	ABAMe	
Pisum sativum seed (10g f.w.)	29.49(0.071)	-	0.0071
Phaseolus vulgaris seed (5g f.w.)	13.62(0.033)	-	0.0065

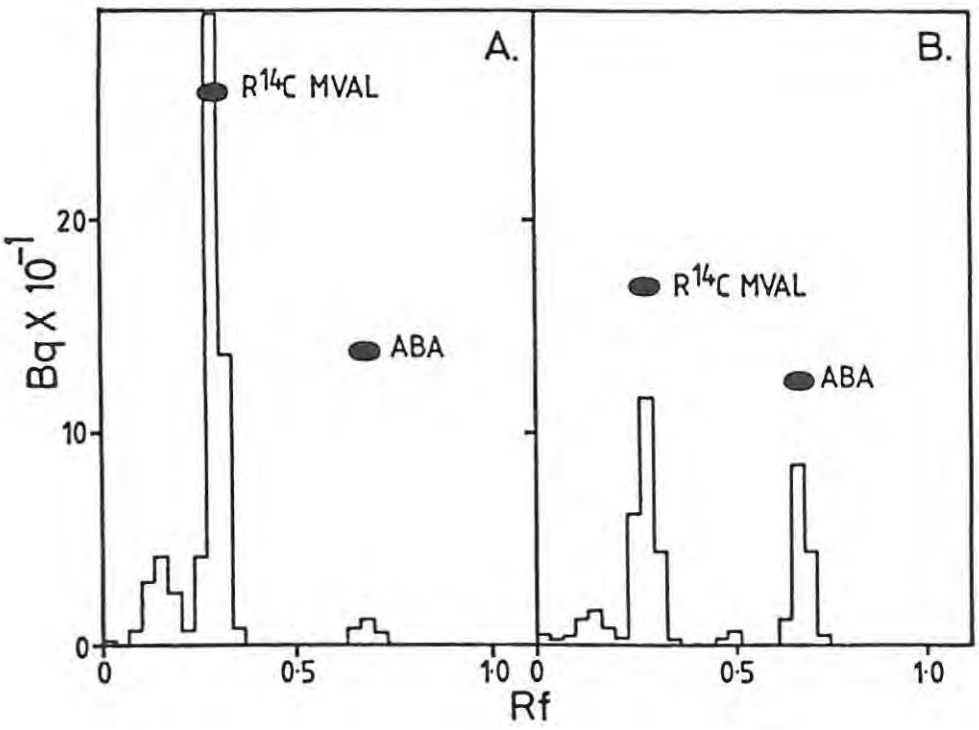

Figure 4.7. The metabolism of mevalonate in stressed leaves of *Hordeum vulgare* cv. Dyan.

TLC separation of the acidic products generated from *R*-[2-^{14}C]-MVAL (188kBq) fed to excised, light-grown leaves of *Hordeum vulgare* (20g fresh weight) *via* the transpiration stream. Following uptake, half of the tissue was subjected to a stream of warm air until it had lost 20% of its original fresh weight. A), turgid and B), stressed tissue was incubated for 24h under constant illumination as described in Chapter 2, Section 2.7 and the diethyl ether-soluble acids separated by TLC on silica gel GF_{254} in toluene/ethyl acetate/acetic acid (50:30:4, v/v) developed (2x) to 15cm.

TABLE 4.7: Attempts to demonstrate the incorporation of label from R-[2-^{14}C]-MVAL into ABA in turgid and wilted, excised leaves of Hordeum vulgare cv. Dyan.

10g Fresh weight of 4,7 and 10d old, light-grown Hordeum vulgare leaf tissue were excised and placed with their proximal ends into glass beakers containing R-[2-^{14}C]-MVA (91.7kBq) and uptake achieved via the transpiration stream under constant illumination (66μmol $m^{-2}s^{-1}$) at 25°C. Following uptake, each tissue batch was divided equally and one half exposed to hot air until it had lost 20% of its original fresh weight. Tissue was incubated in sealed polythene bags (containing wet paper towelling for turgid tissue) for 24h under constant illumination (66μmol $m^{-2}s^{-1}$) at 25°C. Tissue was extracted and ABA in the samples analysed as described in Chapter 2.

			Radioactivity Incorporated (Bq)			
Expt. No.	Tissue age (days)	Treatment	ABA	ABAMe	1',4'-cis diol of ABAMe	1',4'-trans diol of ABAMe
1	10	T	293.5	0.0	N.D.	N.D.
		W	617.2	0.0	0.0	0.0
2	10	T	237.9	237.8	N.D.	N.D.
		W	452.4	447.8	221.4	214.8
3	10	T	122.3	0.0	N.D.	N.D.
		W	188.6	91.3	0.0	0.0
4	10	T	312.1	0.0	N.D.	N.D.
		W	181.2	0.0	0.0	0.0
5	10	T	812.0	0.0	N.D	N.D.
		W	354.9	0.0	0.0	0.0
6	10	T	285.3	0.0	N.D.	N.D.
		W	576.7	23.6	0.0	0.0
7	10	T	267.1	0.0	N.D.	N.D.
		W	291.5	0.0	0.0	0.0
8	7	T	53.9	0.0	N.D.	N.D.
		W	200.9	0.0	0.0	0.0
9	4	T	74.3	0.0	N.D.	N.D.
		W	201.2	0.0	0.0	0.0

N.D. not detected.

T turgid

W wilted

in a water-saturated environment at 25°C, either in light (66μmol m^{-2} s^{-1}) or in darkness. Following the incubation period the tissue was extracted and the levels of radioactivity associated with ABA were determined as previously described. The data presented in Table 4.8 demonstrate a ±30% increase in the accumulation of label in ABA in the tissue sample incubated in darkness.

TABLE 4.8: Effect of light on the biosynthesis of ABA in excised mesocarp tissue from Persea americana cv. Fuerte.

20g Fresh weight blocks of Persea americana mesocarp tissue were incubated with R-[2-^{14}C] MVAL (90kBq) supplied in 200μl of Tween 80/acetone/H_2O (1:1:8, v/v) to the cut surface. Tissue was incubated for 24h under either, conditions of continuous illumination (66μmol $m^{-2}s^{-1}$) or in total darkness at 25°C in a H_2O-saturated environment. Levels of radioactivity incorporated into ABA were determined as previously described.

	Radioactivity incorporated into ABA	
Treatment	Bq (%)	
LIGHT	89.76	(0.0997)
DARK	117.93	(0.1310)

4.2.2.3. Studies on the effects of chemical factors on ABA biosynthesis in ripening fruits of *Persea americana* cv. Fuerte.

4.2.2.3.1. Effect of AMO1618, CCC and ancymidol on the biosynthesis of ABA in ripening fruits of *Persea americana* cv. Fuerte.

Solutions of the GA biosynthesis inhibitors AMO1618, ancymidol and CCC, all at 500μM, were applied to the sliced surface of individual blocks (20g f.w.) of mesocarp tissue from *Persea americana* fruits and incubated for 6h. *R*-[2-^{14}C]-MVAL was then added and the tissue incubated for a further 18h in a H_2O-saturated environment under continuous illumination. Following incubation the tissue was extracted and the purified diethyl ether fractions separated on thin layers of silica gel (GF_{254}), in toluene/ethyl acetate/acetic acid (50:30:4, v/v).

The data presented in Table 4.9 demonstrate that both ancymidol and CCC reduced the incorporation of radioactivity from *R*-[2-^{14}C]-MVAL into ABA by ±80% in *Persea americana* fruits.

In contrast, AMO1618 enhanced the incorporation of label from [^{14}C]-MVAL into ABA in *Persea americana* (Table 4.9), while at higher concentrations (10^{-3}M and above), it had little further effect on this process (Figure 4.8). These results support the data obtained by Milborrow (1976) and data obtained using *Botrytis cinerea* (Hirai *et al*, 1986). However, they contradict results from similar studies in the fungus *Cercospora rosicola* (Norman *et al*, 1983).

Since AMO1618 enhanced ABA biosynthesis in mesocarp of *Persea americana*, presumably by channeling terpenyl pyrophosphate intermediates into ABA, as a result of the inhibition of GA (Oster and West, 1968) and steroid biosynthesis (Douglas and Paleg, 1978), it may prove useful in later cell-free studies on ABA biosynthesis.

4.2.2.3.2. Effect of cytokinins on the biosynthesis of ABA in ripening fruits of *Persea americana* cv. Fuerte.

Cytokinins have been found to inhibit ABA biosynthesis in the fungi *Cercospora rosicola* (Norman *et al*, 1982) and *Botrytis cinerea* (Hirai *et al*, 1986) but it is unknown whether they exert a similar effect on plants. Solutions of several cytokinins, at concentrations of 500μM, which inhibited ABA production in *Cercospora rosicola* (Norman *et al*, 1982), were therefore applied to the sliced surfaces of blocks of mesocarp tissue from ripening fruits of *Persea americana* and incubated for 6h, prior to the addition of *R*-[2-^{14}C]-MVAL. Tissue samples were incubated in the presence of radiolabelled substrate for a further 18h in a water-saturated environment under constant illumination.

Analysis of the diethyl ether soluble acid fractions by TLC on silica gel GF_{254} in toluene/ethyl acetate/acetic acid (50:30:4, v/v), gave the results presented in Table 4.10. These data show that, as in fungi, cytokinins can inhibit ABA biosynthesis from MVA in ripening mesocarp tissue from fruits of *Persea americana* with BA and kinetin being the most effective.

4.2.2.3.3. Effect of inhibitors of translation on ABA biosynthesis in ripening fruits of *Persea americana* cv. Fuerte.

The stress-induced increase in ABA levels in plants was thought to require chloroplast-localised synthesis of ABA (Milborrow, 1974a; 1974b; 1976). If chloroplasts are involved in ABA metabolism in plants, it is reasonable to assume that plastid-synthesized enzymes could be implicated in

TABLE 4.9: The effect of inhibitors of gibberellin biosynthesis on the biosynthesis of ABA from MVAL in Persea americana cv. Fuerte mesocarp tissue.

Blocks of sliced Persea americana mesocarp tissue (20g f.w.) were supplied with solutions of AMO1618, ancymidol and CCC (all 500μM in Tween 80/acetone/H_2O; 1:1:8,v/v) and incubated for 6h in a water-saturated environment at 25°C under constant illumination (66μmol $m^{-2}s^{-1}$). After 6h, R-[2-^{14}C]-MVAL (91.7kBq) was added and the tissue incubated for a further 18 h as above. ABA was extracted and analysed as described in Chapter 2.

Treatment	Radioactivity incorporated		% inhibition or stimulation*
	Bq (%)		
Control	80.56	(0.0895)	0
AMO1618	131.33	(0.1459)	+ 63.00
CCC	16.83	(0.0187)	79.11
Ancymidol	15.77	(0.0175)	80.43

* % inhibition or stimulation relative to control.

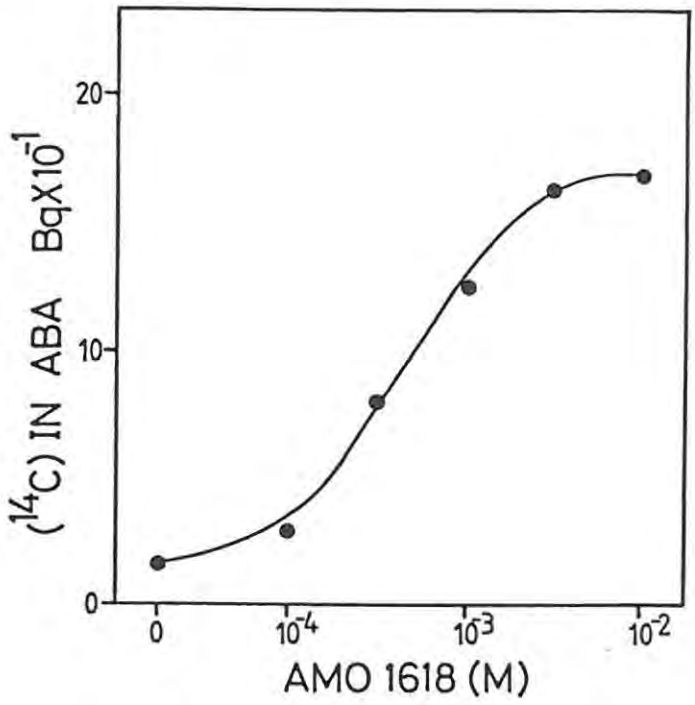

Figure 4.8. The effect of AMO1618 concentration on the incorporation of label from *R*-[2-^{14}C]-MVAL (91kBq) into ABA in *Persea americana* mesocarp tissue (20g fresh weight). Conditions were as described in Figure 4.4.

TABLE 4.10: The effect of cytokinins on the biosynthesis of ABA from MVAL in Persea americana cv Fuerte mesocarp tissue.

Blocks of sliced Persea americana mesocarp tissue (20g f.w.) were supplied with solutions of cytokinins (all at 500μM in Tween 80/acetone/H_2O; 1:1:8,v/v) and incubated for 6h in a water-saturated environment at 25°C under constant illumination (66μmol $m^{-2}s^{-1}$). After 6h, R -[2-^{14}C]-MVAL (91.7kBq) was added and the tissue incubated for a further 18h as above. ABA was extracted and analysed as described in Chapter 2.

Treatment	Radioactivity incorporated Bq (%)		% inhibition*
Control	80.56	(0.0895)	0
Adenine	78.74	(0.0875)	2.27
IPA	13.14	(0.0146)	83.69
BA	9.83	(0.0109)	87.81
Zeatin	23.42	(0.0260)	70.94
Kinetin	11.30	(0.0126)	85.97

* % inhibition relative to control

TABLE 4.11: Effect of protein synthesis inhibitors on ABA biosynthesis in illuminated excised blocks of mesocarp from Persea americana cv. Fuerte.

Sliced Persea americana mesocarp tissue (20g f.w.) were supplied with solutions of lincomycin (0.1 mg/ml), chloramphenicol (1 mg/ml) and cycloheximide (1 mg/ml) in Tween 80/acetone/H_2O (1:1:8, v/v) and incubated for 6h in a H_2O-saturated environment at 25°C under constant illumination (66μmol $m^{-2}s^{-1}$). After 6h, R-[2-^{14}C]-MVAL (90kBq) was added, and the tissue incubated for a further 18h as described above. The incorporation of radioactivity from MVAL into ABA was determined as previously described.

Treatment	Incorporation of Radioactivity into ABA Bq (%)		% inhibition*
Control	97.44	(0.1080)	0
Lincomycin (0.1 mg/ml)	72.29	(0.0803)	25.81
Chloramphenicol (1 mg/ml)	81.28	(0.0903)	16.58
Cycloheximide (1 mg/ml)	22.22	(0.0246)	77.19

* Percentage inhibition relative to control

catalysing some of the steps in this process. It might be possible to detect alterations in ABA biosynthesis using inhibitors of plastid protein synthesis. Therefore, two such inhibitors CAP and LINC, which affect translation on 70s plastid ribosomes (Ellis, 1982) were supplied to illuminated mesocarp slices from *Persea americana*, together with *R*-[2-^{14}C]-MVAL, conditions under which ABA is known to enter chloroplasts *via* a pH gradient (Heilmann *et al*, 1980). In addition, separate tissues were also treated with CHI, a potent inhibitor of protein synthesis on cytoplasmic 80s ribosomes (Galling, 1982), in the presence of *R*-[2-^{14}C]-MVAL, as above.

The results in Table 4.11 show that whereas ABA biosynthesis was not markedly affected by 70s ribosome inhibitors, it was markedly reduced in the presence of cycloheximide. Though the difficulties associated with interpreting data from experiments involving inhibitors of translation are well known (Galling, 1982), the limited effect of 70s ribosome inhibitors on ABA biosynthesis in mesocarp of *Persea americana* suggests that plastid-synthesized proteins are probably not involved in this process. These results have in part been published (Cowan and Railton, 1986).

CHAPTER FIVE

STUDIES ON THE CATABOLISM OF ABSCISIC ACID IN VARIOUS HIGHER PLANT TISSUES.

5.1.INTRODUCTION.

Studies on ABA catabolism were carried out to evaluate the potential of various tissues as sources of enzymes for later cell-free studies on ABA catabolism. Tissues were used in which ABA catabolism had not previously been examined in detail but where good evidence was available concerning their potential for this process, or where studies had been conducted before but under different conditions using different cultivars of the same species. Such information, it was hoped, would be of value for later studies on the effect of environmental factors and applied chemicals on possible changes in the rate of ABA catabolism which might be mediated *via* alterations in the levels of enzymes catalysing the transformations of applied ABA.

Most studies on applied ABA catabolism in vegetative higher plants have been undertaken with dicotyledons (Sembdner *et al*, 1980) but the identity of the catabolites has only been established unequivocally by reliable physico-chemical techniques in very few cases. These dicotyledonous species are *Lycopersicon esculentum* (Milborrow, 1970), *Phaseolus vulgaris* (Zeevaart and Milborrow, 1976), *Xanthium strumarium* (Zeevaart and Boyer, 1982; Boyer and Zeevaart, 1986) and *Pisum sativum* (Tietz *et al*, 1979; Tietz, 1985). In contrast, there is only limited information on the fate of applied ABA in monocotyledons (Cummins, 1973; Gross and Schütte, 1974; Lehmann and Schütte, 1984; Murphy, 1984) and no catabolites have been identified unequivocally by spectroscopic methods.

Initially, Cummins (1973) demonstrated that light-grown leaves of *Hordeum vulgare* converted applied, racemic ABA to unknown polar, acidic catabolites. These products were later tentatively identified as PA and DPA by cochromatography on TLC in studies using leaves of both *Hordeum vulgare* and *Triticum aestivum* (Gross and Schütte, 1974). Lehmann and Schütte (1984), employing intact *Triticum aestivum* seedlings, were only able to detect low levels of the acidic products of ABA catabolism which were not identified. Nevertheless, PA and DPA were tentatively identified as catabolites of applied, racemic ABA in leaves of *Triticum aestivum* by cochromatography and micro-chemical methods, along with the detection of an unidentified acidic, ABA catabolite (Murphy, 1984).

Other studies on ABA catabolism in monocotyledons have focussed on the role of the aleurone layer of *Hordeum vulgare* seeds in this process (Dashek *et al*, 1979; Ho and Uknes, 1982; Uknes and Ho, 1984) where ABA interferes with GA-induced synthesis of various enzymes (Ho, 1983). Little is known however, about the contribution of *Hordeum vulgare* embryos to ABA catabolism which might be important given the finding that both *Triticum aestivum* embryos (Milborrow and Robinson, 1973) and *Hordeum vulgare* embryos (present study, Chapter 4) synthesize ABA from applied MVA.

Likewise, the few detailed investigations on ABA catabolism in dicotyledons, have neglected immature seeds as a likely source of enzymes for ABA degradation. Intact, immature seeds of both *Pisum sativum* and *Phaseolus vulgaris* contain endogenous PA and DPA (Tinelli *et al*, 1973; Walton *et al*, 1973; Frydman *et al*, 1974; Milborrow, 1975b) and have been used to generate cell-free preparations which are highly active in catalysing the oxidations and hydroxylations of *ent*-gibberellane intermediates in the GA biosynthetic pathway (Graebe, 1987). This implies that these organs could also be useful systems in which to study the catabolism of ABA where related chemical modifications of this compound are involved.

Thus, the present investigation examined, in detail, the catabolism of applied (*R,S,*)-ABA in both vegetative and non-vegetative tissues from a monocotyledonous species and various dicotyledonous species.

5.2.RESULTS.

5.2.1.ABSCISIC ACID CATABOLISM IN VEGETATIVE TISSUES.

ABSCISIC ACID CATABOLISM IN A VEGETATIVE MONOCOTYLEDON.

5.2.1.1.Studies on the catabolism of (*R,S,*)-ABA in excised, light-grown leaves of *Hordeum vulgare* cv. Dyan.

When (*R,S,*)-[2-^{14}C]-ABA was fed, *via* the transpiration stream, to excised leaves of *Hordeum vulgare*, the distribution of radioactivity depicted in Figure 5.1 was observed.

Catabolite zone 1 (Rf0-0.1) constituted a highly polar compound which was barely mobile in this

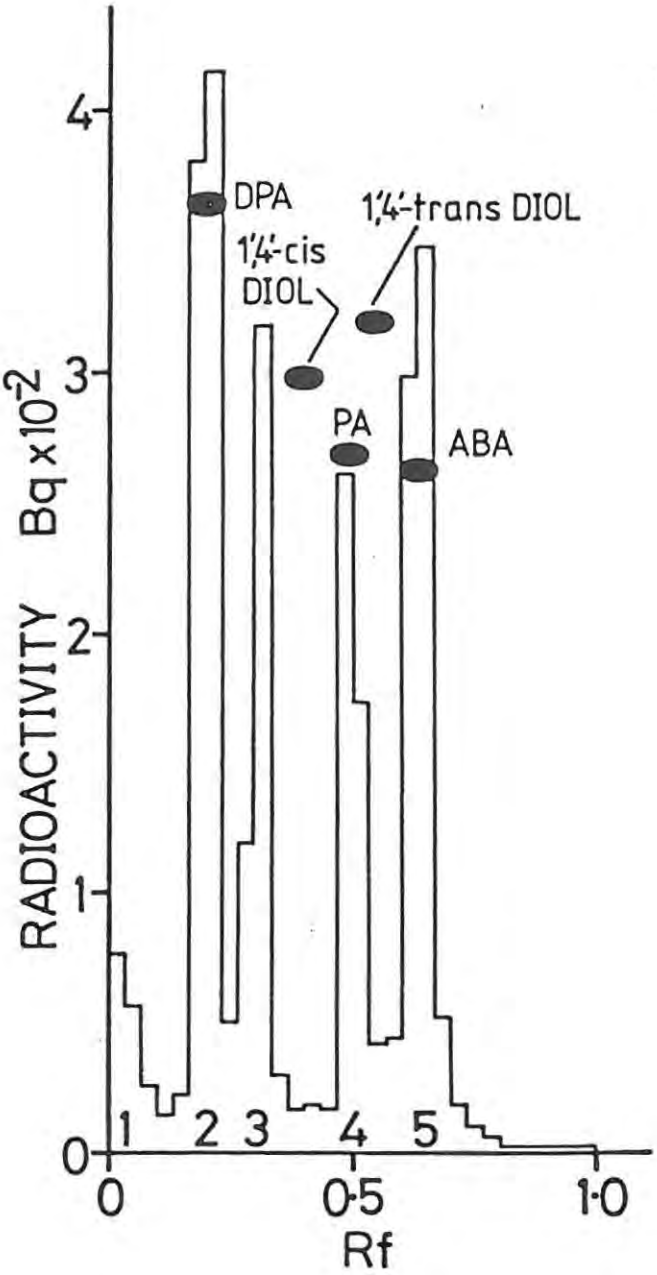

Figure 5.1. Separation by TLC of (*R,S,*)-[2-^{14}C]-ABA and its catabolites from extracts of light-grown *Hordeum vulgare* leaves. Leaves (5g fresh weight) were fed with (*R,S,*)-[2-^{14}C]-ABA (4.2kBq) for 30h and the ethyl acetate-soluble acids separated by TLC on silica gel GF_{254} with toluene/ethyl acetate/acetic acid (50:30:4, v/v) developed 2x to 15cm.

solvent system. Zone 2 (Rf0.2-0.23) displayed similar chromatographic properties to those of authentic DPA while zone 4 (Rf0.5-0.56) co-chromatographed with authentic PA. Radioactive TLC zone 5 co-migrated with authentic, substrate ABA. Catabolite zone 3 (Rf0.33-0.4), which constituted almost 30% of the total radioactivity associated with the acidic catabolites of (*R,S,*)-ABA, did not co-chromatograph with any known acidic product of ABA catabolism reported previously in this solvent system (Zeevaart and Milborrow, 1976; Milborrow, 1983).

5.2.1.1.1. Analysis of the acidic catabolites of (*R,S,*)-[2-^{14}C]-ABA in light-grown leaves of *Hordeum vulgare* cv. Dyan.

In order identify the acidic products of (*R,S,*)-ABA catabolism, (*R,S,*)-[2-^{14}C]-ABA (42kBq) was fed to excised, light-grown leaves of *Hordeum vulgare* (10g fresh weight) and the ethyl acetate-soluble acids separated by TLC, eluted, methylated and analysed by cochromatography and micro chemical methods.

Analysis of putative PAMe and DPAMe, and of the methyl ester of catabolite 3, by TLC gave the results depicted in Figure 5.2. Putative [^{14}C]-PAMe cochromatographed with authentic PAMe as a single radioactive peak (Figure 5.2A). Similarly, putative [^{14}C]-DPAMe migrated as a single radiopeak and cochromatographed with authentic DPAMe (Figure 5.2B). The methyl ester of catabolite 3 chromatographed as a single peak of intermediate polarity between PAMe and DPAMe (Figure 5.2C). These findings suggested that catabolite 3 might be an oxygen containing derivative of either (*R,S,*)-ABA or PA.

The identity of these compounds was further established by examining the products of treatment with either $NaBH_4$ or Jones' reagent as described in Chapter 2 for PA and DPA. The data shown in Figure 5.2 and Figure 5.3 confirmed that both PA and DPA were catabolites of applied ABA in leaves of *Hordeum vulgare*.

Treatment of the methyl ester of catabolite 3 with $NaBH_4$ resulted in reduction of this compound to two, more polar products (Figure 5.3 C), suggesting the presence of an intact keto function at the C-4'position in the cyclohexene ring thereby discounting the possibility that catabolite 3 might be the 1',4'-*cis*-diol of ABA. The methyl ester of catabolite 3 was unaffected by treatment with Jones' reagent (Figure 5.3C), again indicating the presence an intact keto function in the cyclohexene ring and discounting its identity as the 1',4'-*cis* diol of ABA.

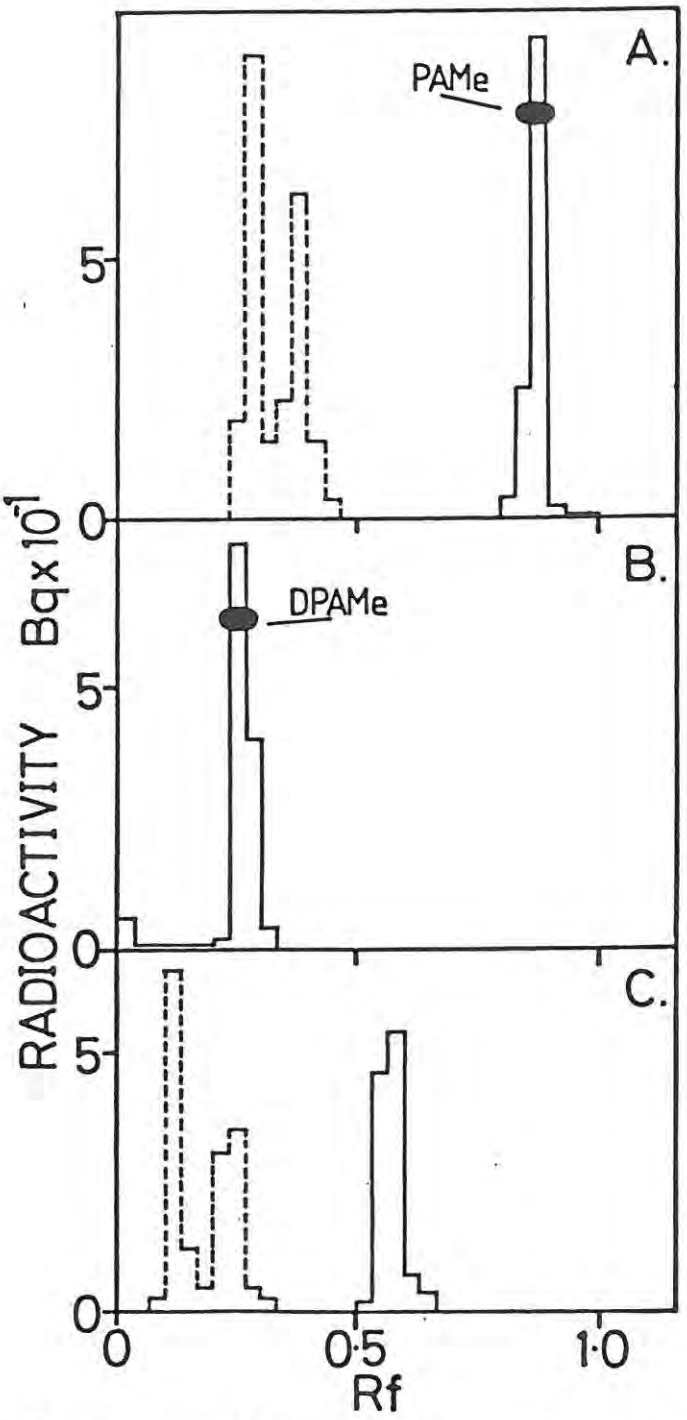

Figure 5.2. Thin layer chromatograms of the methyl esters of the acidic catabolites of (*R,S,*)-[2-^{14}C]-ABA (—) from extracts of light-grown leaves of *Hordeum vulgare* and their reduction products (...) following treatment with $NaBH_4$. (A) Catabolite 4; (B) catabolite 2; (C) catabolite 3. Samples were separated on silica gel GF_{254} in *n*-hexane/ethyl acetate (1:1, v/v) developed 3x to 15cm.

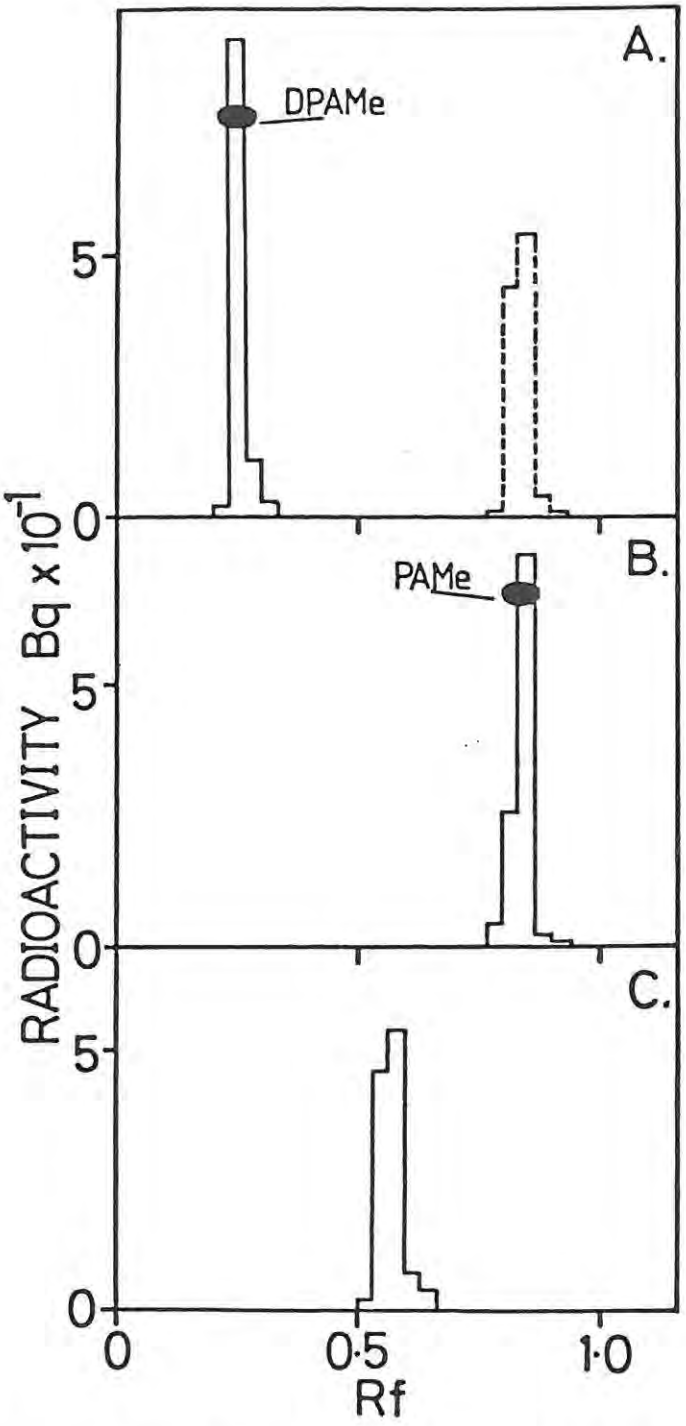

Figure 5.3. Thin layer chromatograms of the methyl esters of the acidic catabolites of (*R,S,*)-[2-^{14}C]-ABA (—) from extracts of light-grown leaves of *Hordeum vulgare* and their oxidation products (...) following treatment with Jones' reagent. (A) Catabolite 2; (B) catabolite 4; (C) catabolite 3. Samples were separated on silica gel GF_{254} in *n*-hexane/ethyl acetate (1:1, v/v) developed 3x to 15cm.

5.2.1.1.2. Radio-GLC analysis of the acidic catabolites of (*R,S,*)-ABA in leaves of *Hordeum vulgare* cv. Dyan.

The radioactive components co-chromatographing with standard ABA, PA and DPA, and catabolite 3 were analysed by radio-GLC on packed columns of either 1% XE-60 or 2% SE-30 and the data are presented in Table 5.1. The radio-peaks which co-chromatographed with authentic ABA, PA and DPA, displayed identical retention times to those of authentic (*R,S,*)-[2-^{14}C]-ABA, [^{14}C]-PA and [^{14}C]-DPA. Methylated, radioactive catabolite 3 displayed an intermediate polarity between PA and DPA when chromatographed on packed columns of either 1% XE-60 or 2% SE-30.

TABLE 5.1: Radio-GLC analysis of the acidic catabolites of (R,S)-[2-^{14}C]-ABA in leaves of Hordeum vulgare cv. Dyan.

(R,S)-[2-^{14}C]-ABA (25kBq) was fed to excised, light-grown leaves of Hordeum vulgare (10g f.w.) via the transpiration stream. Following a 30h incubation period under conditions of continuous illumination (66μmol $m^{-2}s^{-1}$) at 25°C the acidic products were extracted with ethyl acetate pH 2.5 and separated on thin layers of silica gel GF_{254}. The radioactive zones corresponding to ABA, PA and DPA and the radioactive catabolite 3 were eluted from the gel with H_2O-saturated ethyl acetate, methylated and analysed by radio-GLC as described in Chapter 2, Section 2.12.5.

Radioactive zone	Retention time on 2% SE-30 (min)*		Retention time on 1% XE-60 (min)*	
	Authentic	Putative	Authentic	Putative
[^{14}C]-ABAMe	3.8	3.8	2.2	2.2
[^{14}C]-PAMe	4.4	4.4	3.0	3.0
[^{14}C]-3Me	-	4.6	-	3.4
[^{14}C]-DPAMe	5.8	5.8	4.2	4.2

*Packed glass columns (1.8 m x 2 mm i.d.) of either 2% SE-30 or 1% XE-60 on gas-chrom Q (80 - 100 mesh) with Ar as carrier gas at a flow rate of 42 ml/min and oven temp. 180°C.

5.2.1.1.3. Characterisation of PA and DPA as catabolites of (*R,S,*)-ABA in leaves of *Hordeum vulgare* cv. Dyan by combined capillary GC-MS.

Further identification of PA and DPA as catabolites of (*R,S,*)-ABA in excised, light-grown leaves of *Hordeum vulgare* was achieved by feeding mg amounts of (*R,S,*)-ABA to this tissue and analysing the products by GLC and combined capillary GC-MS.

20 mg of (*R,S,*)-ABA to excised leaves of *Hordeum vulgare* and the ethyl acetate-soluble acids were extracted and separated on TLC as in Figure 5.1. The UV-quenching zones, which cochromatographed with authentic, radioactive PA and DPA, were eluted from the silica gel, methylated and examined by GLC using a packed column and then by capillary GC. Catabolite zone 4, which cochromatographed with PA on TLC, produced a single mass peak (Rt = 4.0min) at longer retention time than ABAMe (Figure 5.4A), when analysed as its methyl ester on a packed column of 2% SE-30.

In order to further establish the identity of catabolite zone 4 the methyl ester of this compound was irradiated with UV light and then analysed by GLC using a packed column of 2% SE-30 (see Chapter 2, section 2.17). An additional mass peak was observed at longer retention time (Rt = 5.0min) than ABAMe which displayed identical chromatographic properties to those of authentic *trans*-PAMe.

Further analysis of the methyl ester of catabolite zone 4 by capillary GC-MS yielded the single mass peak shown in Figure 5.4B which generated the electron impact (EI) mass spectrum depicted in Figure 5.5 which was identical to of PAMe (Tietz *et al*, 1979; Dörffling and Tietz, 1983). Likewise, catabolite zone 2, which co-migrated with authentic, radioactive DPA on TLC, produced a single mass peak (Rt = 5.8min), at longer retention time than PAMe, when analysed as the methyl ester on a packed column of 2% SE-30 (Figure 5.6A). Similarly, capillary GC of the methyl ester of catabolite 2 produced a single mass peak (Figure 5.6B) which generated the EI mass spectrum depicted in Figure 5.7 which was consistent with the spectrum already published for DPAMe (Tietz *et al*, 1979; Dörffling and Tietz, 1983).

Thus, as expected, PA and DPA were unequivocally characterised as catabolites of (*R,S,*)-ABA in excised, light-grown leaves of *Hordeum* vulgare.

5.2.1.1.4. The identification of catabolite 3 isolated from feeds of (*R,S,*)-ABA to excised, light-grown leaves of *Hordeum vulgare* cv. Dyan.

The chromatographic behaviour of catabolite 3 suggested that this compound might be an oxygen-containing, acidic catabolite of applied (*R,S,*)-ABA in leaves of *Hordeum vulgare*. In order to obtain further evidence to this effect, excised leaves of *Hordeum vulgare* were allowed to catabolise (*R,S,*)-[2-^{14}C]-ABA in a N_2 atmosphere, and the distribution of radioactivity in the acidic

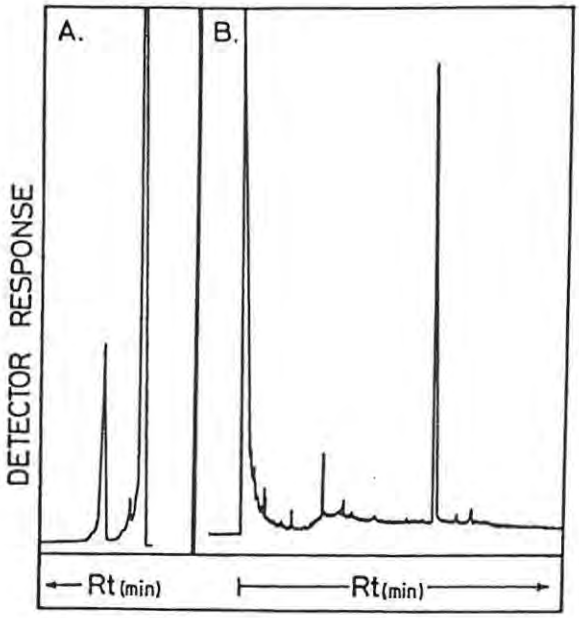

Figure 5.4. Gas chromatograms of the methyl ester of catabolite 4 from TLC separations as in Figure 5.1, from feeds of (*R,S,*)-ABA (4mg) to excised, light-grown leaves of *Hordeum vulgare* (20g f.w.). (A) GLC of esterified catabolite 4 on a packed column of 2% SE-30 and (B), capillary GLC of esterified catabolite 4 on a capillary column coated with OV-1. GLC was carried out using a silanized glass column (1.8m x 2mm i.d.) packed with 2% SE-30 on gaschrom Q (80-100 mesh) with Ar as carrier gas (43ml/min) at an oven temperature of 190°C. Capillary GC was carried out using a wall-coated open tubular (WCOT) capillary column (50m x 0.31mm fused-silica coated with OV-1) with a He flow rate of 1ml/min and temperature programming (start 180°C to 190°C at 2°C min^{-1}).

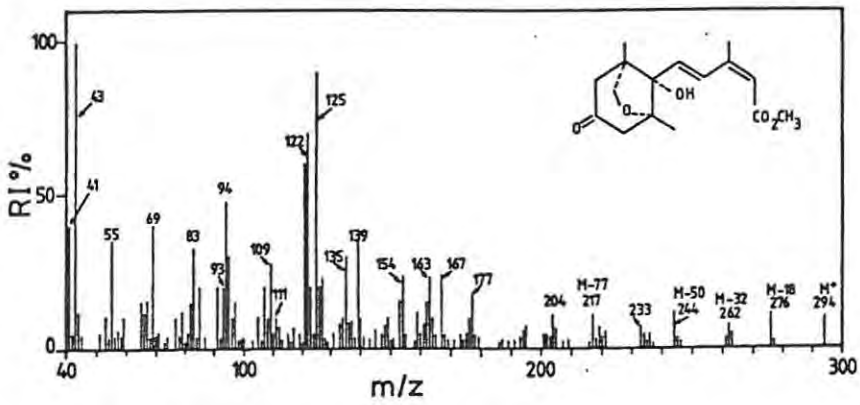

Figure 5.5. Electron impact mass spectrum of catabolite 4 isolated from feeds of (*R,S,*)-ABA to light-grown leaves of *Hordeum vulgare* and separated by capillary GLC on OV-1 as described in Figure 5.4. The mass spectra was obtained at 70eV with a source temperature of 175°C and was recorded at 11.5 sec per mass decade.

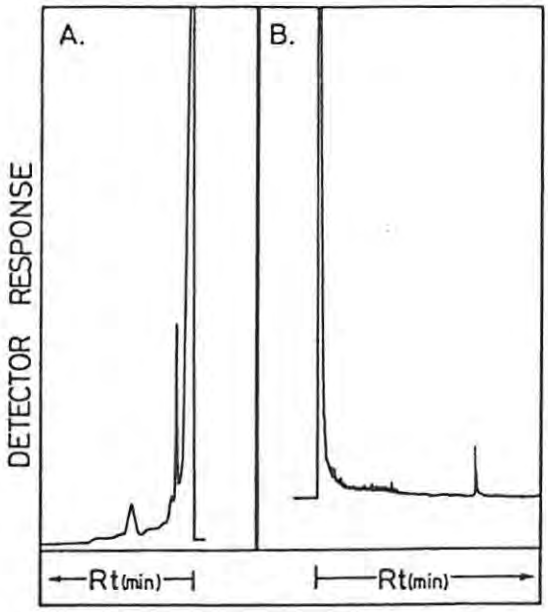

Figure 5.6. Gas chromatograms of the methyl ester of catabolite 2 from TLC separations as in Figure 5.1, from feeds of (*R,S,*)-ABA (4mg) to excised, light-grown leaves of *Hordeum vulgare* (20g f.w.). (A) GLC of esterified catabolite 2 on a packed column of 2% SE-30 and (B), capillary GLC of esterified catabolite 2 on a capillary column coated with OV-1. GLC was carried out using a silanized glass column (1.8m x 2mm i.d.) packed with 2% SE-30 on gaschrom Q (80-100 mesh) with Ar as carrier gas (43ml/min) at an oven temperature of 190°C. Capillary GC was carried out using a wall-coated open tubular (WCOT) capillary column (50m x 0.31mm fused-silica coated with OV-1) with a He flow rate of 1ml/min and temperature programming (start 180°C to 190°C at 2°C min^{-1}).

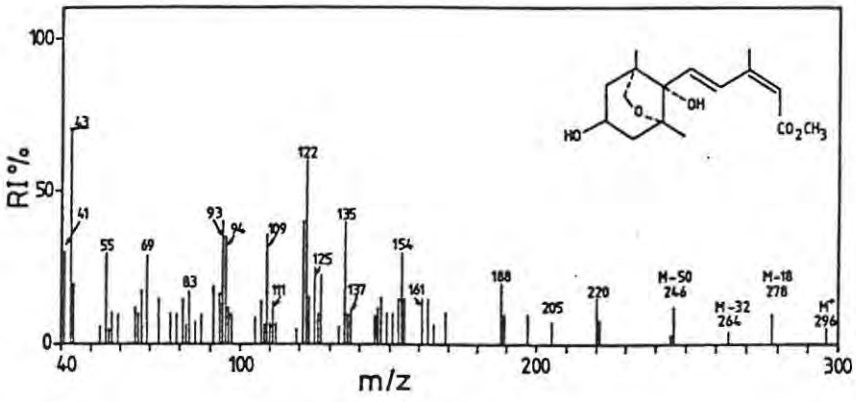

Figure 5.7. Electron impact mass spectrum of catabolite 2 isolated from feeds of (*R,S,*)-ABA to light-grown leaves of *Hordeum vulgare* and separated by capillary GLC on OV-1 as described in Figure 5.6. The mass spectrum was obtained at 70eV with a source temperature of 175°C and was recorded at 11.5 sec per mass decade.

catabolites then determined. The data presented in Table 5.2 demonstrated that the incorporation of radioactivity into catabolite 3 was significantly curtailed in the presence of N_2, suggesting that this product was an oxygen containing derivative of either ABA or PA. Thus, in order to examine further the nature of this product, several feeds of non-radioactive (*R,S,*)-ABA to leaves of *Hordeum vulgare* were carried out.

TABLE 5.2: The effect of anaerobiosis on the catabolism of (R,S)-[2-^{14}C]-ABA by excised, light-grown leaves from 10d old seedlings of Hordeum vulgare cv. Dyan.

Two grams of leaves were placed with their proximal ends in 0.4 ml K_2HPO_4/KH_2PO_4 buffer (10 mM, pH 7.5) containing 2.88kBq (R,S)-[2-^{14}C]-ABA and maintained in either an atmosphere of N_2 or air druing uptake via the transpiration stream (± 1.5h). Further buffer (1 ml) was added to each vial and catabolism allowed to proceed for 25h in air or N_2 at 22°C under continuous illumination (18μmol $m^{-2}s^{-1}$). ABA and its catabolites were extracted and analysed as described in Chapter Two.

	Radioactivity in ABA and its catabolites				
Treatment	DPA	TLC zone 3	PA	ABA	Aqueous fraction
			Bq (%)		
Air	446.4 (17.3)	401.0 (15.6)	494.8 (19.2)	699.0 (27.1)	529.3 (20.6)
N_2	69.1 (2.6)	74.7 (2.9)	343.9 (13.3)	1878.3 (72.9)	208.5 (7.9)

Following feeds of non-radioactive (*R,S,*)-ABA to excised leaves of *Hordeum vulgare* catabolite 3 was isolated, methylated and examined by both GLC, using a packed column of 2% SE-30 (Figure 5.8A) and by capillary GC (Figure 5.8C) on a capillary column coated with OV-1. A large mass peak, X (Rt = 4.9min), was observed, at longer retention time than PAMe (Figure 5.8B), when samples were separated isothermally on a packed column of 2% SE-30 (Figure 5.8A). This was further resolved by capillary GC with temperature programming (Figure 5.8C) to yield a major mass peak (X), again at longer retention time than PAMe. When peak 'X' was analysed by combined capillary GC-MS the EI mass spectrum depicted in Figure 5.9 was generated.

The mass spectrum of catabolite 3 (peak X on capillary GC, Figure 5.8C) gave a weak molecular ion at m/z 294 ($C_{16}H_{22}O_5$), suggesting that this product contained an additional oxygen atom compared with ABAMe. Clearly, the principal fragmentation pattern (see Figure 5.10) involves the cleavage of butylene (C_4H_8) from the cyclohexene ring,a (Gray *et al*, 1974), to give m/z 238(5%), and the subsequent loss of methanol and water to give strong fragment ions at m/z 206(72%) and m/z

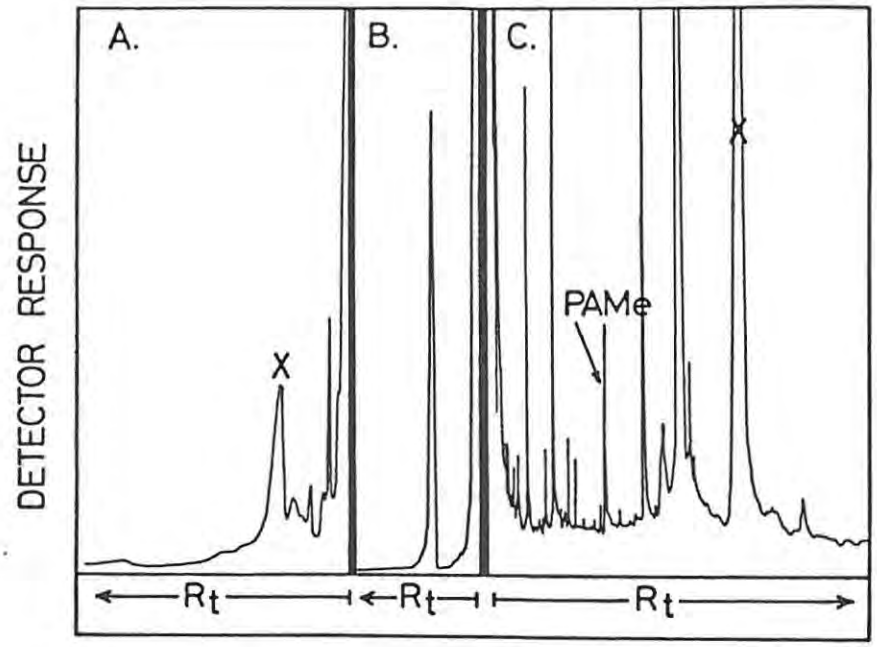

Figure 5.8. Gas chromatograms of esterified catabolite 3 from TLC separation (as in Figure 5.1) of the ethyl acetate-soluble acids following a high dose feed of (*R,S,*)-ABA (20mg) to excised, light-grown leaves (50g f.w.) of *Hordeum vulgare* and of standard PAMe. (A) catabolite 3 chromatographed on a packed column of 2% SE-30; (B) standard PAMe chromatographed on a packed column of 2% SE-30; (C) catabolite 3 and PAMe chromatographed on a capillary column of OV-1. GLC was carried out using a silanized glass column (1.8m x 2mm i.d.) packed with 2% SE-30 on gaschrom Q (80-100 mesh) with Ar as carrier gas (43ml/min) at an oven temperature of 190°C. Capillary GC was carried out using a wall-coated open tubular (WCOT) capillary column (50m x 0.31mm fused-silica coated with OV-1) with a He flow rate of 1ml/min and temperature programming (start 180°C to 190°C at 2°C min^{-1}).

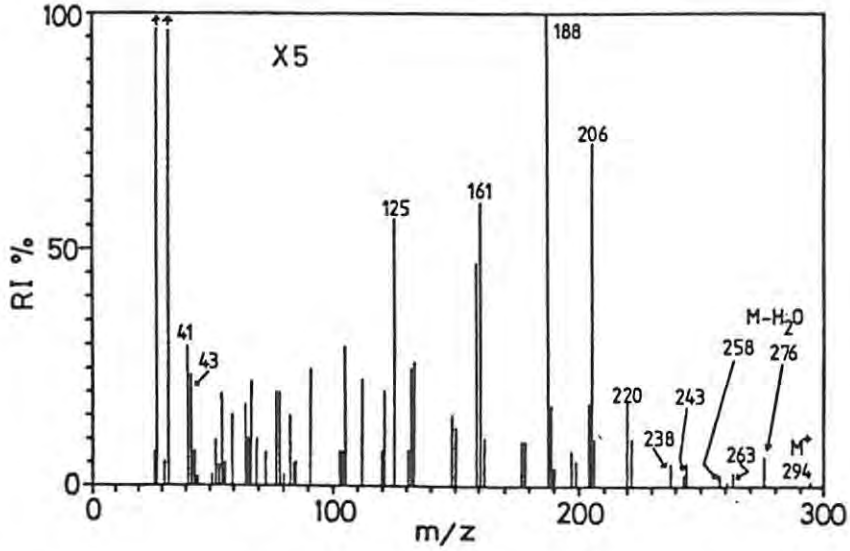

Figure 5.9. Electron impact mass spectrum of peak X (Figure 5.8), separated from catabolite 3 by capillary GC on OV-1. The mass spectrum was obtained at 70eV with a source temperature of 175°C and was recorded at 11.5 sec per mass decade.

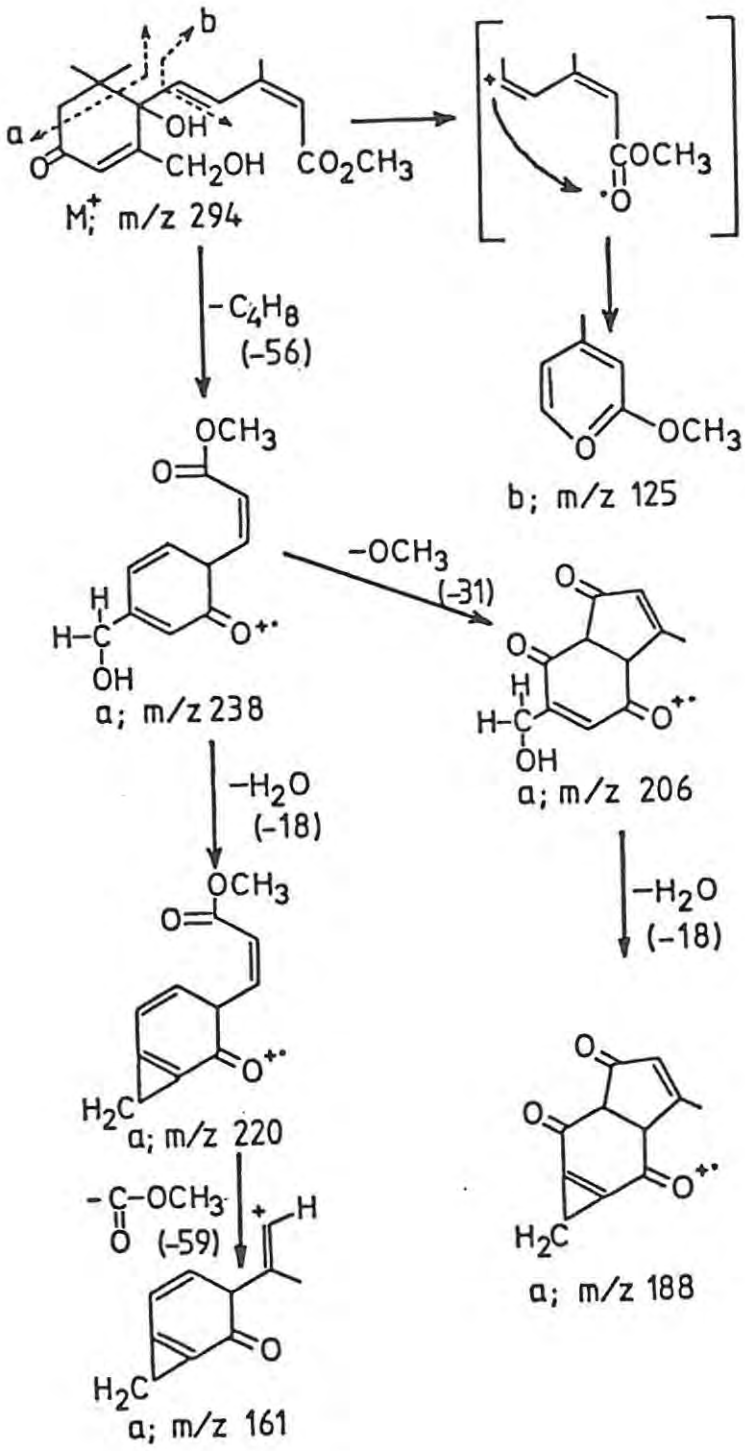

Figure 5.10. Proposed fragmentation pattern of catabolite 3 (component X, Figure 5.8) and its identification as 2'-hydroxymethyl ABA.

188(100%). Both of these fragment ions contain an additional oxygen atom when compared to the corresponding fragments in ABAMe (see Chapter 2, section 2.14), indicating that the 6'-gem-dimethyl groups in catabolite 3 were not modified. Likewise, a strong fragment ion at m/z 125(b; 57%) suggests that the side chain of catabolite 3 was unchanged from that in ABAMe, thus eliminating modification of the 6-methyl group.

The loss of H_2O from m/z 238 to give a fragment ion at m/z 220(18%) and the subsequent loss of acetic acid to give m/z 161(60%), suggests the initial loss of oxygen, in the form of water, from the C-2' position in m/z 238 to give m/z 220. Therefore, catabolite 3 was identical to the ABA catabolite which was isolated from cell suspension cultures of *Nigella damascena* and identified as 2'-hydroxymethyl ABA (7'-hydroxy ABA)[3] (Lehmann *et al*, 1983). Its structure is shown in Figure 5.11. The characterisation of 2'-HMABA as a catabolite of (*R,S,*)-ABA in leaves of *Hordeum vulgare*, has already been reported briefly (Railton and Cowan, 1985a; 1985b).

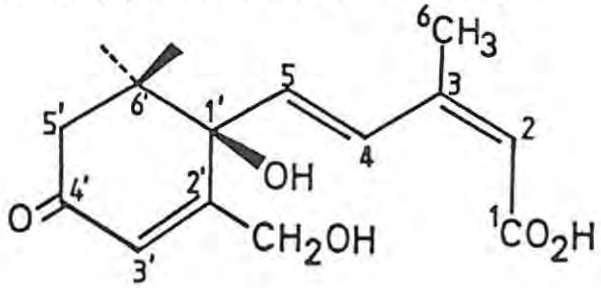

Figure 5.11. Structure of 2'-hydroxymethyl ABA (7'-hydroxy ABA), a major catabolite of (*R,S,*)-ABA catabolism in light-grown leaves of *Hordeum vulgare*.

5.2.1.1.5. Determination of the chirality of 7'-hydroxy ABA.

Since substrate ABA comprised a mixture of both the (*R*)- and (*S*)- enantiomers, the derivation of 7'-hydroxy ABA was unknown. Thus, the chirality of 7'-hydroxy ABA was sought by circular dichroism (CD) analysis.

Determination of the chirality of 7'-hydroxy ABA, as its methyl ester, by CD analysis, gave the result depicted in Figure 5.12. Clearly, 7'-hydroxy ABA displays a marked negative Cotton effect with

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[3]2'-HMABA is renamed 7'-hydroxy ABA in accordance with the newly proposed nomenclature for ABA and its catabolites, which includes the exocyclic methyl groups (Boyer *et al*, 1986). This nomenclature will be used for the remainder of this thesis.

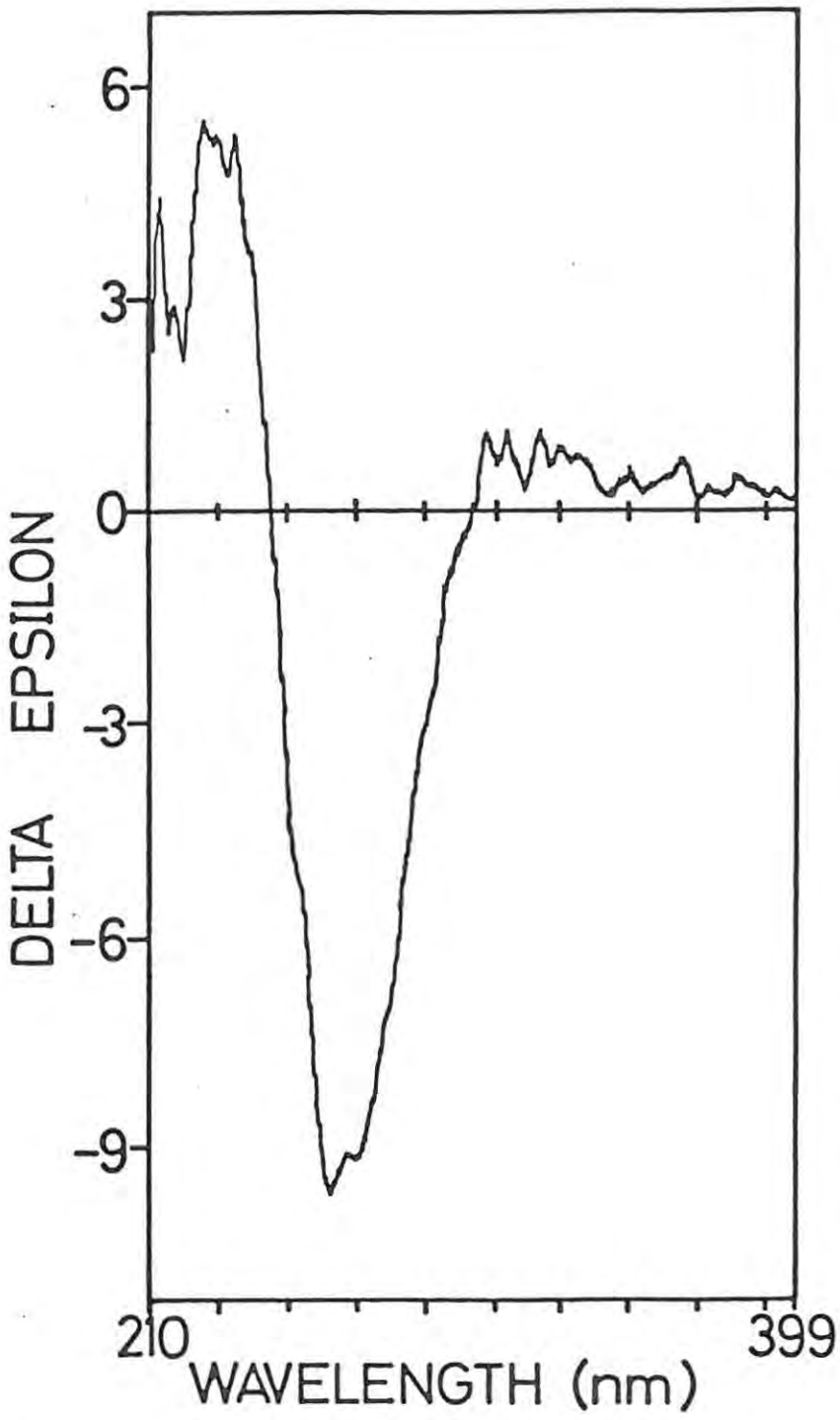

Figure 5.12. Circular dichroism spectrum of 7'-hydroxy ABA measured in methanol. 7'-hydroxy ABA, generated from (*R,S,*)-ABA by excised, light-grown leaves of *Hordeum vulgare* was extracted and purified as described in Chapter 2 and methylated with ethereal diazomethane.

extrema at 224nm ($\Delta\varepsilon$ + 5.63) and 264nm ($\Delta\varepsilon$-9.75). This result suggested that 7'-hydroxy ABA (Figure 5.11) arose predominantly from the catabolism of the unnatural (*R*)- enantiomer of (*R,S,*)-ABA rather than the (*S*)- enantiomer, although the presence of the natural enantiomer cannot be discounted until the relevant feeding studies with (*R*)- and (*S*)-ABA have been carried out.

5.2.1.1.6. Analysis of catabolite 1 and the aqueous phases from (*R,S,*)-ABA feeds to leaves of *Hordeum vulgare* cv. Dyan.

In order to obtain information about the identity of catabolite zone 1, sufficient amounts of this radioactive component were generated by feeding (*R,S,*)-[2-^{14}C]-ABA (42kBq) to excised, light-grown leaves of *Hordeum vulgare*. Following chromatography and elution of this component from the silica gel, catabolite zone 1 was analysed by hydrolysing this fraction with base, as described in Chapter 2, section 2.11.2.3, since in other studies (Boyer and Zeevaart, 1982; Railton and Symon, 1983) similar polar catabolites of ABA were found to be conjugates which could be partially extracted from aqueous phases of ethyl acetate. The results shown in Figure 5.13A and B confirm this suggestion and indicate that ABA and some PA can exist as either glucosylated ether or ester catabolites of (*R,S,*)-ABA in light-grown leaves of *Hordeum vulgare*.

Similar treatment of aqueous phases remaining after partitioning against ethyl acetate, produced the results shown in Figure 5.14A and B. These data show that ABA, PA, 7'-hydroxy ABA and DPA can be transformed into water-soluble, base-labile conjugates by excised leaves of *Hordeum vulgare*, although the detailed chemical nature of these compounds is currently unknown.

Interestingly, base hydrolysis of the aqueous fraction from feeds of (*R,S,*)-[2-^{14}C]-ABA to *Hordeum vulgare* leaves resulted in the release of large amounts of a compound which cochromatographed with *trans*-ABA but which was absent from similar hydrolyses of catabolite 1 (Figure 5.13 B).

5.2.1.1.7. Attempts to distinguish the catabolites of applied (*R,S,*)-ABA from the same endogenous compounds in leaves of *Hordeum vulgare* prior to MS analysis.

In the present study on the catabolism of (*R,S,*)-ABA in leaves of *Hordeum vulgare*, PA, DPA and 7'-hydroxy ABA were unequivocally identified by combined capillary GC-MS as catabolites of applied ABA. However, a problem associated with such studies, and particularly following high-dose feeds of non-radioactive substrate to tissues, is the ability to distinguish the hormone

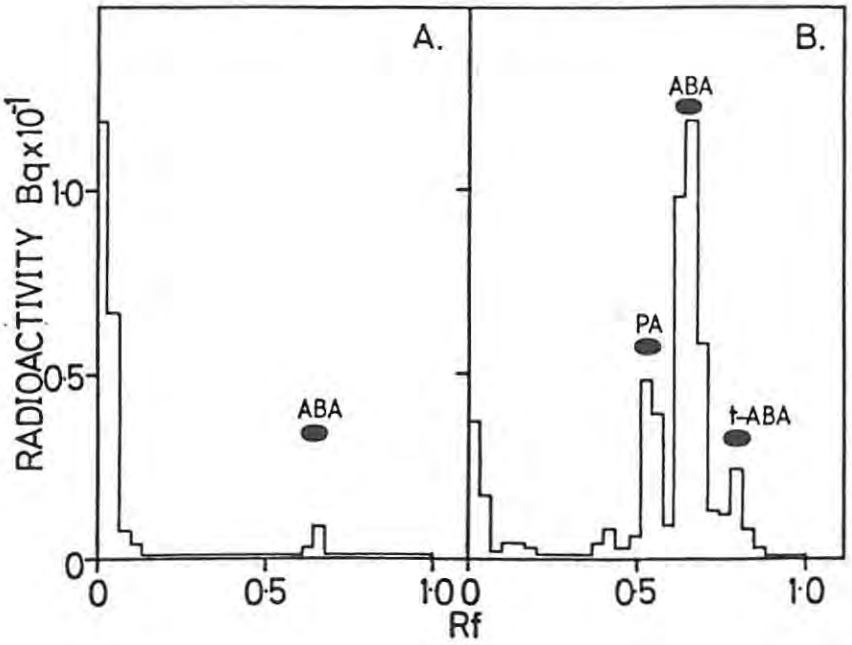

Figure 5.13. Hydrolysis of catabolite 1 (as in Figure 5.1) in the ethyl acetate-soluble acids from excised, light-grown leaves of *Hordeum vulgare* fed with (*R,S,*)-[2-^{14}C]-ABA. (A) Control held at pH7.0 at 60°C for 1h; (B) treated with base at pH11.0 at 60°C for 1h. Samples were processed as described in Chapter 2 and chromatographed on thin layers of silica gel GF_{254} in toluene/ethyl acetate/acetic acid (50:30:4, v/v) developed 2x to 15cm.

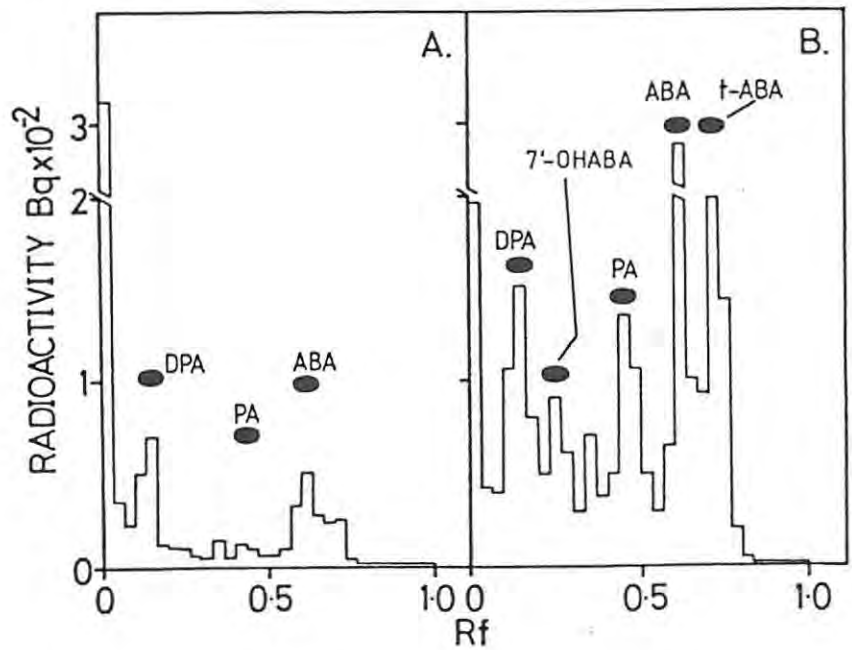

Figure 5.14. Hydrolysis of the aqueous fraction from an extract of excised, light-grown leaves of *Hordeum vulgare* fed with (*R,S,*)-[2-^{14}C]-ABA. (A) Control held at pH7.0 at 60°C for 1h; (B) treated with base at pH11.0 at 60°C for 1h. Samples were processed as described in Chapter 2 and chromatographed on thin layers of silica gel GF_{254} in toluene/ethyl acetate/acetic acid (50:30:4, v/v) developed 2x to 15cm.

catabolites from the same endogenous compounds.

This aspect has been evident in studies on the metabolism of gibberellins in immature seeds of *Pisum sativum* (Sponsel and MacMillan, 1977) and in studies on ABA biosynthesis in the ABA-producing fungus, *Cercospora rosicola* (Neill *et al*, 1982b), where high levels of these endogenous compounds are present. In part, this problem has been overcome by using deuterated substrates. Such compounds generate deuterium-labelled products which are readily discernable from the same endogenous compounds following mass spectral analysis.

In most plants and plant tissues the levels of the endogenous ABA and its catabolites are low and in some cases undetectable (Milborrow, 1978b). Given this proviso, it becomes apparent that the use of deuterated substrates is unnecessary to confirm their identification by MS analysis. This suggestion has been suitably demonstrated in the present study for the characterisation of PA (Figure 5.15).

The PA fraction isolated from *Hordeum vulgare* leaves fed with or without (*R,S,*)-ABA was analysed by GLC on a packed column of 2% SE-30. Clearly, tissue not fed with (*R,S,*)-ABA contained no mass peak in the PA region of the chromatogram (Figure 5.15A) while that fed with (*R,S,*)-ABA readily produced a mass peak (Rt = 4.0min) corresponding to authentic PAMe (Figure 5.15B). Thus, the identification of the catabolites of applied (*R,S,*)-ABA in leaves of *Hordeum vulgare*, by combined capillary GC-MS, was based exclusively on compounds produced from applied (*R,S,*)-ABA.

5.2.1.1.8. Kinetics of (*R,S,*)-ABA catabolism and refeeding studies in excised, light-grown leaves of *Hordeum vulgare* cv. Dyan.

Very few studies (Zeevaart and Milborrow,1976; Railton and Symon, 1983) have been undertaken to verify the metabolic interrelationship between ABA and its catabolites. In order to confirm the metabolic interrelationship between the catabolites of (*R,S,*)-ABA in excised, light-grown leaves of *Hordeum vulgare*, both kinetic analyses and refeeding studies were carried out. The results depicted in Figure 5.16 show that with a decline in the amounts of substrate ABA, PA was produced rapidly and reached its maximum at approximately 3h. The slow decline in PA was followed by a concomitant increase in the amounts of DPA. Incorporation of label into 7'-hydroxy ABA increased rapidly up to 5h but then increased less rapidly after that time. In addition, levels of radioactivity associated with catabolite zone 1 and the incorporation of label into the aqueous phases,

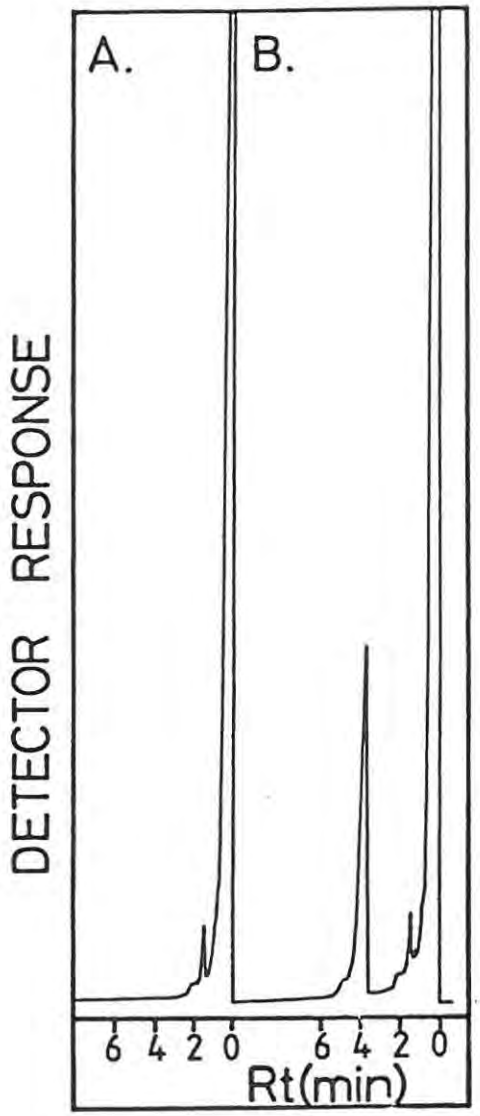

Figure 5.15. Gas chromatograms of the methylated PA zone from a Sep-pak C_{18} purified, ethyl acetate-soluble acid fraction from extracts of excised, light-grown leaves (20g f.w.) of *Hordeum vulgare* fed, (A) without, or (B) with 4mg (*R,S,*)-ABA. Samples ($1/200^{th}$) were separated on a packed column of 2% SE-30 and detected with a flame ionization detector. The large mass peak (Rt = 4.0min) in (B) was characterized by GC-MS as PAMe.

increased steadily during the first 20h of catabolism and more slowly or not at all thereafter.

Confirmation of the metabolic interrelationship between ABA and its acidic catabolites was achieved by refeeding PA, 7'-hydroxy ABA and DPA, generated biosynthetically in light-grown leaves of *Hordeum vulgare*, to excised leaves of this tissue. When [^{14}C]-PA was refed to excised leaves of *Hordeum vulgare* (Figure 5.17A), it was converted predominantly into DPA. In addition, it was also converted in lower yields to a substance, 'M', of intermediate polarity between PA and DPA which appeared similar to a catabolite of PA detected in leaves of *Spinacia oleracea* (Railton and Symon, 1983).

Although catabolite 'M' displayed similar chromatographic properties to those of 7'-hydroxy ABA in this solvent system, its yield from [^{14}C]-PA was usually very low and thus did not interfere with estimates of 7'-hydroxy ABA derived from (*R,S,*)-ABA. When 7'-hydroxy ABA was refed to leaves of *Hordeum vulgare* it generated further, more polar acidic compounds of unknown identity (Figure 5.17B). Leaves of *Hordeum vulgare* were unable to transform label from [^{14}C]-DPA into further acidic products (Figure 5.17C). Furthermore, no evidence was obtained to suggest the catabolism of DPA (Figure 5.17D) even after separation of the ethyl acetate soluble acids by TLC in chloroform/methanol/acetic acid/water (45:15:3:2, v/v) which was used to resolve DPA from its acidic catabolites in *Hordeum vulgare* aleurone layers (Dashek *et al*, 1979).

5.2.1.1.9. The catabolism of (*R,S,*)-[2-^{14}C]-ABAMe in light-grown leaves of *Hordeum vulgare* cv. Dyan.

In order to determine whether light-grown leaves of *Hordeum vulgare* could catabolise ABAMe, (*R,S,*)-[2-^{14}C]-ABAMe was fed to leaves of this species and catabolism allowed to proceed for 30h. The distribution of radioactivity in both the ethyl acetate and the diethyl ether fractions was then determined. The result depicted in Figure 5.18 shows that light-grown leaves of *Hordeum vulgare* efficiently hydrolysed ABAMe to its free acid, which was subsequently catabolised to PA, 7'-hydroxy ABA, DPA and water-soluble conjugates.

ABSCISIC ACID CATABOLISM IN VEGETATIVE DICOTYLEDONS.

5.2.1.2. Studies on the catabolism of (*R,S,*)-ABA in excised seedlings of *Pisum sativum* L. cv Black-eyed Susan and *Phaseolus vulgaris* L. cv Top-crop.

Although the catabolism of applied (*R,S,*)-ABA has already been investigated in detail in light-

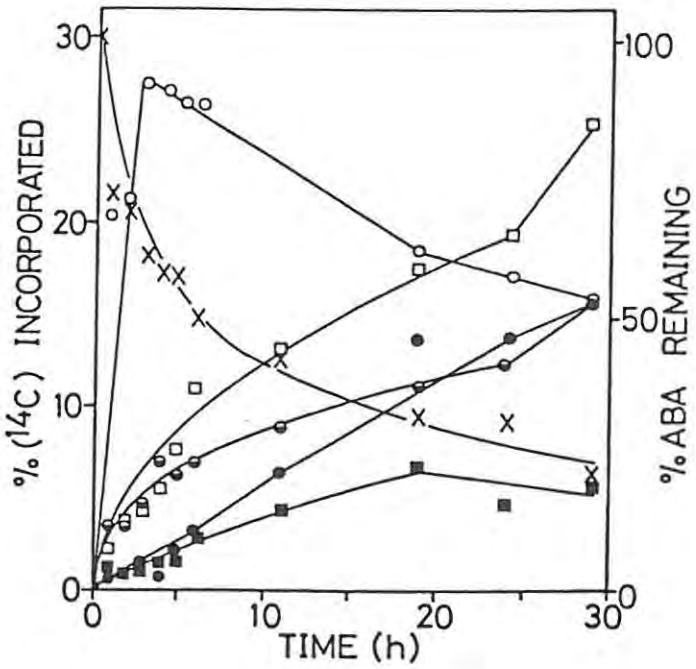

Figure 5.16. Kinetics of (*R,S,*)-[2-^{14}C]-ABA catabolism in excised, light-grown leaves of *Hordeum vulgare*. DPA (●), PA (o), 7'-hydroxy ABA (◒), ABA substrate (X), catabolite 1 (●) and aqueous fraction (◻).

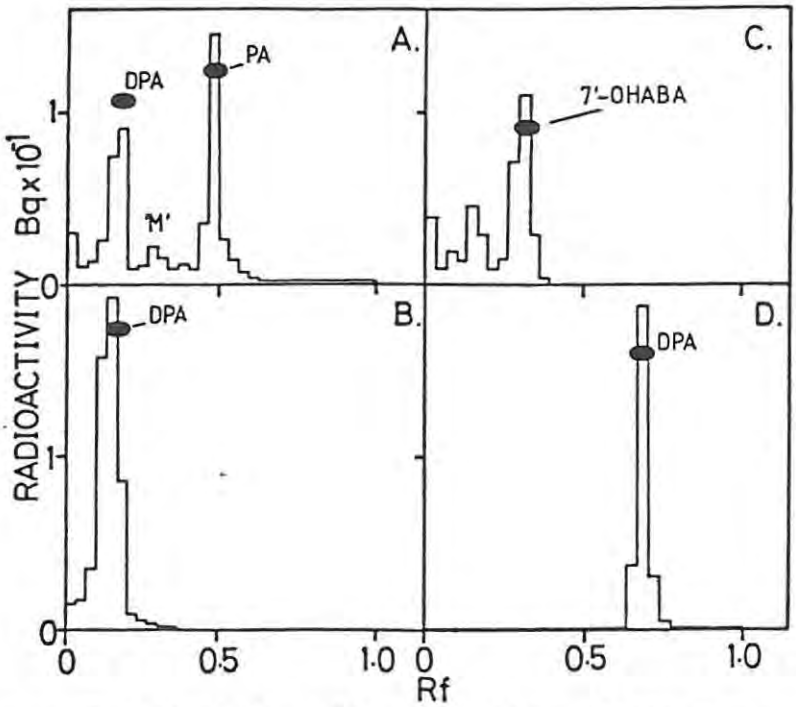

Figure 5.17. Thin layer chromatograms of refeeds of PA, DPA and 7'-hydroxy ABA to excised, light-grown leaves of *Hordeum vulgare*. [^{14}C]-PA (A); [^{14}C]-DPA (B) and [^{14}C]-7'-hydroxy ABA (C), all 500Bq, were fed *via* the transpiration stream to excised leaves (1.0g f.w.) and catabolism allowed to proceed for 30h. The ethyl acetate-soluble acids were separated on silica gel GF_{254} in toluene/ethyl acetate/acetic acid (50:30:4, v/v) developed 2x to 15cm. (D) Ethyl acetate-soluble acids from refeeds of [^{14}C]-DPA (500Bq) to *Hordeum vulgare* leaves separated on thin layers of silica gel GF_{254} in chloroform/methanol/acetic acid/water (45:15:3:2, v/v) developed once to 15cm.

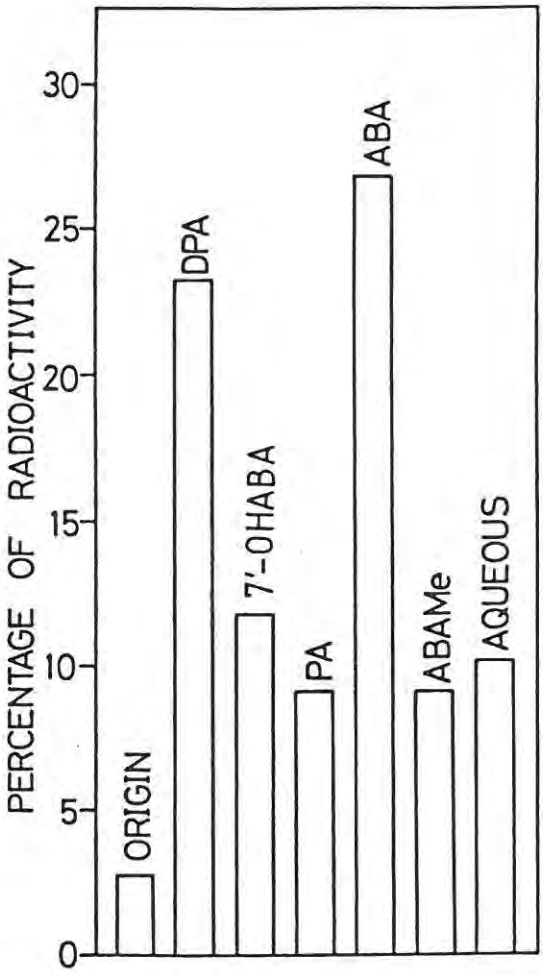

Figure 5.18. Percentage radioactivity in (*R,S,*)-ABAMe and its catabolites in excised, light-grown leaves of *Hordeum vulgare*. (*R,S,*)-[2-^{14}C]-ABAMe (5.8kBq) was supplied to excised leaves (1g f.w.) *via* the transpiration stream and catabolism allowed to proceed for 30h at 25°C under continuous illumination (66μmol m^{-2} s^{-1}). Tissues were extracted and analysed as described in Chapter 2.

grown seedlings of *Pisum sativum* cv. Kleine Rheinlanderin (Tietz *et al*, 1979) and *Phaseolus vulgaris* cv. The Prince (Zeevaart and Milborrow, 1976), including the unequivocal identification of the major catabolites as PA and DPA, the use of other cultivars in the present work, together with the different physiological state of the plants, prompted a re-investigation of some aspects of ABA catabolism in these species. This was deemed necessary in order to assist later studies (Chapter 6) on the effect of environmental factors and applied chemicals on ABA catabolism in these plants.

5.2.1.2.1. Characterisation of (*R,S,*)-ABA catabolites in seedlings of *Pisum sativum* cv. Black-eyed Susan and *Phaseolus vulgaris* cv. Top-crop.

When (*R,S,*)-[2-^{14}C]-ABA was fed to excised, light-grown seedlings of both *Pisum sativum* and *Phaseolus vulgaris* the distribution of radioactivity depicted in Figure 5.19A and B was observed. Radioactivity from (*R,S,*)-ABA was transformed into a range of products, some of which displayed identical chromatographic properties to those of authentic PA, DPA and the 1',4'-*cis* and 1',4'-*trans* diols of ABA.

In addition to the aforementioned products, a further radioactive catabolite of (*R,S,*)-ABA, catabolite 3 (Rf0.3-0.33) was observed which had not been detected previously in ABA catabolic studies using these species. In these studies, levels of the putative 1',4'-*cis* and 1',4'-*trans* diols never exceeded 3% of the total radioactivity incorporated into the acidic catabolites of (*R,S,*)-ABA in *Pisum sativum* seedlings. This was in contrast to data obtained in wilted and then rehydrated seedlings (Tietz *et al*, 1979; Milborrow, 1983) and suggested that these products are minor catabolites of ABA in turgid tissue. Catabolite 3, generated from (*R,S,*)-[2-^{14}C]-ABA feeds to seedlings of both *Pisum sativum* (Figure 5.19A) and *Phaseolus vulgaris* (Figure 5.19B) was absent in similar feeds when non-radioactive ABA was used as substrate.

PA and DPA were tentatively identified as catabolites of (*R,S,*)-ABA by cocochromatography and microchemical derivatisation (as described in Chapter 2). Further identification of PA and DPA as products of (*R,S,*)-ABA catabolism was achieved by radio-GLC using a packed column of 2%SE-30 and the data are presented in Table 5.3. The unequivocal identity of PA and DPA, as catabolites of applied (*R,S,*)-ABA in seedlings of *Pisum sativum* and *Phaseolus vulgaris* was established by GC-MS (as described in Chapter 2). The mass spectra generated were identical to those previously published for a similar investigation using *Pisum sativum* (Tietz *et al*, 1979) and *Phaseolus vulgaris* (Zeevaart and Milborrow, 1976) seedlings.

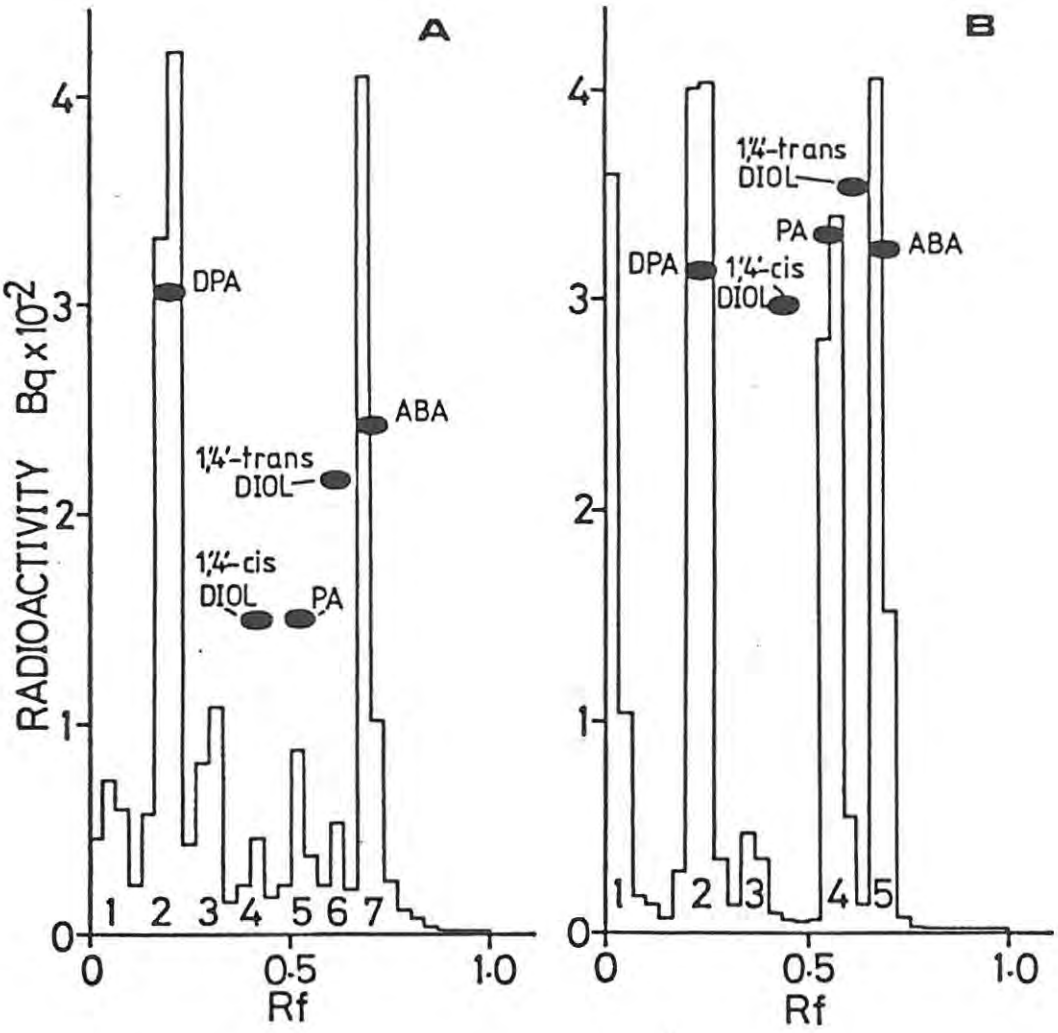

Figure 5.19. Separation by TLC of (*R,S,*)-[2-^{14}C]-ABA and its catabolites from extracts of light-grown (A) *Pisum sativum* cv. Black-eyed Susan and (B) *Phaseolus vulgaris* cv. Top-crop seedlings. Excised, intact seedlings (5g fresh weight) were fed with (*R,S,*)-[2-^{14}C]-ABA (4.2kBq) for 30h and the ethyl acetate-soluble acids separated by TLC on silica gel GF_{254} with toluene/ethyl acetate/acetic acid (50:30:4, v/v) developed 2x to 15cm.

TABLE 5.3: Radio-GLC analysis of the acidic catabolites of (R,S)-[2-^{14}C]-ABA in seedlings of Pisum sativum cv. Black-eyed Susan and Phaseolus vulgaris cv. Top-crop.

(R,S)-[2-^{14}C]-ABA (25kBq) was fed to excised, light-grown seedlings of both Pisum sativum and Phaseolus vulgaris (10g f.w.) via the transpiration stream. Following a 30h incubation period under continuous illumination (66μmol $m^{-2}s^{-1}$) at 25°C, the acidic products were extracted with ethyl acetate at pH 2.5 and separated on thin layers of silica gel GF_{254}. The radioactive zone corresponding to ABA, PA and DPA were eluted from the gel with H_2O-saturated ethyl acetate, methylated and analysed by radio-GLC as described in Chapter 2, Section 2.12.5.

Radioactive component	Authentic	Putative
	Retention time on 2% SE-30 (min)*	
[^{14}C]-ABAMe	3.6	3.6
[^{14}C]-PAMe	4.4	4.4
[^{14}C]-DPAMe	5.4	5.4

*Packed glass column (1.8m x 2 mm i.d.) of 2% SE-30 on gas-chrom Q (80-100 mesh) with Ar carrier gas at a flow rate of 42 ml/min and oven temp. 180°C.

Pisumic acid (PISA) was recently characterised as a product of ABA in wilted and rehydrated seedlings of *Pisum sativum* (Tietz, 1985). This product reportedly co-migrates with DPA as the free acid on TLC in the solvent system; toluene/ethyl acetate/acetic acid (50:30:4, v/v). However, in the present study, no such catabolite was detected even after rechromatography of DPA as its methyl ester on silica gel in *n*-hexane/ethyl acetate (1:1, v/v) developed 3x to 15cm (data not shown), conditions under which PISAMe and DPAMe separate.

Zeevaart and Milborrow (1976) demonstrated that PA was also transformed into 4'-*epi*-DPA in seedlings of *Phaseolus vulgaris* and it was therefore of interest to see whether the cultivar Top-crop also produced this compound. Since DPA and *epi*-DPA cochromatograph on TLC, attempts were made to resolve any *epi*-DPA from DPA by chromatographing the methyl ester of this radioactive component.

[^{14}C]-DPA, isolated from feeds of (*R,S,*)-[2-^{14}C]-ABA to *Phaseolus vulgaris* seedlings, was methylated and analysed by TLC in two different solvent systems. Five developments of [^{14}C]-DPAMe on thin layers of silica gel in ethyl acetate/*n*-hexane (2:1, v/v) to 15cm (Zeevaart and

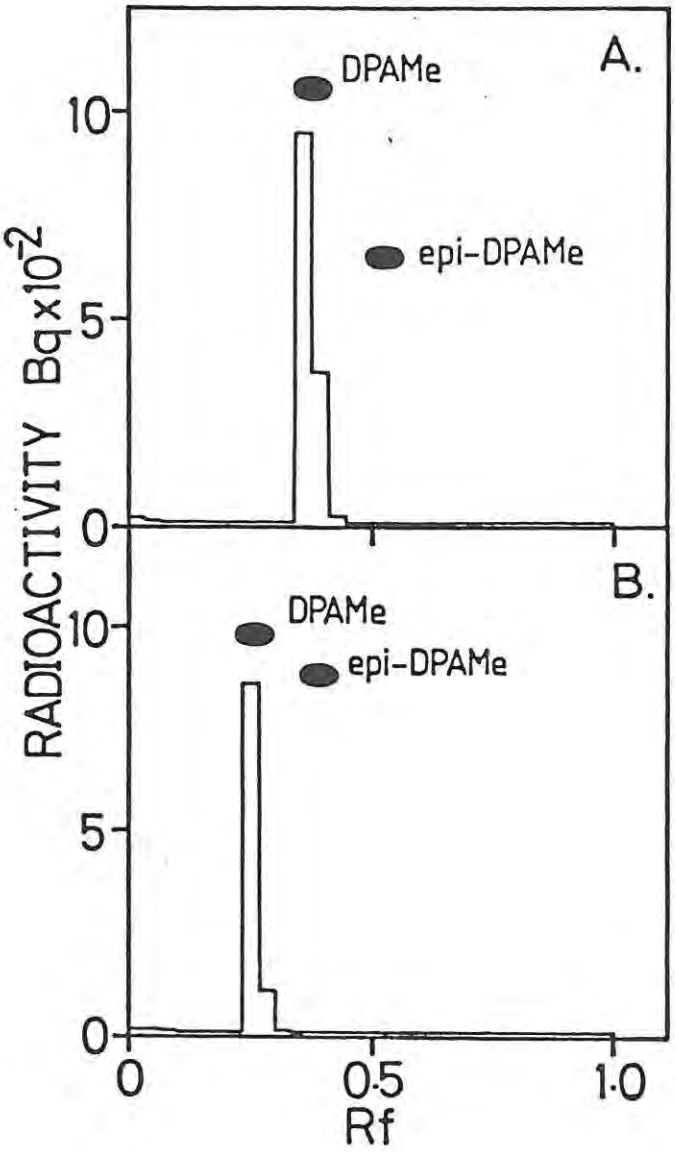

Figure 5.20. Thin layer chromatograms of methylated [^{14}C]-DPA produced in seedlings of *Phaseolus vulgaris* fed with (*R,S*)-[2-^{14}C]-ABA. Samples were chromatographed on silica gel GF_{254} in (A) ethyl acetate/*n*-hexane (1:1, v/v) developed 5x to 15cm and (B) *n*-hexane/ethyl acetate (1:1, v/v) developed 3x to 15cm.

Milborrow, 1976) and three developments of [^{14}C]-DPAMe on thin layers in *n*-hexane/ethyl acetate (1:1, v/v) gave the results depicted in Figure 5.20A and B respectively. The data show that 4'-*epi*-DPA was not present as a component in the labelled DPA fraction isolated from extracts of *Phaseolus vulgaris* seedlings in these experiments.

Analysis of catabolite zone 1 from seedlings of both *Pisum sativum* and *Phaseolus vulgaris* demonstrated that this polar catabolite comprised conjugates that could be partially extracted from the aqueous phase by ethyl acetate (Figure 5.21 and Figure 5.22). Similar treatment of the aqueous phases revealed that ABA and some of its acidic catabolites were transformed into water-soluble, base-labile conjugates in these species (Figure 5.23 and Figure 5.24). Furthermore, the data suggest that applied (*R,S,*)-ABA is transformed into its *trans* isomer, confirming an earlier findig (Milborrow, 1970).

5.2.1.2.2. Kinetics of (*R,S,*)-ABA catabolism and refeeding studies in seedlings of *Pisum sativum* cv. Black-eyed Susan.

In the studies conducted by Tietz *et al* (1979) and Milborrow (1983a) on ABA catabolism in *Pisum sativum* seedlings, no information on the catabolism PA and DPA was presented. Thus, to confirm the metabolic interelationship between the products of (*R,S,*)-ABA catabolism in turgid, light-grown *Pisum sativum* seedlings, both kinetic analyses and refeeding studies were carried out. The results in Figure 5.25 show that DPA was produced rapidly, reaching its maximum between 12 to 24h, while PA was produced more slowly, reaching its maximum by 24h. After 24h, DPA declined more rapidly than PA. Thus, this result implied that some of the DPA produced could have arisen *via* an alternative pathway (Walton *et al*, 1973) as suggested by Tietz (1985) rather than by reduction of PA, since levels of the putative 1',4'-*trans* diol of ABA (catabolite zone 6) increased rapidly within the first 12h and declined rapidly thereafter. However, a more reasonable explanation might be that any PA generated is rapidly reduced to DPA, thus resulting in the low levels of PA accumulating in this tissue. This suggestion has, in part, been substantiated in refeeding studies, since when [^{14}C]-PA is fed to excised, light-grown turgid seedlings of *Pisum sativum*, only 5.68% of the radiolabelled substrate is present following a 30h incubation (see Figure 5.26A).

Levels of the putative 1',4'-*cis* diol of ABA (catabolite zone 4) increased slowly while levels of catabolite zone 3 increased gradually over the 150h incubation period.

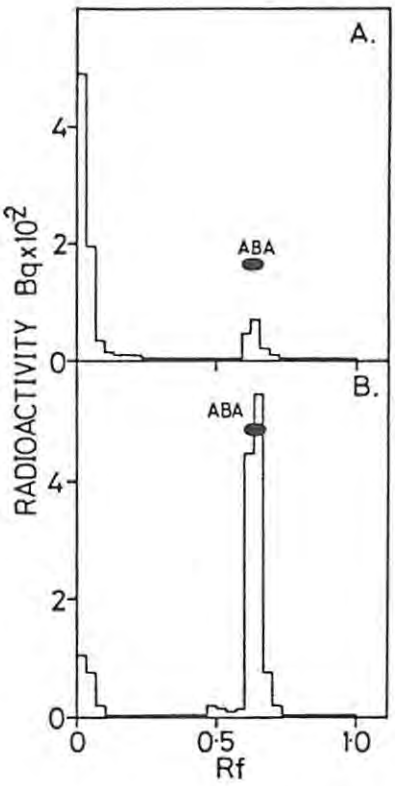

Figure 5.21. Hydrolysis of catabolite 1 (as in Figure 5.19A) in the ethyl acetate-soluble acids from excised, light-grown seedlings of *Pisum sativum* fed with (*R,S,*)-[2-^{14}C]-ABA. (A) Control held at pH 7.0 at 60°C for 1h; (B) treated with base at pH 11.0 at 60°C for 1h. Samples were processed as described in Chapter 2 and chromatographed on thin layers of silica gel GF_{254} in toluene/ethyl acetate/acetic acid (50:30:4, v/v) developed 2x to 15cm.

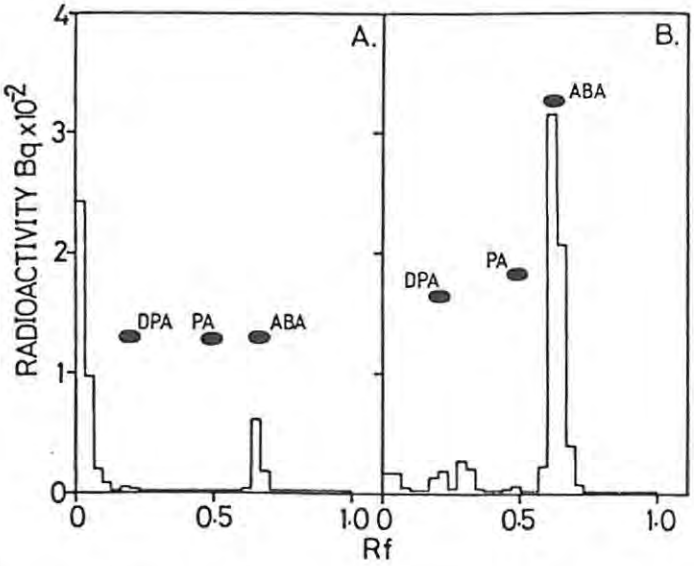

Figure 5.22. Hydrolysis of catabolite 1 (as in Figure 5.19B) in the ethyl acetate-soluble acids from excised, light-grown seedlings of *Phaseolus vulgaris* fed with (*R,S,*)-[2-^{14}C]-ABA. (A) Control held at pH7.0 at 60°C for 1h; (B) treated with base at pH11.0 at 60°C for 1h. Samples were processed as described in Chapter 2 and chromatographed on thin layers of silica gel GF_{254} in toluene/ethyl acetate/acetic acid (50:30:4, v/v) developed 2x to 15cm.

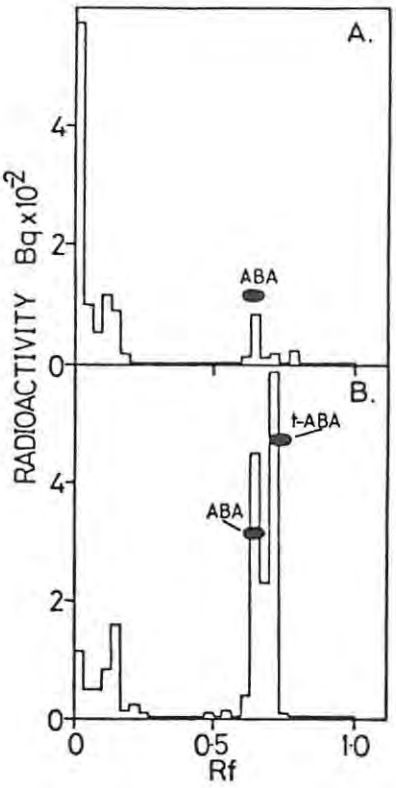

Figure 5.23. Hydrolysis of the aqueous fraction from an extract of excised, light-grown seedlings of *Pisum sativum* fed with (*R,S,*)-[2-^{14}C]-ABA. (A) Control held at pH7.0 at 60°C for 1h; (B) treated with base at pH11.0 at 60°C for 1h. Samples were processed as described in Chapter 2 and chromatographed on thin layers of silica gel GF_{254} in toluene/ethyl acetate/acetic acid (50:30:4, v/v) developed 2x to 15cm.

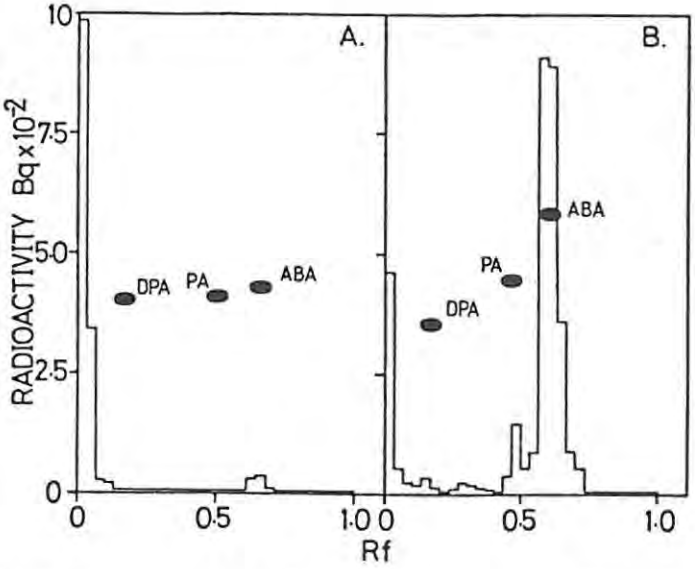

Figure 5.24. Hydrolysis of the aqueous fraction from an extract of excised, light-grown seedlings of *Phaseolus vulgaris* fed with (*R,S,*)-[2-^{14}C]-ABA. (A) Control held at pH7.0 at 60°C for 1h; (B) treated with base at pH11.0 at 60°C for 1h. Samples were processed as described in Chapter 2 and chromatographed on thin layers of silica gel GF_{254} in toluene/ethyl acetate/acetic acid (50:30:4, v/v) developed 2x to 15cm.

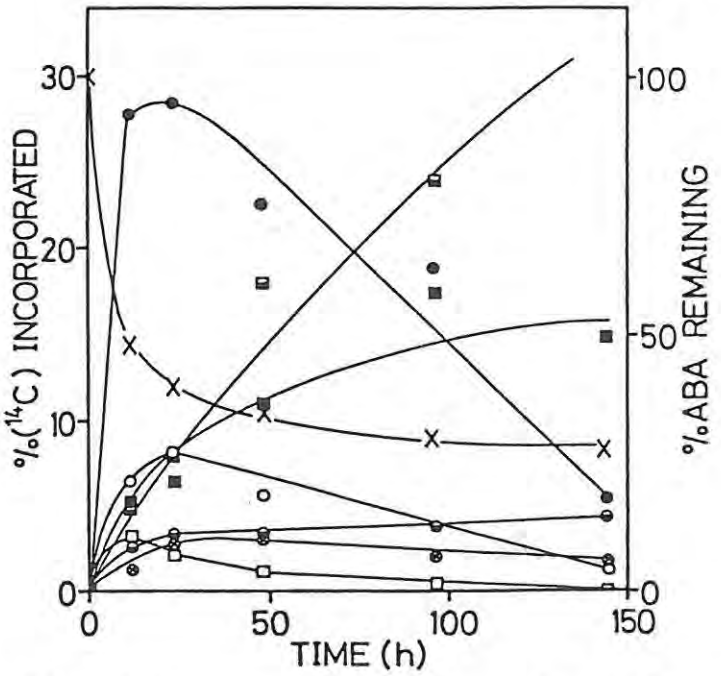

Figure 5.25. Kinetics of (*R,S,*)-[2-^{14}C]-ABA catabolism in excised, light-grown seedlings of *Pisum sativum*. ABA substrate (X), PA (o), DPA (●), catabolite 3 (◒), catabolite 1 (■), 1',4'-*trans* diol (□), 1',4'-*cis* diol (⊗) and aqueous fraction (⊟).

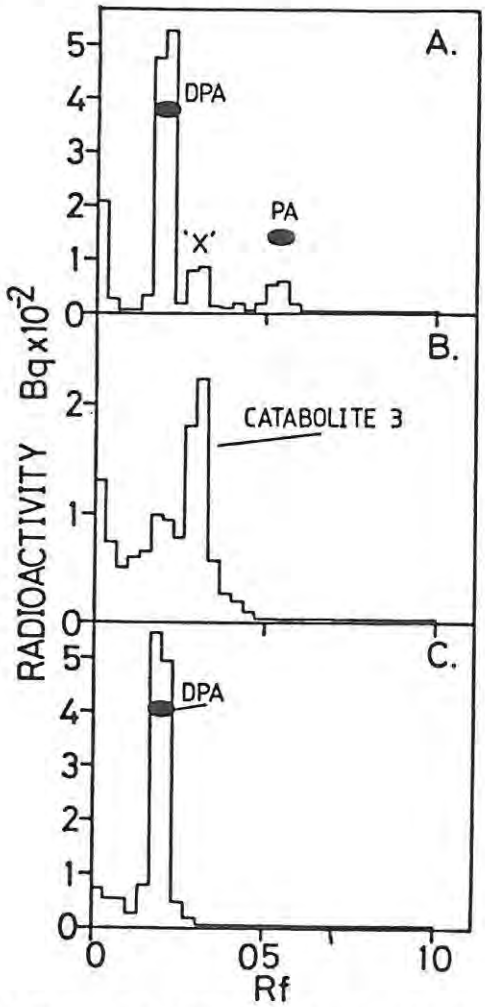

Figure 5.26. Thin layer chromatograms from refeeds of (A) [^{14}C]-PA; (B) [^{14}C]-catabolite 3 and (C) [^{14}C]-DPA to excised, light-grown seedlings of *Pisum sativum*. The ethyl acetate-soluble acids were separated on silica gel GF_{254} in toluene/ethyl acetate/acetic acid (50:30:4, v/v) developed 2x to 15cm. Substrates (each 3.83kBq) were fed to 5g f.w. of tissue as described in Chapter 2.

When [^{14}C]-PA was refed to turgid, light-grown seedlings of *Pisum sativum* (Figure 5.26A), it was converted predominantly into DPA but also in lower yield to a further acidic catabolite, 'X', cochromatographing with catabolite zone 3, detected in ABA feeds. Assuming that the biosynthetically prepared [^{14}C]-PA, fed to *Pisum sativum* seedlings, was derived predominantly from (*S*)- enantiomer of (*R,S,*)-ABA and since the compound derived from ABA (catabolite 3) was produced in equal yield from [^{14}C]-PA, the data suggested that catabolite 3 might be derived from PA. Refeeding [^{14}C]-catabolite 3 to seedlings of *Pisum sativum* (Figure 5.26B) generated a further acidic compound, with similar chromatographic properties to those of DPA in this solvent system. No evidence was obtained for the catabolism of [^{14}C]-DPA into acidic products (Figure 5.26C) while [^{14}C]-PA, [^{14}C]-catabolite 3 and [^{14}C]-DPA were catabolised into water-soluble products (Table 5.4) in high yield in turgid seedlings of *Pisum sativum*.

5.2.1.2.3. The catabolism of PA and DPA in excised, light-grown seedlings of *Phaseolus vulgaris* cv. Top-crop.

To confirm the metabolic interrelationship between PA and DPA refeeding studies were also undertaken using excised seedlings of *Phaseolus vulgaris*. When [^{14}C]-PA was refed to seedlings of *Phaseolus vulgaris* (Figure 5.27A) it was converted into DPA only, suggesting that the minor catabolic product, catabolite 3 (Figure 5.19B), arose from ABA. No evidence was obtained to suggest the catabolism of [^{14}C]-DPA into further acidic products (Figure 5.27B) in excised seedlings of *Phaseolus vulgaris*.

5.2.2.ABSCISIC ACID CATABOLISM IN SEEDS.

5.2.2.1. Studies on the catabolism of (*R,S,*)-ABA in mature seeds of *Hordeum vulgare* L. cv. Dyan.

ABA is known to occur as an endogenous compound in mature seeds of *Hordeum vulgare* (Morris *et al*, 1988). The studies outlined in Chapter 4 (section 4.2.1.2) demonstrated the synthesis of ABA from MVA in mature seed of *Hordeum vulgare*, implying that the enzymes required for ABA biosynthesis were present in this tissue. In contrast, very little is known concerning the catabolism of applied ABA in imbibing embryo and endosperm halves of these seeds. Dashek *et al*, (1979) showed that ABA was not catabolised by endosperm portions and Uknes and Ho (1984) provided only fragmentary data on the catabolism of ABA in embryo tissue. In order to assess both embryos and endosperms as possible sources of the enzymes involved in ABA catabolism, the

TABLE 5.4: The distribution of radioactivity between the ethyl acetate fraction and aqueous phases from feeds of ABA and its catabolites to excised, light-grown seedlings of Pisum sativum cv. Black-eyed Susan.

Seedlings of Pisum sativum (5g f.w.) were supplied with solutions of KPi buffer containing 3.83kBq of either (R,S)-[2-^{14}C]-ABA, [^{14}C]-PA, [^{14}C]-DPA or [^{14}C]-catabolite 3 via the transpiration stream. Once uptake was complete, further buffer was added (4.0 ml) and catabolism allowed to proceed for 24h at 25°C under continuous illumination (66μmol $m^{-2}s^{-1}$). ABA and its catabolites were extracted and analysed as described in Chapter 2.

	Distribution of Radioactivity*	
Substrate	Aqueous	Ethyl acetate
	Bq (%)	
(R,S)-[2-^{14}C]-ABA	1062.77 (27.75)	2770.66 (72.25)
[^{14}C]-PA	1620.12 (42.30)	2212.88 (57.70)
[^{14}C]-3	2367.33 (61.76)	1466.61 (38.24)
[^{14}C]-DPA	2042.56 (53.33)	1790.77 (46.67)

* Data corrected for uptake and losses during extraction and purification.

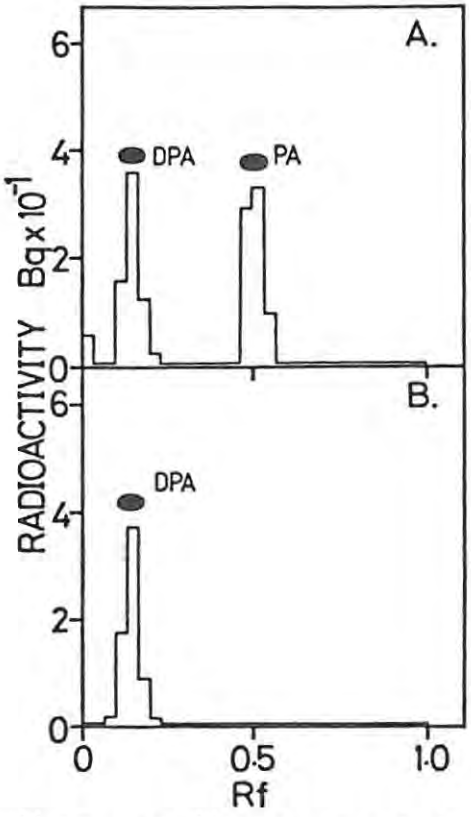

Figure 5.27. Thin layer chromatograms from refeeds of (A) [^{14}C]-PA; (B) [^{14}C]-DPA to excised, light-grown seedlings of *Phaseolus vulgaris*. The ethyl acetate-soluble acids were separated on silica gel GF_{254} in toluene/ethyl acetate/acetic acid (50:30:4, v/v) developed 2x to 15cm. Substrates (each 600Bq) were fed to 5g f.w. of tissue as described in Chapter 2.

ability of these tissues to degrade applied (*R,S,*)-ABA was investigated.

5.2.2.1.1.(*R,S,*)-ABA catabolism in embryo and endosperm portions from mature seeds of *Hordeum vulgare* cv. Dyan.

When embryo and endosperm portions from mature seeds of *Hordeum vulgare* were imbibed in Na-succinate buffer (pH5.0) containing (*R,S,*)-[2-^{14}C]-ABA for 30h incubation periods, the distributions of radioactivity depicted in Figure 5.28A and B, were observed. These data indicate that embryo tissue was far more efficient at degrading ABA than the endosperm portions which, for the main part, only transformed labelled ABA into highly polar products. Tentative identification of the catabolic products was made by reference to authentic ABA, PA, 7'-hydroxy ABA and DPA markers following the separation of the acidic catabolites on thin layers of silica gel GF_{254} in toluene/ethyl acetate/acetic acid (50:30:4, v/v). Thus, the enzymes required for the oxidation of ABA were present in *Hordeum vulgare* embryo tissue and were apparently unaffected by the choice of incubation buffer and the pH of these buffers (Table 5.5). Similarly, the production of polar catabolites from applied (*R,S,*)-ABA in endosperm portions was unaffected by buffer type and the pH of the respective buffers (Table 5.5).

Previous studies on ABA catabolism in isolated aleurone layers (Uknes and Ho, 1984) showed that pretreatment with cold ABA enhanced the conversion of (*R,S,*)-[^{3}H]-ABA into PA. This implied that ABA-induced the synthesis of the enzymes catalysing this step. The advantages of this effect for later studies on ABA catabolism in cell-free extracts are clearly apparent. In an attempt to demonstrate a similar increase in ABA catabolism using aleurone layers from *Hordeum vulgare* cv Dyan, isolated aleurone layers were pretreated with (*R,S,*)-ABA (10^{-3}M) for 24h, prior to labelling with (*R,S,*)-[2-^{14}C]-ABA for a further 24h. The data (Figure 5.29) showed that pretreatment of aleurone layers with (*R,S,*)-ABA (10^{-3}M) actually inhibited the catabolism of (*R,S,*)-[2-^{14}C]-ABA. The amounts of substrate [2-^{14}C]-ABA remaining after incubation were 77.65% in the control and 98.11% in the pretreated sample, confirming the reduced catabolism of applied, radioactive (*R,S,*)-ABA in pretreated tissue.

5.2.2.2. Studies on the catabolism of (*R,S,*)-[2-^{14}C]-ABA in immature seeds of *Pisum sativum* L. and *Phaseolus vulgaris* L.

ABA has been characterised as an endogenous compound in immature seeds of *Pisum sativum* and

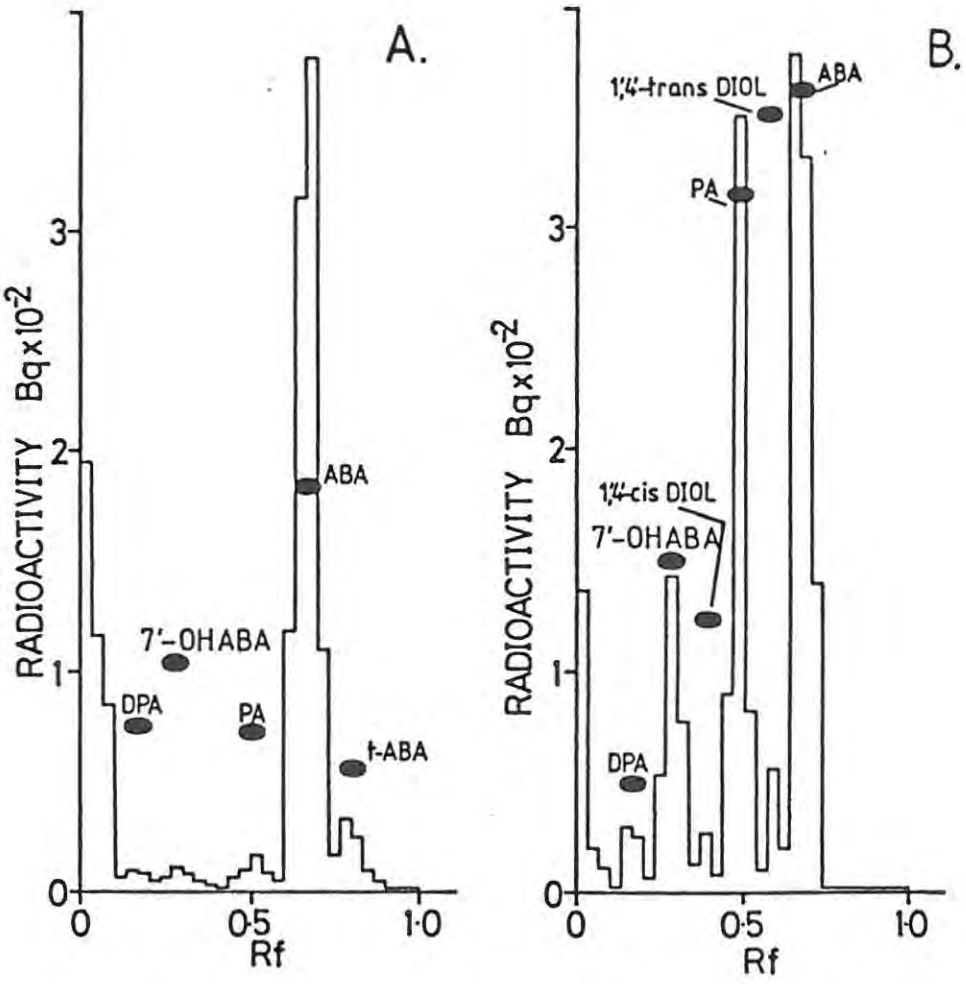

Figure 5.28. TLC separation of (*R,S,*)-[2-^{14}C]-ABA and its acidic catabolites from extracts of *Hordeum vulgare* (A) endosperm and (B) embryo tissue excised from mature seeds. 30 endosperm and embryo halves, prepared as described in Chapter 2, were incubated in 2.0ml of 20mM Na-succinate buffer (pH5.0) containing 20mM $CaCl_2$ and (*R,S,*)-[2-^{14}C]-ABA (4.5kBq) for 30h in darkness at 26°C. The acidic fractions were separated on thin layers of silica gel GF_{254} in toluene/ethyl acetate/acetic acid (50:30:4, v/v) developed 2x to 15cm.

TABLE 5.5 The effect of incubation buffer type and pH on the catabolism of ABA in Hordeum vulgare cv. Dyan embryo and endosperm tissue.

30 Embryo and endosperm halves were incubated in 2.0 ml of either Na-succinate buffer (20mM; pH 5.0) or KPi buffer (10mM; pH 7.5) containing (R,S)-[2-^{14}C]-ABA (4.5kBq). Uptake was facilitated by placing the incubates under vacuum for 1.0 min. Incubations were carried out in total darkness in a metabolic shaker at 26°C for 30h. ABA and its catabolites were extracted and analysed as described in Chapter 2.

		Distribution of Radioactivity				
Tissue	Buffer	Conjugates	DPA	7'hydroxy ABA	PA	Substrate ABA
				Bq (%)		
Embryo	Kpi	1669.95 (37.11)	54.80 (1.22)	222.71 (4.95)	678.71 (15.08)	1873.71 (41.64)
	Na-succinate	1582.48 (35.17)	57.59 (1.28)	258.14 (6.34)	870.17 (19.34)	1704.72 (37.88)
Endosperm	Kpi	1264.50 (28.10)	9.90 (0.22)	8.55 (0.19)	19.35 (0.43)	3197.70 (71.06)
	Na-succinate	1328.40 (29.52)	16.65 (0.37)	13.95 (0.31)	35.10 (0.78)	3105.90 (69.02)

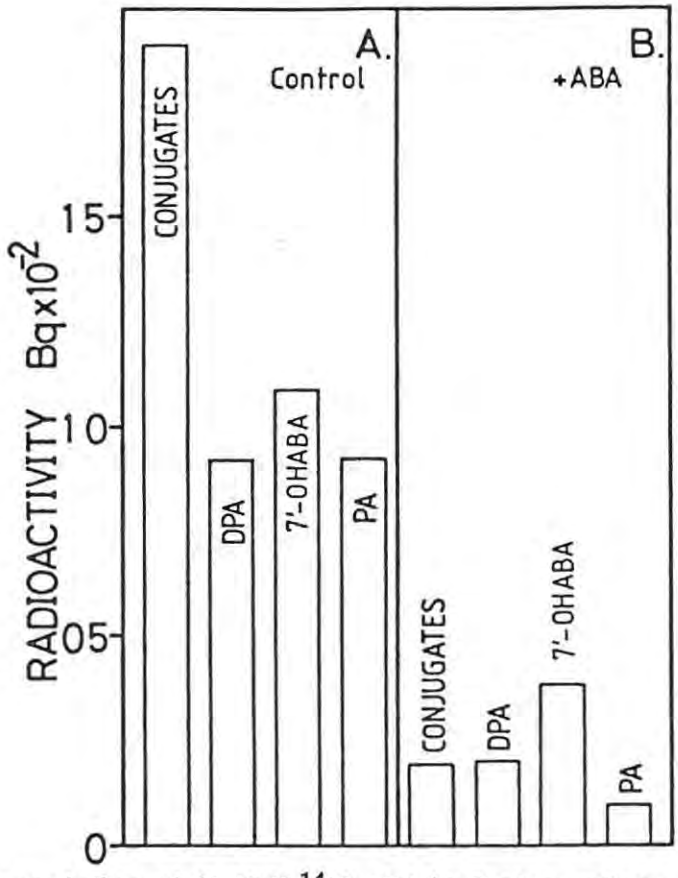

Figure 5.29. The effect of (*R,S,*)-ABA on the catabolism of (*R,S,*)-[2-^{14}C]-ABA by *Hordeum vulgare* aleurone layers. (A) Control; (B) (*R,S,*)-ABA pretreated. Aleurone layers were incubated with or without (*R,S,*)-ABA (10^{-3}M) for 24h. After pre-incubation, fresh buffer containing (*R,S,*)-[2-^{14}C]-ABA (4.5kBq) was added and the tissue incubated for a further 24h. ABA and its catabolites were extracted and analysed as described in Chapter 2.

Phaseolus vulgaris (Isogai *et al*, 1967; Hiraga *et al*, 1974). However, as these seeds develop there is an observable decline in the levels of endogenous ABA (Hsu, 1979; Browning, 1980). This decline might be indicative of an increased rate of catabolism or a reduced rate of synthesis. As there were no published reports of ABA catabolism in these tissues, the catabolism of (*R,S,*)-[2-^{14}C]-ABA was examined in immature seed from two species of Leguminosae.

5.2.2.2.1.(*R,S,*)-ABA catabolism in immature seeds of *Pisum sativum* L. and *Phaseolus vulgaris* L.

When intact, immature seeds of *Pisum sativum* and *Phaseolus vulgaris* were allowed to catabolise (*R,S,*)-[2-^{14}C]-ABA the incorporation of label into the acidic catabolites, shown in Figure 5.30 was observed. The results show that immature seeds of these species possess the capacity to catabolise applied (*R,S,*)-ABA into a range of acidic products. Immature seeds from *Phaseolus vulgaris* appeared more efficient at transforming ABA into its acidic products than immature seeds of *Pisum sativum*. Nevertheless, the acidic catabolites produced displayed similar chromatographic properties to those of authentic PA, DPA and the 1',4'-*cis* diol of ABA.

Tentative identification of the radiopeaks cochromatographing with authentic PA and DPA (Figure 5.30) was achieved by microchemical methods as described in Chapter 2. Thus, PA and DPA were tentatively identified as catabolites of applied, labelled ABA in immature seeds of both *Pisum sativum* and *Phaseolus vulgaris*. The remaining acidic products of (*R,S,*)-ABA catabolism were routinely generated in amounts too low for the micro-chemical manipulations involved.

Preliminary analyses of catabolite zone 1 (Rf0.03-0.10) produced from (*R,S,*)-[2-^{14}C]-ABA feeds to immature seeds of *Pisum sativum* (Figure 5.31) and *Phaseolus vulgaris* (Figure 5.32) demonstrated that immature seeds have the ability to conjugate applied (*R,S,*)-ABA. Similar treatments of the aqueous phases produced the results shown in Figure 5.33A and B and Figure 5.34A and B. These data indicate that immature seed of *Pisum sativum* transforms ABA to a water-soluble, base-labile conjugate, while seed of *Phaseolus vulgaris* conjugated ABA and some PA. In addition, mild alkaline hydrolysis of the aqueous fractions from *Pisum sativum* and *Phaseolus vulgaris* released a compound which co chromatographed with *trans*-ABA which was absent from similar hydrolysates of catabolite zone 1. Similar findings were also reported earlier in ABA catabolic studies in vegetative tissues of these species. However, whether these compounds are produced as glucose ethers or esters is currently unknown.

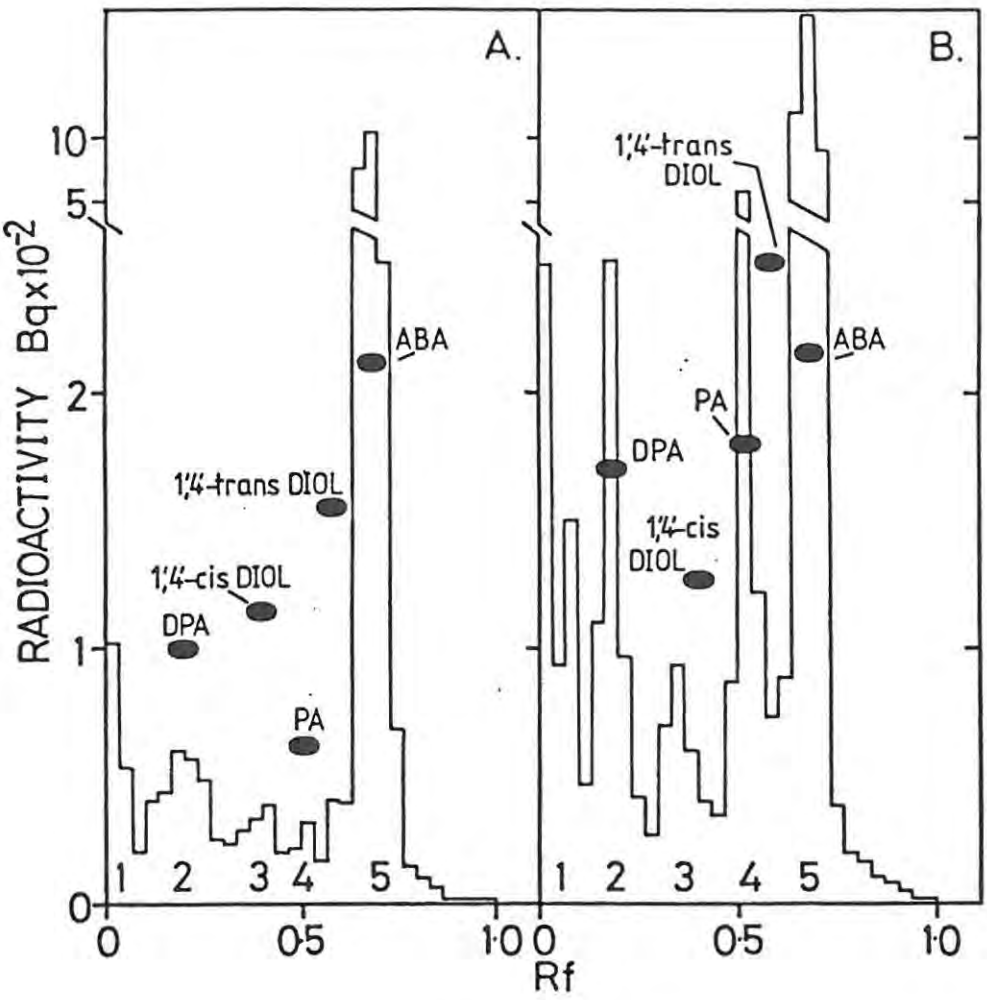

Figure 5.30. Thin layer chromatographic separation of (*R,S,*)-[2-^{14}C]-ABA and its catabolites from extracts of immature seeds of (A) *Pisum sativum* and (B) *Phaseolus vulgaris*. Immature seeds (10g f.w.), excised from the pod, were fed with (*R,S,*)-[2-^{14}C]-ABA (4.2kBq) and incubated in 15.0ml of nutrient media (Nitsch, 1957) for 48h and the ethyl acetate-soluble acids separated by TLC on silica gel GF_{254} with toluene/ethyl acetate/acetic acid (50:30:4, v/v) developed 2x to 15cm.

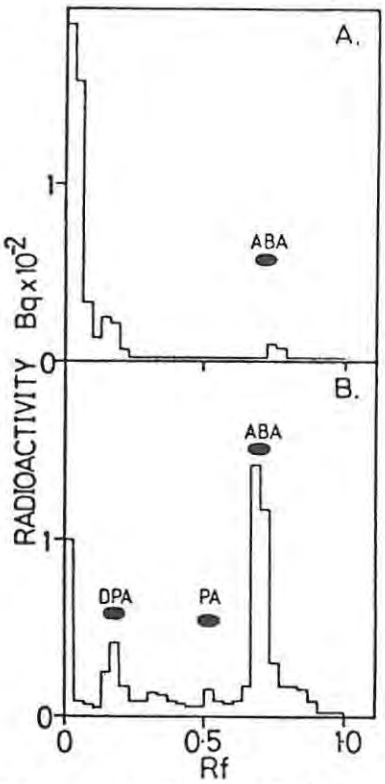

Figure 5.31. Hydrolysis of catabolite 1 (as in Figure 5.30A) in the ethyl acetate-soluble acids from immature seeds of *Pisum sativum* fed with (*R,S,*)-[2-^{14}C]-ABA. (A) Control held at pH7.0 at 60°C for 1h; (B) treated with base at pH11.0 at 60°C for 1h. Samples were processed as described in Chapter 2 and chromatographed on thin layers of silica gel GF_{254} in toluene/ethyl acetate/acetic acid (50:30:4, v/v) developed 2x to 15cm.

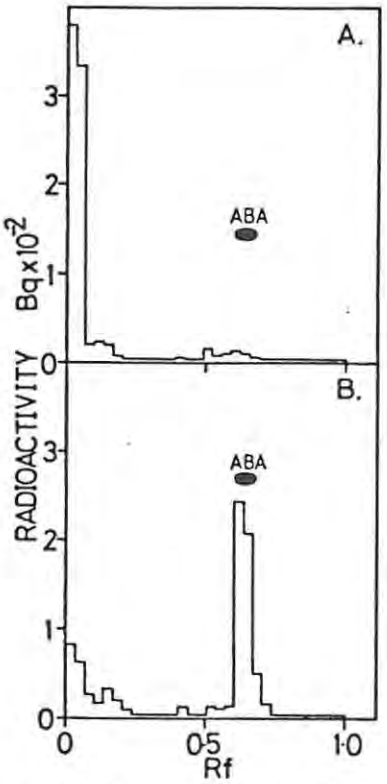

Figure 5.32. Hydrolysis of catabolite 1 (as in Figure 5.30B) in the ethyl acetate-soluble acids from immature seeds of *Phaseolus vulgaris* fed with (*R,S,*)-[2-^{14}C]-ABA. (A) Control held at pH7.0 at 60°C for 1h; (B) treated with base at pH11.0 at 60°C for 1h. Samples were processed as described in Chapter 2 and chromatographed on thin layers of silica gel GF_{254} in toluene/ethyl acetate/acetic acid (50:30:4, v/v) developed 2x to 15cm.

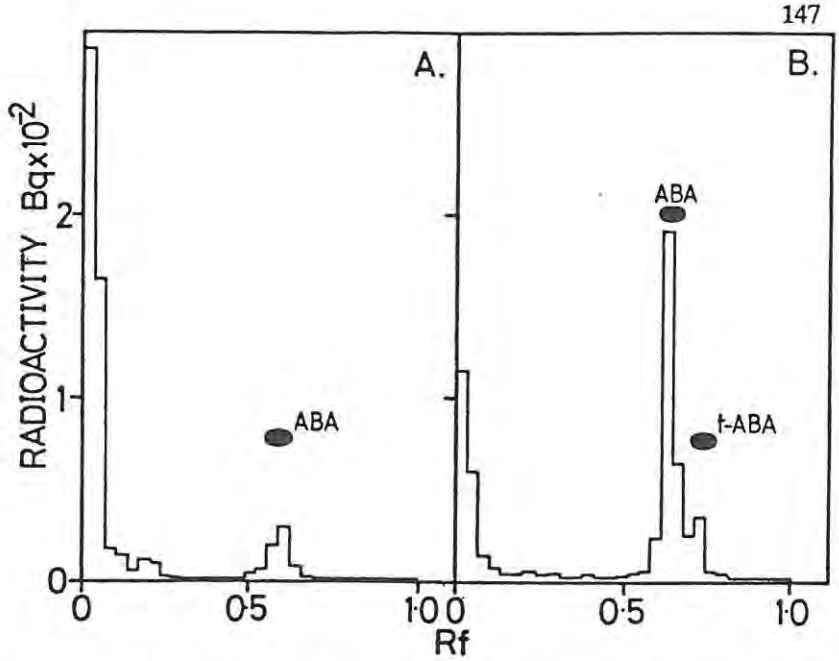

Figure 5.33. Hydrolysis of the aqueous fraction from an extract of immature seeds of *Pisum sativum* fed with (*R,S,*)-[2-^{14}C]-ABA. (A) Control held at pH7.0 at 60°C for 1h; (B) treated with base at pH11.0 at 60°C for 1h. Samples were processed as described in Chapter 2 and chromatographed on thin layers of silica gel GF_{254} in toluene/ethyl acetate/acetic acid (50:30:4, v/v) developed 2x to 15cm.

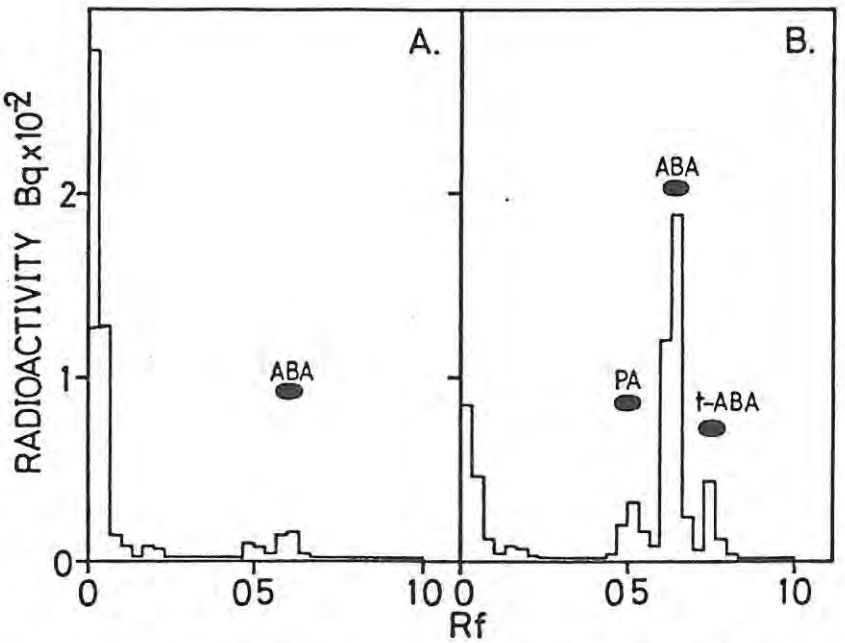

Figure 5.34. Hydrolysis of the aqueous fraction from an extract of immature seeds of *Phaseolus vulgaris* fed with (*R,S,*)-[2-^{14}C]-ABA. (A) Control held at pH7.0 at 60°C for 1h; (B) treated with base at pH11.0 at 60°C for 1h. Samples were processed as described in Chapter 2 and chromatographed on thin layers of silica gel GF_{254} in toluene/ethyl acetate/acetic acid (50:30:4, v/v) developed 2x to 15cm.

5.2.2.2.2. Kinetics of (*R,S,*)-ABA catabolism and refeeding studies in immature seeds of *Pisum sativum* L. and *Phaseolus vulgaris* L.

Kinetic analyses of (*R,S,*)-[2-^{14}C]-ABA catabolism in immature seed of *Pisum sativum* (Figure 5.35A) and *Phaseolus vulgaris* (Figure 5.35B) revealed that DPA was produced rapidly, reaching its maximum at 72h while radioactivity in PA never exceeded that associated with DPA. A decline in PA was followed by further increases in the incorporation of label into DPA. Catabolite 'X', possibly produced from PA, reached its maximum at 48h. Catabolite 1, an ABA conjugate, reached its maximum at 24h and thereafter remained fairly constant in *Pisum sativum* but declined steadily in *Phaseolus vulgaris* immature seed

When [^{14}C]-PA was refed to immature seed of either *Phaseolus vulgaris* or *Pisum sativum*, it was converted predominantly into DPA, as expected, but also into a compound, 'X', of intermediate polarity between PA and DPA (Table 5.6). In all cases, the levels of radioacitivity associated with compound 'X' were higher when [^{14}C]-PA was used as substrate. This suggested that 'X' was derived from PA. In addition, *Phaseolus vulgaris* immature seed catabolised ABA, PA and DPA into a compound, 'X-1', which was more polar than DPA. Since 'X-1' was produced in highest amounts from DPA, it appeared to be derived only from [^{14}C]-DPA. This compound was not present in similar feeds to immature seed of *Pisum sativum*. In all cases, high levels of radioactiviy were associated with the aqueous fractions indicated that ABA and its acidic catabolites were being transformed into water-soluble conjugates.

5.2.2.2.3. Release of acidic products, following the uptake and catabolism of (*R,S,*)-ABA, by immature seeds of *Pisum sativum* L. and *Phaseolus vulgaris* L.

One interesting aspect of the studies on ABA catabolism in immature seeds of *Pisum sativum* and *Phaseolus vulgaris* was the observation that an initial decline in radioactivity in the nutrient medium was followed by an increase during the 96h incubation period (Figure 5.36).

When seeds of *Phaseolus vulgaris* were imbibed in nutrient medium for periods up to 96h the levels of radioactivity in the nutrient medium were always greater than 60% of the total radioactivity fed to the tissue. For immature seeds of *Pisum sativum* the level of radioactivity in the nutrient medium declined over the first 24h and then increased to above 60% of the total radioactivity during the subsequent incubation period. This finding led to further detailed studies *in vitro* in order to

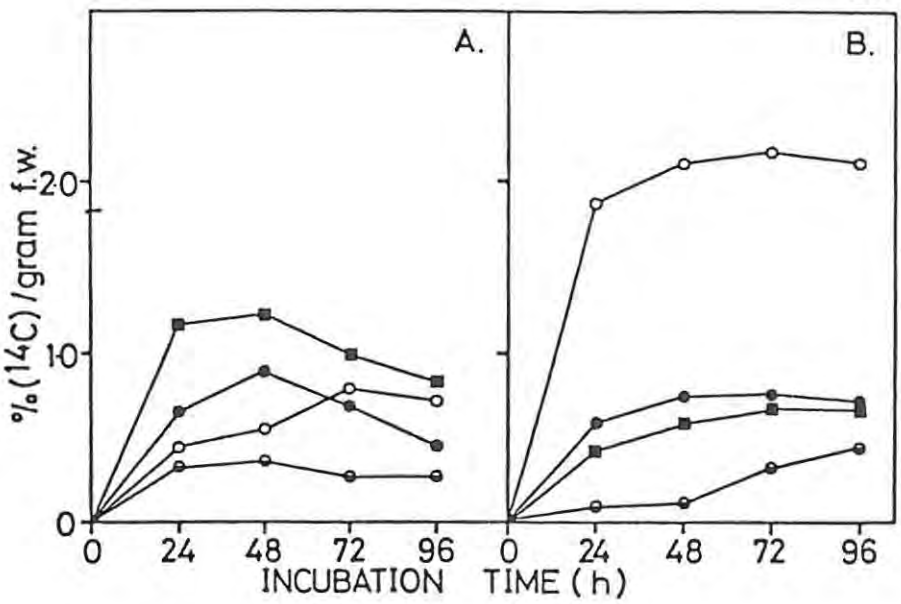

Figure 5.35. The kinetics of (*R,S,*)-[2-^{14}C]-ABA catabolism in immature seeds of (A) *Pisum sativum* and (B) *Phaseolus vulgaris*. DPA (●); PA (o); catabolite 1 (■) and catabolite 3 (◒).

TABLE 5.6: Catabolism of ABA, PA and DPA in immature seeds of Pisum sativum and Phaseolus vulgaris

4.5g Fresh weight of tissue was incubated in 15.0 ml of nutrient medium (Nitsch, 1951), prepared as described in Chapter 2, containing either (R,S)-[2-^{14}C]-ABA (5.83kBq), [^{14}C] -PA (0.83kBq) or [^{14}C]-DPA (0.83kBq). Uptake was facilitated by placing the incubates under vacuum for 1.0 min. Samples were incubated in an orbital shaker at 28°C for 48h under continuous illumination (42μmol $m^{-2}s^{-1}$). ABA, PA, DPA and their catabolites were extracted and analysed as described in Chapter 2.

	Distribution of Radioactivity				
Tissue and Substrate	Conjugates	X-1	DPA	X	PA
	Bq (%)				
Phaseolus vulgaris					
+ ABA	1715.58 (29.41)	90.42 (1.55)	294.00 (5.04)	135.33 (2.32)	477.17 (18.18)
+ PA	146.33 (17.56)	22.92 (2.75)	146.58 (17.59)	55.75 (6.69)	-
+ DPA	187.17 (22.46)	39.78 (4.77)	-	-	-
Pisum sativum					
+ ABA	677.83 (11.62)	-	152.25 (2.61)	61.25 (1.05)	42.58 (0.73)
+ PA	110.42 (13.25)	-	133.92(16.07)	120.58(14.47)	-
+ DPA	322.75 (38.73)	-	-	-	-

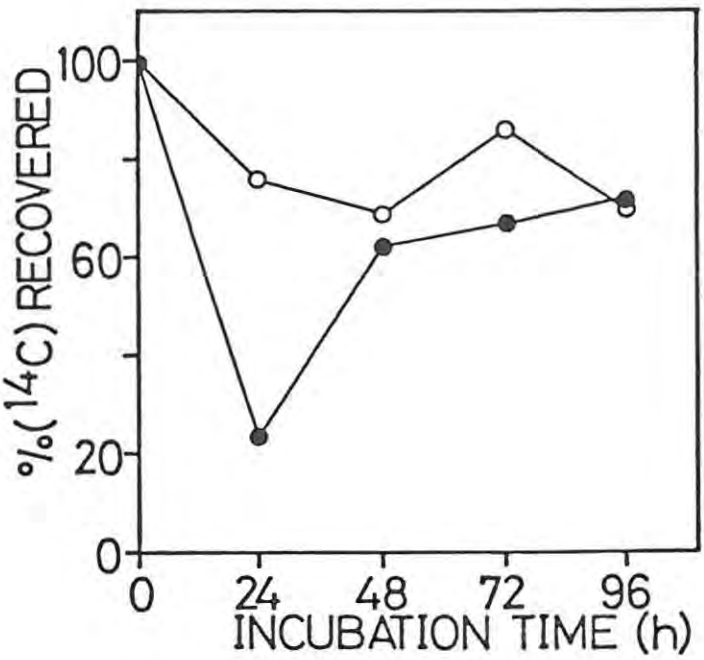

Figure 5.36. The recovery of radioactivity in the nutrient medium following the catabolism of (*R,S,*)-[2-^{14}C]-ABA (3.33kBq) by imbibing immature seeds of *Pisum sativum* (●) and *Phaseolus vulgaris* (o).

examine the uptake of [2-^{14}C]-ABA and leakage of (*R,S,*)-[2-^{14}C]-ABA and its catabolites from the tissue into the nutrient medium.

Following incubations of immature seeds of *Pisum sativum* and *Phaseolus vulgaris* with (*R,S,*)-[2-^{14}C]-ABA, the immature seed was removed from the nutrient medium, thoroughly washed in distilled water and the seed tissue, nutrient media and washings extracted and analysed separately. Radioactivity in the nutrient medium from imbibng seeds of both species declined rapidly during the first 24h of incubation (Figure 5.37A and B). This was associated with a concomitant increase in the levels of radioactivity detected in the tissue extracts prepared from immature seed. After 24h, the levels of radioactivity in the nutrient media increased to ±50% of the total radioactivity supplied, after which time they remained fairly constant throughout the duration of the incubation period. These data show that, with an increase in incubation time, label imbibed by immature seed of both *Pisum sativum* and *Phaseolus vulgaris* moved from the seed back into the nutrient medium.

An examination of the distribution of label between the ethyl acetate-soluble acids, prepared from the nutrient media and tissue extracts (Figure 5.37C and D), and the residual aqueous fractions, from the nutrient media and extracts (Figure 5.37E and F), demonstrated that the increase in radioactivity associated with the nutrient media (Figure 5.37A and B) was due to increased incorporation of label into water-soluble products present in the nutrient media rather than by release of the free acids from the tissue into the nutrient media.

When the ethyl acetate-soluble acids prepared from the nutrient media, remaining after incubation, were analysed, the rates of appearance of the acidic catabolites in the nutrient media depicted in Figure 5.38A and B, were observed. These data showed that with time there was an increase in the rates of appearance of DPA, PA and catabolite 'X'. However, levels of these acids were low suggesting that the bulk of radioactivity in the nutrient medium was associated with water-soluble conjugates. In addition, the amounts of substrate (*R,S,*)-[2-^{14}C]-ABA declined throughout the incubation period, suggesting that once ABA was absorbed by the tissue it was not released into the nutrient medium as the free acid.

A further aspect regarding the catabolism of (*R,S,*)-[2-^{14}C]-ABA in immature seed of *Pisum sativum* and *Phaseolus vulgaris* was that the total recoverable counts declined with increasing incubation time (Figure 5.37A and B). One possible explanation for this result might be that, label was being lost from these systems in the form of [^{14}C]-CO_2. A similar process was purported to

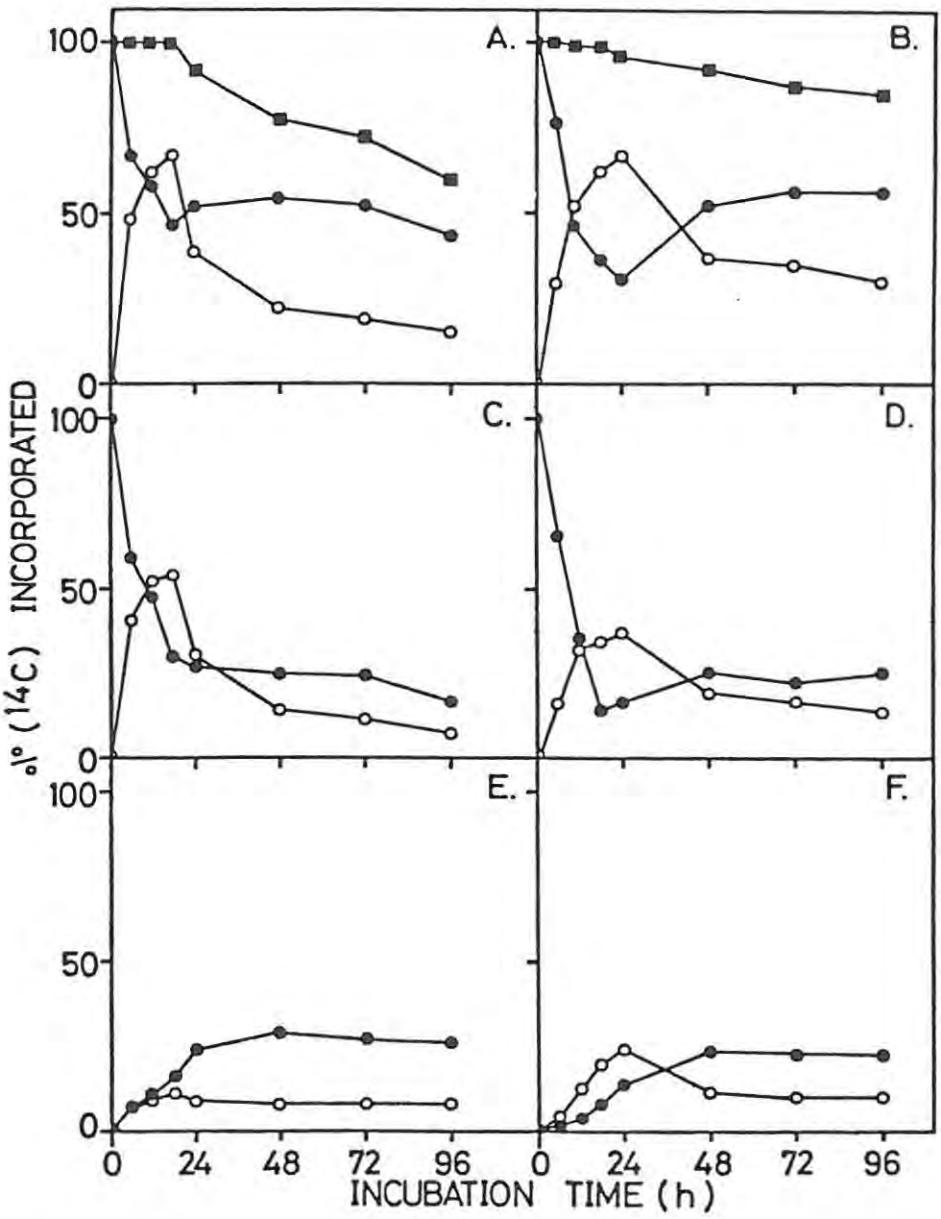

Figure 5.37. The distribution of radioactivity from supplied (*R,S,*)-[2-^{14}C]-ABA (4.2kBq) during uptake and catabolism by immature seeds of *Phaseolus vulgaris* (5g f.w.) and *Pisum sativum* (10g f.w.). (A) Total radioactivity (■) associated with the nutrient medium (●) and tissue extract (o) from *Phaseolus vulgaris* immature seed; (B) total radioactivity (■) associated with the nutrient medium (●) and tissue extract (o) from *Pisum sativum* immature seed; (C) the distribution of label between the ethyl acetate-soluble acids from the nutrient medium (●) and immature seed extract (o) from *Phaseolus vulgaris*; (D) the distribution of label between the ethyl acetate-soluble acids from the nutrient medium (●) and immature seed extract (o) from *Pisum sativum*; (E) the distribution of label in the aqueous fractions from the nutrient medium (●) and immature seed extract (o) from *Phaseolus vulgaris*; (F) the distribution of label in the aqueous fractions from the nutrient medium (●) and immature seed extract (o) from *Pisum sativum*.

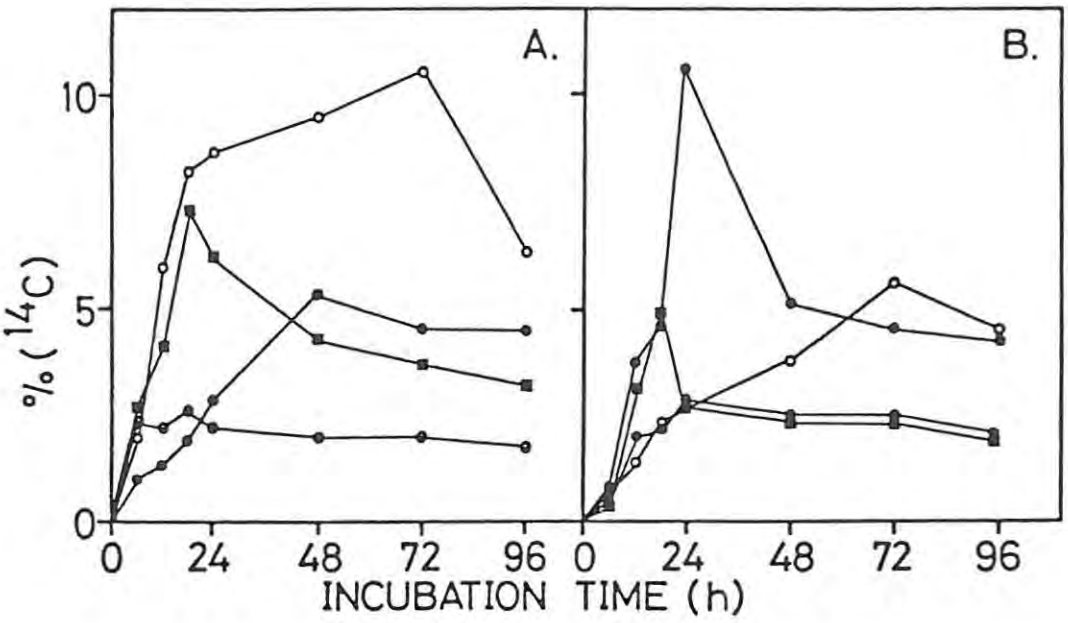

Figure 5.38. The appearance rates of radioactivity in the acidic compounds released from imbibing immature seeds of (A) *Phaseolus vulgaris* and (B) *Pisum sativum* into the nutrient medium during the catabolism of (*R,S,*)-[2-^{14}C]-ABA (4.2kBq). PA (o); DPA (●); catabolite X (◒) and catabolite 1 (■).

occur during the uptake and degredation of [^{14}C]-ABA by *Pyrus* seeds (Rudnicki and Czapski, 1974), although it was later shown to be attributable to bacterial contamination (Milborrow and Vaughan, 1979). However, the micro-chemical characterisation of both PA and DPA, coupled with studies where the inhibitors of prokaryotic ribosomal protein synthesis, CAP and LINC (see Chapter 6), were included during incubation and did not significantly alter the % [^{14}C] incorporated, suggest that bacterial contamination was not responsible for the overall losses in the recovery of radioactivity. In addition, although Milborrow and Vaughan (1979) demonstrated the catabolism of [^{14}C]-ABA by bacterial microflora to unknown products, no PA or DPA was produced.

5.2.3.ABSCISIC ACID CATABOLISM IN RIPENING FRUITS OF *PERSEA AMERICANA*.

5.2.3.1. The catabolism of (*R,S,*)-[2-^{14}C]-ABA in ripening fruits of *Persea americana* cv. Fuerte.

The catabolism of (*R,S,*)-ABA was examined in ripening fruits of *Persea americana* since it is well known that mesocarp tissue from this species can synthesize ABA from MVA (Noddle and Robinson, 1969; Milborrow and Noddle, 1970; Milborrow and Robinson ,1973; Milborrow, 1978b; Cowan and Railton, 1986). Milborrow (1974c) mentioned briefly in a conference proceedings that ripening mesocarp tissue from fruits of *Persea americana* transformed applied ABA into acidic products but no details were provided.

Therefore, (*R,S,*)-ABA was fed to sliced mesocarp tissue of *Persea americana* and the distribution of radioactivity in the acidic products is shown in Figure 5.39. The tentative identification of the catabolites and of substrate ABA, was established by comparing the Rf values of the radio peaks with those of the authentic ABA, PA and DPA markers. Thus, in mesocarp tissue from *Persea americana*, ABA was apparently transformed predominantly into PA and DPA, and to a lesser extent, into a further acidic catabolite with similar chromatographic properties to those of 7'-hydroxy ABA. In addition, a radioactive zone cochromatographing with *trans*-ABA was observed. No radioactive peak was associated with the 1',4'-*trans* diol standard, suggesting that the 1',4'-*trans* diol of ABA was not produced as a catabolite of (*R,S,*)-[2-^{14}C]-ABA in *Persea americana* mesocarp tissue in these studies.

In order to determine whether mesocarp from *Persea americana* could produce water-soluble conjugates from applied, radiolabelled (*R,S,*)-ABA the aqueous phase from feeds of (*R,S,*)-[2-^{14}C]-ABA to mesocarp tissue was treated with base and the products analysed as described previously

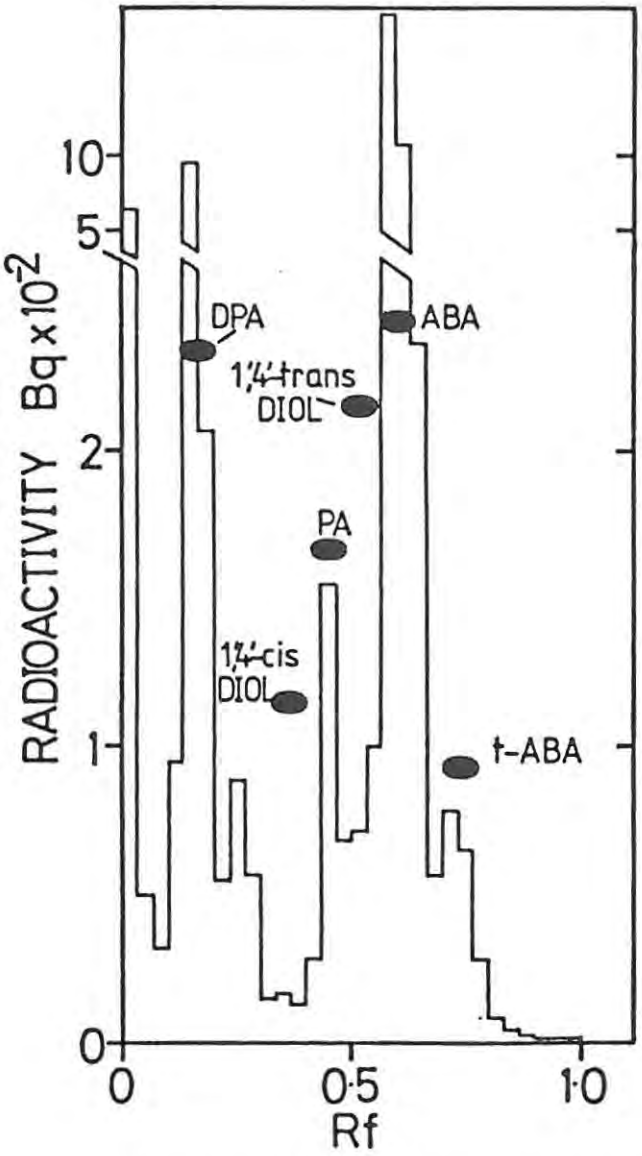

Figure 5.39. Thin layer chromatographic separation of (*R,S,*)-[2-^{14}C]-ABA and its catabolites from extracts of the mesocarp tissue from *Persea americana* cv. Fuerte fruits. 20g Blocks of mesocarp tissue were fed with (*R,S,*)-[2-^{14}C]-ABA (4.2kBq) in 0.2ml of Tween 80/acetone/water (1:1:8, v/v) and allowed to catabolize it for 24h in a water saturated environment under continuous illumination. The ethyl acetate-soluble acids separated by TLC on silica gel GF_{254} with toluene/ethyl acetate/acetic acid (50:30:4, v/v) developed 2x to 15cm.

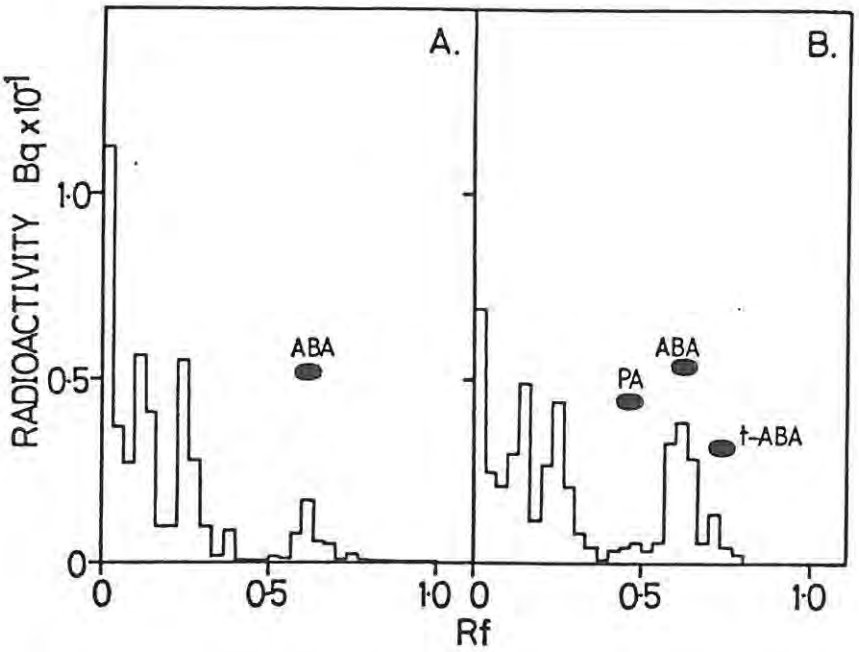

Figure 5.40. Hydrolysis of the aqueous fraction from an extract of excised *Persea americana* mesocarp tissue fed with (*R,S,*)-[2-^{14}C]-ABA. (A) Control held at pH7.0 at 60°C for 1h; (B) treated with base at pH11.0 at 60°C for 1h. Samples were processed as described in Chapter 2 and chromatographed on thin layers of silica gel GF_{254} in toluene/ethyl acetate/acetic acid (50:30:4, v/v) developed 2x to 15cm.

TABLE 5.7: Catabolism of ABA, PA and DPA in excised mesocarp tissue from ripening Persea americana cv. Fuerte fruits.

20g f.w. blocks of mesocarp tissue were fed with (R,S)-[2-^{14}C]-ABA (4.2kBq), [^{14}C]-PA (1.0 kBq) and [^{14}C]-DPA (1.0kBq) in 200µl Tween 80/acetone/water (1:1:8, v/v). Tissues were incubated for 24h at 25°C under continuous illumination (66µmol m^{-2}s^{-1}) in a water-saturated environment. ABA, PA, DPA and their catabolites were extracted and analysed as described in Chapter 2.

	Distribution of Radioactivity				
Substrate	Conjugates	DPA	X	PA	ABA
	Bq (%)				
(R,S)-[2-^{14}C]-ABA	1890.0 (45.00)	428.4 (10.20)	100.8 (2.40)	75.6 (1.80)	2251.2 (53.60)
[^{14}C]-PA	158.2 (15.82)	210.8 (21.08)	-	631.0 (63.10)	-
[^{14}C]-DPA	254.2 (25.42)	745.8 (74.58)	-	-	-

(see Chapter 2). Some PA, ABA and a compound co-chromatographing with the *trans* isomer of ABA were released from a bound form (Figure 5.40). Refeeding studies (Table 5.7) using [^{14}C]-PA and [^{14}C]-DPA confirmed the expected metabolic relationship between these compounds and further showed that they could be transformed into water-soluble, base-labile conjugates.

CHAPTER SIX

STUDIES ON THE MODIFICATION OF ABSCISIC ACID CATABOLISM BY ENVIRONMENTAL AND CHEMICAL FACTORS.

6.1. INTRODUCTION.

The regulation of ABA levels in plant tissues is a two-part process, where the concentration present in the tissue is balanced by both its synthesis and its breakdown or sequestration as conjugates. Studies on ABA biosynthesis in higher plant tissues, outlined in Chapter 4, have shown that both chemical and environmental factors may modify this process. Thus, it is not unreasonable to speculate that these factors modify ABA biosynthesis by regulating the levels and/or activity of the enzymes required for ABA metabolism. Similarly, the stage of tissue development could also play a role in modifying ABA metabolism. However, whether such factors also regulate the levels of ABA in plants by exerting an influence on ABA catabolism is unclear and therefore studies were undertaken to examine this aspect.

6.1.1. Tissue age.

Several studies have suggested that enzyme levels in plants vary in relation to tissue age, and these include the enzymes involved in lipid oxidation (Priestley and Leopold, 1979), camphor biosynthesis (Croteau *et al*, 1981), nitrogen metabolism (Sherrard and Dalling, 1979), photosynthesis (Suzuki *et al*, 1987), sucrose and mannitol synthesis (Davis *et al*, 1988), intermediates in the GA biosynthetic pathway (Railton *et al*, 1984) and the metabolism of GAs (Pharis and King, 1985). Similarly, tissue age may play a role in mediating ABA catabolism, possibly as a result of altered levels of the ABA-catabolising enzymes.

Studies on the effects of tissue age on ABA metabolism in plants are limited. Nevertheless, it is a general observation that ABA is present in higher concentrations in younger tissues than in older tissues (Lorah, 1974; Sivakamuran and Hall, 1978; Raschke and Zeevaart, 1976), and the ability to accumulate ABA in response to wilting also declines with increasing leaf age, while the levels of PA and DPA remain unchanged (Zeevaart, 1977). In addition, ABA is extensively catabolised in

growing seeds of *Triticum aestivum* (King, 1979) and radiolabelled ABA is rapidly catabolised in the apical leaves and released axillary buds of *Vicia faba* (Everat-Bourbouloux, 1982). Thus, these findings indicate that young tissue might be a rich source of the enzymes for the catabolism of ABA in plants.

6.1.2. Light.

Light may play a key role in regulating the endogenous ABA levels in plant tissues (outlined in Chapter 4). When plant tissues are actively photosynthesizing the bulk of their endogenous ABA is purported to be localised within the chloroplasts (Loveys, 1977; Heilmann *et al*, 1980), and since chloroplasts do not appear to catabolise ABA (Hartung *et al*, 1980; Cowan and Railton, 1986) it has been suggested (Loveys and Milborrow, 1984) that it should be possible to demonstrate an effect of light on the catabolism of ABA in plant tissue other than that of photosynthesis. However, only a few studies have been directed at investigating the effects of light on the catabolism of applied radiolabelled ABA (Loveys, 1979; Phillips *et al*, 1980; Zeevaart, 1983; Loveys and Milborrow, 1984).

Previously, Cummins (1973) demonstrated that the pattern of radiolabelled ABA degradation was similar in *Hordeum vulgare* leaves kept in either light or darkness, with very little breakdown occurring. Loveys (1979) obtained evidence to suggest that the production of DPA, from applied radiolabelled ABA in seedlings of *Lycopersicon esculentum*, depended largely on the quality of light to which the plants were exposed. It was suggested that far-red light enhanced the conversion of ABA to 8'-hydroxy ABA but not to PA and DPA, whereas red light enhanced the conversion of ABA to PA and further catabolism to DPA. In addition, far-red enriched light accelarated the glucosylation of ABA and the catabolism of ABA to PA (Loveys, 1979; Phillips *et al*, 1980). Thus, the effect of light on the catabolism of applied, radiolabelled (*R,S,*)-ABA was investigated in vegetative and non-vegetative plant tissues.

6.1.3. Water stress.

A further factor which might modify the catabolism of ABA in plants is water-stress. One of the most clearly defined effects of water-stress is the increase of ABA levels in plants (Wright, 1969; Wright and Hiron, 1969; Hiron and Wright, 1973; Mansfield *et al*, 1978; Henson and Quarrie, 1981; Milborrow, 1983b; Davies and Mansfield, 1983; Loveys and Milborrow, 1984; Quarrie and Lister, 1984b). However, the underlying biochemical mechanism of this process has only been investigated

in a few species and remains a contentious issue.

Water-stress results in elevated levels of endogenous PA and DPA in leaves of *Phaseolus vulgaris* (Harrison and Walton, 1975) and in leaves of *Xanthium strumarium* (Zeevaart, 1983). In addition, Pierce and Raschke (1981), showed that in *Phaseolus vulgaris* leaves the rate of conversion of endogenous ABA to PA rose steadily following the imposition of water-stress. Similar results were obtained with *Xanthium strumarium* leaves (Zeevaart, 1980). Thus, the results of these studies suggest that water-stress enhances the catabolism of ABA in these tissues.

Unlike the aforementioned studies, the effect of water-stress on the catabolism of applied, radiolabelled ABA has only been examined in a few species. No significant differences were noted in the levels of PA and DPA produced in turgid and water-stressed *Phaseolus vulgaris* leaves (Harrison and Walton, 1975) or in the catabolism of labelled ABA substrate in wilted and non-wilted leaves of *Xanthium strumarium* (Cornish and Zeevaart, 1984). Similarly, in stressed and non-stressed leaves of *Triticum aestivum* there was little difference in the amounts of PA and DPA produced from labelled ABA (Lehmann and Schütte, 1984; Murphy, 1984). The apparent inability of water-stress to influence the catabolism of applied ABA in the above tissues, suggests that stress-induced increases in ABA levels might be regulated by processes other than ABA catabolism.

6.1.4. Chemical factors.

Studies with ABA-producing fungi have shown that both cytokinins and inhibitors of GA biosynthesis will also inhibit the biosynthesis of ABA (Norman *et al*, 1982; 1983; Hirai *et al*, 1986). The data obtained in Chapter 4 confirmed that these compounds could also interfere with ABA biosynthesis in *Persea americana* mesocarp and therefore possibly in other tissues as well.

Nothing is known of the effects of cytokinins on ABA catabolism in plants. However, cytokinins have been shown to enhance the conversion of GA_9 to GA_{20} in *Pisum sativum* seedlings (Railton, 1974) and the conversion of GA_4 in *Lactuca sativa* seedlings (Durley *et al*, 1976). Whether a similar situation holds for the conversion of ABA to PA and DPA in plants remains to be determined. Likewise, whether the inhibitors of GA biosynthesis, ancymidol, AMO1618 and CCC, are capable of inhibiting the catabolism of ABA in higher plants is currently unknown.

The numerous studies on ABA catabolism in plant tissues (see Chapter 1) give no information

regarding enzyme stability or whether active nucleic acid and/or protein biosynthesis is required for (*R,S,*)-ABA catabolism. Thus, very little information regarding the effects of inhibitors of translation on the catabolism of ABA has been forthcoming. To date, only one report on the effect of these inhibitors on ABA catabolism has been published. Uknes and Ho, (1984) demonstrated that CHI inhibited the conversion of ABA to PA, DPA and conjugates in *Hordeum vulgare* aleurone layers. However, nothing is known of the effects of similar compounds on the catabolism of radiolabelled ABA in excised but otherwise intact plant tissues. Thus, in order to determine whether continued protein synthesis was required for ABA catabolism, the effect of inhibitors of protein biosynthesis on this process was examined.

6.2 RESULTS.

6.2.1.Studies on the effect of tissue age on (*R,S,*)-ABA catabolism.

VEGETATIVE TISSUES.

6.2.1.1. Effect of leaf age on (*R,S,*)-ABA catabolism in light-grown *Hordeum vulgare* cv. Dyan seedlings.

In order to see whether leaf age played a role in regulating the catabolism of applied, radiolabelled (*R,S,*)-ABA in monocotyledonous species this aspect was investigated using *Hordeum vulgare* leaves.

When (*R,S,*)-[2-^{14}C]-ABA was fed to excised, light-grown *Hordeum vulgare* leaves, harvested from seedlings of increasing ages, and the distribution of radioactivity between the catabolites of ABA determined, the result presented in Figure 6.1 was obtained. This indicates that ABA was catabolised more efficiently in older leaves than in younger leaves. Although young leaves accumulated aqueous conjugates, their ability to transform ABA into its acidic catabolites was markedly reduced.

6.2.1.2. The effect of leaf age on (*R,S,*)-ABA catabolism in etiolated leaves of *Hordeum vulgare* cv. Dyan.

Since ABA was catabolised more efficiently in older, light-grown leaves of *Hordeum vulgare* it was

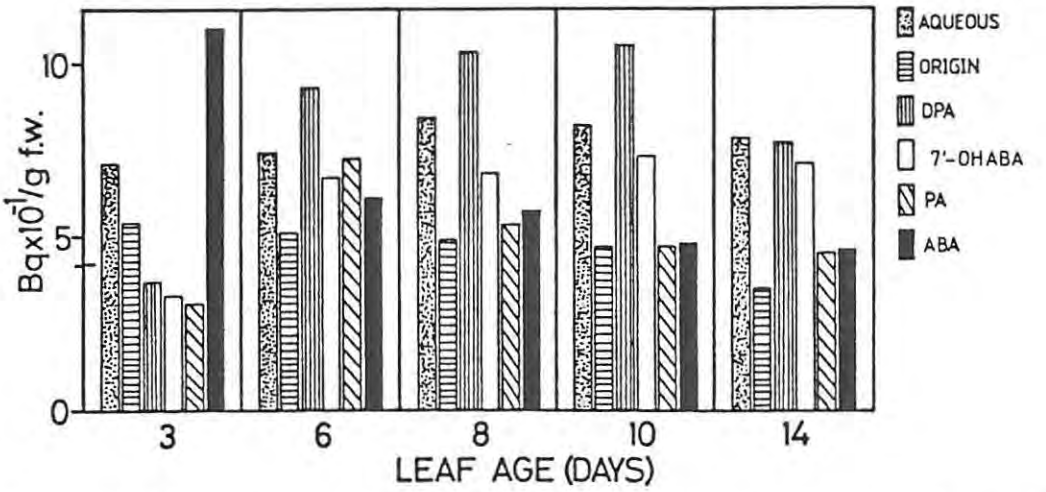

Figure 6.1. The effect of tissue age on the catabolism of (*R,S,*)-ABA by excised, light-grown leaves of *Hordeum vulgare*. Excised, light-grown leaves (1g f.w.) were fed with (*R,S,*)-[2-^{14}C]-ABA (4.2kBq) *via* the transpiration stream and catabolism allowed to proceed for 30h at 25°C. ABA and its catabolites were extracted and analysed as described in Chapter 2.

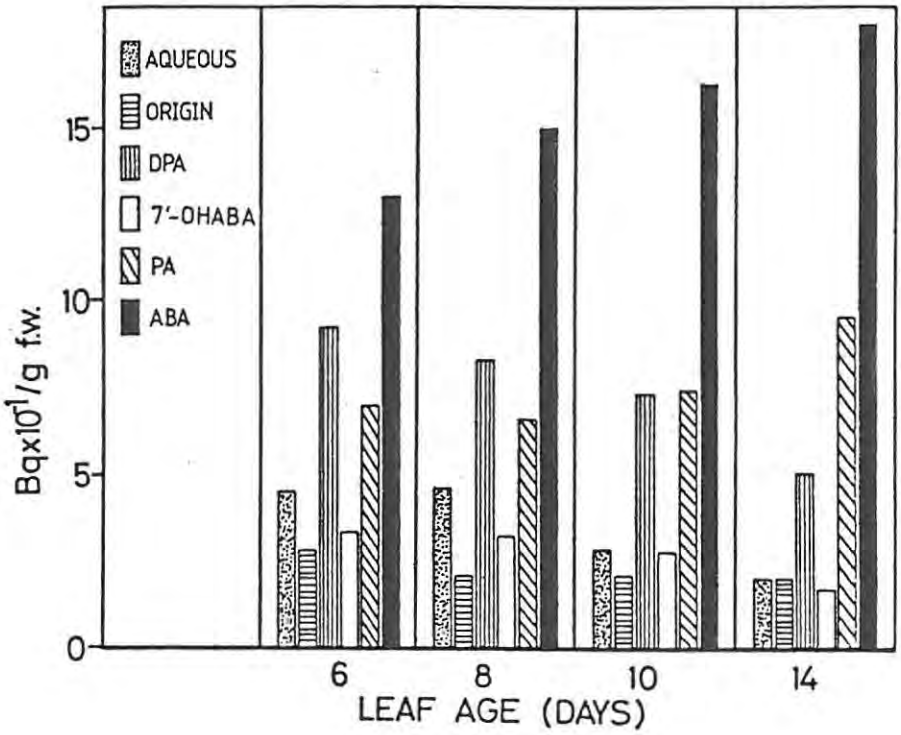

Figure 6.2. The effect of tissue age on the catabolism of (*R,S,*)-ABA by excised, etiolated leaves of *Hordeum vulgare*. Excised, etiolated leaves (1g f.w.) were fed with (*R,S,*)-[2-^{14}C]-ABA (4.2kBq) *via* the transpiration stream and catabolism allowed to proceed for 30h at 25°C in total darkness. ABA and its catabolites were extracted and analysed as described in Chapter 2.

of interest to investigate this aspect in leaves from etiolated seedlings of this species.

The catabolism of (*R,S,*)-[2-^{14}C]-ABA in etiolated *Hordeum vulgare* leaves of increasing tissue age was determined and the results are depicted in Figure 6.2. Etiolated leaves from younger seedlings were more able to catabolise (*R,S,*)-ABA than were their older counterparts, with leaves from 6d old seedlings exhibiting the greatest ability to catabolise applied, radiolabelled (*R,S,*)-ABA. This is in marked contrast to the situation in light-grown leaves of *Hordeum vulgare*.

6.2.1.3. The effect of leaf age on PA catabolism in light-grown leaves of *Hordeum vulgare* cv. Dyan.

In an attempt to determine whether the reduced ability of young leaves to catabolise ABA also reflected their competence to transform PA into DPA and water-soluble conjugates the catabolism of applied PA was examined. If PA were transformed with similar efficiency in tissue of all ages, this might indicate that younger leaves had a lowered capacity for converting ABA to PA than their older counterparts.

Applied [^{14}C]-PA was catabolised more efficiently in older tissue (Figure 6.3). Since younger leaves of *Hordeum vulgare* catabolised both ABA and PA, *albeit* in lower yields than older leaves, the results suggest that young leaves were less able to catabolise both ABA and PA.

6.2.1.4. (*R,S,*)-ABA catabolism in *Hordeum vulgare* cv. Dyan leaf sections.

A compounding factor in experiments on leaf age and (*R,S,*)-ABA catabolism in monocots is the gradation in tissue age along the length of the leaves, with the older tissue at the tip region and younger tissue closer to the intercalary meristem. Thus, 1cm sections of tissue, taken along the length of leaves from 10d old *Hordeum vulgare* seedlings, were excised and fed with (*R,S,*)-[2-^{14}C]-ABA and the efficiency of catabolism determined. Analysis of the ethyl acetate soluble acids gave the results depicted in Figure 6.4. Similar to intact leaf tissue, sections from older tissue catabolised (*R,S,*)-ABA more efficiently than young tissue close to the intercalary meristems.

6.2.1.5. Effect of leaf age on (*R,S,*)-ABA catabolism in *Phaseolus vulgaris* cv. Top-crop seedlings.

In order to examine the effect of leaf age on the catabolism of ABA in dicotyledons, (*R,S,*)-[2-^{14}C]-ABA was fed to excised light-grown leaves of *Phaseolus vulgaris* of increasing age. The data,

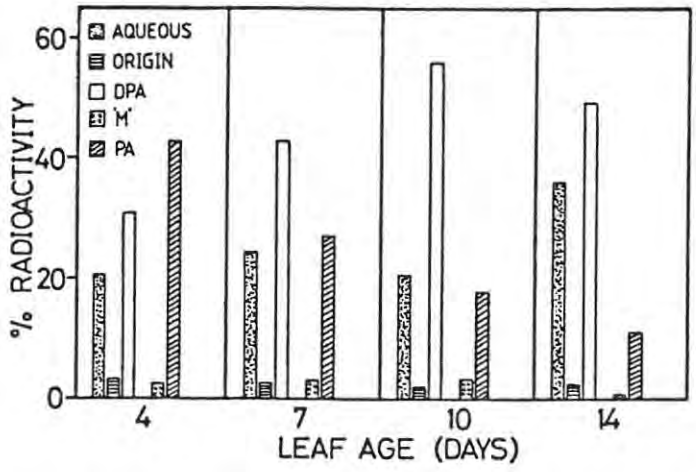

Figure 6.3. The effect of tissue age on the catabolism of PA by excised, light-grown leaves of *Hordeum vulgare*. Excised, light-grown leaves (1g f.w.) were fed with [^{14}C]-PA (0.84kBq) *via* the transpiration stream and catabolism allowed to proceed for 30h at 25^{o}C. ABA and its catabolites were extracted and analysed as described in Chapter 2.

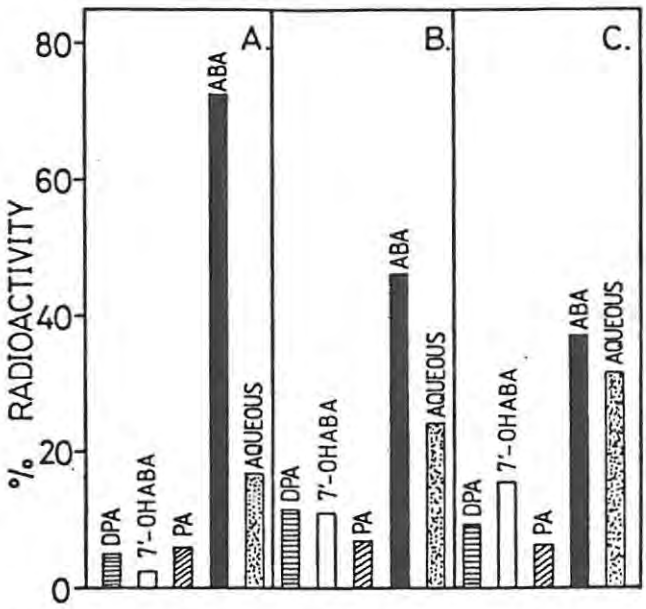

Figure 6.4. The catabolism of (*R,S,*)-ABA in leaf sections of increasing age along the length of excised, light-grown *Hordeum vulgare* seedlings. (A) Basal section; (B) intermediate section and (C) tip section. 1.0cm Leaf setions (0.2g f.w.) were excised from the leaf base (youngest), the tip of the leaf (oldest) and the intermediate region. Sections were floated on 2.0ml K_2HPO_4/KH_2PO_4 (10mM, pH7.5) containing (*R,S,*)-[2-^{14}C]-ABA (1.3kBq). Uptake was facilitated under vacuum (2min) and the leaf sections incubated in an orbital shaker for 48h at 25^{o}C under constant illumination (4.8μmol m^{-2} s^{-1}). ABA and its catabolites were extracted and analysed as described in Chapter 2.

(Figure 6.5) indicated that leaves from mature seedlings catabolised (*R,S,*)-ABA more efficiently although, young leaves incorporated radioactivity into conjugates as efficiently as mature leaves, while leaves of intermediary ages (8 and 10d old seedlings) were less able to incorporate label from (*R,S,*)-ABA into its acidic catabolites and aqueous conjugates. Thus, mature leaves of *Phaseolus vulgaris* appear to catabolise applied, radiolabelled ABA more efficiently than younger leaves of this species. After these studies had been completed, Cornish and Zeevaart (1984) reported similar findings in *Xanthium strumarium*.

NON-VEGETATIVE TISSUES.

6.2.1.6. The effect of seed size on (*R,S,*)-ABA catabolism in immature seeds of *Pisum sativum* cv. Progress No.9.

Other than the study reported in Chapter 5, no published information is currently available on the catabolism of ABA in immature seeds of *Pisum sativum*. It has been shown that the metabolism of gibberellins in immature seeds of *Pisum sativum* is biphasic and to some extent dependent on the stage of development, and hence organ size (Graebe, 1969; 1986; Sponsel, 1983; 1985). Thus, the effect of seed size, and hence developmental stage, on the catabolism of (*R,S,*)-ABA in immature seeds of *Pisum sativum* was investigated. Immature seeds of *Pisum sativum* cv. Progress No.9 were harvested at 10 (small seeds; young tissue) and 21 days (large seeds; old tissue) following anthesis and incubated in the presence of radiolabelled (*R,S,*)-ABA and the efficiency of these organs to catabolise ABA determined.

The data presented in Table 6.1 indicates that 10d old, small, immature seeds catabolised (*R,S,*)-ABA more readily than did the 21d old, larger immature seeds. This, is therefore in contrast to the findings in vegetative tissues and suggests that young, immature seeds contain higher levels of the enzymes required for ABA catabolism.

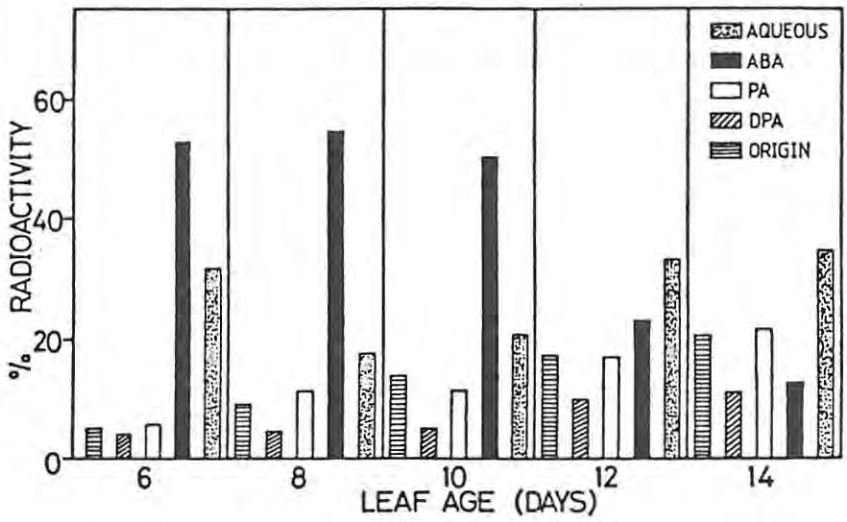

Figure 6.5. The catabolism of (*R,S,*)-ABA in excised, light-grown expanding leaves of *Phaseolus vulgaris*. The first true leaves from 6d (0.14g f.w.), 8d (0.96g f.w.), 10d (1.4g f.w.), 12d (3.0g f.w.) and 14d (4.2g f.w.) old seedlings were fed with (*R,S,*)-[2-^{14}C]-ABA (2.6kBq) *via* the transpiration stream and catabolism allowed to proceed for 30h at 25^{o}C under continuous illumination (66μmol m^{-2} s^{-1}). ABA and its catabolites were extracted and analysed as described in Chapter 2.

TABLE 6.1: The catabolism of ABA in immature seed of Pisum sativum cv. Progress No. 9 harvested 10 and 21 days following anthesis.

Immature seed harvested at 10d (13.9g f.w.) and 21d (21.5g f.w.) were washed in distilled water and placed in 100ml Erlenmyer flasks containing 20ml nutrient medium (Nitsch, 1951) and (R,S)-[2-^{14}C]-ABA (8.3kBq). Uptake was facilitated by placing the flasks under vacuum for 1 min and the tissue incubated under constant illumination (14.4μmol m^{-2}s^{-1}) at 28°C in a metabolic shaker for 48h. ABA and its catabolites were extracted and analysed as described in Chapter 2.

Tissue Age (days)	Distribution of Radioactivity					
	Origin	DPA	Catabolite 3	PA	ABA	Aqueous conjugates
	Bq (%)					
10	722.7 (8.8)	435.5 (5.2)	85.5 (1.0)	74.5 (0.8)	5132.8 (61.6)	1887.5(22.6
21	348.8 (4.2)	92.4 (1.1)	25.5 (0.3)	76.1 (0.9)	6567.6 (78.8)	1222.8(14.7

6.2.2. Studies on the effect of light on (*R,S,*)-ABA catabolism.

VEGETATIVE TISSUE.

6.2.2.1. (*R,S,*)-ABA catabolism in light-grown and etiolated seedlings of *Hordeum vulgare* cv. Dyan, *Pisum sativum* cv. Black-eyed Susan and *Phaseolus vulgaris* cv. Top-crop.

In further defining the conditions which might influence the catabolism of ABA in plants, the effect of light on this process was investigated. Initially, a preliminary study was undertaken in order to examine the ability of 10d old, light-grown and etiolated seedlings to catabolise (*R,S,*)-ABA. [2-^{14}C]-ABA was fed to both excised leaves of etiolated and light-grown *Hordeum vulgare* seedlings and to excised shoots from etiolated and light-grown *Phaseolus vulgaris* and *Pisum sativum* seedlings and the incorporation of label into the products determined.

The results presented in Table 6.2 show that light had no apparent effect on the catabolism of radiolabelled (*R,S,*)-ABA in excised shoots of either *Phaseolus vulgaris* or *Pisum sativum*. However, marked differences in the catabolism of ABA were observed between etiolated and light-grown leaves of *Hordeum vulgare*. Light-grown leaves appeared more efficient at transforming labelled (*R,S,*)-ABA into aqueous conjugates and 7'-hydroxy ABA. The increased production of radiolabelled aqueous conjugates suggested that light-grown leaves of *Hordeum vulgare* possessed a greater capacity to catabolise (*R,S,*)-ABA. Therefore, further studies were carried out using this species.

TABLE 6.2: Comparison of the catabolism of (R,S)-ABA by light-grown and etiolated excised, intact seedlings.

5g fresh weight of tissue from excised, 10d old light-grown, or 10d old etiolated seedlings of either Hordeum vulgare cv. Dyan, Pisum sativum cv. Black-eyed Susan or Phaseolus vulgaris cv. Top-crop were stood with proximal ends in 0.4 ml K_2HPO_4/KH_2PO_4 buffer (10mM; pH 7.5) containing (R,S)-[2-^{14}C]-ABA (4.2 kBq) at 25°C in either darkness or under continuous illumination (66μmol $m^{-2}s^{-1}$). Once all the substrate had been taken up via the transpiration stream, catabolism was allowed to proceed for 30h. ABA and its catabolites were extracted and analysed as described in Chapter 2.

		Radioactivity in ABA and its catabolites					
Tissue	Light-grown or Etiolated	Conjugates	DPA	7'-hydroxy ABA	'3'	PA	ABA
		Bq (%)					
Hordeum vulgare	L	1608.6(38.3)	1066.8(25.4)	777.0(18.5)	-	368.4(9.2)	348.6(8.3)
	E	718.2(17.1)	1155.0(27.5)	331.8(7.9)	-	302.4(7.2)	1663.2(39.6)
Pisum sativum	L	1356.6(31.3)	1197.0(28.5)	-	205.8(4.9)	134.4(3.2)	1306.2(31.1)
	E	1218.0(29.0)	877.8(20.9)	-	176.4(4.2)	100.8(2.4)	1827.0(43.5)
Phaseolus	L	2074.8(39.4)	936.6(22.3)	-	-	554.4(13.2)	1054.2(25.1)
vulgaris	E	1495.2(35.6)	911.4(21.7)	-	-	583.8(13.9)	1209.6(28.8)

6.2.2.2. Kinetic analyses and refeeding studies in light-grown and etiolated *Hordeum vulgare* cv. Dyan leaves.

Kinetic studies revealed that with a decline in levels of substrate (*R,S,*)-[2-^{14}C]-ABA (Figure 6.6E) there was a concomitant increase in the levels of PA (Figure 6.6A), 7'-hydroxy ABA (Figure 6.6B), DPA (Figure 6.6C) and water-soluble conjugates (Figure 6.6D) in both light-grown and etiolated leaves of *Hordeum vulgare*. However, the rate of incorporation of label, from substrate (*R,S,*)-ABA into the acidic catabolites was markedly reduced in etiolated leaves. In order to examine this aspect further, biosynthetically prepared PA, 7'-hydroxy ABA and DPA were fed to excised, etiolated and light-grown *Hordeum vulgare* leaves and the efficiency of catabolism determined.

When [^{14}C]-PA was refed to leaves of *Hordeum vulgare* (Figure 6.7A) it was catabolised more rapidly in light-grown tissues than in their etiolated counterparts. Similarly, 7'-hydroxy ABA (Figure 6.7B) was catabolised more rapidly in light-grown tissues, although the physiological significance of this result is uncertain in the absence of evidence that this compound is native to *Hordeum vulgare*. Little difference was observed in the catabolism of [^{14}C]-DPA (Figure 6.7C) between light-grown and etiolated leaves. These results indicate that light stimulated the catabolism of (*R,S,*)-ABA to PA in leaves of *Hordeum vulgare*. In addition, light would appear to be a necessary requirement for the enhanced conjugation of applied, radiolabelled ABA and its acidic catabolites in this tissue, perhaps as a result of the availability of sugars from photosynthesis, but possibly also as a result of light-mediated changes in enzyme levels and/or activity.

NON-VEGETATIVE TISSUES.

The effect of light on the catabolism of (*R,S,*)-[2-^{14}C]-ABA was also investigated in immature seed of *Pisum sativum* and *Phaseolus vulgaris* and in *Persea americana* mesocarp.

6.2.2.3. Effect of light on (*R,S,*)-ABA catabolism in immature seed of *Pisum sativum* L. and *Phaseolus vulgaris* L.

(*R,S,*)-[2-^{14}C]-ABA was added to 15ml of nutrient medium containing immature seeds of either *Pisum sativum* or *Phaseolus vulgaris* and the tissues incubated under conditions of continuous

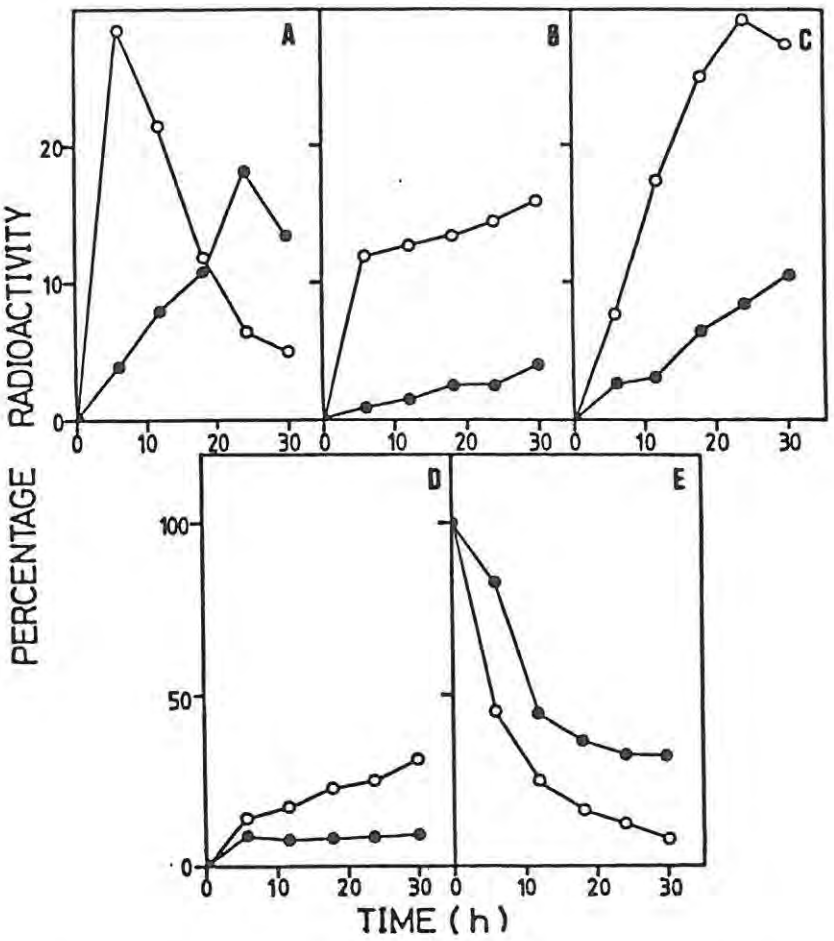

Figure 6.6. Kinetics of (*R,S,*)-ABA catabolism in excised, light-grown (o) and etiolated (●) *Hordeum vulgare* leaves. (*R,S,*)-[2-^{14}C]-ABA (2.4kBq) was fed to 10d old excised, light-grown and etiolated leaves *via* the transpiration stream. Light-grown tissue was incubated at 25°C under continuous illumination (66μmol m^{-2} s^{-1}) while etiolated tissue was incubated in total darkness under the same conditions for varying lengths of time. ABA and its catabolites were extracted and analysed as described in Chapter 2.

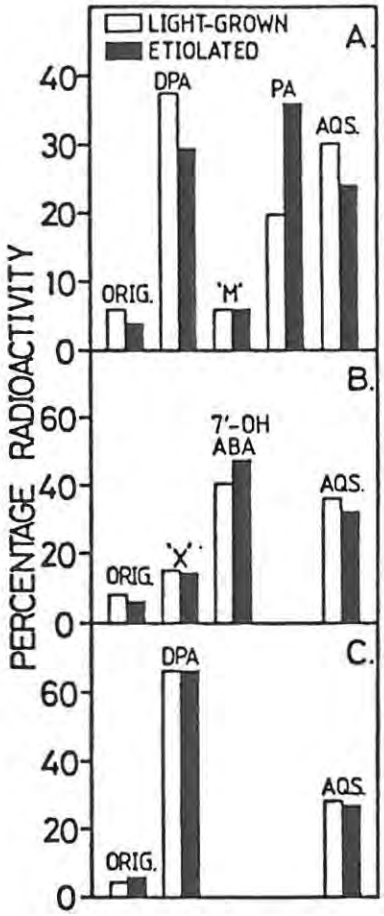

Figure 6.7. The catabolism of PA, 7'-hydroxy ABA and DPA in excised, light-grown and etiolated *Hordeum vulgare* seedlings. [^{14}C]-PA, [^{14}C]-7'-hydroxy ABA and [^{14}C]-DPA (all 2.5kBq) were fed to excised, light-grown and etiolated leaves *via* the transpiration stream and catabolism allowed to proceed for 30h at 25^{o}C. Light-grown tissues were incubated under continuous illumination (66μmol m^{-2} s^{-1}) and etiolated tissues in total darkness. The catabolites of PA, 7'-hydroxy ABA and DPA were extracted and analysed as described in Chapter 2.

illumination (42μmol m^{-2} s^{-1}) or in total darkness. After a 48h incubation period the tissues were extracted and and the efficiency of catabolism determined.

In all cases, continuous illumination resulted in enhanced (*R,S,*)-ABA catabolism (Table 6.3) and suggested that light may be involved in the regulation of (*R,S,*)-ABA catabolism in immature seeds of *Pisum sativum* and *Phaseolus vulgaris*

6.2.2.4. Effect of light on (*R,S,*)-ABA catabolism in *Persea americana* cv. Fuerte mesocarp tissue.

The efficiency of ABA catabolism in ripening fruits of *Persea americana* which were incubated under conditions of either constant illumination (66μmol m^{-2} s^{-1}) or in total darkness was determined and gave the results shown in Table 6.4. Applied, radiolabelled ABA was more efficiently catabolised in tissues maintained under conditions of constant illumination. These results, together with those on ABA biosynthesis in Chapter 4, illustrate that while light reduced the incorporation of MVA into ABA by ±30%, it enhanced the catabolism of ABA in *Persea americana* mesocarp by ±45%. The general enhancement of ABA catabolism in the light-grown tissues used in this study could be indicative of light-regulated increases in some of the enzymes involved in ABA catabolism.

6.2.3.Studies on the effect of water-stress on the catabolism of (*R,S,*)-ABA in plant tissues.

The few studies which have been carried out on the catabolism of ABA in relation to water-stress in plants, employed *Phaseolus vulgaris* (Harrison and Walton, 1975), *Xanthium strumarium* (Cornish and Zeevaart, 1984) and *Triticum aestivum* (Lehmann and Schütte, 1984; Murphy, 1984) seedlings and showed that ABA catabolism was unchanged following wilting. Therefore, attempts were made to investigate the effects of water-stress on ABA catabolism in excised leaves of *Hordeum vulgare* in which the effect of wilting on ABA catabolism is unknown.

6.2.3.1. Effect of water stress on the catabolism of (*R,S,*)-ABA in light- grown leaves of *Hordeum vulgare* cv. Dyan.

Excised leaves of *Hordeum vulgare* were fed with (*R,S,*)-[2-^{14}C]-ABA and following uptake of the labelled substrate, were wilted until they had lost 12% of their original fresh weight. Stressed and non-stressed leaves were then incubated for a 30h period after which their capacity to catabolise

TABLE 6.3: The effect of light on ABA catabolism in immature seeds of Pisum sativum and Phaseolus vulgaris.

(R,S)-[2-^{14}C]-ABA (3.66 kBq) was added to 15.0 ml of nutrient medium (Nitsch, 1951) containing 10g f.w. of tissue. Samples were incubated for 48h in a orbital shaker at 28°C under continuous illumination (66µmol $m^{-2}s^{-1}$) or in darkness. Following incubation ABA and its catabolites were extracted and analysed as described in Chapter 2.

		Distribution of Radioactivity %				
Tissue	Treatment	Conjugates	DPA	'3'	PA	ABA
Pisum sativum	L	39.6	17.0	5.9	2.3	35.2
	D	28.5	8.1	2.1	1.3	59.7
Phaseolus vulgaris	L	46.6	12.0	7.9	10.1	23.4
	D	27.2	5.8	3.0	4.8	59.2

TABLE 6.4: Effect of light on ABA catabolism in Persea americana cv. Fuerte mesocarp.

20 gram blocks of Persea americana mesocarp, excised from ripening fruits, were fed with (R,S)-[2-^{14}C]-ABA (3.66 kBq) to the cut surface in 0.2 ml Tween 80/acetone/water (1:1:8, v/v) and incubated for 24h at 25°C in a water-saturated environment under conditions of continuous illumination (66µmol $m^{-2}s^{-1}$) or in total darkness. ABA and its catabolites were extracted and analysed as described in Chapter 2.

	Distribution of Radioactivity				
Treatment	Conjugates	DPA	'3'	PA	ABA
			Bq (%)		
Light	1427.4 (39.0)	373.3 (10.2)	87.8 (2.4)	73.2 (2.0)	1698.2 (46.4)
Dark	563.6 (15.4)	58.6 (1.6)	29.3 (0.8)	43.9 (1.2)	2957.3 (80.8)

applied, radiolabelled ABA was determined.

Unlike the results reported by Murphy (1984) for *Triticum aestivum*, the solvent system chloroform/methanol/water (75:22:3, v/v) did not adequately resolve the catabolites of (*R,S,*)-[2-^{14}C]-ABA (Figure 6.8A and B) and thus the solvent system toluene/ethyl acetate/acetic acid (50:30:4, v/v) was routinely used in these studies. The results shown in Figure 6.9 demonstrate that the catabolism of ABA in stressed tissue was markedly different from that in their turgid counterparts. In contrast, there was little difference in the catabolism of applied, radiolabelled (*R,S,*)-ABA between stressed and non-stressed *Phaseolus vulgaris* seedlings (Table 6.5), which confirmed the results obtained by Harrison and Walton (1975). This suggested that the stress-induced increases in endogenous ABA levels in *Phaseolus vulgaris* (Harrison and Walton, 1975; Zeevaart and Milborrow, 1976) were probably not due to the inhibition of ABA catabolism in response to wilting in this tissue.

In excised leaves of *Hordeum vulgare* however, water-stress reduced the incorporation of radioactivity into DPA by 88.5%, 7'-hydroxy ABA by 77.3% and water-soluble conjugates by 47.0%. This decrease in these catabolites was accompanied by a decrease in substrate (*R,S,*)-ABA while PA rose by 20% in stressed tissue. This suggested that the reduced amounts of substrate were due to the the enhanced conversion of ABA to PA which appeared to be stimulated by water-stress, whereas the conversion of ABA to 7'-hydroxy ABA and aqueous conjugates was inhibited. However, higher incorporation into PA could have resulted from the rapid transformation of ABA to PA during the uptake period, particularly given the kinetics for *Hordeum vulgare* leaves shown in Chapter 5, prior to the imposition of stress, or due to the inhibition of PA reduction to DPA.

In order to investigate these possibilities, firstly the amount of PA produced during the uptake of labelled ABA was determined. The data presented in Figure 6.10 shows that the amount of PA produced during uptake was significantly less than that present at the end of the incubation period in stressed tissues (Figure 6.9B). These data therefore precluded an effect of water-stress on the conversion of ABA to PA and suggested that PA, in stressed tissues, might increase due to stress-inhibited reduction of PA to DPA.

Secondly, kinetic studies on the fate of applied, radiolabelled (*R,S,*)-ABA in stressed and non-stressed leaves of *Hordeum vulgare* were undertaken in order to clarify the effect of water-stress on ABA catabolism and in particular the reduction of PA to DPA. The data presented in Figure 6.11 suggested that water-stress inhibited the reduction of PA to DPA. Following the onset of

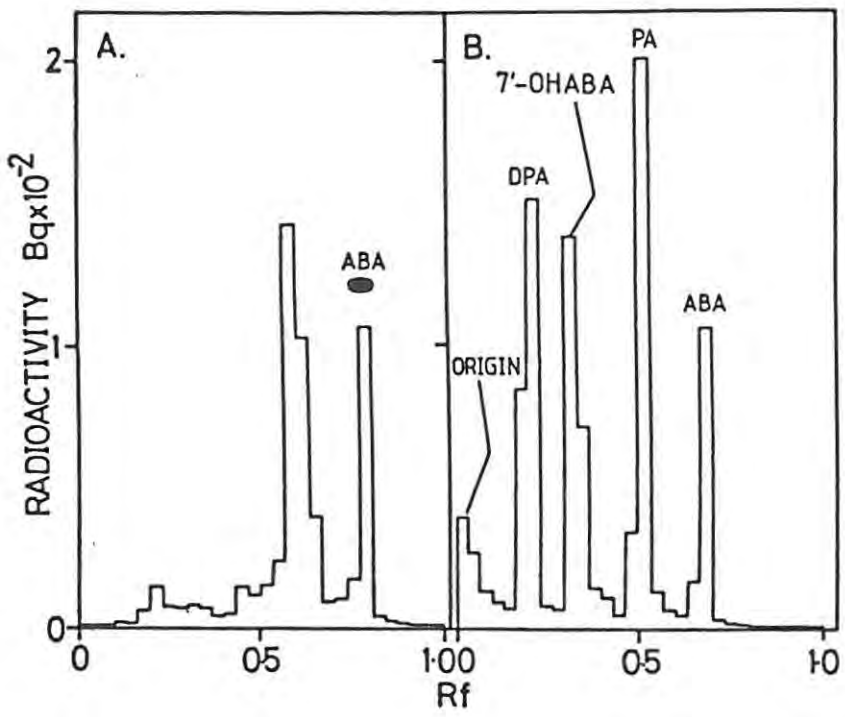

Figure 6.8. Thin layer chromatograms of the radioactive, ethyl acetate-soluble acids from extracts of excised leaves of *Hordeum vulgare*. Leaves were fed with (*R,S,*)-[2-^{14}C]-ABA (2.3kBq) and catabolism allowed to proceed for 30h after which the ethyl acetate-soluble acids were separated by TLC on silica gel GF_{254} in either, (A) chloroform/methanol/water (75:22:3, v/v), or (B) toluene/ethyl acetate/acetic acid (50:30:4, v/v) as described in Chapter 2.

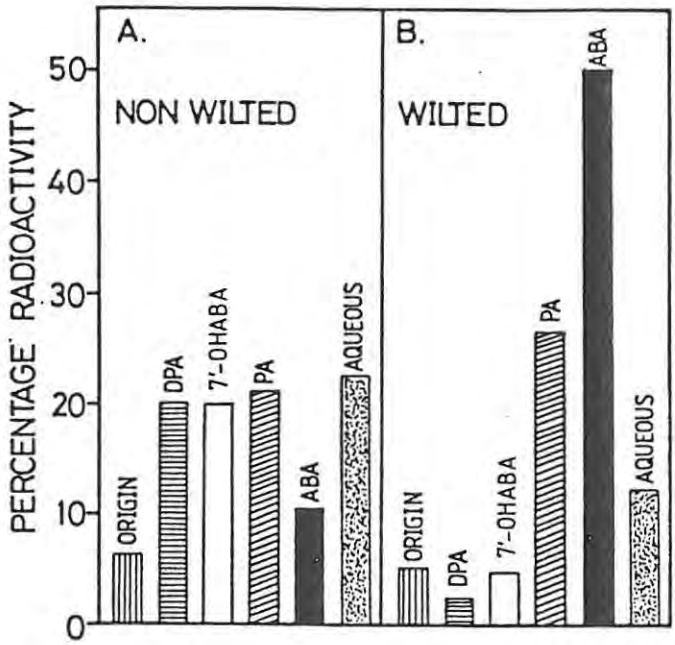

Figure 6.9. Percentage radioactivity in ABA and its catabolites after feeds of (*R,S,*)-ABA to leaves of *Hordeum vulgare* which were then wilted. (*R,S,*)-[2-^{14}C]-ABA was taken up *via* the transpiration stream. Following uptake (1-2h) one batch of tissue was wilted until it had lost 12% of its original fresh weight. Catabolism was then allowed to proceed for 30h at 25°C under continuous illumination (66μmol m^{-2} s^{-1}). Tissues were incubated in clear, plastic bags. Non-wilted tissues were maitained under identical conditions in plastic bags containing wetted strips of paper towel. ABA and its catabolites were processed as described in Chapter 2.

TABLE 6.5: The effect of water stress on the catabolism of (R,S)-ABA in excised, intact Phaseolus vulgaris cv. Top-crop seedlings.

Excised, light-grown seedlings of Phaseolus vulgaris (5g f.w.) were fed with (R,S)-[2-^{14}C]-ABA (2 kBq) via the transpiration stream in 0.2 ml K_2HPO_4/KH_2PO_4 buffer (10 mM, pH 7.5). Following uptake one batch of tissue was transferred to a stream of warm air until it had lost 15% of its original fresh weight. Samples were incubated in polythene bags for 20h under constant illumination at 25°C. Turgid samples contained moistened tissue paper and were incubated in a water-saturated environment. ABA and its catabolites were extracted and processed as described in Chapter 2.

	Distribution of Radioactivity			
Treatment	Aqueous conjugates	DPA	PA	ABA substrate remaining
	Bq (%)			
Turgid	1098.4 (54.92)	419.2 (20.96)	267.6 (13.38)	216.8 (10.84)
15% Wilt	1111.8 (55.59)	437.8 (21.89)	223.8 (11.19)	116.6 (8.33)

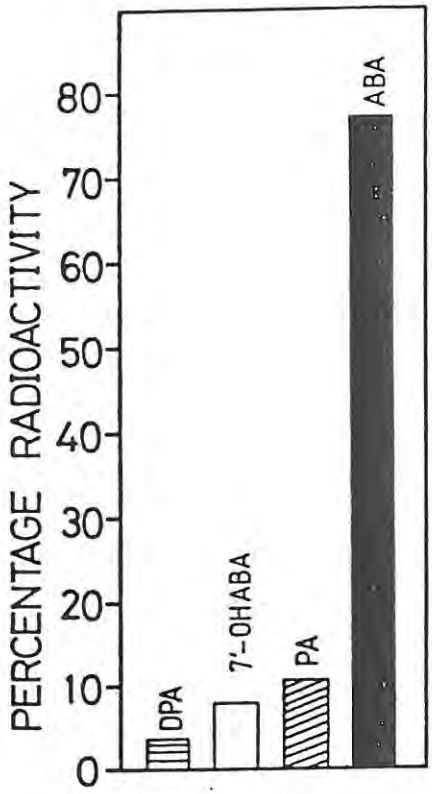

Figure 6.10. Percentage radioactivity in ABA and its catabolites at the end of the uptake period (2h) after supplying (*R,S,*)-[2-^{14}C]-ABA to turgid, excised leaves of *Hordeum vulgare* as in Figure 6.9. ABA and its catabolites were extracted and analysed as described in Chapter 2.

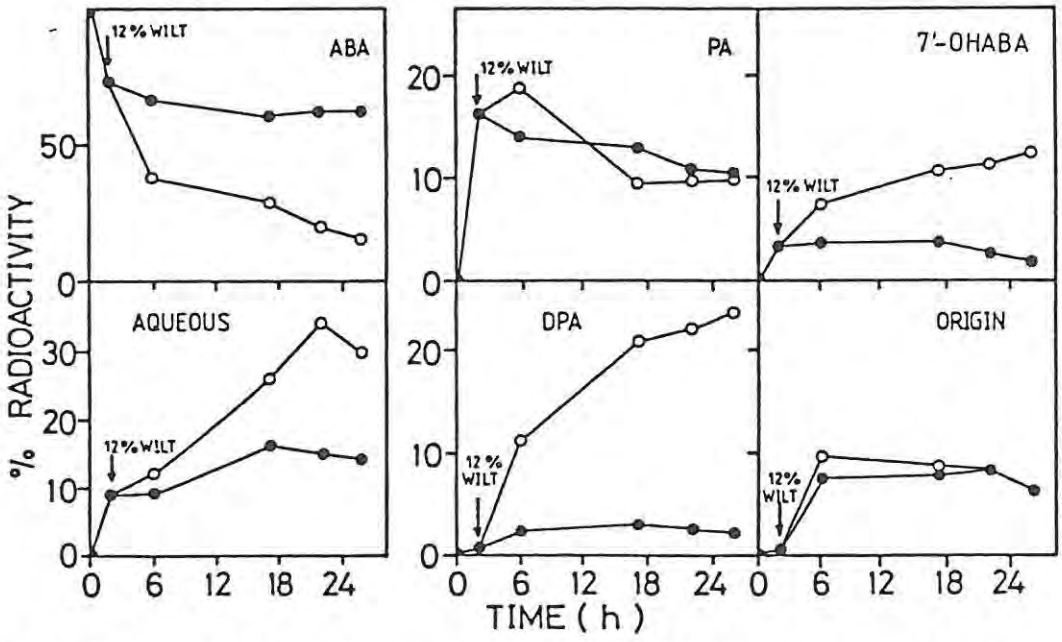

Figure 6.11. The kinetics of (*R,S,*)-ABA catabolism in tugid (o) and wilted (●) leaves of *Hordeum vulgare*. (*R,S,*)-[2-^{14}C]-ABA (2.3kBq) was supplied to turgid leaves *via* the transpiration stream. Following uptake one batch of tissue was wilted until it had lost 12% of its original fresh weight. Tissues were incubated for varying lengths of time as described in Figure 6.9 and ABA and its catabolites extracted and analysed as described in Chapter 2.

stress the production of DPA from PA was rapidly curtailed. Although incorporation into PA in turgid leaves was higher than that in wilted leaves immediately after the onset of stress, by 30h this stuation had been reversed. Furthermore, these data revealed the stress-induced inhibition of the transformation of labelled ABA to 7'-hydroxy ABA and aqueous conjugates. The conversion of ABA to PA appeared less affected since the rate of PA disappearance was greater for non-stressed leaves. Thus, the effects of water stress on PA catabolism in leaves of *Hordeum vulgare* were examined in more detail.

6.2.3.2. Effect of water stress on the catabolism of [^{14}C]-PA in leaves of *Hordeum vulgare* cv. Dyan.

The catabolism of [^{14}C]-PA was investigated in stressed and non-stressed *Hordeum vulgare* leaves. Following uptake of labelled PA, leaf tissue was wilted until it had lost 12% of its original fresh weight and its capacity to catabolise PA determined.

The results presented in Figure 6.12 show that the reduction of PA to DPA was inhibited by 71.5% in stressed tissues. In addition, water-stress did not effect the production of water-soluble conjugates from PA. Thus, these results suggest that in leaves of *Hordeum vulgare*, water-stress inhibited the reduction of PA to DPA and the production of water-soluble conjugates of ABA.

6.2.3.3. Effect of (*R,S,*)-ABA on the catabolism of (*R,S,*)-[2-^{14}C]-ABA in leaves of *Hordeum vulgare* cv. Dyan.

An additional factor which may have contributed to the observed effects of water-stress on (*R,S,*)-ABA catabolism in leaves of *Hordeum vulgare* was that the labelled substrate would be diluted by elevated levels of endogenous ABA which might have led to the apparent reduction in catabolism in wilted tissues. In order to examine this possibility, the endogenous pool of ABA was artificially increased by feeding, simultaneously, non-radioactive and radioactive (*R,S,*)-ABA to excised, light-grown leaves of *Hordeum vulgare* and catabolism followed.

The results presented in Table 6.6 show that when (*R,S,*)-[2-^{14}C]-ABA was fed to tissues in the presence of non-radioactive (*R,S,*)-ABA, at levels similar to those reported for ABA in stressed leaves of other monocotyledons (Quarrie, 1981), little difference was observed in the levels of acidic catabolites generated. Nevertheless, 1μg of (*R,S,*)-ABA did reduce the levels of radioactivity incorporated into the aqueous fraction. However, increasing the amounts of "cold" (*R,S,*)-ABA well

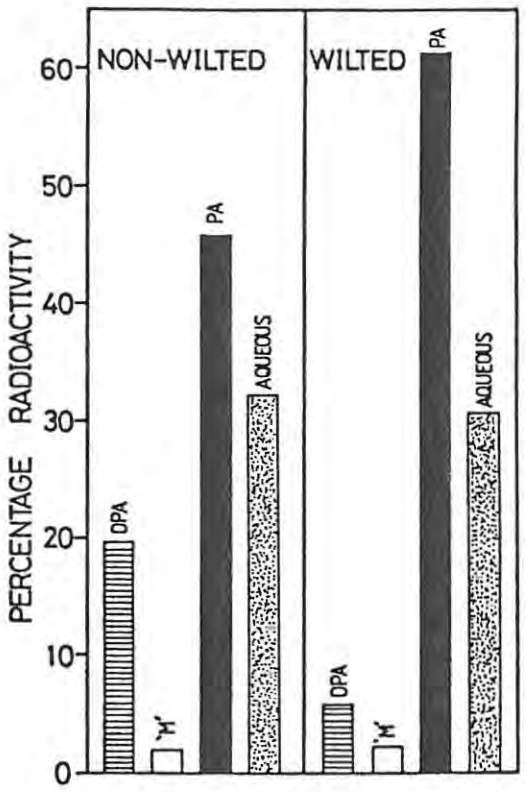

Figure 6.12. Percentage of radioactivity in PA and its catabolites in turgid and wilted leaves of *Hordeum vulgare*. [^{14}C]-PA (500Bq) was supplied to turgid, excised leaves and after uptake, one batch of tissue was wilted until it had lost 12% of its original fresh weight, while an identical batch was kept turgid. Catabolism was allowed to proceed for 30h after which the distribution of radioactivity in PA and its catabolites was determined as described in Chapter 2.

TABLE 6.6: The effect of dilution with non-radioactive (R,S)-ABA on the catabolism of (R,S)-[2-^{14}C]-ABA by turgid, excised, light-grown leaves of Hordeum vulgare cv. Dyan.

(R,S)-[2-^{14}C]-ABA (2.3kBq, 636 ng) was mixed with (R,S)-ABA (1µg), fed to turgid leaves of Hordeum vulgare via the transpiration stream and the tissues incubated for 30h under continuous illumination (66µmol m^{-2} s^{-1}) at 25°C. The radioactive ABA catabolites were extracted and analysed as described in Chapter 2.

Treatment	Origin	DPA	7'-hydroxy ABA	PA	ABA	Aqueous conjugates
			Distribution of radioactivity (%)			
Control	5.3	18.8	11.9	19.4	25.2	19.4
+ 1µg (R,S)-ABA	5.9	19.2	11.7	20.8	27.4	14.9

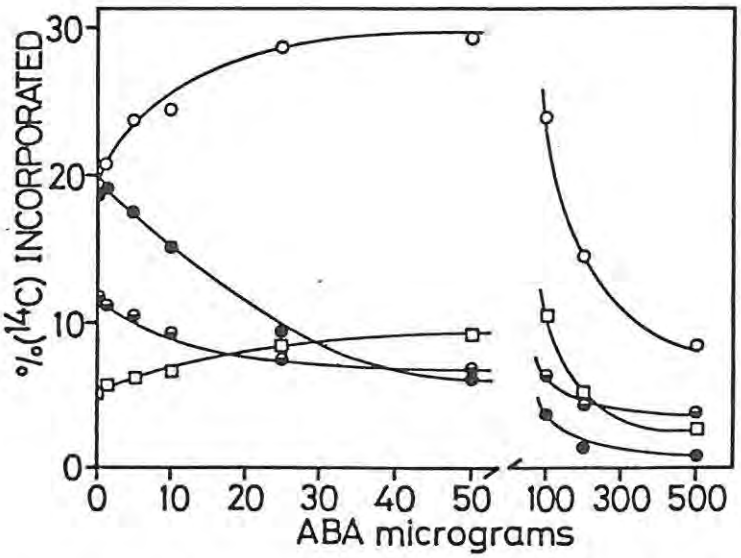

Figure 6.13. Effect of dilution with non-radioactive (*R,S,*)-ABA on the catabolism of (*R,S,*)-[2-^{14}C]-ABA by turgid, light-grown leaves of *Hordeum vulgare*. (*R,S,*)-[2-^{14}C]-ABA (2.3kBq; 636ng) was mixed with increasing amounts of (*R,S,*)-ABA, fed to turgid leaves (1g f.w.) *via* the transpiration stream and the tissue incubated for 30h at 25°C under cotinuous illumination (66µmol m^{-2} s^{-1}). ABA and its catabolites were extracted and analysed as described in Chapter 2. PA (o); DPA (●); 7'-hydroxy ABA (◒) and catabolite 1 (◘).

above the levels normally found in water-stressed leaves, demonstrated marked changes in the of applied, radiolabelled ABA in turgid leaves of *Hordeum vulgare* (Figure 6.13).

The production of both DPA from PA, and 7'-hydroxy ABA from ABA were markedly reduced while the incorporation of label from ABA into PA was enhanced in tissues fed simultaneously with "cold" ABA up to 50μg. The reduced incorporation of label into DPA and the enhanced levels of PA substantiate the earlier findings, where increases in endogenous levels of ABA due to the imposition of water-stress significantly reduced the production of DPA. Interestingly, only at levels above 100μg of "cold" (*R,S,*)-ABA was the production of PA from applied, labelled ABA significantly reduced. This suggests that stress-induced increases in endogenous levels of ABA did not result in the dilution of applied, labelled substate and that at high levels of applied "cold" ABA other factors were responsible for the observed changes in the catabolism of (*R,S,*)-[2-^{14}C]-ABA in turgid leaves of *Hordeum vulgare*.

6.2.4.Studies on the effect of chemical factors on the catabolism of (*R,S,*)-ABA in plant tissues.

The inhibition of ABA biosynthesis in *Persea americana* by cytokinins, ancymidol and CCC (see Chapter 4) might be an indirect effect due to enhanced catabolism. Thus, it was of interest to examine their effects on ABA catabolism in *Persea americana* mesocarp and other plant tissues. In addition, studies employing inhibitors of protein biosynthesis (see Chapter 4) suggested that cytoplasmic protein biosynthesis was necessary for ABA biosynthesis in plants. This in turn suggested that the enzymes involved in ABA biosynthesis were labile. Whether a similar situation holds for the catabolism of ABA is at present unknown, although Uknes and Ho (1984) have provided some information to suggest that it is required for this process in *Hordeum vulgare* aleurone layers. Thus, the effect of these inhibitors on the catabolism of applied, radiolabelled ABA was examined in both vegetative and non-vegetative higher plant tissues.

VEGETATIVE TISSUES.

6.2.4.1 Effect of chemical factors on (*R,S,*)-ABA catabolism in excised, light-grown leaves of *Hordeum vulgare* cv. Dyan.

6.2.4.1.1. Effect of cytokinins on the catabolism of (*R,S,*)-ABA in excised leaves of *Hordeum vulgare* cv. Dyan.

In order to determine whether cytokinins could influence the catabolism of ABA in higher plants the effects of these hormones on the catabolism of applied, radiolabelled (*R,S,*)-ABA was first of all investigated in light-grown leaves of *Hordeum vulgare*. Excised, light-grown leaves of *Hordeum vulgare* were pretreated with cytokinins *via* the transpiration stream and their efficiency to catabolise (*R,S,*)-ABA was then determined and the results are presented in Table 6.7. Although Skoog *et al* (1967) established a hierarchy of effectiveness for the various terminal substituents of cytokinins in callus bioassays: benzyl > furfuryl = phenyl > cyclohexyl, results from this study (Table 6.7) show marked variations. Zeatin and kinetin appeared to stimulate (*R,S,*)-ABA catabolism while BA and IPA appeared to retard the catabolism of (*R,S,*)-[2-^{14}C]-ABA. Adenine was used as a control to assess the role of the purine portion of the cytokinin molecules in the catabolism of (*R,S,*)-ABA.

In order to clarify these results, increasing concentrations of kinetin, which appeared to enhance (*R,S,*)-ABA catabolism, and increasing concentrations of IPA, which appeared to retard (*R,S,*)-ABA catabolism, were fed to leaves of *Hordeum vulgare* and the efficiency of (*R,S,*)-[2-^{14}C]-ABA catabolism determined (Table 6.8). Kinetin, at low concentrations, enhanced the catabolism of (*R,S,*)-ABA by ±150% and in particular the transformation of PA to DPA. However, with increasing concentrations of kinetin there was an observed decline in the levels of DPA produced while the levels of PA increased. This suggested that high concentrations of kinetin retarded the catabolism of (*R,S,*)-ABA by influencing the transformation of PA to DPA. With a decline in the oxidation of (*R,S,*)-ABA there was a concomitant increase in the levels of water-soluble conjugates produced. In the main, this result suggested that kinetin at low concentrations stimulated catabolism *via* oxidation, while at higher concentrations kinetin stimulated conjugation.

In contrast, IPA appeared to retard the catabolism of (*R,S,*)-ABA to PA and DPA at low and high

TABLE 6.7: The effect of cytokinins on the catabolism of (R,S)-ABA in excised, light-grown Hordeum vulgare cv. Dyan leaves.

Cytokinins (500μM) were fed to excised, light-grown 10d old Hordeum vulgare leaves via the transpiration stream in 0.5 ml K_2HPO_4/KH_2PO_4 buffer (10mM, pH 7.5). After 12h (R,S)-[2-^{14}C]-ABA (3.3kBq) was added to the small volume of uptake buffer remaining. Following uptake of the labelled substrate a further 4.0 ml of buffer was added and the tissue incubated for 30h at 25°C under continuous illumination (66μmol $m^{-2}s^{-1}$). ABA and its catabolites were extracted and analysed as described in Chapter 2.

	Distribution of Radioactivity					
Treatment	Origin	DPA	7'-hydroxy ABA	PA	ABA	Aqueous conjugates
			Bq (%)			
Control	475.2 (14.4)	158.4 (4.8)	491.7 (14.9)	191.4 (5.8)	726.0 (22.0)	1237.5(37.5)
Adenine	544.5 (16.5)	122.1 (3.7)	435.6 (13.2)	231.0 (7.0)	620.4 (18.8)	1300.2(39.4)
Zeatin	458.7 (13.9)	419.1(12.7)	234.3 (7.1)	438.9(13.3)	372.9 (11.3)	1359.6(41.2)
Kinetin	481.8 (14.6)	227.7 (6.9)	310.2 (9.4)	458.7(13.9)	105.6 (3.2)	1692.9(51.3)
BA	257.4 (7.8)	79.2 (2.4)	290.4 (8.8)	405.9(12.3)	1481.7 (44.9)	660.0(20.0)
IPA	161.7 (4.9)	92.4 (2.8)	161.7 (4.9)	300.3 (9.1)	2003.1 (60.7)	465.3(14.1)

TABLE 6.8: The effect of cytokinin concentration on the catabolism of (R,S)-ABA in leaves of Hordeum vulgare cv. Dyan.

Varying concentrations to kinetin and IPA were fed to excised, light-grown leaves of Hordeum vulgare via the transpiration stream as described in Table 6.7. After 12h (R,S)-[2-^{14}C]-ABA (3.3 kBq) was added. Following uptake the tissues were incubated for 30h at 25°C under constant illumination. The distribution of radioactivity in the catabolites of ABA was determined as described in Chapter 2.

		% Radioactivity relative to control[a]			
Treatment (mM)		Aqueous conjugates	DPA	7'hydroxy ABA	PA
Control		100	100	100	100
Kinetin	0.01	227.5	202.9	123.3	50.3
	0.05	257.5	209.2	149.9	59.8
	0.10	286.8	193.9	132.6	69.7
	0.20	374.4	181.6	102.7	94.7
	0.50*	385.9	121.7	92.4	139.9
	1.00*	403.1	60.6	85.0	212.9
IPA	0.01	214.2	147.6	107.4	75.7
	0.05	202.7	179.3	130.5	65.9
	0.10	216.3	198.7	118.0	51.8
	0.20	257.6	159.7	96.4	83.5
	0.50*	309.0	134.7	80.7	111.7
	1.00*	312.0	76.8	75.0	158.1

[a] Data for control presented in Table 6.7

* Where necessary a small volume of ethanol (0.25 ml) was added to facilitate solubility.

concentrations, while enhancing the oxidation of (*R,S,*)-ABA at intermediate concentrations (Table 6.8). The reason for this is unclear but might be due to the rapid metabolism of IPA to zeatin and dihydrozeatin (Sembdner *et al*, 1980), which could have given rise to this result. Low concentrations of IPA might in turn result in less zeatin being available to exert an effect whereas at higher concetrations feedback inhibition might result in the reduced production of zeatin thus retarding ABA catabolism. Therefore, it is possible that the effects of IPA on ABA catabolism might be dependent on the rate of transformation of IPA to zeatin in this tissue since zeatin significantly enhanced the catabolism of ABA in leaves of *Hordeum vulgare* by ±50% (Table 6.7). Nevertheless, IPA enhanced the incorporation of radioactivity into water-soluble products, the levels of which increased with increasing IPA concentration. One point of concern was that ethanol had been used to solubilise cytokinins above 0.2mM and this might have had a deleterious effect on ABA catabolism. However, data from control samples indicated that the small amounts of ethanol used (0.25ml) had no apparent adverse effects on ABA catabolism.

6.2.4.1.2. Effect of AMO1618, ancymidol and CCC on (*R,S,*)-ABA catabolism in leaves of *Hordeum vulgare* cv. Dyan.

Inhibitors of GA biosynthesis have been shown to inhibit ABA biosynthesis in ABA-producing fungi (Norman *et al*, 1983; Hirai *et al*, 1986) and in fruits of *Persea americana* (see Chapter 4). However, nothing is known of their effects on ABA catabolism in vegetative plants. Thus, the effect of three inhibitors of GA biosynthesis, AMO1618, CCC and ancymidol, on ABA catabolism was investigated in excised, light-grown leaves of *Hordeum vulgare*. These were chosen for their different mechanisms of action. AMO1618 and CCC inhibits kaurene synthetase (Dennis *et al*, 1965; Frost and West, 1977), a cyclase, whereas ancymidol inhibits the mixed function oxidase, kaurene oxidase (Coolbaugh, 1984).

Treatment of light-grown leaves of *Hordeum vulgare* with AMO1618 and ancymidol had little effect on the incorporation of label from (*R,S,*)-[2-^{14}C]-ABA into PA and DPA (Table 6.9), while CCC reduced the production of both PA and DPA by ±20%. In addition, CCC, ancymidol and AMO1618 markedly reduced the production of 7'-hydroxy ABA by more than 50%, and CCC and ancymidol treatments reduced the incorporation of label into the aqueous fractions by 51% and 43.4% respectively.

TABLE 6.9: The effect of AM01618, CCC and ancymidol on the catabolism of (R,S)-ABA in excised, light-grown leaves of Hordeum vulgare cv. Dyan.

Excised leaves (2g f.w.) were placed in 0.4 ml K_2HPO_4/KH_2PO_4 buffer (10mM, pH 7.5) containing AM01618 (500μM), CCC (500μM) and ancymidol (500μM). After 6h (R,S)-[2-^{14}C]-ABA (4.2kBq) was added to the residual uptake buffer. Once uptake of the labelled substrate was complete a further 4.0ml of buffer was added and catabolism allowed to proceed for 30h at 25°C under continuous illumination (66μmol $m^{-2}s^{-1}$). ABA and its catabolites were extracted and analysed as described in Chapter 2.

Treatment	Radioactivity in ABA and its catabolites					
	Origin	DPA	7'-hydroxy ABA	PA	ABA	Aqueous conjugates
	Bq (%)					
Control	201.6 (4.8)	848.4 (20.2)	705.6 (16.8)	1121.4 (26.7)	268.8 (6.4)	1054.2(25.1)
AM01618	340.2 (8.1)	911.4 (21.7)	340.2 (8.1)	701.4 (16.7)	722.4(17.2)	1184.4(28.2)
CCC	117.6 (2.8)	625.8 (14.9)	176.4 (4.2)	470.4 (11.2)	2297.4(54.7)	516.6(12.3)
Ancymidol	218.4 (5.2)	1209.5 (28.8)	268.8 (6.4)	777.0 (18.5)	1129.8(26.9)	596.4(14.2)

TABLE 6.10: Effect of protein synthesis inhibitors on the catabolism of (R,S)-[2-^{14}C]-ABA in excised, light-grown leaves of Hordeum vulgare cv. Dyan.

Leaves (2g f.w.) from 10 d old seedlings of Hordeum vulgare were placed with their proximal ends in 0.5ml K_2HPO_4-KH_2PO_4 buffer (10mM, pH 7.5) containing either cycloheximide (10 μg/ml) lincomycin (100 μg/ml), chloramphenicol (1 mg/ml) or buffer alone under constant illumination (66μmol $m^{-2}s^{-1}$) at 25°C until all solution had been taken up (± 2h). Further buffer was then added and leaves were left for a further 6h under the same conditions before being transferred to identical solns (0.4 ml) containing (R,S)-[2-^{14}C]-ABA (5.0kBq). Once all the labelled ABA had been taken up, the leaves were leflt to catabolize this substrate for 30h under the same conditions. ABA and its catabolites were extracted and analysed as described in Chapter 2.

Treatment	Radioactivity in ABA and its catabolites					
	Origin	DPA	7'-hydroxy ABA	PA	ABA	Aqueous fraction
	Bq (%)					
Control	241.5 (5.1)	1004.3 (21.5)	866.7 (18.6)	548.6 (11.7)	732.9(15.7)	1263.1(27.1)
Lincomycin	264.5 (5.6)	948.0 (20.4)	660.0 (14.2)	565.0 (12.1)	852.0(18.3)	1357.0(29.2)
Chloramphenicol	210.0 (4.5)	1234.0 (26.5)	694.0 (14.9)	555.0 (11.9)	627.0(13.4)	1326.0(28.5)
Cycloheximide	353.0 (7.5)	131.0 (2.8)	465.0 (10.0)	408.0 (8.7)	2597.0(55.8)	692.0(14.8)

6.2.4.1.3. Effect of lincomycin, chloramphenicol and cycloheximide on (*R,S,*)-ABA catabolism in excised *Hordeum vulgare* cv. Dyan leaves.

The stability of the enzymes catalysing ABA catabolism and whether active protein biosynthesis is indeed necessary for this process in leaves of *Hordeum vulgare* is unknown. In order to address this aspect, excised leaves from light-grown seedlings of *Hordeum vulgare* were pretreated with either LIN, CAP or CHI prior to feeding radiolabelled, substrate (*R,S,*)-ABA and determining their effects on the catabolism of this hormone.

The results presented in Table 6.10 show that LIN and CAP did not significantly inhibit the catabolism of applied radiolabelled ABA in this tissue. However, the production of 7'-hydroxy ABA was inhibited by 24% in the case of LIN and 20% by CAP which might be indicative of some plastid protein biosynthesis being required for the production of this catabolite. In contrast to 70s ribosomal protein synthesis inhibitors, CHI was inhibitory to (*R,S,*)-ABA catabolism at concentrations as low as 1μg/ml (Figure 6.14) and significantly inhibited the production of all (*R,S,*)-ABA catabolites. Treatment with CHI reduced the incorporation of label into DPA by 87%, into 7'-hydroxy ABA by 47%, into PA by 26% and conjugates in the aqueous phases by 45% (Table 6.10).

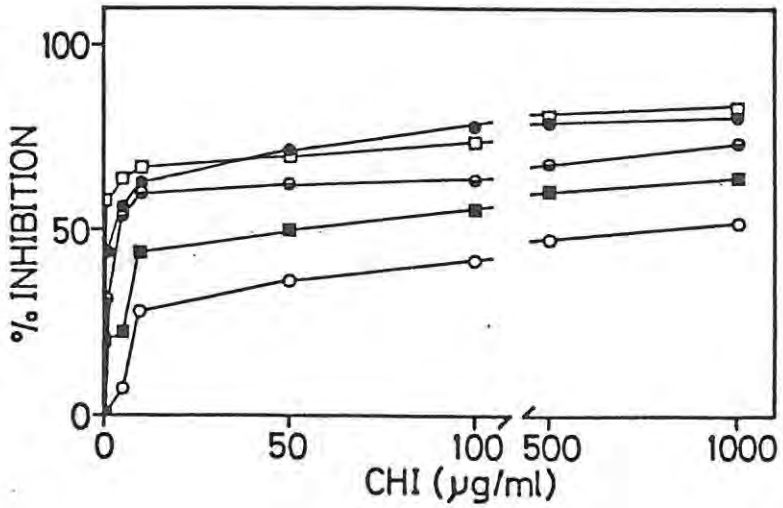

Figure 6.14. Effect of cycloheximide concentration on the inhibition of (*R,S,*)-ABA catabolism in excised, light-grown leaves of *Hordeum vulgare*. Increasing concentrations of CHI were fed to excised leaves *via* the transpiration stream at 25°C under continuous illumination (66μmol m^{-2} s^{-1}). Following uptake (± 2h) the tissue was transfered to fresh buffer containing (*R,S,*)-[2-^{14}C]-ABA (2.7kBq). Once the labelled substrate had been taken up, further buffer was added and the tissue was incubated as above for 30h. ABA and its catabolites were extracted and analysed as described in Chapter 2.

In order to examine further the effects of CHI on the enzymes involved in the catabolism of ABA, the catabolism of [^{14}C]-PA was investigated in leaves of *Hordeum vulgare* pretreated with CHI. The data presented in Table 6.11 show that the marked decrease in DPA levels in CHI-treated tissue arose as a direct result of the inhibition of the biochemical reduction of PA to DPA rather than by a reduction in PA levels brought about by the CHI inhibition of the oxidation (*R,S,*)-ABA to PA.

A further aspect regarding the effect of CHI on the catabolism of (*R,S,*)-ABA in leaves of *Hordeum vulgare* was that it inhibited the production of aqueous conjugates (Table 6.10). In contrast, LIN and CAP did not appear to affect this process. Thus, the residual aqueous fractions, remaining after extraction of the acids, were examined in order to determine the effects of inhibitors of protein biosynthesis on the production of water-soluble, base-labile conjugates.

Mild alkaline hydrolysis of the aqueous fractions from leaves treated with and without inhibitors produced the results shown in Table 6.12. LIN and CAP did not affect the range of conjugates produced although the levels of these compounds were somewhat higher in treated tissues compared to non-treated tissues. In contrast, CHI totally inhibited the conjugation of DPA and 7'-hydroxy ABA and markedly reduced the levels of PA conjugation but had no effect on ABA conjugation in this tissue. This finding was further substantiated in studies on [^{14}C]-PA catabolism in CHI-treated and non-treated leaf tissue (Table 6.11) and suggested that the enzyme(s) required for the conjugation of ABA catabolites were highly labile and rapidly turned over whereas those required for ABA conjugation *per se* were more stable.

6.2.4.2. Effect of chemical factors on the catabolism of (*R,S,*)-ABA in excised *Phaseolus vulgaris* cv. Top-crop seedlings.

6.2.4.2.1. Effect of cytokinins on (*R,S,*)-ABA catabolism in excised *Phaseolus vulgaris* cv. Top-crop seedlings.

Since cytokinins influenced significantly ABA catabolism in *Hordeum vulgare* leaves, it was of interest to investigate the effects of these compounds on the catabolism of applied, radiolabelled (*R,S,*)-ABA in other plant species.

Since the catabolism of ABA has been studied in detail in light-grown plants of *Phaseolus vulgaris* cv.

TABLE 6.11: Effect of Cycloheximide on the catabolism of [^{14}C] -PA in excised, light-grown leaves of Hordeum vulgare cv. Dyan.

Leaves (2g f.w.) from 10 d old seedlings of Hordeum vulgare were stood in 0.5ml K_2HPO_4-KH_2PO_4 buffer (10mM, pH 7.5) with or without cycloheximide (10µg/ml). Once all soln had been taken up, leaves were maintained under constant illumination (66µmol $m^{-2}s^{-1}$) for 6h in the presence of buffer alone. Leaves were then transferred to 0.4ml of the same buffer containing [^{14}C]-PA (4.2 kBq) and all label was taken up via the transpiration stream. Additional buffer was added (1.0 ml) and leaves were allowed to catabolise [^{14}C] -PA for 30h under identical conditions. PA and its catabolites were extracted and analysed as described in Chapter 2.

	Radioactivity in PA and its catabolites				
Treatment	Origin	DPA	'M'	PA	Aqueous fraction
			Bq (%)		
Control	125.5 (3.0)	1134.8 (27.5)	472.5 (11.4)	1035.3 (25.1)	1356.6 (32.8)
Cycloheximide	49.2 (1.1)	201.3 (4.8)	386.4 (9.3)	3144.1 (76.3)	338.4 (8.2)

TABLE 6.12: Effect of protein synthesis inhibitors on the conjugation of (R,S)-[2-^{14}C]-ABA and its catabolites in excised, light-grown leaves of Hordeum vulgare cv. Dyan.

Leaves (2g f.w.) from 10d old seedlings of Hordeum vulgare were placed in 0.5ml K_2HPO_4-KH_2PO_4 buffer (10mM, pH 7.5) with or without cycloheximide (10µg/ml), chloramphenicol (1mg/ml) and lincomycin (100µg/ml). When all the soln had been taken up, leaves were maintained under constant illumination (66µmol $m^{-2}s^{-1}$) at 25°C for 12h in the presence of buffer alone. Leaves were then transferred to 0.4ml of the same buffer containing (R,S)-[2-^{14}C]-ABA (5.0 kBq). Once all labelled ABA had been taken up, leaves were allowed to catabolise this substrate for 30h under identical conditions after which time samples were analysed for the conjugates of ABA and its acidic catabolites as described in Chapter 2.

	Radioactivity in conjugates of ABA and its catabolites			
Treatment	DPA	7'-hydroxy ABA	PA	ABA
		Bq (%)		
Control	143.9 (8.5)	119.0 (7.1)	309.6 (18.5)	1108.9 (65.9)
Cycloheximide	0.0 (0.0)	0.0 (0.0)	60.0 (3.6)	1620.0 (96.4)
Lincomycin	144.0 (5.4)	150.0 (5.5)	480.0 (17.5)	1960.0 (71.6)
Chloramphenicol	99.0 (5.4)	170.0 (9.2)	530.0 (28.6)	1050.0 (56.8)

The Prince (Zeevaart and Milborrow, 1976) and cv. Top-crop (Chapter 5), foliar application of cytokinins were made to *Phaseolus vulgaris* plants and the catabolism of applied, radiolabelled (*R,S,*)-ABA examined (Table 6.13). Treatment of seedlings with various cytokinins did not significantly alter the incorporation of label from (*R,S,*)-[2-^{14}C]-ABA into its acidic catabolites, although levels of substrate (*R,S,*)-ABA and PA were lower in BA treated tissue.

In addition, the residual label remaining in the aqueous fractions (Table 6.13) was higher from cytokinin-treated tissue. This suggested that cytokinins may enhance the conjugation of substrate (*R,S,*)-ABA. Hydrolysis of the aqueous fractions, demonstrated only slight increases in the amounts of released, free ABA in cytokinin-treated seedlings (Table 6.14). In order to confirm that the catabolism of (*R,S,*)-[2-^{14}C]-ABA was enhanced in BA-treated seedlings, refeeding studies were undertaken using *Phaseolus vulgaris* seedlings given foliar applications of BA. When [^{14}C]-PA was fed to such seedlings it was more rapidly catabolised in BA-treated plants than in untreated seedlings (Figure 6.15A). By comparison, little difference was noted in the catabolism of [^{14}C]-DPA between treated and non-treated tissues (Figure 6.15B). This suggests that in *Phaseolus vulgaris* seedlings, BA altered the catabolism of (*R,S,*)-ABA by enhancing the conversion of PA to DPA and aqueous conjugates, which might be indicative of increases in the activity or levels of the enzymes catalysing these steps.

6.2.4.2.2. Effect of AMO1618, ancymidol and CCC on (*R,S,*)-ABA catabolism in *Phaseolus vulgaris* cv. Top-crop seedlings.

Although the GA biosynthesis inhibitors, AMO1618, CCC and ancymidol did not appear to alter ABA catabolism in excised leaves of *Hordeum vulgare*, it was nevertheless of interest to examine their effects on ABA catabolism in a dicotyledon.

(*R,S,*)-[2-^{14}C]-ABA was fed to excised apical leaves of light-grown *Phaseolus vulgaris* seedlings pretreated with either AMO1618, CCC or ancymidol, as foliar applications with simultaneous soil drenches, and the efficiency of (*R,S,*)-ABA catabolism determined (Table 6.15). AMO1618, CCC and ancymidol appeared to have no significant effect on the catabolism of (*R,S,*)-ABA although AMO1618, CCC and ancymidol appeared to enhance the incorporation of label into water-soluble conjugates. A possible explanation for the limited effect of these compounds on the oxidation of (*R,S,*)-ABA is that they might be detoxified rapidly, thereby reducing their effect. It is well known that CCC is actively metabolised in plants (Lawrence, 1984) and recently a catabolite of

TABLE 6.13: Catabolism of (R,S)-ABA in excised, light-grown seedlings of Phaseolus vulgarus cv. Top crop pretreated with cytokinins.

Seedlings of Phaseolus vulgaris were treated with adenine, BA, zeatin, IPA and kinetin (all 500 μM in Tween 80/H_2O, 0.01%) as a foliar spray (200 ml) and soil drench (200 ml) for 4 consecutive days prior to harvesting. Excised seedlings were fed with (R,S)-[2-^{14}C]-ABA (4.2 kBq) via the transpiration stream and catabolism allowed to continue for 30h at 25°C under constant illumination (66μmol $m^{-2}s^{-1}$). ABA and its catabolites were extracted and analysed as described in Chapter 2.

	Distribution of Radioactivity				
Treatment	Origin	DPA	PA	ABA	Aqueous conjugates
			Bq (%)		
Control	702.6 (16.7)	1084.0 (25.8)	531.4 (12.7)	519.0 (12.4)	1363.0 (32.4)
Adenine	892.9 (21.3)	929.9 (22.1)	511.6 (12.2)	469.1 (11.2)	1396.5 (33.2)
BA	798.7 (19.0)	1035.2 (24.6)	379.5 (9.0)	408.2 (9.8)	1578.4 (37.6)
Zeatin	808.9 (19.3)	1018.9 (24.3)	357.6 (8.5)	484.7 (11.5)	1529.9 (36.4)
IPA	816.2 (19.4)	1095.2 (26.1)	527.9 (12.6)	495.9 (11.8)	1264.8 (30.1)
Kinetin	671.8 (16.1)	1065.9 (25.4)	419.0 (9.9)	655.3 (15.6)	1388.0 (33.0)

TABLE 6.14: Effect of cytokinin pretreatment on the conjugation of (R,S)-[2-^{14}C]-ABA and its catabolites in excised, light-grown seedlings of Phaseolus vulgaris cv. Top crop.

Seedlings of Phaseolus vulgaris were pretreated with solutions of adenine, BA, zeatin, IPA and kinetin (all at 500μM in Tween 80/H_2O, 0.01%) as a foliar spray (200 ml) and soil drench (200 ml) for 4 consecutive days prior to harvesting and maintained at 25°C under constant illumination. Excised seedlings (10g f.w.) were fed (R,S)-[2-^{14}C]-ABA (4.2 kBq) via the transpiration stream and catabolism allowed to proceed for 30h under identical conditions to those described above. Samples were analysed for the conjugates of ABA and its catabolites as described in Chapter 2.

	Radioactivity in ABA and its catabolites*		
Treatment	PA	ABA	t-ABA
		Bq (%)	
Control	ND	147.4 (3.51)	28.1 (0.67)
Adenine	ND	136.5 (3.25)	26.5 (0.63)
BA	ND	183.9 (4.38)	15.5 (0.37)
Zeatin	ND	170.5 (4.06)	15.1 (0.36)
IPA	ND	170.1 (4.05)	20.2 (0.48)
Kinetin	ND	184.4 (4.39)	16.0 (0.38)

* Corrected for controls at pH 7.0

ND = not detected

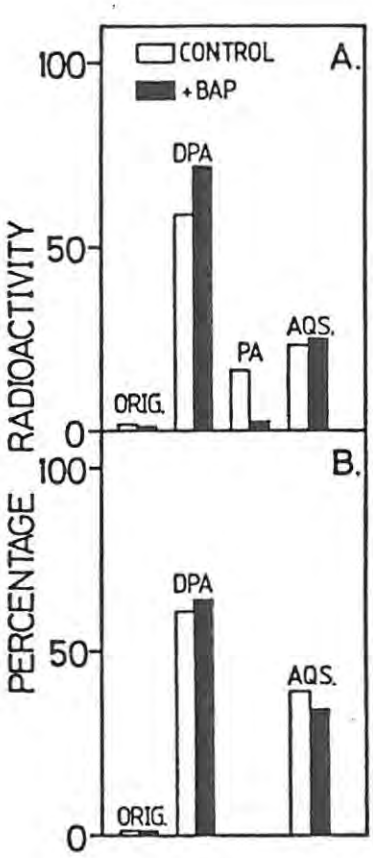

Figure 6.15. The catabolism of PA and DPA in excised, light-grown seedlings of *Phaseolus vulgaris* pretreated with benzyladenine. Seedlings were treated with 200ml BA (500μM in Tween 80/H_2O, 0.01%) as a soil drench and 200ml as a foliar spray for 5 consecutive days prior to harvesting. [^{14}C]-PA and [^{14}C]-DPA (all 4.2kBq) were fed to excised seedlings (10g f.w.) *via* the transpiration stream and catabolism allowed to proceed for 24h at 25°C under continuous illumination (66μmol m^{-2} s^{-1}). The distribution of radioactivity in the catabolites was determined as described in Chapter 2.

TABLE 6.15: The effect of AM01618, CCC and ancymidol on the catabolism of (R,S)-ABA in Phaseolus vulgaris cv. Top crop seedlings.

Seedlings of Phaseolus vulgaris (10 d old) were treated with 200ml (500μM) AM01618, 200ml (500μM) CCC and 100ml (400 μM) ancymidol as a soil-drench for three consecutive days prior to harvesting. Excised seedlings (2 shoots/incubate) were then stood in 0.5ml of K_2HPO_4/KH_2PO_4 buffer (10mM, pH 7.5) containing (R,S)-[2-^{14}C] -ABA (3.67 kBq). Once uptake was complete, further buffer (5.0 ml) was added and catabolism allowed to proceed for 24h at 25°C under continuous illumination (66μmol $m^{-2}s^{-1}$). ABA and its catabolites were extracted and analysed as described in Chapter 2.

Treatment	Fresh weight (g)	Distribution of Radioactivity /g fresh weight				
		Origin	DPA	PA	ABA	Aqueous conjugates
			Bq			
Control	6.2	61.7	120.2	116.0	95.1	157.8
AM01618	6.0	76.3	104.0	130.3	59.3	197.7
CCC	7.0	29.5	81.1	124.7	84.3	161.5
Ancymidol	4.9	81.7	94.2	183.4	110.8	211.1

TABLE 6.16: Effect of AM01618, ancymidol and CCC applied via the transpiration stream on the catabolism of (R,S)-ABA in Phaseolus vulgaris cv. Top crop seedlings.

Seedlings of Phaseolus vulgaris (10 d old) were treated with 200ml (500μM) AM01618, 200ml (500μM) CCC and 100ml (500 μM) ancymidol as a soil-drench for three consecutive days prior to harvesting. Excised 14d old Phaseolus vulgaris seedlings (6.0g f.w.) were fed with (R,S)-[2-^{14}C] -ABA (3.67 kBq) in 0.5ml of either AM01618 (500 μM), CCC (500μM) or ancymidol (500μM) dissolved in 10mM KPi buffer (pH 7.5) via the transpiration stream. Following uptake tissue was incubated for 24h under constant illumination (66μmol $m^{-2}s^{-1}$) at 25°C. ABA and its catabolites were extracted and analysed as described in Chapter 2.

Treatment	Distribution of Radioactivity			
	Conjugates	DPA	PA	ABA
	Bq (%)			
Control	233.59 (19.15)	127.90 (10.48)	123.50 (10.12)	2211.16 (60.25)
AM01618	284.57 (23.33)	108.04 (8.86)	135.28 (11.09)	2081.62 (56.72)
Ancymidol	253.56 (20.78)	81.52 (6.62)	158.78 (13.02)	2186.58 (59.58)
CCC	236.82 (19.41)	98.79 (8.09)	152.01 (12.46)	2214.47 (60.34)

ancymidol was detected in studies of ABA biosynthesis in *Cercospora rosicola* (Norman *et al*, 1986), suggesting that it might also be catabolised by higher plants.

Attempts were therefore made to limit the possibility of catabolism of these compounds by feeding them to excised, intact seedlings of *Phaseolus vulgaris via* the transpiration stream prior to the addition of radiolabelled substrate. Thus, AMO1618, CCC and ancymidol were supplied to excised, intact seedlings of *Phaseolus vulgaris via* the transpiration stream and the tissue incubated for 3h prior to the addition of radiolabelled (*R,S,*)-ABA. Following a 24h incubation period, under continuous illumination, the tissue was extracted and analysed and the results are shown in Table 6.16. In contrast to the foliar applications of these compounds, AMO1618, CCC and ancymidol appeared to reduce the catabolism of radiolabelled (*R,S,*)-ABA by reducing the conversion of PA to DPA thus resulting in an increase in PA. CCC and ancymidol were more effective at reducing the conversion of PA to DPA than was AMO1618. These inhibitors of GA biosynthesis did not appear to influence the production of aqueous conjugates when applied *via* the transpiration stream and appeared to effect only the production of the acidic catabolites of (*R,S,*)-ABA in this tissue.

6.2.4.2.3. Effect of cycloheximide, lincomycin and chloramphenicol on (*R,S,*)-ABA catabolism in excised *Phaseolus vulgaris* cv. Top-crop seedlings.

In order to determine whether continued enzyme synthesis was required for ABA catabolism in seedlings of *Phaseolus vulgaris*, the effect of inhibitors of protein biosynthesis on this process was

TABLE 6.17: The effect of protein synthesis inhibitors of the catabolism of (R,S)-ABA in excised, light-grown seedlings of Phaseolus vulgarus cv. Top crop.

Excised shoots (7.0g f.w.) of 10 d old Phaseolus vulgaris were pretreated with lincomycin (0.1 mg/ml), chloramphenicol (1 mg/ml) and cycloheximide (1 mg/ml) via the transpiration stream as described for Table 6.10. Following uptake, seedlings were transferred to identical solutions containing (R,S)-[2-^{14}C]-ABA (3.42kBq). Once all the labelled substrate had been taken up, the shoots were left to catabolise this substrate for 24h under the same conditions. ABA and its catabolites were extracted and analysed as described in Chapter 2.

	Radioactivity in ABA and its catabolites			
Treatment	DPA	PA	ABA	Aqueous conjugates
		Bq (%)		
Control	872.1 (25.5)	759.2 (22.2)	533.5 (15.6)	1255.1 (36.7)
Lincomycin	1084.1 (31.7)	656.4 (19.5)	355.7 (10.4)	1313.3 (38.4)
Chloramphenicol	824.2 (24.1)	779.8 (22.8)	383.0 (11.2)	1432.9 (41.9)
Cycloheximide	482.2 (14.1)	615.6 (18.0)	1173.1 (34.3)	1135.4 (33.2)

examined. As in excised leaves of *Hordeum vulgare*, CHI also inhibited the catabolism of (*R,S,*)-[2-^{14}C]-ABA in light-grown seedlings of *Phaseolus vulgaris* (Table 6.17). In contrast, LIN and CAP had no effect on ABA catabolism in light-grown seedlings of *Phaseolus vulgaris* whereas CHI reduced significantly the production of DPA. The limited effects of 70s ribosome inhibitors in this vegetative dicotyledon suggest that plastid-synthesized proteins are probably not required for ABA catabolism while continued cytoplasmic protein biosynthesis appears to be necessary for this process.

6.2.4.3. Effect of chemical factors on (*R,S,*)-ABA catabolism in excised *Pisum sativum* cv. Black-eyed Susan seedlings.

6.2.4.3.1. Effect of cytokinins on the catabolism of (*R,S,*)-ABA in excised *Pisum sativum* cv. Black-eyed Susan seedlings.

In order to examine the effect of cytokinins on ABA catabolism in other plant species, seedlings of *Pisum sativum* were pre-treated with BA, which had exerted the greatest effect in plants of *Phaseolus vulgaris*, and the efficiency of (*R,S,*)-[2-^{14}C]-ABA catabolism determined in excised seedlings from both treated and non-treated tissues. The results presented in Table 6.18 indicated that BA-treated tissues were less able to catabolise (*R,S,*)-[2-^{14}C]-ABA than were their non-treated counterparts. In particular, BA treatment of *Pisum sativum* seedlings reduced the ability of these tissues to incorporate label from (*R,S,*)-ABA and its catabolites, into water-soluble aqueous conjugates.

In order to confirm this suggestion the residual aqueous fractions were treated with base and the distribution of radioactivity in the free acids determined (Table 6.19). This result indicates that non-treated tissue conjugated applied, radiolabelled (*R,S,*)-ABA and its catabolites more readily than BA-treated seedlings. Thus, it appeared that BA treatment reduced the ability of this tissue to conjugate applied (*R,S,*)-ABA and its catabolites. Nevertheless, this does not imply that treatment with BA inhibited the transformation of (*R,S,*)-ABA into its acidic catabolites. On the contrary, BA may enhance the conversion of ABA to PA and DPA in seedlings of *Pisum sativum* and only interfere with the conjugation of these catabolites.

TABLE 6.18: The catabolism of (R,S)-[2-^{14}C]-ABA in seedlings of Pisum sativum cv. Black-eyed Susan, pretreated with benzyladenine.

Seedlings of Pisum sativum which had been pretreated with BA (500μM in 0.01% Tween 80/H_2O) as described in Chapter 2, were excised and placed with their proximal ends into 0.5ml K_2HPO_4/KH_2PO_4 buffer (10 mM pH 7.5) containing (R,S)-[2-^{14}C]-ABA (4.2 kBq). Following uptake, a further 4.0ml of buffer was added and the tissue allowed to catabolize the substrate for 24h under conditions of continuous illumination. ABA and its acidic catabolites were extracted and analysed as described in Chapter 2.

	Distribution of Radioactivity					
Treatment	Origin	DPA	Catabolite 3	PA	ABA	Aqueous conjugates
			Bq (1%)			
Control	159.6 (3.8)	766.2 (18.1)	138.6 (3.3)	113.4 (2.7)	600.6(14.3)	2427.6(57.8)
BA	327.6 (7.8)	1268.4 (30.2)	184.8 (4.4)	205.8 (4.9)	1289.4(30.7)	924.0(22.0)

TABLE 6.19: Hydrolysis of the aqueous fractions from (R,S)-ABA catabolic studies in Pisum sativum cv. Black-eyed Susan seedlings treated with BA.

Aqueous fractions from extracts of Pisum sativum seedlings (treated as in Table 6.18) were treated with base (5N NaOH) at pH 11.0) at 60°C for 1h. ABA and its catabolites were extracted and analysed as described in Chapter 2.

	Radioactivity released by hydrolysis*				
Treatment	DPA	Catabolite 3	PA	ABA	Aqueous conjugates
			Bq (% of total aqueous)		
Control	83.8 (3.45)	108.9 (4.49)	120.4 (4.96)	218.2 (8.99)	17.2 (0.71)
BA	-	0.3 (0.03)	2.2 (0.24)	25.0 (2.71)	5.5 (0.60)

* Corrected for controls held at pH 7.0.

6.2.4.3.2. Effect of chloramphenicol, lincomycin and cycloheximide on the catabolism of (*R,S,*)-ABA in *Pisum sativum* cv. Black-eyed Susan seedlings.

It was previously demonstrated (see Section 6.2.4.1.3. and 6.2.4.2.4.) that cytoplasmic protein biosynthesis was required for the catabolism of (*R,S,*)-ABA in leaves of *Hordeum vulgare* and *Phaseolus vulgaris*. In order to determine whether the same applied for seedlings of *Pisum sativum*, the effect of inhibitors of protein biosynthesis on the catabolism of applied, radiolabelled ABA in this tissue was examined.

Following pretreatment of excised seedlings of *Pisum sativum* with inhibitors of protein biosynthesis and application of radiolabelled substrate, the tissue was extracted and the incorporation of radioactivity into the catabolites of ABA determined. The data presented in Table 6.20 show that treatment with CHI markedly reduced the incorporation of label from ABA into its acidic catabolites but did not affect the incorporation of label into aqueous conjugates. In contrast to results obtained in similar studies using other tissues, inhibitors of 70s ribosomal translation also retarded the catabolism of ABA in light-grown seedlings of *Pisum sativum*. In particular, incorporation of label into both PA and DPA were inhibited by LIN, and incorporation into DPA inhibited by CAP.

TABLE 6.20: The effect of protein synthesis inhibitors on the catabolism of (R,S)-ABA in excised, light-grown seedlings of Pisum sativum cv. Black-eyed Susan.

Excised shoots (2.0g f.w.) of 10d old Pisum sativum were pretreated with lincomycin (0.1 mg/ml), chloramphenicol (1 mg/ml) and cycloheximide (1 mg/ml) via the transpiration stream as described in Table 6.10. Following uptake seedlings were transferred to identical solutions containing (R,S)-[2-^{14}C]-ABA (2.78kBq). Once all the labelled substrate had been taken up, the shoots were left to catabolise this substrate for 24h under the same conditions. ABA and its catabolites were extracted and analysed as described in Chapter 2.

	Radioactivity in ABA and its catabolites				
Treatment	DPA	PA	Catabolite 3	ABA	Aqueous conjugates
			Bq (%)		
Control	294.7 (10.6)	127.9 (4.6)	61.2 (2.2)	1206.5 (43.4)	1089.7 (39.2)
Lincomycin	191.8 (6.9)	72.3 (2.6)	75.0 (2.7)	1145.3 (41.2)	1295.5 (46.6)
Chloramphenicol	169.6 (6.1)	136.2 (4.9)	63.9 (2.3)	1123.1 (40.4)	1287.1 (46.3)
Cycloheximide	13.9 (0.5)	11.1 (0.4)	11.1 (0.4)	1462.3 (52.6)	1281.6 (46.1)

NON-VEGETATIVE TISSUES.

6.2.4.4. Effect of chemical factors on (*R,S,*)-ABA catabolism in immature seeds of *Pisum sativum* L. and *Phaseolus vulgaris* L.

6.2.4.4.1. Effect of cytokinins on (*R,S,*)-ABA catabolism in immature seeds of *Phaseolus vulgaris* L. and *Pisum sativum* L.

Cytokinins were shown to influence the catabolism of applied, radiolabelled (*R,S,*)-ABA in vegetative tissues, an effect which might result from altered levels of the enzymes catalysing this process. Thus, an attempt was made to determine whether these compounds also influenced ABA catabolism in non-vegetative tissues. Immature seeds of *Pisum sativum* and *Phaseolus vulgaris* were pretreated with cytokinins prior to the addition of radiolabelled substrate. Following a 24h incubation period the catabolites of ABA were extracted and analysed by TLC on silica gel GF_{254} in toluene/ethyl acetate/acetic acid (50:30:4, v/v) and the results are shown in Table 6.21. BA and zeatin reduced the catabolism of (*R,S,*)-[2-^{14}C]-ABA in immature seeds of *Pisum sativum* by ±75%. In immature seeds of *Phaseolus vulgaris* zeatin reduced ABA catabolism by ±15% whereas BA reduced catabolism by nearly 50% (Table 6.21).

6.2.4.4.2. Effect of AMO1618, ancymidol and CCC on (*R,S,*)-ABA catabolism in immature seeds of *Phaseolus vulgaris* L. and *Pisum sativum* L.

In order to determine whether inhibitors of GA biosynthesis influenced the catabolism of (*R,S,*)-ABA in immature seeds, the effect of AMO1618, CCC and ancymidol on this process was investigated.

When immature seed of *Pisum sativum* and *Phaseolus vulgaris* were treated with AMO1618, CCC and ancymidol *in vitro* and the efficiency of (*R,S,*)-[2-^{14}C]-ABA catabolism determined, the inhibitory effects were more pronounced than those observed in similar experiments using vegetative tissue. The data presented in Table 6.22 show that CCC and ancymidol inhibited the catabolism of (*R,S,*)-ABA significantly in both *Pisum sativum* and *Phaseolus vulgaris* immature seed. In contrast, AMO1618 while inhibiting (*R,S,*)-ABA catabolism in *Pisum sativum*, appeared to stimulate its catabolism in *Phaseolus vulgaris* immature seed. The reason for the contrasting effects of AMO1618 on the catabolism of (*R,S,*)-ABA in immature seeds of these species is unknown.

TABLE 6.21: The catabolism of (R,S)-ABA in immature seeds of Pisum sativum L. and Phaseolus vulgaris L. pretreated with cytokinins.

Immature seeds of Pisum sativum (10g f.w.) and Phaseolus vulgaris (5g f.w.) were incubated in 15ml nutrient medium (Nitsch, 1951) containing either adenine (500 μM), zeatin (500 μM) or 6-BA (500 μM) for 6h in an orbital shaker at 28°C under constant illumination (42μmol $m^{-2}s^{-1}$). Following pretreatment, (R,S)-[2-^{14}C] -ABA (4.2kBq) was added and the tissue incubated as above for a further 24h period. The distribution of radioactivity in the catabolites of ABA was determined as described in Chapter 2.

	% Radioactivity relative to control			
Tissue and Treatment	Aqueous conjugates	DPA	Catabolite 3	PA
Pisum sativum				
Control	100	100	100	100
+ Adenine	112.7	119.3	95.0	83.1
+ Zeatin	25.6	29.2	25.5	12.7
+ BA	32.9	17.9	60.5	15.2
Phaseolus vulgaris				
Control	100	100	100	100
+ Adenine	182.0	328.2	36.6	57.7
+ Zeatin	112.9	44.4	81.1	100
+ BA	172.9	6.1	2.6	9.9

TABLE 6.22: The effect of AM01618, CCC and ancymidol on the catabolism of (R,S)-ABA in immature seeds of Pisum sativum L. and Phaseolus vulgaris L.

Immature seeds of Pisum sativum (10g f.w.) and Phaseolus vulgaris (5g f.w.) in 15ml nutrient medium were pretreated with AM01618 (500 μM), CCC (500 μM) and ancymidol (500 μM) for 6h at 28°C under continuous illumination (42μmol $m^{-2}s^{-1}$). Following pretreatment (R,S)-[2-^{14}C]-ABA (4.2kBq) was added and the samples incubated as above for a further 24h. The distribution of radioactivity in the catabolites of ABA was determined as described in Chapter 2.

	% Radioactivity relative to control			
Tissue and Treatment	Aqueous conjugates	DPA	Catabolite 3	PA
Pisum sativum				
Control	100	100	100	100
AM01618	73.4	84.1	0	55.3
Ancymidol	48.3	51.3	30.5	76.9
CCC	61.4	48.7	35.7	69.4
Phaseolus vulgaris				
Control	100	100	100	100
AM01618	118.7	217.9	111.8	99.8
Ancymidol	41.3	10.7	75.6	10.8
CCC	78.6	65.2	93.3	85.

6.2.4.4.3. Effect of cycloheximide, chloramphenicol and lincomycin on (*R,S,*)-ABA catabolism in immature seeds of *Phaseolus vulgaris* L. and *Pisum sativum* L.

CHI retarded the catabolism of applied, radiolabelled ABA in vegetative tissues suggesting that cytoplasmic protein sythesis was required for this process. In order to determine whether the same applied for immature seeds, the influence of inhibitors of protein synthesis on ABA catabolism in immature seeds was examined.

The catabolism of applied, radiolabelled (*R,S,*)-ABA was not influenced by either LIN or CAP (Table 6.23). However, CHI inhibited significantly the catabolism of (*R,S,*)-[2-^{14}C]-ABA in excised, illuminated immature seed of *Pisum sativum* and *Phaseolus vulgaris* (Table 6.23). These results suggested that the enzymes involved in the catabolism of ABA were labile and possibly produced by cytoplasmically localised protein biosynthesis. In addition, it should be noted that since CAP and LINC did not significantly influence the catabolism of (*R,S,*)-[2-^{14}C]-ABA in immature seed *in vitro* when compared to non-treated tissue, that any catabolism of labelled substrate was thus not due to microbial contamination (see Chapter 5).

6.2.4.5. Effect of chemical factors on (*R,S,*)-ABA catabolism in mesocarp tissue from ripening *Persea americana* cv. Fuerte fruits.

6.2.4.5.1. Effect of cytokinins on the catabolism of (*R,S,*)-ABA in *Persea americana* cv. Fuerte mesocarp tissue.

Cytokinins inhibited the biosynthesis of ABA in *Persea americana* mesocarp tissue (see Chapter 4). Since this apparent inhibition could have resulted from cytokinin-enhanced catabolism of ABA it was necessary to examine the effect of a cytokinin on the catabolism of applied, radiolabelled ABA in this tissue. In addition, cytokinins may retard the catabolism of ABA thus providing a useful biochemical tool for use in cell-free studies on this process. BA, which retarded ABA biosynthesis most effectively, was therefore selected for use in these studies.

Persea americana mesocarp was treated with BA for 6h prior to the addition of (*R,S,*)-[2-^{14}C]-ABA and then incubated for a further 18h under continuous illumination. The result shown in Table 6.24 indicate that BA treatment enhanced the catabolism of ABA in this tissue by about 25%.

TABLE 6.23: The effect of inhibitors of protein synthesis on the catabolism of (R,S)-ABA in immature seeds of Pisum sativum L. and Phaseolus vulgaris L.

Excised, intact immature seed of Pisum sativum (10g f.w.) and Phaseolus vulgaris (5g f.w.) were incubated in 15ml nutrient medium (Nitsch, 1951) containing either lincomycin (0.1 mg/ml), chloramphenicol (1 mg/ml) or cycloheximide (1 mg/ml) for 6h in an orbital shaker at 28°C under constant illumination (42μmol $m^{-2}s^{-1}$). Following pretreatment (R,S)-[2-^{14}C]-ABA (4.2 kBq) was added and catabolism allowed to proceed for a further 24h under the identical conditions to those already described. ABA and its catabolites were extracted and analysed as described in Chapter 2.

	Distribution of Radioactivity					
Tissue and treatment	Origin	DPA	Catabolite 3	PA	ABA	Aqueous conjugates
	Bq (%)					
Pisum sativum						
Control	33.6 (0.8)	667.0 (13.5)	205.8 (4.9)	550.2 (13.1)	2213.4 (52.7)	630.0 (15.0)
Lincomycin	67.2 (1.6)	726.6 (17.3)	210.0 (5.0)	621.6 (14.8)	2179.8 (51.9)	403.2 (9.4)
Chloramphenicol	25.2 (0.6)	596.4 (14.2)	159.6 (3.8)	550.2 (13.1)	2326.8 (55.4)	541.8 (12.9)
Cycloheximide	46.2 (1.1)	33.6 (0.8)	29.4 (0.7)	29.4 (0.7)	3519.6 (83.8)	541.8 (12.9)
Phaseolus vulgaris						
Control	180.6 (4.3)	772.8 (18.4)	50.4 (1.2)	823.2 (19.6)	1785.0 (42.5)	588.0 (14.0)
Lincomycin	210.0 (5.0)	831.6 (19.8)	54.6 (1.3)	835.8 (19.9)	1738.8 (41.4)	529.2 (12.6)
Chloramphenicol	184.8 (4.4)	793.8 (18.9)	46.2 (1.1)	789.6 (18.8)	2091.6 (49.8)	294.0 (7.0)
Cycloheximide	50.4 (1.2)	12.6 (0.3)	12.6 (0.3)	46.2 (1.1)	3729.6 (88.8)	348.6 (8.3)

6.2.4.5.2. Effect of AMO1618, CCC and ancymidol on the catabolism of (*R,S,*)-ABA in *Persea americana* cv. Fuerte mesocarp tissue.

AMO1618, CCC and ancymidol were shown to inhibit ABA biosynthesis in *Persea americana* mesocarp tissue. (see Chapter 4). Since a similar argument to that used for cytokinins may apply to these results it was pertinent to examine the effects of these inhibitors of GA biosynthesis on the catabolism of applied, radiolabelled ABA in this tissue.

Excised blocks of *Persea americana* mesocarp tissue were infiltrated with solutions of AMO1618, CCC and anymidol and incubated for 6h prior to the addition of radioactive substrate. (*R,S,*)-[2-^{14}C]-ABA was then applied to the tissue and the tissue incubated for a further 18h after which the ethyl acetate-soluble acids were analysed on thin layers of silica gel GF_{254} in toluene/ethyl acetate/acetic acid (50:30:4, v/v) and the results are shown in Table 6.25.

AMO1618, CCC and ancymidol reduced (by ±25%) the capacity of *Persea americana* mesocarp tissue to catabolise applied, radiolabelled ABA.

6.2.4.5.3. Effect of chloramphenicol, lincomycin and cycloheximide on the catabolism of (*R,S,*)-ABA in *Persea americana* cv. Fuerte mesocarp tissue.

In order to determine whether continued protein biosynthesis was reqired for the catabolism of applied, radiolabelled ABA the effect of inhibitors of protein biosynthesis on (*R,S,*)-ABA catabolism in *Persea americana* mesocarp was examined.

Slices of mesocarp tissue were pretreated with solutions of LIN, CAP and CHI, at the concentrations used previously, for 6h prior to the addition of (*R,S,*)-[2-^{14}C]-ABA. After a further 18h the tissue was extracted and the efficiency of ABA catabolism determined. The results shown in Table 6.26 demonstrate that CHI reduced the catabolism of (*R,S,*)-[2-^{14}C]-ABA to PA and DPA and other acidic catabolites in the ripening mesocarp of *Persea americana* (Table 6.26) while having little effect on the production of aqueous conjugates. These findings have, in part, already been published (Cowan and Railton, 1986). Inhibitors of 70s ribosomal protein synthesis had little or no effect on the catabolism of applied, radiolabelled ABA in *Persea americana* mesocarp. Together these results further imply that chloroplasts are not a major site of ABA catabolism in this tissue.

TABLE 6.24: The effect of benzyl adenine on the catabolism of (R,S)-ABA in Persea americana cv. Fuerte mesocarp.

BA (500 μM) was applied to the sliced surface of Persea americana mesocarp in 0.2 ml Tween 80/acetone/water (1:1:8, v/v) and incubated in a water-saturated environment at 25°C under constant illumination (66μmol $m^{-2}s^{-1}$) for 6h. Following pretreatment (R,S)-[2-^{14}C]-ABA (4.2 kBq) was applied to the treated and non-treated control and the tissue incubated for the remainder of the 24h period, as above. ABA and its catabolites were extracted and analysed as described in Chapter 2.

	Distribution of Radioactivity				
Treatment	Aqueous conjugates	DPA	Catabolite 3	PA	ABA
		Bq (%)			
Control	1008.0 (24.0)	109.2 (2.6)	75.6 (1.8)	134.4 (3.2)	2872.8 (68.4)
BA	1478.4 (35.2)	722.4 (17.2)	117.6 (2.8)	142.8 (3.4)	1738.8 (41.4)

TABLE 6.25: The effect of AM01618, CCC and ancymidol on the catabolism of (R,S)-ABA in Persea americana cv. Fuerte mesocarp.

Mesocarp tissue (20g f.w.) was treated with AM01618 (500 μM), CCC (500 μM) and ancymidol (500 μM) in Tween 80/acetone/water (1:1:8, v/v) to the cut surface of the tissue. Following a 6h pre-incubation period (R,S)-[2-^{14}C]-ABA (4.2 kBq) was added in Tween 80/acetone/water (1:1:8, v/v) and the tissue incubated for the remainder of the 24h incubation period in a water-saturated environment at 25°C under continuous illumination (66μmol $m^{-2}s^{-1}$). ABA and its catabolites were extracted and analysed as described in Chapter 2.

	Distribution of Radioactivity					
Treatment	Origin	DPA	Catabolite 3	PA	ABA	Aqueous conjugates
			Bq (%)			
Control	109.2 (2.6)	109.2 (2.6)	75.6 (1.8)	134.4 (3.2)	2872.8 (68.4)	907.2 (21.6)
AM01618	67.2 (1.6)	92.4 (2.2)	67.2 (1.6)	109.2 (2.6)	3460.8 (82.4)	403.2 (9.6)
Ancymidol	8.4 (0.2)	16.8 (0.4)	16.8 (0.4)	25.2 (0.6)	3897.6 (92.8)	235.2 (5.6)
CCC	16.8 (0.4)	25.2 (0.6)	50.4 (1.2)	42.0 (1.0)	3603.6 (85.8)	462.0 (11.0)

TABLE 6.26: The effect of protein synthesis inhibitors on the catabolism of (R,S)-ABA in illuminated, excised blocks of mesocarp of Persea americana cv. Fuerte.

Mesocarp tissue (20g f.w.) from Persea americana were treated with solutions (0.2ml) of lincomycin (0.1 mg/ml), chloramphenicol (1 mg/ml) and cycloheximide (1 mg/ml) in Tween 80/acetone/H_2O (1:1:8, v/v) to the sliced surface and incubated in a H_2O-saturated environment at 25°C under constant illumination (66μmol $m^{-2}s^{-1}$). Following pretreatment, (R,S)-[2-^{14}C]-ABA (4.2 kBq) in Tween 80/acetone/H_2O (1:1:8, v/v) was added and the tissue incubated for 24h as described above. ABA and its catabolites were extracted and analysed as described in Chapter 2.

	Radioactivity in ABA and its catabolites					
Treatment	Origin	DPA	Catabolite 3	PA	ABA	Aqueous conjugates
	Bq (%)					
Control	239.4(5.7)	357.0(8.5)	25.2(0.6)	71.4(1.7)	1730.4(41.2)	1776.6(42.3)
Lincomycin	226.8(5.4)	357.0(8.5)	21.0(0.5)	67.4(1.6)	1692.6(40.3)	1835.4(43.7)
Chloramphenicol	226.8(5.4)	352.8(8.4)	25.2(0.6)	100.8(2.4)	1793.4(42.7)	1701.0(40.5)
Cycloheximide	37.8(0.9)	25.2(0.6)	8.4(0.2)	37.8(0.9)	2167.2(51.6)	1923.6(45.8)

CHAPTER SEVEN

STUDIES ON THE BIOSYNTHESIS AND CATABOLISM OF ABSCISIC ACID IN CELL-FREE PREPARATIONS FROM HIGHER PLANT TISSUES.

7.1. INTRODUCTION

Most available information concerning the biosynthesis and catabolism of ABA has been derived from studies using intact plants and excised plant parts. The studies outlined in the preceding chapters (see Chapters 4,5 and 6) were carried out in order to determine the best conditions under which it might be possible to develop an ABA-metabolising cell-free system. By using treatments which modified ABA biosynthesis and catabolism in intact tissues, it was hoped that such alterations in metabolism might reflect changes in the levels and/or activity of enzymes catalysing some of the steps in these processes. Such information might lend itself to the development of cell-free systems from tissues in which the enzymes of ABA metabolism were most active.

It is well known that 3*R*-MVA is the natural precursor of terpenoids and the only substrate recognised by MVA kinase (EC 2.7.1.36). MVA kinase has been detected in homogenates of germinating *Pisum sativum* seeds (Green and Baisted, 1972), in numerous other plant tissues (Spurgeon and Porter, 1980) and more recently, in leaves of *Nepeta cataria* (Arebalo and Mitchell, 1984). In addition, it is now well established that C-5 to C-20 terpenyl pyrophosphates are intermediates in the biosynthesis of higher terpenoids in plants. Terpenyl pyrophosphates have been synthesized using enzyme preparations from *Lycopersicon esculentum* fruit plastids (Jungalwala and Porter, 1967), immature seed of *Echinocystis macrocarpa* (*Marah macrocarpus*) (Oster and West, 1968), juice from *Citrus sinensis* (Potty and Bruemmer, 1970; Chayet *et al*, 1973), *Pisum sativum* cotyledons (Moore and Coolbaugh, 1976) and in *Cucurbita pepo* endosperm and *Avena sativa* etioplasts (Stieger *et al*, 1985).

Furthermore, prenyltransferase, often designated as geranyl pyrophosphate (GPP) synthetase or farnesyl pyrophosphate (FPP) synthetase, plays a fundamental role in terpenoid metabolism by coupling IPP with either dimethylallyl pyrophosphate (DMAPP) or GPP to form GPP and FPP, respectively. This enzyme (EC 2.5.1.1) has been partially purified from *Ricinus communis* (Green and West, 1974), *Gossypium hirsutum* seed (Adams and Heinstein, 1973) and germinating seed

and seedlings of *Pisum sativum* (Green and Baisted, 1972; Allen and Banthorpe, 1981).

FPP has been implicated as the key intermediate in the biosynthesis of sesquiterpenoids (Loomis and Croteau, 1980; Banthorpe and Charlwood, 1980), while attempts have been made to develop the concept that IPP is a central intermediate in plant polyprenoid biosynthesis (Kreuz and Kleinig, 1981; 1984; Lutke-Brinkhaus and Kleinig, 1987). Nevertheless, the biosynthesis of the eudesmanoid phytoalexin, lubimin, a sesquiterpenoid, has been adequately demonstrated in cell-free extracts of *Solanum tuberosum* from both [2-^{14}C]-MVA and [1-^{14}C]-IPP (Coolbear and Threlfall, 1985). Furthermore, the study presented in Chapter 4 demonstrated that certain plant tissues were capable of synthesizing ABA from applied mevalonate, showing that they possessed the enzymes required to synthesize ABA from terpenyl pyrophosphate intermediates.

Only one report has been published on the cell-free biosynthesis of ABA. Milborrow (1974b) demonstrated that lysed chloroplast preparations from the mesocarp tissue of ripening *Persea americana* fruits would incorporate label from [^{14}C]-mevalonate into ABA. The reaction appeared to require light, oxygen and a comprehensive range of cofactors. Although ABA occurs in chloroplasts (Railton *et al*, 1974c; Loveys, 1977), contrary to earlier reports (Milborrow, 1974b; 1974c; 1976), recent evidence suggests that these organelles are not the major site of ABA biosynthesis and that the enzymes responsible for ABA metabolism are localised in the cytoplasm of mesophyll cells, at least in leaves of *Spinacia oleracea* (Hartung *et al*, 1980; 1981). In addition, Cowan and Railton (1986) were unable to demonstrate the synthesis of ABA from MVAL, MVA or IPP in lysed chloroplast preparations from either *Persea americana* or *Pisum sativum* and additional evidence was obtained to suggest that the catabolism of ABA in these preparations arose from contaminating, cytoplasmic enzymes. Further circumstantial evidence in support of the hypothesis for the cytoplasmic synthesis of ABA has recently been obtained in studies on the localisation of ABA in *Chenopodium polysternum* using immunoelectron-microscopy (Sossountzov *et al*, 1986).

Likewise, there is only a single report of a cell-free system capable of catabolising ABA (Gillard and Walton, 1976). A membranous fraction obtained from a low-speed, particulate preparation of *Echinocystis lobata* liquid endosperm, catalysed the hydroxylation of ABA to PA and its reduction to DPA. The hydroxylation of ABA required O_2 and NADPH or, less effectively, NADH and was inhibited in the presence of CO. In addition, the reaction was shown to be highly specific for the naturally-occurring (*S*)-enantiomer of ABA. Although this report appeared in the literature over

ten years ago, no attempts have been made to corroborate these findings and no further reports have been forthcoming regarding studies on the cell-free catabolism of ABA. Thus, it would appear that efforts to develop an ABA-catabolising cell-free system need to be focussed on other plant tissues as potential sources of the enzymes involved in this process.

In contrast to studies on the cell-free metabolism of ABA, the biosynthesis of GAs has been demonstrated in cell-free preparations from *Pisum sativum* immature embryos (Greabe *et al*, 1972; Kamiya and Greabe, 1983), *Cucurbita maxima* endosperm (Hedden *et al*, 1983), *Phaseolus vulgaris* immature seed (Kamiya *et al*, 1984; Takahashi *et al*, 1986), *Phaseolus coccineus* immature seed (Turnbull *et al*, 1986) and more recently in extracts of leaves from *Spinacia oleracea* (Gilmour *et al*, 1986). In addition, the GA 2β-hydroxylases have been partially purified and characterised from extracts of seeds of *Pisum sativum* and *Phaseolus vulgaris* (Smith and MacMillan, 1984; 1986).

The early stages of ABA biosynthesis from MVA to FPP should be catalysed by soluble enzymes requiring ATP and either Mg^{2+} or Mn^{2+} similar to GA biosynthesis (Hedden and Graebe, 1982). However, very little is known concerning both the early and the later stages of ABA biosynthesis in higher plants. Thus, it is clearly necessary to develop a cell-free system, capable of biosynthesizing and catabolising ABA, in order to investigate its biochemistry in more detail. In addition, the controversial issues surrounding the role of chloroplasts in ABA metabolism emphasizes the need to develop a cell-free system capable of metabolising ABA.

7.2. RESULTS.

7.2.1.Attempts to demonstrate the cell-free metabolism of ABA in extracts of *Hordeum vulgare* cv. Dyan embryos.

7.2.1.1.Attempts to demonstrate the biosynthesis of ABA in cell-free extracts of *Hordeum vulgare* cv. Dyan embryos.

The synthesis of terpenyl pyrophosphates, including FPP, from MVA in cell-free extracts of *Hordeum vulgare* cv. Zephyr seed embryos has been demonstrated (Davies *et al*, 1975). Furthermore, this is a non-photosynthetic tissue which therefore might preclude the involvement of the postulated carotenoid origin of ABA (see Chapter 1). Since intact embryo portions from seeds of *Hordeum vulgare* cv. Dyan incorporated radioactivity from *R*-[^{14}C]-MVAL into ABA (see

Chapter 4), cell-free extracts from embryos of this cultivar were examined for their ability to synthesize FPP and subsequently ABA.

7.2.1.1.1. Biosynthesis of terpenyl pyrophosphates from *R*-[2-^{14}C]-MVA and [1-^{14}C]-IPP in cell-free extracts of *Hordeum vulgare* cv. Dyan embryos.

Embryo cell-free systems were prepared as described by Davies *et al* (1975) (see Chapter 2) and were incubated with either *R*-[2-^{14}C]-MVA or [1-^{14}C]-IPP as substrates in phosphate buffer (0.1M, pH7.5) and in the presence of ATP(10mM), GSH(10mM), $MgCl_2$(6mM), NaF(5mM) and AMO1618 (1mM), which stimulated ABA biosynthesis in *Persea americana* mesocarp (Milborrow, 1976; and data presented in Chapter 4). Each anaerobic incubation was terminated after 2h, the protein precipitated and the supernatant chromatographed on a DEAE-cellulose column using increasing concentrations of $(NH_4)_2CO_3$ to give the elution profiles depicted in Figure 7.1 A, B and C.

Figure 7.1A depicts the elution profile obtained by separating the products from a heat-inactivated enzyme preparation incubated with *R*-[2-^{14}C]-MVA. The elution profiles depicted in Figure 7.1A and B represent the products of 3*R*-[2-^{14}C]-MVA (Figure 7.1B) and [1-^{14}C]-IPP (Figure 7.1C) metabolism in the *Hordeum vulgare* embryo cell-free extracts. The radioactive components were tentatively identified by comparison of their elution properties with those of authentic MVA and IPP and those reported for similar separations on DEAE-cellulose columns (Dugan *et al*, 1968; Potty and Bruemmer, 1973; Davies *et al*, 1975; Moore and Coolbaugh, 1976) and by paper chromatography (Dugan *et al*, 1968; Davies *et al*, 1975).

Analysis of the radioactive components by paper chromatography resulted in the tentative identification of the eluted compounds as follows: 3, [^{14}C]-IPP and 4, [^{14}C]-FPP (Table 7.1). Component 5, from feeds of [1-^{14}C]-IPP to *Hordeum vulgare* embryo cell-free extracts (Figure 7.1C) was tentatively identified as geranylgeranyl pyrophosphate (GGPP) from its elution properties on ion-exchange chromatography. Compounds 1 and 2 were tentatively identified as substrate *R*-[2-^{14}C]-MVA and the unhydrolysed lactone, *R*-[2-^{14}C]-MVAL, from similar separations on DEAE-cellulose columns of authentic *R*-[2-^{14}C]-MVA and *R*-[2-^{14}C]-MVAL (see Chapter 2).

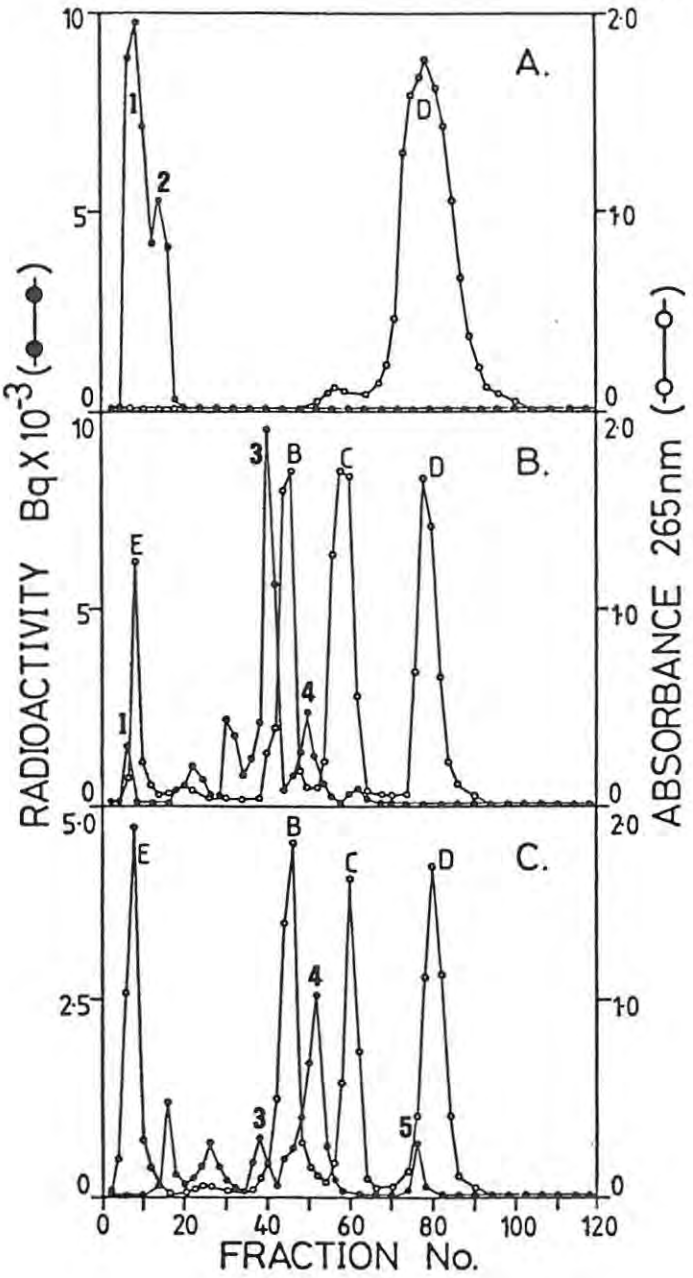

Figure 7.1. DEAE-column chromatographic separations of terpenyl pyrophosphates synthesized in a cell-free system from imbibed embryos of *Hordeum vulgare* cv. Dyan.

Separation of the radioactive components of the incubation mixture generated from feeds of (B) *R*-[2-^{14}C]-MVA (188kBq) and (C) [1-^{14}C]-IPP (94kBq) to cell-free extracts prepared from *Hordeum vulgare* cv. Dyan embryo tissues (see Section 2.7.5.2.2 for details) and incubated as described in Chapter 2. (A) The elution profile generated from a heat- treated control (100^{o}C x 10min), fed with *R*-[2-^{14}C]-MVA (188kBq). Components were separated on DEAE-cellulose columns (1.8 x 13.5cm) developed with 0-0.2M $(NH_4)_2CO_3$ and 5.0ml fractions were collected. Column effluent was monitored for radioactivity (●) and absorbance at 265nm (o). Alternate baseline points have been omitted. 1, MVA; 2, MVAL; 3, IPP; 4, FPP; 5, GGPP; B, AMP; C, ADP; D, ATP; E, Adenosine.

TABLE 7.1: Identification of putative terpenyl pyrophosphates from DEAE-cellulose column fractions.

An aliquot of the putative IPP and FPP zones, generated from either R-[2-^{14}C]-MVA (Fig. 7.5B) or [1-^{14}C]-IPP (Fig. 7.5C in cell-free systems prepared from Hordeum vulgare embryo tissue and separated by DEAE-cellulose column chromatography, was separated on Whatman No. 1 paper chromatograms developed to 35 cm in a decending manner with iso-propanol/iso-butanol/NH_3 (sp. gr. 0.91)/H_2O (40:20:1:39, v/v).

Substrate	Pooled column fraction*	Rf x 100	Tentative Identification
R-[2-^{14}C]-MVA	3	51	IPP[a]
	4	83	FPP
[1-^{14}C]-IPP	3	51	IPP[a]
	4	83	FPP
	5	ND	ND

* Pooled column fractions from DEAE-cellulose column separations (see Figure 7.5).

[a] co-chromatographed with a known standard

ND not determined

TABLE 7.2: Identification of terpenols from putative terpenyl pyrophosphates.

Aliquots of the putative IPP and FPP zones, generated from either R-[2-^{14}C]-MVA (Fig. 7.1B) or [1-^{14}C]-IPP (Fig. 7.1C) were hydrolysed with acid at 60°C for 60 min or alkaline phosphatase as described in Chapter 2. The products of hydrolysis were extracted with diethyl ether and chromatographed by TLC on silica gel G (type 60) in n-propanol/NH_3 (sp. gr. 0.91)/H_2O (6:3:1, v/v) developed once to 10 cm.

Substrate	Pooled column fraction	Products of hydrolysis* Rf x 100 on TLC		Rf x 100 on TLC of standard compound
		Acid	Alkaline Phosphatase	
R-[2-^{14}C]-MVA	3	81	81	[^{14}C]-isopentenol*
	4	90	90	(±) -farnesol[a]
[1-^{14}C]-IPP	3	81	81	[^{14}C]-isopentenol*
	4	90	90	(±) -farnesol[a]
	5	ND	ND	

* detected by liquid scintillation spectrometry.

[a] detected by exposure to iodine vapours (x 45 min) in a sealed chromatography tank.

ND = not determined.

7.2.1.1.2. Identification of IPP and FPP as products of *R*-[2-^{14}C]-MVA and [1-^{14}C]-IPP metabolism in cell-free extracts of *Hordeum vulgare* cv. Dyan embryos.

The identification of putative IPP and FPP was further established from the terpenol moieties released by hydrolysis of the pyrophosphate residues following treatment with alkaline phosphatase and/or acid.

Aliquots of the pooled fractions of compounds 3 and 4, produced from either *R*-[2-^{14}C]-MVA or [1-^{14}C]-IPP were treated with either alkaline phosphatase or hydrolysed in the presence of acid (1N, HCl added until pH1.0) and the products extracted into diethyl ether and analysed by TLC on silica gel G(Type 60) in *n*-propanol/NH_3 /H_2O (6:3:1, v/v) developed once to 10cm (Shechter, 1973). The terpenol moiety of compound 3, derived from feeds of *R*-[2-^{14}C]-MVA and [1-^{14}C]-IPP to *Hordeum vulgare* embryo cell-free extracts, cochromatographed with standard [1-^{14}C]-isopentenol, while that of compound 4, from similar feeds, cochromatographed with authentic (*R,S,*)-farnesol (Table 7.2).

The incorporation of radioactivity into IPP, FPP,and putative GGPP from substrates MVA and IPP is presented in Table 7.3 and indicates that these cell-free homogenates actively synthesize terpenyl pyrophosphates thus confirming the finding of Davies *et al* (1975) and suggesting that the enzymes

TABLE 7.3: Biosynthesis of [^{14}C]-IPP, [^{14}C]-FPP and putative [^{14}C]-GGPP in cell-free extracts of Hordeum vulgare cv. Dyan embryo.

Dissected embryo portions (2.0g f.w.), excised from Hordeum vulgare seeds, were imbibed in aerated tap-water for 12 h and homogenized on ice using a mortar and pestle in 5 ml 0.1M K_2HPO_4/KH_2PO_4 buffer (pH 7.5) aliquots of the 10 000 g supernatant (equivalent to 20 mg protein) were incubated with either R-[2-^{14}C]-MVA (183 kBq) or [1-^{14}C]-IPP (73.3 kBq) with the appropriate cofactors (ATP, 10mM; $MgCl_2$, 6mM; GSH, 10mM; NaF, 5mM) and AM01618 (1mM) in phosphate buffer (pH 7.5) in a total volume of 5.0 ml. Enzyme preparations were incubated in an N_2 atmosphere at 24°C for 2h under constant illumination (14.4μmol $m^{-2}s^{-1}$). Reactions were terminated by heating at 80°C x 2 min and the products analysed by DEAE-cellulose column chromatography as described in Chapter 2.

	Radioactivity incorporated into terpenyl pyrophosphates		
Substrate	IPP	FPP	GGPP*
	kBq (%)		
R-[2-^{14}C]-MVA	20.8 (11.35)	3.2 (1.72)	-
[1-^{14}C]-IPP	-	3.7 (5.05)	1.04 (1.42)

* GGPP was tentatively identified from its elution properties and sequence from DEAE-cellulose column chromatography.

required for the conversion of substrates MVA and IPP, into FPP are active in cell-free homogenates prepared from imbibing *Hordeum vulgare* embryo tissue. Thus, knowledge that cell-free extracts of *Hordeum vulgare* embryo tissue have the ability to synthesize intermediates in the terpenoid biosynthetic pathway, particularly the sesquiterpenoid precursor FPP, provided an ideal tool with which to examine the possible synthesis of ABA in similar cell-free systems.

7.2.1.1.3. Biosynthesis of ABA from *R*-[2-^{14}C]-MVA and [1-^{14}C]-IPP in cell-free extracts of *Hordeum vulgare* cv. Dyan embryos.

7.2.1.1.3.1. Biosynthesis of ABA from *R*-[2-^{14}C]-MVA.

R-[2-^{14}C]-MVA was incubated in cell-free extracts of *Hordeum vulgare* embryo tissue in the presence of AMO1618, NADPH (0.5μM) and air for 2h and the diethyl ether-soluble acids analysed for ABA. The results are depicted in Figure 7.2.

Figure 7.2A depicts the distribution of radioactivity generated from *R*-[2-^{14}C]-MVA feeds to cell-free extracts of *Hordeum vulgare* embryos. These data show a zone of radioactivity cochromatographing with authentic (*R,S,*)-ABA. In order to discount the possibility that this zone of radioactivity arose due to spill-over from the pH8.5 diethyl ether fraction, this fraction was analysed by TLC on silica gel GF_{254} in toluene/ethyl acetate/acetic acid (50:30:4, v/v). The result depicted in Figure 7.2D demonstrated that no major radioactive peak co-chromatographed with ABA. This suggested that the radio-peak corresponding in Rf to that of authentic (*R,S,*)-ABA was an acid and derived from MVA.

Examination of the methyl ester of the putative ABA by TLC in *n*-hexane/ethyl acetate (1:1, v/v) gave the result depicted in Figure 7.2B and showed that the putative ABAMe cochromatographed with authentic (*R,S,*)-ABAMe. Reduction of the putative ABAMe with $NaBH_4$ generated an equal mixture of two, more polar, radioactive products which cochromatographed with authentic standards of the 1',4'-*cis* and 1',4'-*trans* diols of ABAMe (Figure 7.2C), further establishing the identity of the putative ABAMe according to the criteria of Noddle and Robinson (1969) and Milborrow and Noddle (1970).

Initially, attempts were made to increase the incorporation of label from MVA into ABA by

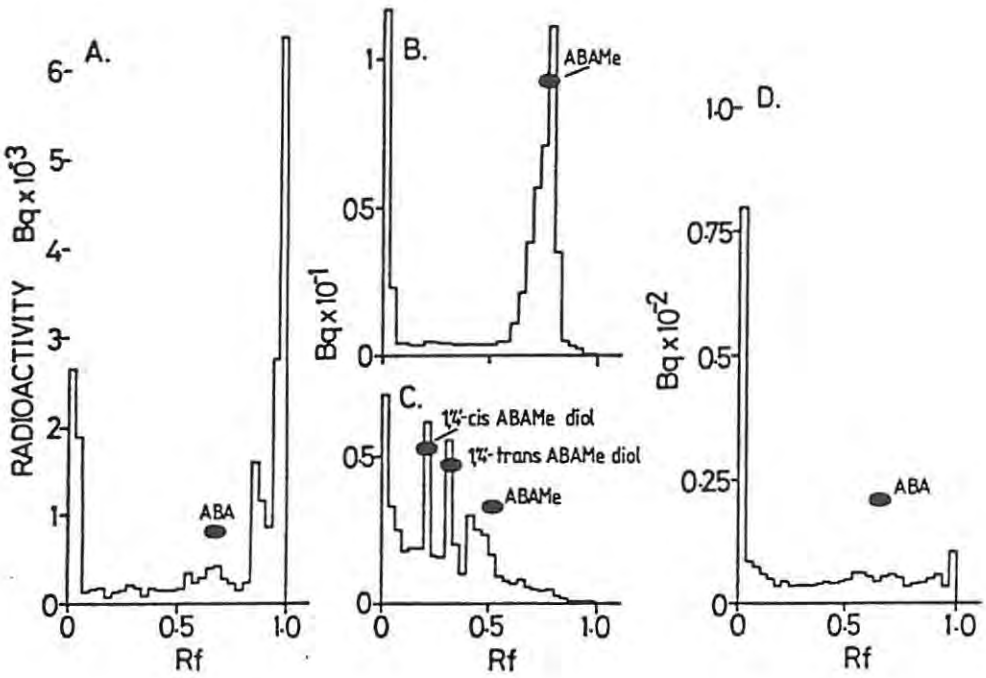

Figure 7.2. Biosynthesis of ABA from *R*-MVA in a cell-free system prepared from *Hordeum vulgare* cv. Dyan embryos.

Cell-free extracts were prepared and incubated as described in Table 7.5. (A) TLC separation of the diethyl ether-soluble acids on silica gel GF_{254} in toluene/ethyl acetate/acetic acid (50:30:4, v/v) developed 2x to 15cm; (B) TLC separation of the methyl ester of putative ABA from A), on silica gel GF_{254} in *n*-hexane/ethyl acetate (1:1, v/v) developed once to 15cm; (C) TLC separation of the $NaBH_4$ reduction products of B), on silica gel GF_{254} in benzene/ethyl acetate/acetic acid (15:3:1, v/v) developed once to 15cm and (D) TLC separation of the diethyl ether (pH 8.5) fraction on silica gel GF_{254} in toluene/ethyl acetate/acetic acid (50:30:4, v/v) developed 2x to 15cm.

including non-radioactive (*R,S,*)-ABA in incubations to act as a "cold-pool trap". When cell-free extracts were treated this way the results in Table 7.4 were obtained. These data demonstrated that the addition of (*R,S,*)-ABA resulted in an 8 fold increase in incorporation of radioactivity into newly biosynthesized ABA.

In order to determine the optimal amount of cold ABA needed to achieve maximum incorporation of radioactivity into ABA, increasing amounts of (*R,S,*)-ABA were added and the levels of radioactivity associated with newly biosynthesized ABA determined. Increasing the levels of cold (*R,S,*)-ABA up to 100μg resulted in increased incorporation into ABA. However, amounts of (*R,S,*)-ABA above 100μg actually retarded the incorporation of label from [^{14}C]-MVA into ABA (Figure 7.3). This suggested that high levels of ABA may interfere with the enzymes required for its own synthesis and could exert feedback inhibition in these cell-free extracts similar to that observed in studies on the ABA-producing fungus *Cercospora rosicola* (Norman *et al*, 1985c). In addition, where total feedback inhibition in the fungus was achieved at 400μM ABA only half this concentration was required to bring about total feedback inhibition in the *Hordeum vulgare* embryo cell-free system. Thus, in all further experiments 75μg of (*R,S,*)-ABA, which enhanced maximally the incorporation of label from MVA into ABA, was routinely included in incubations.

The results of five seperate experiments to demonstrate the biosynthesis of ABA from MVA in the presence of a "cold-pool trap" of ABA are presented in Table 7.5 and show, clearly, the reproducibility of the system. Kinetic studies (Figure 7.4) showed that the production of ABA from MVA was linear during the first 3h after which, incorporation of radioactivity declined gradually, probably as a result of product catabolism.

7.2.1.1.3.2. Biosynthesis of ABA from [1-^{14}C]-IPP.

In order to determine whether ABA produced from MVA could be similarly generated from a terpenyl pyrophosphate intermediate, [1-^{14}C]-IPP was incubated in cell-free extracts from *Hordeum vulgare* embryos. The results depicted in Figure 7.5 confirmed that label from [1-^{14}C]-IPP could be incorporated into ABA in this system.

Examination of the methyl ester of putative ABA by TLC on silica gel GF_{254} in *n*-hexane/ethyl acetate (1:1, v/v) gave the result shown in Figure 7.5B and demonstrated that putative ABAMe co-migrated as a single radioactive peak with authentic (*R,S,*)-ABAMe. Reduction of putative ABAMe

TABLE 7.4: Effect of (R,S)-ABA on the biosynthesis of ABA from MVA in cell-free extracts of Hordeum vulgare cv. Dyan embryo tissue.

Cell-free homogenates of Hordeum vulgare were prepared as described in Chapter 2 and incubated in the presence of the appropriate cofacters, AM01618 (1.0mM), NADPH (0.5μM), R-[2-^{14}C]-MVA (90 kBq) in 0.1M K_2HPO_4/KH_2PO_4 buffer (pH 7.5) in a total volume of 4.0 ml, with or without 75 μg (R,S)-ABA. Total protein was 20 mg/incubate. Extracts were incubated at 28°C for 2h in a metabolic shaker under constant illumination and the reaction terminated by the addition of an equal volume of ice-cold methanol. ABA was extracted and analysed as described in Chapter 2.

Treatment	Radioactivity incorporated into ABA*
	Bq (%)
+ (R,S)-ABA (75 μg)	123.17 (0.134)
- (R,S)-ABA	15.18 (0.016)

* Data corrected for heat inactivated controls.

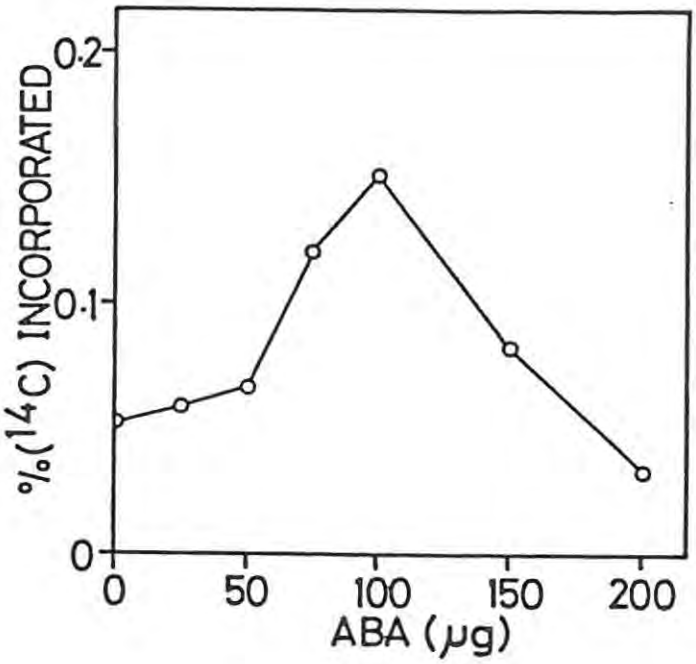

Figure 7.3. The effect of (*R,S,*)-ABA, added as a "cold-pool" trap, on the biosynthesis of ABA from *R*-MVA in a cell-free extract prepared from *Hordeum vulgare* cv. Dyan embryos. Extracts were prepared as described in Chapter 2 and incubated with *R*-[2-^{14}C]-MVA (90kBq), increasing amounts of (*R,S,*)-ABA and all the other additions as previously described. ABA was extracted and analysed as described in Chapter 2.

TABLE 7.5: The biosynthesis of ABA from MVA in cell-free extracts of Hordeum vulgare cv. Dyan embryo tissue.

Embryo portions of Hordeum vulgare seeds were imbibed in aerated tap-water for 12 h and homogenized in ice-cold 0.1M K_2HPO_4/KH_2PO_4 buffer, pH 7.5 (1.5 ml/g f.w.). 2.0 ml Aliquots of the 10 000 g supernatant (equivalent to 20 mg protein) were incubated with R-[2-^{14}C]-MVA (90 kBq) in a mixture of the appropriate cofactors in 0.1M KPi buffer containing 75 µg (R,S)-ABA, 0.5 µM NADPH and AM01618 (1 mM) in a total volume of 4.0 ml. Reactions were incubated at 28°C in a metabolic shaker for 2 h under continuous illumination (14.4µmol $m^{-2}s^{-1}$). Reactions were terminated by the addition of an equal volume of ice-cold methanol and ABA extracted and analysed as described in Chapter 2.

	Incorporation of Radioactivity into ABA			
Experiment No.	ABA	ABAMe	1',4'-cis diol of ABAMe	1',4'-trans diol of ABAMe
		Bq (%)		
1	125.55 (0.139)	120.56 (0.134)	46.55 (0.052)	63.05 (0.070)
2	108.61 (0.121)	98.22 (0.109)	39.14 (0.043)	51.86 (0.057)
3	108.67 (0.121)	104.53 (0.116)	47.88 (0.053)	48.39 (0.054)
4	126.75 (0.141)	103.06 (0.115)	44.60 (0.049)	51.62 (0.057)
5	117.38 (0.130)	106.59 (0.118)	44.54 (0.049)	53.73 (0.059)

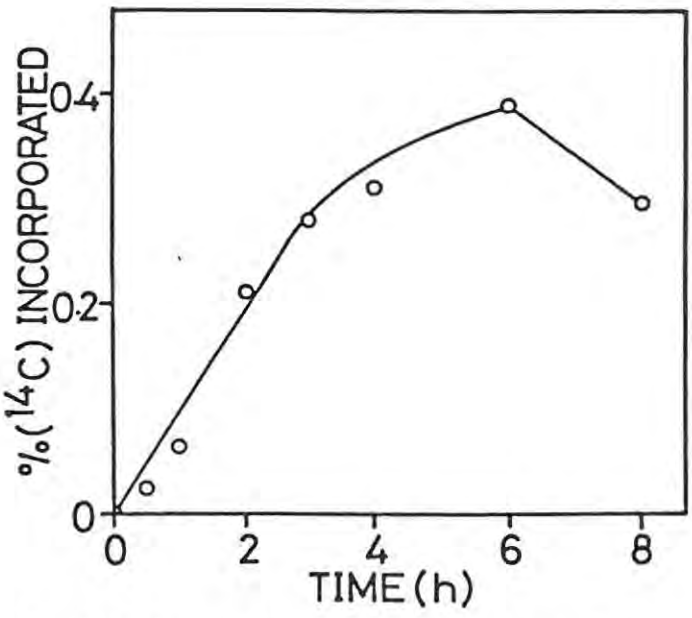

Figure 7.4. Kinetics of ABA biosynthesis from *R*-MVA in cell-free extracts of *Hordeum vulgare* cv. Dyan embryos. All reaction mixtures contained 20mg of protein, *R*-[2-^{14}C]-MVA (90kBq) and all the other additions as previously described. Reactions were carried out at 28°C for 2h under continuous illumination (14.4µmol m^{-2} s^{-1}). ABA was extracted and analysed as described in Chapter 2.

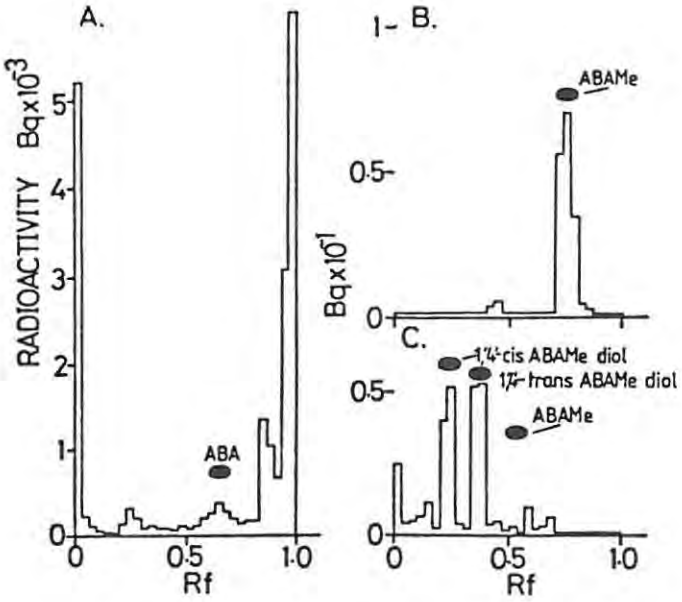

Figure 7.5. Biosynthesis of ABA from IPP in a cell-free system prepared from *Hordeum vulgare* cv. Dyan embryos.

Cell-free extracts were prepared and incubated as described in Table 7.6. (A) TLC separation of the diethyl ether-soluble acids on silica gel GF_{254} in toluene/ethyl acetate/acetic acid (50:30:4, v/v) developed 2x to 15cm; (B) TLC separation of the methyl ester of putative ABA from A), on silica gel GF_{254} in *n*-hexane/ethyl acetate (1:1, v/v) developed once to 15cm; (C) TLC separation of the $NaBH_4$ reduction products of B), on silica gel GF_{254} in benzene/ethyl acetate/acetic acid (15:3:1, v/v) developed once to 15cm.

TABLE 7.6: **The biosynthesis of ABA from IPP in cell-free extracts of Hordeum vulgare cv. Dyan embryo tissue.**

Embryo portions of Hordeum vulgare seeds were imbibed in aerated tap-water for 12 h and homogenized in ice-cold K_2HPO_4/KH_2PO_4 buffer, (0.1M; pH 7.5) (1.5 ml/g f.w.). 2.0 ml Aliquots of the 10 000 g supernatant (equivalent to 20 mg protein) were incubated with [1-^{14}C]-IPP (90 kBq) in a mixture of the appropriate cofactors in 0.1M KPi buffer, containing 75 µg (R,S)-ABA, 0.5 µM NADPH and 1.0mM AMO1618 in a total volume of 4.0 ml. Reactions were incubated at 28°C in a metabolic shaker for 2 h under continuous illumination (14.4µmol $m^{-2}s^{-1}$) and terminated by the addition of an equal volume of ice-cold methanol. ABA was extracted and analysed as described in Chapter 2.

	Incorporation of Radioactivity into ABA			
Experiment No.	ABA	ABAMe	1',4'-cis diol of ABAMe	1',4'-trans diol of ABAMe
		Bq (%)		
1	230.13 (0.256)	199.12 (0.221)	82.93 (0.092)	103.15 (0.115)
2	162.39 (0.180)	122.69 (0.136)	60.21 (0.067)	59.25 (0.066)

with $NaBH_4$ generated a mixture of the 1',4'-*cis* and 1',4'-*trans* diols of ABAMe (Figure 7.5C), further establishing the identity of putative ABAMe.

In addition, two further experiments produced identical results, (Table 7.6), again demonstrating the reproducibility of this cell-free system, to incorporate label from [1-^{14}C]-IPP into ABA.

7.2.1.1.4. Evidence for the enzyme-catalysed biosynthesis of ABA from MVA in *Hordeum vulgare* embryo cell-free systems.

When the pH optimum of the reaction was determined, it was found to peak sharply between pH7.4 and pH7.6 (Figure 7.6). Increasing the concentration of protein resulted in increased incorporation of label from MVA into ABA (Figure 7.7), which was indicative of an enzyme-catalysed reaction. The small differences in the incorporation of label from MVA into ABA in incubates containing between 10 and 20mg of protein might be due to increases in associated inhibitory compounds. The inclusion of trypsin, a plant protease (Ryan, 1973), significantly retarded the biosynthesis of ABA by almost 50% (see Table 7.9), further demonstrating the protein requirement for these reactions. In addition, no incorporation of radioactivity from MVA into ABA was observed in heat-inactivated extracts. Thus, the above data provide good evidence to suggest that the conversion of MVA into ABA in cell-free extracts of *Hordeum vulgare* embryos occurs as a result of an enzyme-catalysed reaction.

7.2.1.1.5. Effect of protein synthesis inhibitors on the biosynthesis of ABA in cell-free extracts of *Hordeum vulgare* cv. Dyan embryos.

In order to obtain information on whether the enzymes required for ABA biosynthesis were pre-existing or were synthesized *de novo* during imbibition, *Hordeum vulgare* embryo portions were imbibed in a solution of CHI prior to homogenisation and incubation with *R*-[2-^{14}C]-MVA. In addition, the effect of CAP was also studied in order to negate the possibility that any enzyme activity arose from microbial contamination.

The results presented in Table 7.7 showed that active protein biosynthesis, during imbibition, was required for the cell-free biosynthesis of ABA which precludes the activation of the ABA-biosynthesizing enzymes from a pre-existing enzyme pool. In addition, CAP included in the incubations, had no effect on the levels of radioactivity incorporated into ABA, from either [2-^{14}C]-

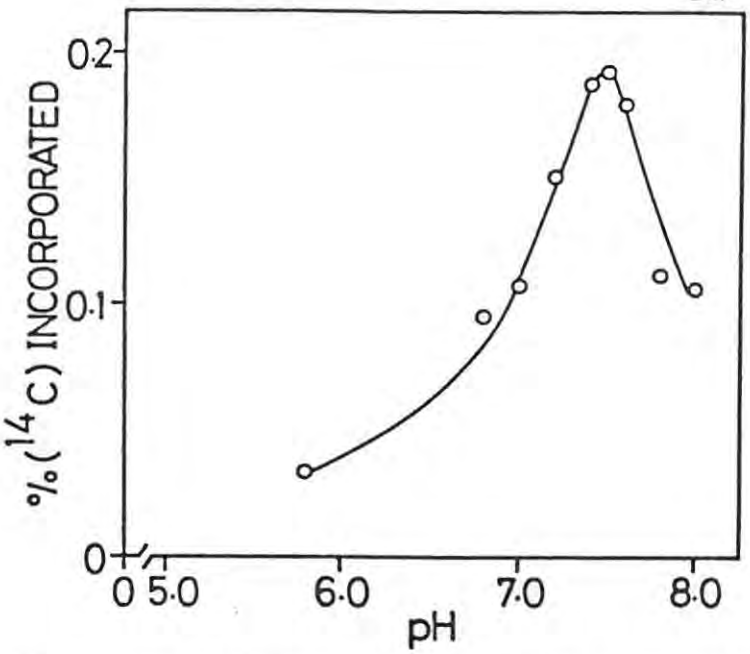

Figure 7.6. The effect of pH on the biosynthesis of ABA from *R*-MVA in cell-free extracts of *Hordeum vulgare* cv. Dyan embryos. Reaction mixtures contained 20mg protein, *R*-[2-^{14}C]-MVA (90kBq), NADPH (0.5μM) and all the other additions as previously described. Reactions were carried out at 28°C for 2h under continuous illumination (14.4μmol m^{-2} s^{-1}). ABA was extracted and analysed as described in Chapter 2.

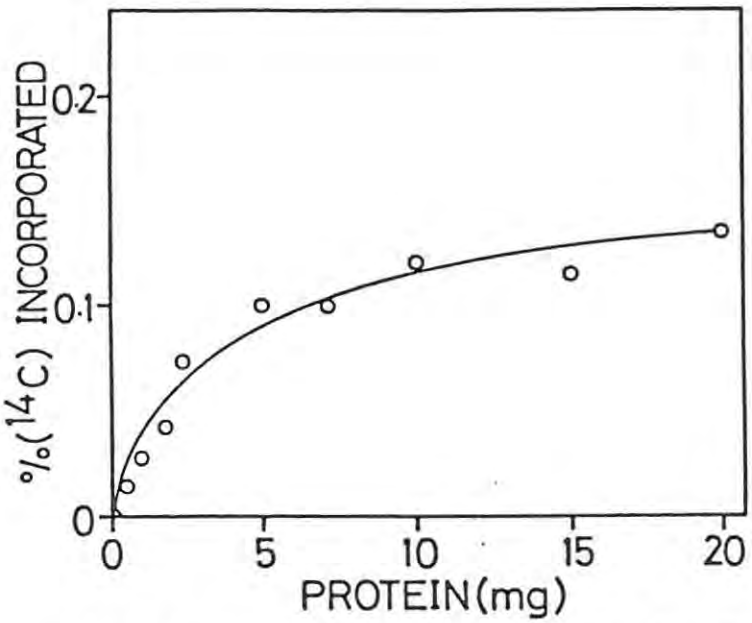

Figure 7.7. The effect of protein concentration on the biosynthesis of ABA from *R*-MVA in cell-free extracts of *Hordeum vulgare* cv. Dyan embryos. Protein concentration was determined using the Bradford dye-binding assay. Incubation procedures were as described for Table 7.5. Reactions were carried out at 28°C for 2h under continuous illumination (14.4μmol m^{-2} s^{-1}). ABA was extracted and analysed as described in Chapter 2.

TABLE 7.7: Effect of inhibitors of protein synthesis on the synthesis of ABA from MVA and IPP in cell-free extracts of Hordeum vulgare cv. Dyan embryo tissue.

Dissected embryo portions of Hordeum vulgare seed were imbibed in water or aqueous solutions of cycloheximide (1 mg/ml) for 12 h prior to homogenization and centrifugation. 2.0 ml Aliquots of the 10 000 g supernatant (equivalent to 20 mg protein) were incubated with the appropriate cofactors (Table 7.8) and either [2-^{14}C]-MVA (90 kBq) or [1-^{14}C]-IPP (90 kBq) in 0.1 M K_2HPO_4/KH_2PO_4 buffer (pH 7.5) in a total volume of 4.0 ml. AMO1618 (1 mM) and (R,S)-ABA (75 µg) were included as described previously and chloramphenicol (1 mg/ml) was added to the incubation medium where specified. Reactions were carried out at 28°C for 2 h in a metabolic shaker under constant illumination and terminated by the addition of an equal volume of ice-cold methanol. ABA was extracted and analysed as described in Chapter 2.

		Radioactivity in ABA		
Substrate	Treatment	Bq	%	% relative to control
R-[2-^{14}C]-MVA	Control	128.58	0.143	100.00
R-[2-^{14}C]-MVA	CHI imbibed	3.87	0.004	2.79
R-[2-^{14}C]-MVA	+ CAP*	139.01	0.154	107.69
[1-^{14}C]-IPP	Control	111.44	0.124	100.00
[1-^{14}C]-IPP	+ CAP*	110.87	0.123	99.12

* CAP included during incubation only.

TABLE 7.8: Phosphatase and Protease activity associated with cell-free extracts of Hordeum vulgare cv. Dyan embryo tissue.

Cell-free extracts were prepared from imbibed Hordeum vulgare embryo tissue and the levels of alkaline phosphatase, acid and alkaline protease activity determined as described in Chapter 2.

		Enzyme Activity	
Enzyme Assay	Protein concentration* µg/ml	µg L-tryptophan released/ ml[a]	ng Alkaline phosphatase[b]
Alkaline phosphatase	100	-	0.35
Alkaline protease	100	45.00	-
Acid protease	100	9.38	-

* Determined from a standard curve of BSA using Bradfords' Reagent.

[a] Determined from a standard curve for L-tryptophan at A_{280}.

[b] Determined from a standard curve for stock solutions of alkaline phosphatase (sep. act. 35 U/mg).

MVA or [1-^{14}C]-IPP, suggesting that metabolism of the radioactive substrates was not due to microbial contamination.

7.2.1.1.6. Determination of phosphatase and protease activity in cell-free extracts of *Hordeum vulgare* cv. Dyan embryos.

A compounding factor in these cell-free studies might be the presence of high levels of phosphatase and protease activity which would reduce the capacity for ABA biosynthesis by hydrolysing terpenyl pyrophosphate intermediates and enzymes associated with this biosynthetic route. Proteases have been detected in extracts of germinating *Hordeum vulgare* seeds (Mikola and Kolehmaninen, 1972).

Analysis of *Hordeum vulgare* embryo supernatants for the presence of these enzymes, demonstrated low levels of alkaline phosphatase and acid protease activity (less than 10% of total protein) but considerably higher levels of alkaline protease activity (Table 7.8).

7.2.1.1.7. Effect of inhibitors of protease activity on the biosynthesis of ABA in cell-free extracts of *Hordeum vulgare* cv. Dyan embryos.

Since significant alkaline protease activity was detected in cell-free extracts of *Hordeum vulgare* embryos, the effects of various inhibitors of protease activity on ABA biosynthesis in this system were investigated.

The results in Table 7.9 showed that BSA, added to provide an alternative substrate for proteases, had no effect on the incorporation of label from MVA into ABA. The inhibitors of proteolytic activity, trypsin inhibitor, pepstatin and PMSF were only marginally effective against associated protease activity and had little effect on ABA biosynthesis from MVA. However, leupeptin which has been used advantageously in other studies to suppress proteolytic activity (Alpi and Beevers, 1981), markedly enhanced the incorporation of label from MVA into ABA and was thus included routinely in such extracts.

7.2.1.1.8. Chemical requirements for the biosynthesis of ABA in cell-free extracts of *Hordeum vulgare* cv. Dyan embryos.

In order to establish the chemical requirements for the optimum cell-free biosynthesis of ABA, extracts of *Hordeum vulgare* embryos were incubated with *R*-[2-^{14}C]-MVA and various combinations of ATP, $MgCl_2$, NADPH, NaF, GSH and AMO1618.

The results presented in Table 7.10 demonstrated that the reaction was highly dependent on ATP, Mg^{2+} and NADPH as cofactors and on the addition of AMO1618. The omission of both sodium

TABLE 7.9: Effect of inhibitors of protease activity, BSA and trypsin on the synthesis of ABA from MVA in cell-free extracts of Hordeum vulgare cv. Dyan embryo tissue.

Cell-free extracts of Hordeum vulgare embryo tissue were prepared as previously described. 2.0 ml aliquots of the 10 000 g supernatant (equivalent to 20 mg protein) were incubated with the appropriate cofactors (Table 7.8) and R-[2-^{14}C]-MVA (90 kBq), NADPH (0.5µM), AMO1618 (1.0 mM) and (R,S)- ABA (75 µg) in 0.1M K_2HPO_4/KH_2PO_4 buffer (pH 7.5) in a total volume of 4.0 ml containing either BSA (1.0 mg/ml), trypsin (1.0 mg/ml) trypsin inhibitor (0.6 mg/ml), pepstatin (0.01 M), leupeptin (0.002 M) or PMSF (0.01 M). Reactions were carried out at 28°C for 2 h in a metabolic shaker under continuous illumination (14.4µmol $m^{-2}s^{-1}$) and terminated by the addition of an equal volume of ice-cold methanol. ABA was extracted and analysed as described in Chapter 2.

	Radioactivity in ABA		
Treatment	Bq	%	% Radioactivity relative to control
Control	115.83	0.129	100.00
+ BSA	113.53	0.126	97.67
+ Trypsin	65.18	0.072	55.81
+ Trypsin inhibitor	145.02	0.161	124.81
+ Pepstatin	116.14	0.129	100.00
+ Leupeptin	285.57	0.317	245.74
+ PMSF	121.53	0.135	104.65

fluoride (NaF), added to reduce phosphatase activity, and glutathione (GSH), added as a sulphydryl protectant, had no effect on the incorporation of label from [^{14}C]-MVA into ABA. A requirement for AMO1618, which is known to inhibit the cyclisation of GGPP to *ent*-kaurene (Fall and West, 1971) and the cyclisation of sqalene 2,3-epoxide (Douglas *et al*, 1972; 1974), suggested that a build-up in the levels of terpenyl pyrophosphates could be responsible for channelling FPP into ABA, although it might stimulate directly an enzyme-catalysed step. Increasing the concentration of AMO1618 up to 2mM was shown to result in increased incorporation of label from [^{14}C]-MVA into ABA (Figure 7.8). A requirement for NADPH implied that there was a post-FPP reductive step and the reaction was shown to be dependent on NADPH at concentrations of between 0.5 and 1.0μM (Figure 7.9). A possible reason for the reduced incorporation of MVA into ABA at NADPH concentrations above 0.5μM might be that the catabolism of ABA, which involves a reductive step requiring this cofactor, was enhanced at these higher concentrations of cofactor.

TABLE 7.10: Cofactor requirements for the synthesis of ABA from MVA in cell-free extracts of Hordeum vulgare cv. Dyan embryo tissue.

Cell-free homogenates of Hordeum vulgare embryo were prepared as described in Chapter 2. 2.0 ml aliquots of 10 000 g supernatant (equivalent to 20 mg protein) were incubated with various combinations of cofactors in a total volume of 4.0 ml 0.1 M K_2HPO_4/KH_2PO_4 buffer (pH 7.5). All incubates contained R-[2-^{14}C]-MVA (90 kBq) and (R,S)-ABA (75 μg). In addition, the effect of AMO1618 on ABA biosynthesis was similarly examined. Extracts were incubated at 28°C for 2 h under constant illumination (14.4μmol $m^{-2}s^{-1}$) in a metabolic shaker. Reactions were terminated by the addition of an equal volume of ice-cold methanol and ABA extracted and analysed as described in Chapter 2.

	Radioactivity incorporated into ABA*		
Treatment	Bq	%	% inhibition
Control	83.83	0.093	0.00
- cofactors	1.47	0.001	98.92
- ATP	0	0	100.00
- $MgCl_2$	0	0	100.00
- NADPH	21.28	0.024	75.38
- NaF	88.70	0.098	0.00
- GSH	87.97	0.098	0.00
- AMO1618	4.60	0.005	95.51

* Data corrected for heat inactivated control.

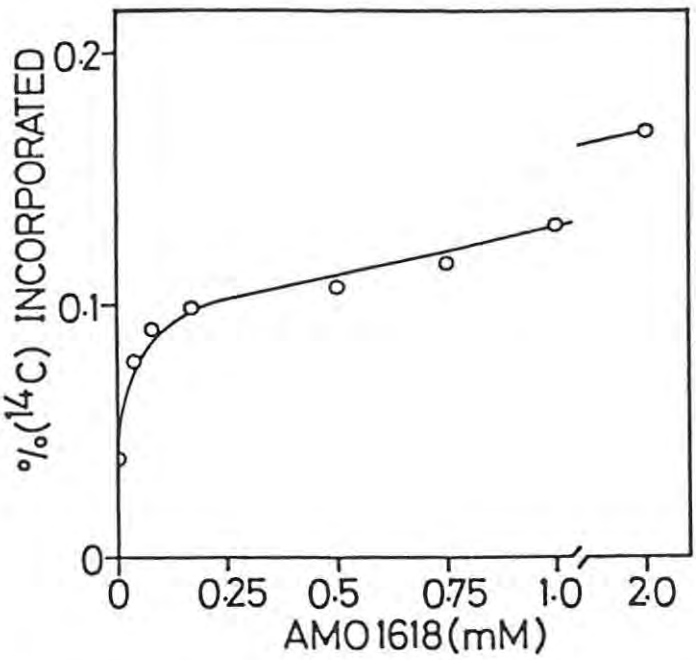

Figure 7.8. The effect of AMO1618 concentration on the biosynthesis of ABA from *R*-MVA in cell-free extracts of *Hordeum vulgare* cv. Dyan embryos. Reaction mixtures contained 20mg protein, *R*-[2-^{14}C]-MVA (90kBq), increasing amounts of AMO1618 and all the other additions as previously described. Reactions were carried out at 28°C for 2h under continuous illumination (14.4μmol m^{-2} s^{-1}). ABA was extracted and analysed as described in Chapter 2.

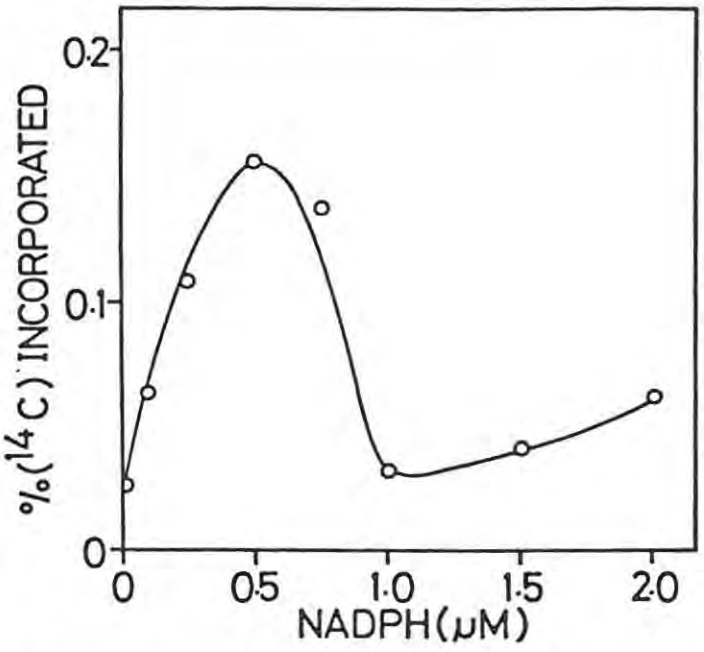

Figure 7.9. The effect of NADPH concentration on the biosynthesis of ABA from *R*-MVA in cell-free extracts of *Hordeum vulgare* cv. Dyan embryos. Reaction mixtures contained 20mg protein, *R*-[2-^{14}C]-MVA (90kBq), increasing amounts of NADPH and all the other additions as previously described. Reactions were carried out at 28°C for 2h under continuous illumination (14.4μmol m^{-2} s^{-1}). ABA was extracted and analysed as described in Chapter 2.

7.2.1.1.9. Effect of anaerobiosis and CO on the biosynthesis of ABA in cell-free extracts of *Hordeum vulgare* cv. Dyan embryos.

A requirement for oxygen would be expected for the transformation of FPP to ABA. Since such oxidations might be mediated *via* mixed function oxidases, the effect of carbon monoxide, a potent inhibitor of these enzymes (Nebert and Gonzalez, 1987), on the synthesis of ABA in *Hordeum vulgare* embryo cell-free extracts was also examined.

Aliquots of *Hordeum vulgare* embryo supernatants were incubated in phosphate buffer (pH7.5) containing the required cofactors and *R*-[2-^{14}C]-MVA in either, air, N_2 or a CO atmosphere for 2h periods. Analysis of the diethyl ether-soluble acids demonstrated that anaerobiosis inhibited the production of ABA by 95% demonstrating that oxygen was needed for the biosynthesis of ABA (Table 7.11). Treatment with CO resulted in a 90% reduction in the capacity of these extracts to synthesize ABA. Thus, the requirement for NADPH, O_2, a pH optimum of 7.5 and the marked inhibition by CO was consistent with the properties of cytochrome P450-dependent mixed function oxidases (Russell, 1971; Potts *et al*, 1974; Rich and Bendall, 1975; Hasson and West, 1976a; 1976b; Reichhart *et al*, 1980; Benveniste *et al*, 1982; Hendry, 1986), although centrifugation studies to reveal the expected particulate nature of this activity would need to be carried out to confirm this suggestion.

7.2.1.1.10. Effect of 2-0xoglutarate, Fe^{2+} and ascorbate on ABA biosynthesis in cell-free extracts of *Hordeum vulgare* cv. Dyan embryos.

It is now well established that soluble oxidases are required in the latter stages of GA biosynthesis (Hedden and Graebe, 1982). These enzymes require 2-oxoglutarate, Fe^{2+}, O_2 and are stimulated by the presence of ascorbate (Hedden and Graebe, 1982; Kamiya *et al*, 1983; Graebe, 1986) and the reaction mechanism involves oxidation of 2-oxoglutarate by an enzyme-bound Fe(ll).O_2 complex resulting in the formation of an oxonium species, the ultimate oxidant (Hedden and Graebe, 1982).

In order to establish whether soluble oxidases might also be involved in the synthesis of ABA in extracts of *Hordeum vulgare*, aliquots of the embryo supernatant were incubated with either *R*-[2-^{14}C]-MVA or [1-^{14}C]-IPP and the appropriate chemical additions in phosphate buffer (pH7.5) with or

TABLE 7.11: Effect of CO and anaerobiosis on the synthesis of ABA from MVA in cell-free extracts of Hordeum vulgare cv. Dyan embryo tissue.

Cell-free homogenates of Hordeum vulgare embryos were prepared as described in Chapter 2. 2.0 ml Aliquots of the 10 000 g supernatant (equivalent to 20 mg protein) were incubated in the presence of cofactors (ATP, 10 mM; $MgCl_2$, 6 mM; GSH, 10 mM; NaF, 5 mM), AMO1618 (1.0mM), NADPH (0.5μM), (R,S)- ABA (75 μg) and R-[2-^{14}C]-MVA (90 kBq) in 0.1M K_2HPO_4/KH_2PO_4 buffer (pH 7.5) in a total volume of 4.0 ml. Reactions were carried out at 28°C for 2 h in a metabolic shaker in atmospheres of either air, N_2, or CO under continuous illumination (14.4μmol $m^{-2}s^{-1}$). Reactions were terminated by the addition of an equal volume of ice-cold methanol and ABA extracted and analysed as described in Chapter 2.

	Radioactivity incorporated into ABA		
Treatment	Bq	%	% inhibition relative to control
Control	115.67	0.129	0.0
N_2	4.76	0.005	96.12
CO	13.61	0.015	88.37

TABLE 7.12: Effect of 2-oxoglutarate, Fe^{2+} and ascorbate on the synthesis of ABA from MVA and IPP in cell-free extracts of Hordeum vulgare cv. Dyan embryo tissue.

Cell-free homogenates of Hordeum vulgare embryos were prepared as described in Chapter 2. 2.0 ml Aliquots of the 10 000 g supernatant (equivalent to 20 mg protein) were incubated in the presence of cofactors (ATP, 10 mM; $MgCl_2$, 6 mM; GSH, 10 mM; NaF, 5 mM), AMO1618 (1.0mM), NADPH (0.5μM), (R,S)- ABA (75 μg) and either R-[2-^{14}C]-MVA (90 kBq) or [1- ^{14}C]-IPP (90 kBq) as substrate in 0.1M K_2HPO_4/KH_2PO_4 buffer (pH 7.5) in a total volume of 4.0 ml. Reactions were carried out at 28°C for 2 h in a metabolic shaker under continuous illumination (14.4μmol $m^{-2}s^{-1}$). Reactions were terminated by the addition of an equal volume of ice-cold methanol and ABA extracted and analysed as described in Chapter 2.

		Radioactivity incorporated in ABA		
Substrate	Hydroxylating system	Bq	%	% increase relative to control
R-[2-^{14}C]-MVA	-	128.58	0.143	-
R-[2-^{14}C]-MVA	+	144.23	0.160	10.63
[1-^{14}C]-IPP	-	111.44	0.123	-
[1-^{14}C]-IPP	+	152.79	0.169	27.22

without 2-oxoglutarate, $FeSO_4$ and ascorbate for 2h. The data presented in Table 7.12 demonstrated that the inclusion of the hydroxylating system in the incubation medium enhanced the incorporation of label from MVA and IPP into ABA by 10.63% and 27.22% respectively which suggests that some of the steps in ABA biosynthesis in *Hordeum vulgare* embryos might be catalysed by these enzymes.

7.2.1.1.11. Effect of inhibitors of GA biosynthesis on ABA biosynthesis in cell-free extracts of *Hordeum vulgare* cv. Dyan embryos.

CCC and ancymidol, two potent inhibitors of GA biosynthesis (Dennis *et al*, 1965; Coolbaugh *et al*, 1978), have been shown to inhibit the biosynthesis of ABA in the ABA-producing fungi *Cercospora rosicola* (Norman *et al*, 1983) and *Botrytis cinerea* (Hirai *et al*, 1986) and to inhibit the biosynthesis of ABA from mevalonate in ripening *Persea americana* mesocarp tissue (see Chapter 4). In contrast, another inhibitor of GA biosynthesis, AMO1618, which was shown to inhibit ABA biosynthesis in *Cercospora rosicola* (Norman *et al*, 1983), enhanced the incorporation of label from mevalonate into ABA in *Persea americana* mesocarp (see Chapter 4) and in cell-free extracts of *Hordeum vulgare* (section 7.2.1.4.5) and therefore the effects of ancymidol and CCC on the biosynthesis of ABA in this system were examined.

Ancymidol and CCC were added to separate incubation media containing the appropriate chemical additives and [2-^{14}C]-MVA and their effect on ABA biosynthesis determined. The results presented in Table 7.13 demonstrated that both ancymidol and CCC, in the presence of AMO1618, inhibited the incorporation of label from MVA into ABA by 63.31% and 66.67% respectively.

Ancymidol inhibits GA biosynthesis by interacting with the enzymes catalysing the oxidation of *ent*-kaurene (Coolbaugh *et al*, 1978). The inhibition of ABA biosynthesis by ancymidol, coupled with the evidence presented in Section 7.2.1.1.9 further suggests that similar mixed function oxidase enzymes might be involved in ABA biosynthesis. CCC is an effective inhibitor of the A-activity of kaurene synthetase (Frost and West, 1977) responsible for the cyclisation of GGPP to copalyl pyrophosphate. Since CCC was effective at reducing ABA biosynthesis in these cell-free extracts, it is possible that CCC inhibits a post-FPP cyclisation step in the biosynthesis of ABA.

TABLE 7.13: Effect of inhibitors of gibberellin biosynthesis on the synthesis of ABA from MVA in cell-free extracts of Hordeum vulgare cv. Dyan embryo tissue.

CCC and ancymidol (at 500 μM) were included in the incubation medium along with NADPH (0.5 μM), AMO1618 (1.0 mM), (R,S)-ABA (75 μg) and R-[2-^{14}C] -MVA (90 kBq) and enzyme (equivalent to 20 mg protein) in a total volume of 4.0 ml of K_2HPO_4/KH_2PO_4 buffer (0.1M; pH 7.5). Reactions were carried out at 28°C for 2 h in a metabolic shaker under conditions of constant illumination (14.4μmol $m^{-2}s^{-1}$) and were terminated by the addition of an equal volume of ice-cold methanol. ABA was extracted and analysed as described in Chapter 2.

	Radioactivity in ABA		
Treatment	Bq	%	% inhibition relative to control
Control	115.83	0.129	0.00
+ CCC	38.69	0.043	66.67
+ Ancymidol	42.50	0.047	63.31

TABLE 7.14: Effect of cytokinins on the synthesis of ABA from MVA in cell-free extracts of Hordeum vulgare cv. Dyan embryo tissue.

Adenine, zeatin, kinetin, BA and IPA (all at 500 μM) were included in the incubation medium containing the appropriate cofactors, NADPH (0.5 μM), AMO1618 (1.0 mM), (R,S)-ABA (75 μg) and R-[2-^{14}C]-MVA (90 kBq) and enzyme (equivalent to 20 mg protein) in a total volume of 4.0 ml of K_2HPO_4/KH_2PO_4 buffer (0.1 M, pH 7.5). Reactions were carried out at 28°C for 2h in a metabolic shaker under conditions of constant illumination (14.4μmol $m^{-2}s^{-1}$) and were terminated by the addition of an equal volume of ice-cold methanol. ABA was extracted and analysed as described in Chapter 2.

	Radioactivity in ABA		
Treatment	Bq	%	% inhibition relative to control
Control	115.83	0.129	0.00
+ Adenine	118.38	0.132	0.00
+ Zeatin	36.36	0.040	68.68
+ Kinetin	32.99	0.037	71.59
+ BA	11.90	0.013	89.75
+ IPA	20.34	0.023	82.49

7.2.1.1.12. Effect of cytokinins on ABA biosynthesis in cell-free extracts of *Hordeum vulgare* cv. Dyan embryos.

Several cytokinins have been shown to inhibit ABA biosynthesis in the fungi *Cercospora rosicola* (Norman *et al*, 1982) and *Botrytis cinerea* (Hirai *et al*, 1986) and also in *Persea americana* mesocarp (see Chapter 4). In order to examine whether cytokinins inhibited the cell-free synthesis of ABA, zeatin, kinetin, BA and IPA were included in the incubation media and their effects on the incorporation of label, from *R*-[2-^{14}C]-MVA into ABA determined. Adenine was included in this experiment as a control. The data presented in Table 7.14 demonstrated that all the cytokinins tested inhibited ABA biosynthesis, with BA and kinetin being the most effective.

Based on superficial, structural similarities between ancymidol and cytokinins, which also inhibit the oxidation of *ent*-kaurene, Coolbaugh (1984) suggested that their mechanism of inhibition was similar and possibly due to interaction with cytochrome P450 mixed function oxidases. Thus, cytokinin-inhibition of ABA biosynthesis in cell-free extracts of *Hordeum vulgare* embryos may be indicative of similar enzymes being involved.

7.2.1.2. Attempts to demonstrate the catabolism of (*R,S,*)-ABA in cell-free extracts of *Hordeum vulgare* cv. Dyan embryo tissue.

Studies in Chapter 5 showed that intact embryos of *Hordeum vulgare* were able to catabolise (*R,S,*)-[2-^{14}C]-ABA to PA, DPA and 7'-hydroxy ABA. Kinetic data from studies on ABA biosynthesis (Figure 7.4) in cell-free extracts of *Hordeum vulgare* embryos showed that after an initial increase in incorporation of label from MVA into ABA, the rate of incorporation began to decline. This suggested that ABA catabolism was perhaps occurring in these cell-free preparations also.

Since these cell-free kinetic studies on ABA biosynthesis had been carried out in the presence of the soluble oxidase cofactors, 2-oxoglutarate-Fe^{2+}-ascorbate, these cofactors together with NADPH were included in incubates designed to examine the catabolism of applied (*R,S,*)-[2-^{14}C]-ABA.

Cell-free extracts of *Hordeum vulgare* embryo tissue were incubated with the appropriate cofactors and (*R,S,*)-[2-^{14}C]-ABA in the presence of air. AMO1618 was not included in incubations during

studies on ABA catabolism. The results presented in Table 7.15 demonstrated that in the presence of the 2-oxoglutarate-$FeSO_4$-ascorbate system, radioactivity from (*R,S,*)-[2-^{14}C]-ABA was transformed into 4 acidic products. Catabolite 1 (Rf0-0.06) behaved as a polar compound. Catabolite 2 (Rf0.13-0.20) co-migrated with authentic DPA, while catabolite 4 (Rf0.46-0.53) cochromatographed with authentic PA. A further acidic product, catabolite 3 (Rf0.26-0.36), displayed similar chromatographic properties to those of 7'-hydroxy ABA, characterised as a major catabolite of (*R,S,*)-ABA in leaves of *Hordeum vulgare* (see Chapter 5) and detected as a catabolite of applied (*R,S,*)-[2-^{14}C]-ABA in intact embryo tissue and aleurone layers of this species.

One notable aspect of this experiment was the apparent inability of cell-free extracts to incorporate radioactivity into putative DPA in the absence of 2-oxoglutarate, $FeSO_4$ and ascorbate (Table 7.15). This suggested that catabolite 2 might not be DPA but an oxidation product of ABA or putative PA which required 2-oxoglutarate, $FeSO_4$ and ascorbate as cofactors.

7.2.1.2.1. Kinetics of (*R,S,*)-ABA catabolism in cell-free extracts of *Hordeum vulgare* cv. Dyan embryos.

Kinetic studies on the rates of incorporation of radioactivity from (*R,S,*)-[2-^{14}C]-ABA into its acidic catabolites demonstrated that these were produced rapidly up to 6h after which their levels declined gradually (Figure 7.10). This decline was accompanied by a considerable increase in incorporation of radioactivity into the aqueous fraction, suggesting that cell-free extracts of *Hordeum vulgare* embryos were capable of conjugating ABA and its acidic catabolites.

7.2.1.2.2. Identification of the acidic products of (*R,S,*)-ABA catabolism in cell-free extracts of *Hordeum vulgare* cv. Dyan embryo tissue.

Several feeds of (*R,S,*)-[2-^{14}C]-ABA to cell-free extracts were carried out, using 6h incubation periods, in order to generate sufficient amounts of products for further analysis by microchemical methods as detailed in Chapter 2. The results (Figure 7.11 and Figure 7.12) showed that PA and 7'-hydroxy ABA were products of (*R,S,*)-ABA catabolism in cell-free extracts of *Hordeum vulgare* embryos. However, oxidation of the methyl ester of catabolite 2 with Jones' reagent did not alter the chromatographic properties of this compound, suggesting that it was not DPA (Figure 7.12A), confirming an earlier suggestion.

TABLE 7.15: Catabolism of (R,S)-ABA in cell-free extracts of Hordeum vulgare cv. Dyan embryo tissue.

100 imbibed embryos, dissected from Hordeum vulgare seed were homogenized using a mortar and pestle and acid washed sand in 0.1M K_2HPO_4/KH_2PO_4 buffer (pH 7.5) on ice for 5 min. 2.0 ml aliquots of the 10 000 g supernatant (equivalent to 25 mg protein) were incubated with the appropriate cofactors (see Chapter 2) with or without 2-oxoglutarate (0.5 mM), $FeSO_4$ (0.5 mM) and ascorbate (5.0 mM) all in 0.1M K_2HPO_4/KH_2PO_4 buffer (pH 7.5). Reactions were initiated by the addition of (R,S)-[2-^{14}C]-ABA (3.3 kBq) and NADPH (0.5 µM) and the 4.0 ml samples incubated at 28°C for 6h under constant illumination in a metabolic shaker. Reactions were terminated by the addition of an equal volume of ice-cold methanol and ABA and its catabolites extracted and analysed as described in Chapter 2.

	Radioactivity incorporated into catabolites*				
Treatment	1 (Rf 0-0.06)	2 (Rf 0.13-0.2)	3 (Rf 0.26-0.33)	4 (Rf 0.46-0.53)	Aqueous fraction
	Bq (%)				
Control	0.0 (0)	0.0 (0)	0.0 (0)	18.52 (0.56)	388.24 (11.65)
$FeSO_4$ + 2-oxoglutarate + ascorbate	157.84(4.74)	97.16 (2.92)	89.55 (2.69)	32.49 (0.97)	470.85 (14.13)

* Data corrected for heat inactivated controls.

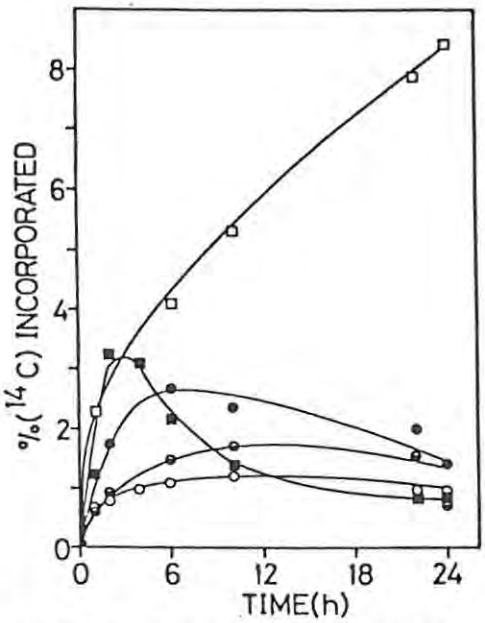

Figure 7.10. Kinetics of (*R,S,*)-[2-^{14}C]-ABA catabolism in cell-free extracts of *Hordeum vulgare* cv. Dyan embryos. Cell-free extracts were supplied with (*R,S,*)-[2-^{14}C]-ABA (4.2kBq), NADPH (0.5µM), 2-oxoglutarate (0.5mM), ascorbate (5.0mM) and $FeSO_4$ (0.5mM) in a total of 4.0ml containing 20mg of protein. Samples were incubated for varying lengths of time at 28°C under continuous illumination (42µmol m^{-2} s^{-1}). ABA and its catabolites were processed as described in Chapter 2. Putative PA (o), putative DPA (●), putative 7'-hydroxy ABA (⊖), catabolite 1 (■) and aqueous phase (□).

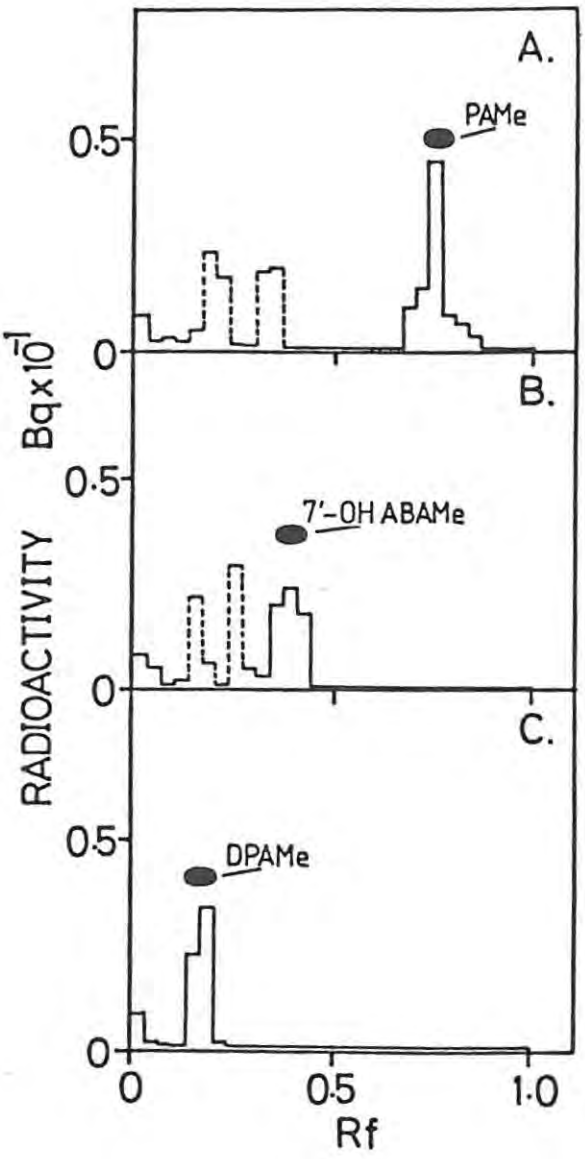

Figure 7.11. Thin layer chromatograms of the methyl esters of the acidic catabolites of (*R,S,*)-[2-^{14}C]-ABA (—) from cell-free extracts of *Hordeum vulgare* cv. Dyan embryos and their reduction products (...) following treatment with $NaBH_4$. (A) Catabolite 5; (B) catabolite 2; (C) catabolite 3. Samples were separated on silica gel GF_{254} in *n*-hexane/ethyl acetate (1:1, v/v) developed 3x to 15cm.

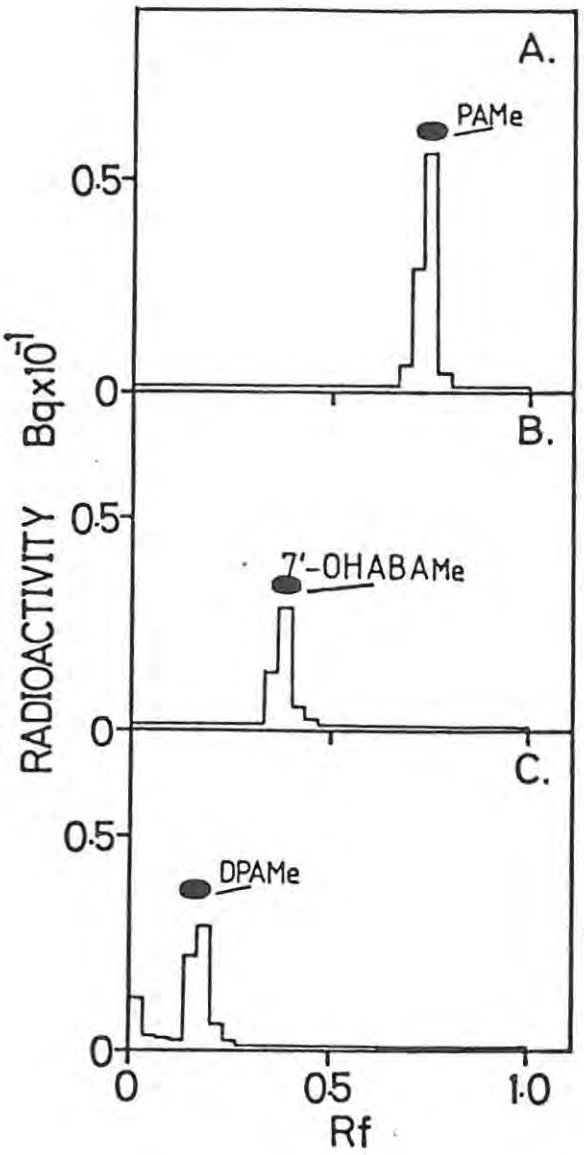

Figure 7.12. Thin layer chromatograms of the methyl esters of the acidic catabolites of (*R,S,*)-[2-^{14}C]-ABA (—) from cell-free extracts of *Hordeum vulgare* cv. Dyan embryos and their oxidation products (...) following treatment with Jones' reagent. (A) Catabolite 5; (B) catabolite 2; (C) catabolite 3. Samples were separated on silica gel GF_{254} in *n*-hexane/ethyl acetate (1:1, v/v) developed 3x to 15cm.

A preliminary analysis of acidic catabolite 1 was carried out by hydrolysing this fraction with base as described in Chapter 2, since studies on intact tissues (Chapter 5) demonstrated that similar polar catabolites of ABA were found to be conjugates which could be partially extracted from aqueous phases by ethyl acetate. The results shown in Figure 7.13A and B demonstrated the ability of cell-free homogenates of *Hordeum vulgare* embryo tissue to conjugate ABA. Similar treatment of the aqueous phases remaining after partitioning against ethyl acetate, produced the results shown in Figure 7.14 A and B. These data indicate that ABA can be transformed readily into a water-soluble, base-labile conjugate by *Hordeum vulgare* embryo cell-free extracts. Thus, it would appear that cell-free extracts of *Hordeum vulgare* embryo tissue contain the enzymes necessary to hydroxylate ABA and convert both it, and to a lesser extent, its acidic catabolites into water-soluble, base-labile conjugates.

7.2.1.2.3. Cofactor requirements for the catabolism of (*R,S,*)-ABA in cell- free extracts of *Hordeum vulgare* embryo tissue.

Having tentatively identified the catabolites of (*R,S,*)-ABA in cell-free systems of *Hordeum vulgare* embryo, the cofactor requirements of this system were examined. The results presented in Table 7.16 demonstrated that 2-oxoglutarate, $FeSO_4$, ATP and NADPH were required for the catabolism of (*R,S,*)-[2-^{14}C]-ABA, whereas ascorbate did not appear to be necessary for ABA catabolism. The conversion of (*R,S,*)-ABA to the compounds tentatively identified as PA and 7'-hydroxy ABA appeared to require 2-oxoglutarate, $FeSO_4$ and NADPH, rather than ascorbate. It has been established that NADPH can substitute for ascorbate (Hedden and Graebe, 1982). This finding indicated that NADPH was the specific reducing agent required by this system.

7.2.1.2.4. Effect of cycloheximide on the catabolism of ABA in cell-free extracts of *Hordeum vulgare* embryos.

In order to determine whether active protein biosynthesis, during imbibition, was required for ABA catabolism in cell-free extracts of *Hordeum vulgare* embryos as it was for ABA biosynthesis, embryos were imbibed in CHI prior to the preparation of a cell-free system and incubation with (*R,S,*)-[2-^{14}C]-ABA.

Cell-free extracts were incubated in exactly the same way as previously described. The results in Table 7.17 indicate that a cell-free system prepared from CHI-imbibed tissue was unable to

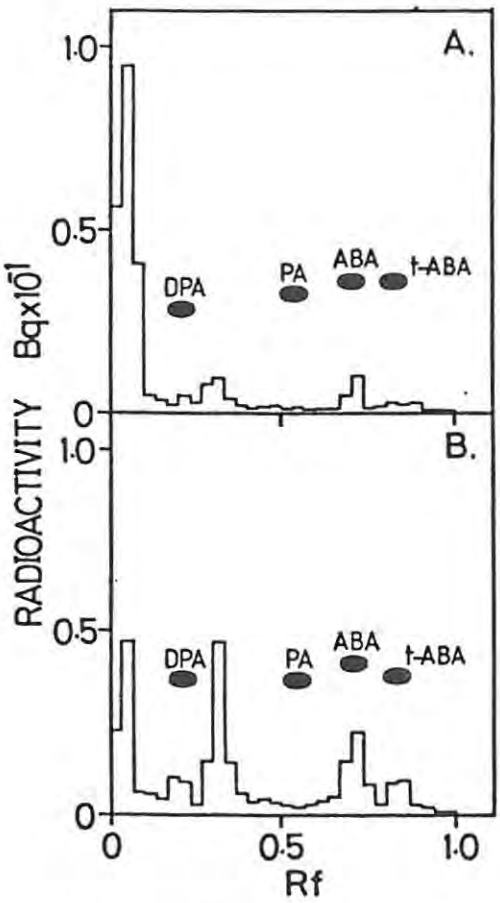

Figure 7.13. Hydrolysis of Catabolite 1 (as in Table 7.15) in the ethyl acetate-soluble acids from cell-free extracts of *Hordeum vulgare* cv. Dyan embryos fed with (*R,S,*)-[2-^{14}C]-ABA. (A) Control held at pH 7.0 at 60^{o}C for 1h; (B) Treated with base at pH 11.0 at 60^{o}C for 1h. Samples were processed as described in Chapter 2 and chromatographed on thin layers of silica gel GF_{254} in toluene/ethyl acetate/acetic acid (50:30:4, v/v) developed 2x to 15cm.

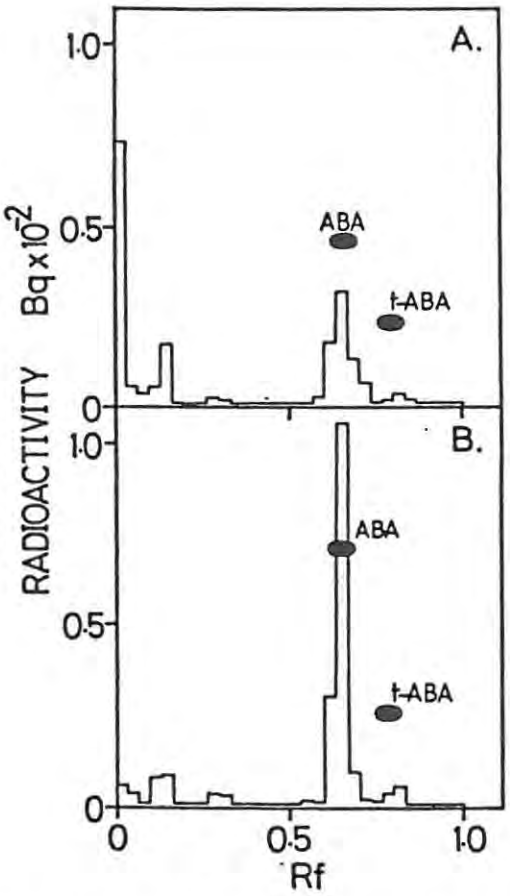

Figure 7.14. Hydrolysis of the aqueous fraction from cell-free extracts of *Hordeum vulgare* cv. Dyan embryos fed with (*R,S,*)-[2-^{14}C]-ABA. (A) Control held at pH 7.0 at 60°C for 1h; (B) Treated with base at pH 11.0 at 60°C for 1h. Samples were processed as described in Chapter 2 and chromatographed on thin layers of silica gel GF_{254} in toluene/ethyl acetate/acetic acid (50:30:4, v/v) developed 2x to 15cm.

TABLE 7.16: Cofactor requirements for the catabolism of (R,S)-ABA in cell-free extracts of Hordeum vulgare cv. Dyan embryo tissue.

Cell-free extracts of Hordeum vulgare embryo tissue were prepared as described in Table 7.15 and aliquots of the 10 000 g supernatant (equivalent to 25 mg protein) incubated with various combinations of cofactors at the following concentrations: ATP (10 mM), GSH (10 mM), $MgCl_2$ (6 mM), KF (5 mM), 2-oxoglutarate (0.5 mM), $FeSO_4$ (0.5 mM) ascorbate (5 mM) and NADPH (0.5 µM) in 0.1 M K_2HPO_4/KH_2PO_4 buffer (pH 7.5) and (R,S)-[2-^{14}C]-ABA (3.3 kBq) in a total volume of 4.0 ml. Reactions were carried out at 28°C for 6 h in a metabolic shaker under constant illumination (42µmol $m^{-2}s^{-1}$) and terminated by the addition of an equal volume of ice-cold methanol. ABA and its catabolites were extracted and analysed as described in Chapter 2.

	Radioactivity incorporated into catabolites				
Cofactors	Origin	Putative DPA	7'-hydroxy ABA	PA	Aqueous fraction
			Bq (%)		
All cofactors	73.6 (2.21)	90.1 (2.70)	50.3 (1.51)	36.3 (1.09)	136.9 (4.11)
- Ascorbate	69.6 (2.09)	90.1 (2.70)	50.3 (1.51)	35.3 (1.06)	136.9 (4.11)
- 2-oxoglutarate	52.7 (1.58)	0.0 (0)	0.0 (0)	11.2 (0.34)	94.9 (2.85)
- $FeSO_4$	16.4 (0.49)	21.9 (0.66)	0.0 (0)	13.2 (0.39)	29.7 (0.89)
- ATP	10.2 (0.31)	16.1 (0.48)	14.5 (0.43)	27.8 (0.83)	18.2 (0.55)
- NADPH	26.3 (0.79)	4.5 (0.14)	14.2 (0.43)	5.9 (0.18)	92.9 (2.79)

TABLE 7.17: Effect of cycloheximide on the catabolism of (R,S)-ABA in cell-free extracts of Hordeum vulgare cv. Dyan embryo tissue.

Dissected Hordeum vulgare embryos were imbibed in water with or without cycloheximide (1 mg/ml) for 12h. Tissue was homogenized in 0.1 M K_2HPO_4/KH_2PO_4 buffer (pH 7.5) at 0°C. Aliquots of the 10 000 g supernatant (equivalent to 25 mg protein) were incubated with the appropriate cofactors (see Table 7.16) and (R,S)-[2-^{14}C]-ABA (3.3 kBq). Samples were incubated at 28°C for 6h in a metabolic shaker under constant illumination (42µmol $m^{-2}s^{-1}$) and terminated by the addition of an equal volume of ice-cold methanol. ABA and its catabolites were extracted and analysed as described in Chapter 2.

	Radioactivity incorporated into catabolites				
Treatment	Origin	DPA	7'-hydroxy ABA	PA	Aqueous fraction
			Bq (%)		
Control	73.6 (2.21)	90.1 (2.70)	50.3 (1.51)	36.3 (1.09)	136.9 (4.11)
+ CHI	28.4 (0.85)	1.9 (0.06)	9.4 (0.28)	2.1 (0.06)	70.9 (2.12)

catabolise ABA, suggesting that protein biosynthesis during imbibition was required for the cell-free catabolism of ABA in extracts of *Hordeum vulgare* embryos.

7.2.1.2.5. Catabolism of (*R,S,*)-ABAMe in cell-free extracts of *Hordeum vulgare* embryos.

Excised, light-grown leaves of *Hordeum vulgare* were able to hydrolyse ABAMe efficiently (Chapter 5) and therefore similar esterase activity might be present in cell-free extracts of mature seeds of *Hordeum vulgare*. Thus, *Hordeum vulgare* embryo cell-free extracts were incubated with ABAMe and the results are presented in Table 7.18. These findings show that ABAMe was catabolised to the same range of acidic catabolites as ABA itself *albeit* in lower yields. This suggested the presence of an esterase that hydrolysed ABAMe to ABA which was then catabolised to PA, 7'-hydroxy ABA and the unidentified acidic product cochromatographing with DPA. Although the presence of esterase activity could have reduced conjugation, incorporation of label into the aqueous fraction and origin peak (Table 7.18) suggested that this esterase was ineffective at hydrolysing conjugates of both ABA and its acidic catabolites. A similar situation was observed in light-grown leaves of *Hordeum vulgare* cv. Dyan (see Chapter 5).

TABLE 7.18: Catabolism of (R,S)-ABAMe in cell-free extracts of Hordeum vulgare cv. Dyan embryo tissue.

Cell-free extracts of Hordeum vulgare embryos were prepared as described in Table 7.16. Aliquots of the 10 000 g supernatant (equivalent to 25 mg protein) were incubated with NADPH (0.5 µM) and the appropriate chemical additions (see Table 7.16) with either (R,S)-[2-^{14}C]-ABA (3.3 kBq) or (R,S)-[2-^{14}C]-ABAMe (3.3 kBq). Samples were incubated at 28°C for 6h in a metabolic shaker under continuous illumination (42µmol $m^{-2}s^{-1}$) and terminated by the addition of an equal volume of ice-cold methanol. ABA and its catabolites were extracted and analysed as described in Chapter 2.

	Radioactivity incorporated				
Substrate	Origin	Putative DPA	7'-hydroxy ABA	PA	Aqueous conjugates
		Bq (%)			
(R,S)- 2-^{14}C -ABA	73.6 (2.21)	90.1 (2.70)	50.3 (1.51)	36.3 (1.09)	136.9 (4.11)
(R,S)- 2-^{14}C -ABAMe	21.7 (0.65)	25.8 (0.78)	35.6 (1.07)	17.1 (0.51)	100.6 (3.02)

7.2.2.Attempts to demonstrate the metabolism of ABA in cell-free extracts of ripening *Persea americana* Mill. cv. Fuerte mesocarp tissue.

7.2.2.1. Attempts to demonstrate the biosynthesis of ABA in cell-free extracts of ripening *Persea americana* cv. Fuerte mesocarp tissue.

Previous studies in Chapter 4 demonstrated that mesocarp tissue from ripened fruits of *Persea americana* actively biosynthesized ABA from applied mevalonate. Therefore, this tissue was examined as a possible source of enzymes for the cell-free biosynthesis of ABA. Although chloroplast lysates from the mesocarp of fruits of *Persea americana* were purported to synthesize ABA from [2-^{14}C]-mevalonate (Milborrow, 1974b) recent findings have demonstrated to the contrary (Hartung *et al*, 1981; Cowan and Railton, 1986) and evidence was obtained to suggest that cytoplasmic enzymes were responsible for the ABA metabolism observed. Thus, this investigation was carried out using homogenates prepared from the mesocarp tissue of *Persea americana* fruits which were then incubated in a similar manner to that described by Milborrow (1974b), and their ability to incorporate label from *R*-[2-^{14}C]-MVA into ABA determined.

When the diethyl ether-soluble acid fractions from [2-^{14}C]-MVA feeds to cell-free homogenates of *Persea americana* mesocarp, incubated with cofactors (Milborrow, 1974b), were separated by TLC on silica gel GF_{254} in toluene/ethyl acetate/acetic acid (50:30:4, v/v), low levels of radioactivity were observed in zones cochromatographing with authentic (*R,S,*)-ABA (Table 7.19). Nevertheless,

TABLE 7.19: The biosynthesis of putative ABA from MVA by cell-free extracts prepared from Persea americana cv. Fuerte mesocarp.

80g f.w. of Persea americana mesocarp was homogenized in 20 mM K_2HPO_4/KH_2PO_4 buffer (pH 7.5) containing ATP (2.0 mM) and $MgCl_2$ (2.0 mM). 9.0 ml Aliquots of the 27 000 g supernatant (equivalent to 20 mg protein) were incubated with R-[2-^{14}C]-MVA (90 kBq) with or without aqueous solutions of cofactors (ATP, 0.02M; $MgCl_2$, 0.05 M; 2-mercaptoethanol, 0.2 M and mixtures of FAD, FMN, NAD, NADH, NADP and NADPH all 0.02M in 1.0 ml of 20 mM K_2HPO_4/KH_2PO_4 buffer, pH 7.5). Control incubates contained NADPH (0.5 µM) only. Samples were incubated for 17 h in a metabolic shaker at 28°C under constant illumination (42µmol $m^{-2}s^{-1}$) and terminated by the addition of an equal volume of ice-cold methanol. ABA was extracted and purified as described in Chapter 2.

	Incorporation of radioactivity into ABA* (n=3)			
Treatment	ABA	ABAMe	1',4'-cis diol of ABAMe	1',4'-trans diol of ABAMe
	Bq (%)			
Control	45.7 ± 2.2 (0.051 ± 0.002)	0 (0)	0 (0)	0 (0)
+ Cofactors	128.5 ± 4.0 (0.143 ± 0.004)	0 (0)	0 (0)	0 (0)

* Data corrected for heat inactivated enzyme preparations.

the levels of radioactivity in the ABA zone from extracts incubated in the presence of cofactors, were almost twice those of similar incubations carried out without the addition of cofactors. However, attempts to confirm the identity of putative ABA by derivatisation, microchemical analyses and cochromatography, showed that no radioactive ABA derivatives were present. Cell-free homogenates of *Persea americana* mesocarp tissue therefore appeared unable to incorporate label from [2-^{14}C]-MVA into ABA under the conditions employed.

7.2.2.1.1. Tentative identification of the 1',4'-*trans* diol of ABA as a product of *R*-[2-^{14}C]-MVA metabolism in cell-free extracts of *Persea americana* cv. Fuerte mesocarp.

Figure 7.15 depicts the distribution of radioactivity on TLC, of the diethyl ether-soluble acid fraction from incubations of extracts of *Persea americana* mesocarp with [2-^{14}C]-MVA. Of interest was the radioactive peak, designated zone 1 (Rf 0.56-0.63), which displayed similar chromatographic properties to those of the 1',4'-*trans* diol of ABA. A similar peak of radioactivity was observed previously in feeds of *R*-[2-^{14}C]-MVAL to intact, ripened *Persea americana* mesocarp (Chapter 4). Analysis of the methyl ester of zone 1 on TLC in *n*-hexane/ethyl acetate (1:1, v/v) showed that it migrated as a single radioactive peak, cochromatographing with authentic, 1',4'-*trans* ABAMe diol (Figure 7.16). However, attempts to confirm this identification by MnO_2 oxidation proved difficult since radioactive metabolite zone 1 was always produced in very low amounts.

7.2.2.2. Attempts to demonstrate (*R,S,*)-ABA catabolism in cell-free extracts of *Persea americana* cv. Fuerte mesocarp.

7.2.2.2.1. Analysis of the ethyl acetate-soluble acid fraction from feeds of (*R,S,*)-[2-^{14}C]-ABA to cell-free extracts of *Persea americana* cv. Fuerte fruit.

The inability to demonstrate the cell-free biosynthesis of ABA in extracts of *Persea americana* mesocarp might have been caused by high rates of ABA catabolism. This would prevent the build-up of any radioactive ABA, produced from labelled MVA. Thus, it was necessary to investigate the capacity of these extracts to catabolise ABA.

When cell-free homogenates, prepared from the ripening mesocarp of fruits of *Persea americana* were incubated in the presence of the same cofactor solution used in biosynthetic studies (see Table

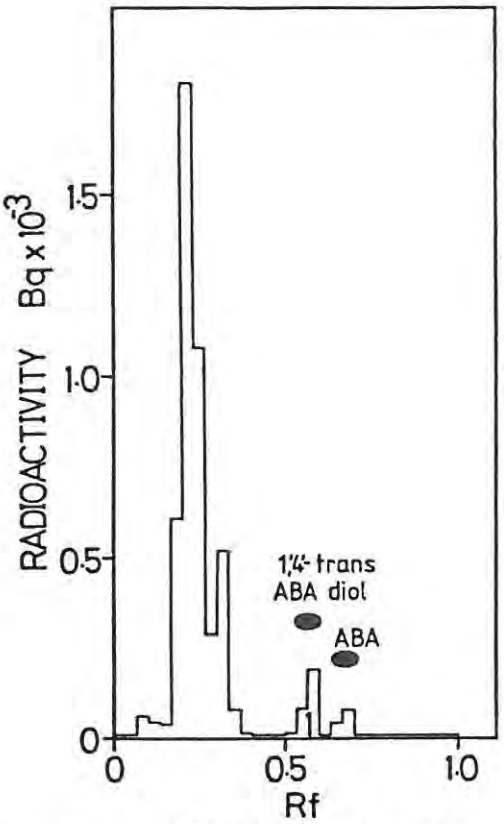

Figure 7.15. Distribution of radioactivity on TLC following feeds of [^{14}C]-MVA to cell-free homogenates of *Persea americana* mesocarp. Preparation of tissue homogenates and incubation procedures as described in Table 7.19. The acidic products of MVA metabolism were extracted into diethyl ether and separated by TLC on silica gel GF_{254} in toluene/ethyl acetate/acetic acid (50:30:4, v/v), developed 2x to 15cm. Data corrected for heat-inactivated control.

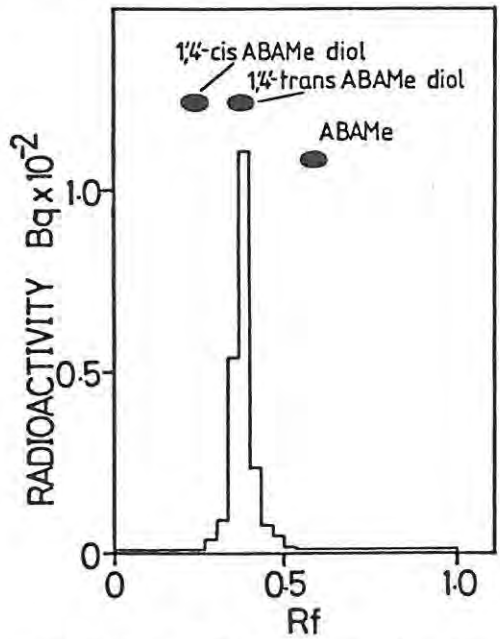

Figure 7.16. TLC analysis of the methyl ester of metabolite zone 1 (Figure 7.15) generated from *R*-[2-^{14}C]-MVA fed to cell-free extracts of *Persea americana* mesocarp tissue. Zone 1 was eluted from the silica gel with water-saturated ethyl acetate, following TLC on slica gel GF_{254} in toluene/ethyl acetate/acetic acid (50:30:4, v/v), methylated with ethereal diazomethane and re-chromatographed on silica gel GF_{254} in *n*-hexane/ethyl acetate (1:1, v/v) developed once to 15cm.

7.19) and (*R,S,*)-[2-^{14}C]-ABA, the distribution of radioactivity depicted in Figure 7.17 was observed. (*R,S,*)-[2-^{14}C]-ABA was catabolised into several acidic products with similar chromatographic properties to those of PA, DPA, 1',4'-*cis* diol and 1',4'-*trans* diol of ABA.

Catabolite zone 1 (Rf0-0.13) appeared similar to the compound routinely detected in ABA feeds to intact tissues (Chapter 5), which, when treated with base resulted in the release of putative ABA. Catabolite zone 2 (Rf0.10-0.26) and catabolite 4 (Rf0.46-0.53) displayed similar chromatographic properties to those of standard DPA and PA, respectively, while catabolite zones 3 (Rf0.36-0.4) and 5 (Rf0.56-0.6) co-migrated with authentic standards of the 1',4'-*cis* and 1',4'-*trans* diols of ABA. The levels of radioactivity associated with these acidic catabolites were low (less than 0.2% of the total radioactivity applied) and thus derivatisation and micro-chemical analyses to confirm their identities were not possible.

Nevertheless, one striking aspect of these studies was the amount of radioactivity associated with catabolite zone 1. This suggests that cell-free extracts of *Persea americana* mesocarp rapidly catabolised (*R,S,*)-[2-^{14}C]-ABA into conjugates, which may also explain the low levels of radioactivity incorporated into the acidic catabolites.

Since almost 20% of the total radioactivity fed to the tissue homogenate remained in the residual aqueous fraction, this fraction was also examined for conjugates of ABA and its acidic catabolites. Base-hydrolysed aqueous fractions, remaining after feeds of (*R,S,*)-[2-^{14}C]-ABA to cell-free extracts of *Persea americana* mesocarp and their heat-inactivated controls gave the results depicted in Figure 7.18A and B.

These results showed that compounds with similar chromatographic properties to those of ABA and its *trans* isomer were released from the aqueous fraction by treatment with base. Similarly, several other acidic products were also released, although their identity is unknown. The relatively low levels of radioactivity incorporated into the products of ABA catabolism in extracts of *Persea americana* precluded further studies with this tissue, and cell-free preparations from other tissues already studied in Chapters 4 and 5 were therefore examined.

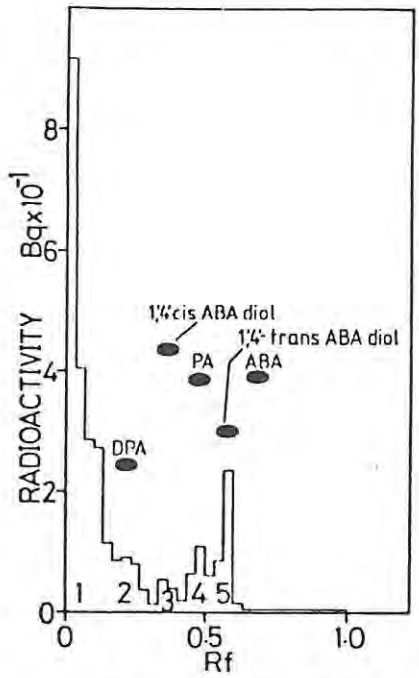

Figure 7.17. TLC separation of the acidic products of (*R,S,*)-ABA from cell-free extracts of *Persea americana* mesocarp. Extracts (equivalent to 50mg protein) were incubated with NADPH (0.5μM) and (*R,S,*)-[2-^{14}C]-ABA (4.2kBq) for 20h at 28°C in a metabolic shaker under continuous illumination (14.4μmol m^{-2} s^{-1}) and the ethyl acetate-soluble acids separated by TLC on silica gel GF_{254} in toluene/ethyl acetate/acetic acid (50:30:4, v/v) developed 2x to 15cm. Data corrected for heat-inactivated control (100°C x 10min).

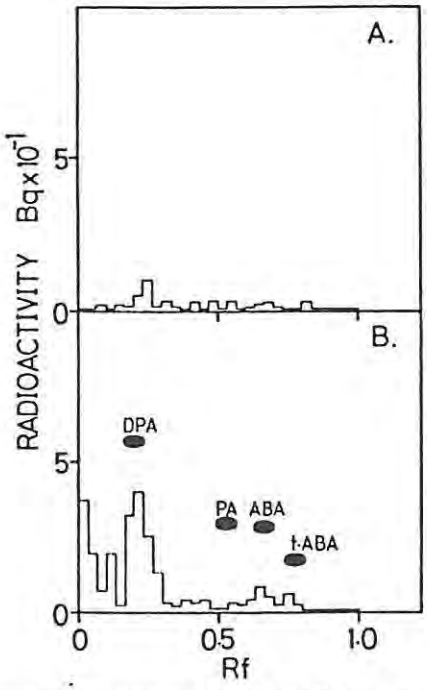

Figure 7.18. Hydrolysis of the aqueous fraction from cell-free extracts prepared as described in Figure 7.17, of excised *Persea americana* mesocarp tissue fed with (*R,S,*)-[2-^{14}C]-ABA. (A) Heat-inactivated control and (B) active enzyme preparation. All data corrected for controls held at pH 7.0 at 60°C for 1h. Samples were processed as described in Chapter 2 and chromatographed on thin layers of silica gel GF_{254} in toluene/ethyl acetate/acetic acid (50:30:4, v/v) developed 2x to 15cm.

7.2.3. Attempts to demonstrate the cell-free metabolism of ABA in extracts prepared from immature seeds of *Phaseolus vulgaris* L.

7.2.3.1. Attempts to demonstrate the cell-free biosynthesis of ABA in extracts of immature *Phaseolus vulgaris* L. seed.

Although excised but otherwise intact immature seeds of *Phaseolus vulgaris* were unable to incorporate label from mevalonate into ABA *in vitro* (see Chapter 4), there is substantial evidence which shows that cell-free preparations of these tissues can synthesize terpenoids. The biosynthesis of GAs has been demonstrated in cell-free extracts prepared from immature seed of *Phaseolus vulgaris* (Kamiya *et al*, 1984; Takahashi *et al*, 1986). The inability of intact immature seed of this species to synthesize ABA from applied MVA may have been due to permeability problems which resulted in the substrate being unable to reach the subcellular site of ABA biosynthesis. Thus, the ability of cell-free extracts of immature *Phaseolus vulgaris* seed to synthesize ABA was investigated, employing the techniques described in Chapter 2.

The results presented in Table 7.20 showed the presence of a radioactive component which cochromatographed with ABA. Derivatisation and micro-chemical manipulations demonstrated that only a small portion of the label in putative ABA was retained as ABAMe, while no label was retained as either the 1',4'-*cis* or 1',4'-*trans* diol of ABAMe (Table 7.20). Thus the bulk of radioactivity associated with putative ABA was probably due to some other acidic product with similar chromatographic properties to those of ABA.

7.2.3.1.1. Effect of inhibitors of protease activity and 2-mercaptoethanol on the capacity of cell-free extracts of *Phaseolus vulgaris* L. immature seed to synthesize ABA.

One possible reason for the apparent inability of cell-free extracts from immature seeds of *Phaseolus vulgaris* to incorporate label from MVA into ABA could be associated protease activity. High levels of protease activity have been detected in *Phaseolus vulgaris* (Ryan, 1973) tissues, and leupeptin has been shown to be a potent inhibitor of protease activity (Alpi and Beevers, 1981; Cowan and Railton, 1986). However, leupeptin, added during tissue homogenisation and incubation, had no effect on the levels of radioactivity associated with the putative ABA zone (Table 7.21). In addition, 2-mercaptoethanol, added as a sulphydryl protectant, did not enhance the incorporation

TABLE 7.20: The biosynthesis of putative ABA from MVA by cell-free systems prepared from immature seeds of Phaseolus vulgaris L.

Immature seed of Phaseolus vulgaris (30g f.w.) was homogenized on ice in 20 mM K_2HPO_4/KH_2PO_4 buffer (pH 7.5) containing ATP (2.0 mM), $MgCl_2$ (2.0 mM) and PVP (1 g/10g f.w.). Aliquots (2 each, equivalent to 20 mg protein) of the 27 000 g supernatant were incubated with R-[2-^{14}C]-MVA (33 kBq) and NADPH (0.5 μM) in a total volume of 10 ml. Incubations were carried out at 28°C under constant illumination (42μmol $m^{-2}s^{-1}$) in an orbital shaker for 20h. Reactions were initiated by the addition of labelled substrate and terminated by the addition of an equal volume of ice-cold methanol. ABA was extracted and analysed as described in Chapter 2 from the pooled incubates.

	Incorporation of radioactivity into ABA			
Incubate	ABA	ABAMe	1',4'-cis diol of ABAMe	1',4'-trans diol of ABAMe
	Bq (%)			
Enzyme prep.	139.6 (0.141)	28.1 (0.028)	0 (0)	0 (0)
Heated control*	25.1 (0.025)	- -	- -	- -

* Enzyme preparations were inactivated in a boiling water bath for 10 min.

TABLE 7.21: Effect of leupeptin and 2-mercaptoethanol on the synthesis of putative ABA from MVA in cell-free extracts of Phaseolus vulgaris L. immature seed.

Immature seed of Phaseolus vulgaris (5g f.w.) was homogenized on ice in K_2HPO_4/KH_2PO_4 buffer (20 mM, pH 7.5) with PVP (1 g/10g f.w.) either with or without the addition of leupeptin (0.02 M) and 2-mercaptoethanol (0.2 M). 9.0 ml Aliquots of the 27 000 g supernatant (equivalent to 6.5 mg protein) were incubated with NADPH (0.5 μM) and R-[2-^{14}C]-MVA (50 kBq). Where leupeptin and mercaptoethanol had been used in the grinding medium they were included in the incubation at the same concentrations. Incubations were carried out at 28°C in an orbital shaker under constant illumination (66μmol $m^{-2}s^{-1}$) for 20 h. Reactions were initiated by the addition of labelled substrate and terminated by the addition of an equal volume of ice-cold methanol. ABA was extracted and analysed as described in Chapter 2.

Treatment	Radioactivity incorporated into putative ABA*	
	Bq	(%)
Control	58.2	0.116
+ 2-mercaptoethanol	69.3	0.139
+ Leupeptin	37.4	0.075

* Data corrected for heat inactivated controls.

of label from MVA into the radioactive zone cochromatographing with ABA either (Table 7.21).

7.2.3.1.2.Effect of 2-oxoglutarate, Fe^{2+}, and ascorbate on the biosynthesis of putative ABA from *R*-[2-^{14}C]-MVA in cell-free extracts of *Phaseolus vulgaris* L. immature seed.

When cell-free extracts of immature seed of *Phaseolus vulgaris* were incubated with MVA and a mixture of 2-oxoglutarate, $FeSO_4$ and ascorbate, no increase in incorporation of radioactivity into the ABA zone was observed (Table 7.22). Therefore, in these experiments cell-free extracts from immature seed of *Phaseolus vulgaris* were unable to synthesize ABA from supplied *R*-[2-^{14}C]-MVA even in the presence of 2-oxoglutarate, $FeSO_4$ and ascorbate.

TABLE 7.22: Effect of 2-oxoglutarate, $FeSO_4$ and ascorbate on the biosynthesis of putative ABA from MVA in cell-free extracts of immature seed of Phaseolus vulgaris L.

Immature seed of Phaseolus vulgaris (5g f.w.) was homogenized on ice in K_2HPO_4/KH_2PO_4 buffer (20 mM, pH 7.5) with PVP (1 g/10g f.w.). 9.0 ml aliquots of the 27 000 g supernatant (equivalent to 8.5 mg protein) were incubated with NADPH (0.5 μM), R-[2-^{14}C]-MVA (50 kBq) with or without 2-oxoglutarate (0.5 mM), $FeSo_4$ (0.5 mM) and ascorbate (5.0 mM). Incubations were carried out at 28°C under constant illumination (42μmol $m^{-2}s^{-1}$) in an orbital shaker. Reactions were initiated by the addition of labelled substrate and terminated by the addition of an equal volume of ice-cold methanol. ABA was extracted and analysed as described in Chapter 2.

	Radioactivity incorporated into putative ABA		
Treatment	Bq	(%)	% increase
Control	62.8	0.126	-
+ 2-oxoglutarate $FeSO_4$ and ascorbate	64.3	0.129	2.38

* Data corrected for heat-inactivated controls.

7.2.3.2.Attempts to demonstrate the catabolism of (*R,S,*)-ABA in cell-free extracts of immature seed of *Phaseolus vulgaris* L.

Since excised, but otherwise intact immature seed of *Phaseolus vulgaris* were able to transform (*R,S,*)-[2-^{14}C]-ABA to products with similar properties to those of PA and DPA (Chapter 5) this tissue was examined as a possible source of the enzymes for ABA catabolism.

When cell-free homogenates of immature seeds of *Phaseolus vulgaris* were incubated with (*R,S,*)-[2-^{14}C]-ABA and NADPH in air, the distribution of radioactivity depicted in Figure 7.19 was observed. 4 Radioactive components were detected, two of which displayed similar chromatographic properties to those of authentic DPA and PA. A further acidic catabolite, zone 3, appeared to be a mixed peak, a component of which cochromatographed with authentic 1',4'-*cis* diol of ABA.

7.2.3.2.1.Effect of inhibitors of protease activity on the catabolism of (*R,S,*)-ABA in cell-free extracts of *Phaseolus vulgaris* L. immature seed.

Attempts were made to enhance the production of the acidic catabolites of ABA in this system for subsequent derivatisation and micro-chemical analyses by examining the effects of inhibitors of protease activity on the catabolism of (*R,S,*)-[2-^{14}C]-ABA in cell-free extracts of *Phaseolus vulgaris* immature seed.

Trypsin inhibitor, leupeptin, pepstatin and PMSF were added during homogenisation of the tissue and incubation of the cell-free supernatant, and their effect on (*R,S,*)-[2-^{14}C]-ABA catabolism determined. The results presented in Table 7.23 indicated that the addition of leupeptin significantly enhanced the incorporation of label from (*R,S,*)-[2-^{14}C]-ABA into its acidic catabolites by up to 100%.

7.2.3.2.2. Effect of 2-mercaptoethanol on (*R,S,*)-ABA catabolism in cell-free extracts of *Phaseolus vulgaris* L. immature seed.

In further attempts to enhance the production of the ABA catabolites the effect of 2-mercaptoethanol on the catabolism of (*R,S,*)-ABA was examined in cell-free extracts of *Phaseolus vulgaris* immature seed.

Cell-free extracts were prepared from immature *Phaseolus vulgaris* seed in KPi buffer containing leupeptin (0.002M) and incubated with or without the addition of 2-mercaptoethanol. The results presented in Table 7.24 showed that the addition of 2-mercaptoethanol enhanced the conversion of (*R,S,*)-[2-^{14}C]-ABA into its acidic products by 100%.

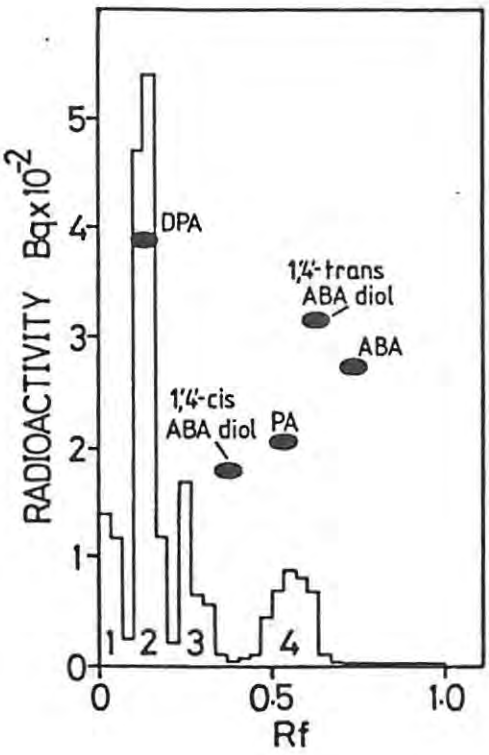

Figure 7.19. TLC separation of the acidic products of (*R,S,*)-ABA from cell-free extracts of immature seeds of *Phaseolus vulgaris*. Extracts (equivalent to 20mg protein) were incubated with NADPH (0.5μM) and (*R,S,*)-[2-^{14}C]-ABA (20kBq) in a total volume of 20ml for 20h at 28°C in a metabolic shaker under continuous illumination (42μmol m^{-2} s^{-1}) and the ethyl acetate-soluble acids separated by TLC on silica gel GF_{254} in toluene/ethyl acetate/acetic acid (50:30:4, v/v) developed 2x to 15cm. Data corrected for heat-inactivated control (100°C x 10min).

TABLE 7.23: Effect of inhibitors of protease activity on the catabolism of (R,S)-ABA in cell-free extracts of Phaseolus vulgaris L. immature seed.

Cell-free extracts were prepared from immature seeds of Phaseolus vulgaris (5g f.w.) by tissue homogenization in 0.1 M K_2HPO_4/KH_2PO_4 buffer (pH 7.5) containing $MgCl_2$ (2.0 mM) at 0°C with PVP (1 g/10g f.w.). During homogenization either leupeptin (0.002 M), pepstatin (0.01 M), trypsin inhibitor (1.74 mg/ml) or PMSF (0.01 M) were included. Aliquots of the 27 000 g supernatant (equivalent to 20 mg protein) were incubated with (R,S)-[2-^{14}C]-ABA (4.2 kBq) and NADPH (0.5 µM) in a total volume of 5.0 ml. Protease inhibitors were included in the incubation mixtures at the concentrations specified above. Samples were incubated at 28°C for 24 h in an orbital shaker under constant illumination (42µmol $m^{-2}s^{-1}$) and terminated by the addition of an equal volume of ice-cold methanol. The catabolites of ABA were extracted and analysed as described in Chapter 2.

	Radioactivity incorporated into catabolites*			
Treatment	1 (Rf 0–0.06)	2 (Rf 0.13–0.2)	3 (Rf 0.26–0.33)	4 (Rf 0.46–0.63)
	Bq (%)			
Control	80.47 (1.92)	81.76 (1.95)	52.72 (1.26)	56.49 (1.35)
Leupeptin	44.15 (1.05)	179.24 (4.27)	69.06 (1.64)	124.47 (2.96)
PMSF	59.41 (1.41)	71.95 (1.71)	35.20 (0.84)	43.09 (1.03)
Pepstatin	93.69 (2.23)	40.62 (0.97)	32.14 (0.77)	59.55 (1.42)

* Data corrected for heat inactivated controls.

TABLE 7.24: Effect of 2-Mercaptoethanol on the cell-free catabolism of (R,S)-ABA in extracts of Phaseolus vulgaris L. immature seed.

5g Fresh weight batches of immature seed of Phaseolus vulgaris were homogenized on ice in 0.1M K_2HPO_4/KH_2PO_4 buffer (pH 7.5) containing $MgCl_2$ (2.0 mM) and either leupeptin (0.002 M) or 2-mercaptoethanol (0.02 M). Aliquots of the (R,S)-[2-^{14}C]-ABA (4.2 kBq), NADPH (0.5 µM) and either leupeptin or mercaptoethanol at the concentrations specified above in a total volume of 5 ml. Samples were incubated at 28°C for 24 h in an orbital shaker under constant illumination (42µmol $m^{-2}s^{-1}$). The catabolites of ABA were extracted and analysed as described in Chapter 2.

	Radioactivity incorporated into catabolites*			
Treatment	1 (Rf 0–0.06)	2 (Rf 0.13 –0.2)	3 (Rf 0.26 – 0.33)	4 (Rf 0.46–0.63)
	Bq (%)			
Control	66.78 (1.59)	60.15 (1.43)	59.83 (1.19)	36.19 (0.86)
Leupeptin	48.16 (1.15)	159.16 (3.79)	70.48 (1.68)	108.36 (2.58)
Leupeptin + mercaptoethanol	42.11 (1.00)	269.43 (6.42)	159.09 (3.79)	252.69 (6.02)

* Data corrected for heat inactivated controls.

7.2.3.2.3. Tentative identification of PA and DPA as catabolites of (*R,S,*)-[2-^{14}C]-ABA in cell-free extracts of *Phaseolus vulgaris* L. immature seed.

Several feeds of (*R,S,*)-[2-^{14}C]-ABA to cell-free homogenates of *Phaseolus vulgaris* immature seed were undertaken in order to generate sufficient amounts of the acidic catabolites for further analysis by cochromatography. TLC of the methyl esters of putative PA (catabolite 4) and DPA (catabolite 2) gave the results depicted in Figure 7.20. Putative PAMe cochromatographed with authentic PAMe (Figure 7.20A) while putative DPAMe migrated as a single peak, cochromatographing with authentic DPAMe (Figure 7.20B). The levels of radioactivity associated with catabolite 3 were too low to afford derivatisation and micro-chemical manipulation. In addition, due to the limited amounts of the radioactive acidic catabolites produced from (*R,S,*)-[2-^{14}C]-ABA, treatments of putative PAMe with $NaBH_4$ and putative DPAMe with Jones' reagent could not be carried out. Nevertheless, circumstantial evidence suggests that these cell-free extracts can transform label from (*R,S,*)-[2-^{14}C]-ABA into acidic compounds, two of which were tentatively identified as PA and DPA.

7.2.3.2.4. Effect of 2-oxoglutarate, $FeSO_4$ and ascorbate on the catabolism of (*R,S,*)-[2-^{14}C]-ABA in cell-free extracts of *Phaseolus vulgaris* L. immature seed.

In order to determine whether or not soluble oxidases were required for ABA catabolism in immature seed of *Phaseolus vulgaris*, the effect of 2- oxoglutarate, $FeSO_4$ and ascorbate on the catabolism of (*R,S,*)-[2-^{14}C]-ABA, was examined.

The result presented in Table 7.25 suggest that 2-oxoglutarate, $FeSO_4$ and ascorbate were not required as cofactors for (*R,S,*)-ABA catabolism in this system. This suggests that the hydroxylation of ABA to PA might occur in the particulate fraction of these extracts, but further studies would need to be done to establish this fact.

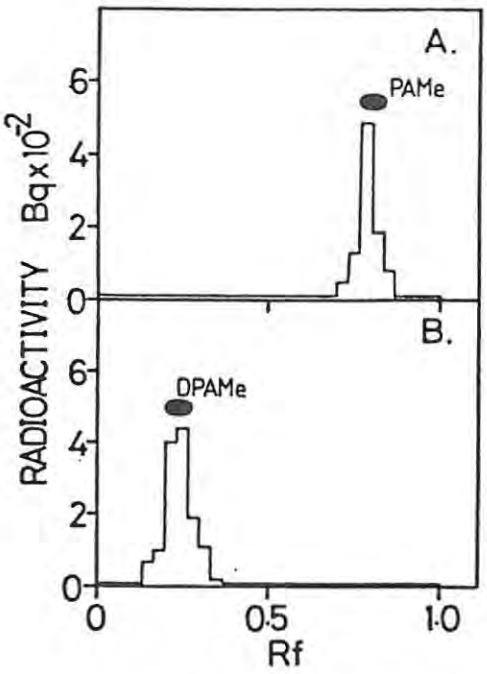

Figure 7.20. Thin layer chromatograms of the methyl esters of the acidic products of (*R,S,*)-ABA catabolism in cell-free extracts from immature seeds of *Phaseolus vulgaris*. (A) Catabolite 4 and (B) catabolite 2. Samples were separated on silica gel GF_{254} in *n*-hexane/ethyl acetate (1:1, v/v) developed 3x to 15cm.

TABLE 7.25: Effect of 2-oxoglutarate, $FeSO_4$ and ascorbate on the catabolism of (R,S)-ABA in cell-free extracts of immature seed of Phaseolus vulgaris L.

5 g fresh weight batches of immature seed of Phaseolus vulgaris were homogenized on ice in 0.1M K_2HPO_4/KH_2PO_4 buffer (pH 7.5) containing $MgCl_2$ (2.0 mM), leupeptin (0.002 M) and 2-mercaptoethanol (0.02M). Aliquots of the 27 000 g supernatant (equivalent to 20 mg protein) were incubated with (R,S)-[2-^{14}C]-ABA (4.2 kBq), NADPH (0.5 µM) with or without 2-oxoglutarate (0.5 mM), $FeSo_4$ (0.5 mM) and ascorbate (5.0 mM). Samples (10 ml total volume) were incubated at 28°C for 24h in an orbital shaker under constant illumination (42µmol $m^{-2}s^{-1}$). Reactions were terminated by the addition of an equal volume of ice-cold methanol and ABA and its catabolites, extracted and analysed as described in Chapter 2.

Treatment	Radioactivity incorporated into catabolites*			
	1 (Rf 0-0.06)	2 (Rf 0.13-0.2)	3 (Rf 0.26-0.33)	4 (Rf 0.46-0.63)
	Bq (%)			
Control	42.11 (1.00)	269.43 (6.42)	159.09 (3.79)	252.69 (6.02)
+ 2-oxoglutarate +$FeSO_4$ and ascorbate	43.40 (1.03)	235.48 (5.61)	175.03 (4.17)	261.75 (6.23)

* Data corrected for heat-inactivated controls.

7.2.4.Attempts to demonstrate the cell-free metabolism of ABA in extracts of *Pisum sativum* L. immature seed.

7.2.4.1. Attempts to demonstrate the cell-free biosynthesis of ABA in extracts of *Pisum sativum* L. immature seed.

It has been shown that cell-free extracts of immature *Pisum sativum* seed can synthesize terpenyl pyrophosphates and intermediates in GA biosynthesis (Graebe, 1968; Moore and Coolbaugh, 1976). In addition, the complete biosynthesis of GAs has been demonstrated in cell-free systems prepared from immature *Pisum sativum* seed (Ropers *et al*, 1978; Kamiya and Graebe, 1983). Although intact immature seeds of *Pisum sativum* were unable to synthesize ABA (see Chapter 4), MVA may not have reached the site of ABA biosynthesis. Therefore, the ability of homogenates of immature seeds of *Pisum sativum* to synthesize ABA was investigated. Cell-free homogenates, incubated with *R*-[2-^{14}C]-MVA and NADPH in air were unable to synthesize ABA (Table 7.26).

7.2.4.2. Attempts to demonstrate the catabolism of (*R,S,*)-ABA in cell- free extracts of immature seed of *Pisum sativum* L.

Cell-free extracts of immature seeds of *Pisum sativum* catabolised (*R,S,*)-[2-^{14}C]-ABA into 5 radioactive products and the distribution of radioactivity depicted in Figure 7.21, was observed. Attempts to identify these products proved unsuccessful due to variations in the quantities of products produced from (*R,S,*)-[2-^{14}C]-ABA in subsequent experiments. In addition, these efforts were further hindered since in all experiments undertaken the levels of radioactivity in the respective catabolic products never exceeded 0.5% of the total radioactivity fed to each incubate. Thus attempts were made to enhance the incorporation of label from substrate (*R,S,*)-[2-^{14}C]-ABA into its acidic catabolites in cell-free extracts of *Pisum sativum* immature seed.

7.2.4.2.1. Effect of inhibitors of protease activity on (*R,S,*)-ABA catabolism in cell-free extracts of immature seed of *Pisum sativum* L.

High levels of protease activity have been detected in *Pisum sativum* tissues (Ellerman, 1974; Collier and Murray, 1977; Noble and Dalling, 1982) and this factor might have resulted in the low conversion of ABA into its acidic catabolites. Thus, the effect of various inhibitors of protease

TABLE 7.26: Attempts to demonstrate the biosynthesis of ABA in a cell-free system prepared from immature seed of Pisum sativum L.

Immature seed of Pisum sativum (20g f.w.) was homogenized on ice in 20 mM K_2HPO_4/KH_2PO_4 buffer (pH 7.5) containing ATP (2.0 mM); $MgCl_2$ (2.0 mM) and PVP (1 g/10g f.w.). 9.0 ml Aliquots (equivalent to 20 mg protein) of the 27 000 g supernatant were incubated with R-[2-^{14}C]-MVA (33 kBq) and NADPH (0.5 µM) in a total volume of 10 ml. Incubations were carried out at 28°C under constant illumination (42µmol $m^{-2}s^{-1}$) in an orbital shaker for 20 h. Reactions were initiated by the addition of labelled substrate and terminated by the addition of an equal volume of ice-cold methanol. ABA was extracted and analysed as described in Chapter 2.

Expt. No.	Incubate	Incorporation of radioactivity into putative ABA			
		ABA	ABAMe	1',4'-cis diol of ABAMe	1',4'-trans diol of ABAMe
		Bq (%)			
1	Enzyme Prep.	0.0 (0.0)	N.D.	N.D.	N.D.
	Heated control*	0.0 (0.0)	N.D.	N.D.	N.D.
2	Enzyme Prep.	0.0 (0.0)	N.D.	N.D.	N.D.
	Heated control*	0.0 (0.0)	N.D.	N.D.	N.D.

N.D. = not determined.

* Enzyme preparations were inactivated in a boiling water bath for 10 min.

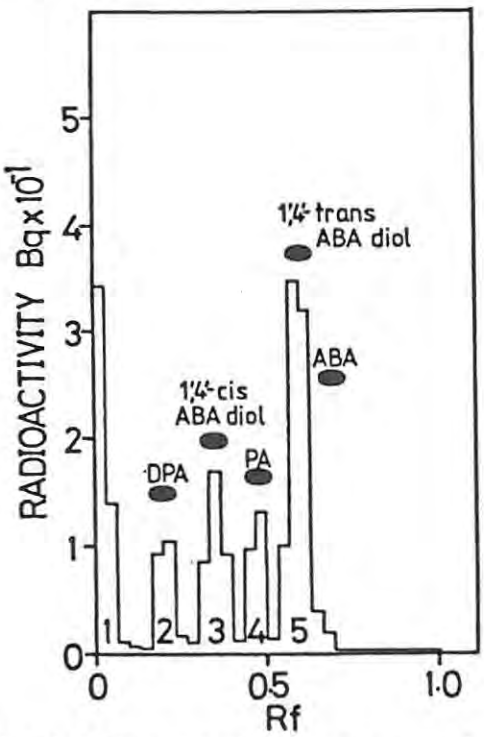

Figure 7.21. TLC separation of the acidic products of (*R,S,*)-ABA from cell-free extracts of immature seeds of *Pisum sativum*. Extracts (equivalent to 50mg protein) were incubated with NADPH (0.5µM) and (*R,S,*)-[2-^{14}C]-ABA (6.6kBq) for 24h at 28°C in a metabolic shaker under continuous illumination (42µmol m^{-2} s^{-1}) and the ethyl acetate-soluble acids separated by TLC on silica gel GF_{254} in toluene/ethyl acetate/acetic acid (50:30:4, v/v) developed 2x to 15cm. Data corrected for heat-inactivated control (100°C x 10min).

activity on the catabolism of (*R,S,*)-[2-^{14}C]-ABA in cell-free extracts of *Pisum sativum* immature seed was examined and their effects on ABA catabolism are shown in Table 7.27.

These results demonstrate that leupeptin enhanced the incorporation of label from (*R,S,*)-[2-^{14}C]-ABA into its acidic catabolic products by over 50%.

7.2.4.2.2. Effect of 2-mercaptoethanol on (*R,S,*)-ABA catabolism in cell-free extracts of immature *Pisum sativum* L. seed.

The effect of 2-mercaptoethanol on the catabolism of (*R,S,*)-[2-^{14}C]-ABA in cell-free extracts of *Pisum sativum* immature seeds was examined since it significantly enhanced the catabolism of ABA in cell-free extracts of immature seeds of *Phaseolus vulgaris* (see Section 7.2.3.1.1) The data in Table 7.28 indicated that the addition of 2-mercaptoethanol resulted in a 5 fold increase production of catabolite zone 3 (see Figure 7.44).

7.2.4.2.3. Tentative identification of the 1',4'-*cis* diol of ABA as a catabolite of (*R,S,*)-ABA in cell-free extracts of immature *Pisum sativum* L. seed.

Catabolite 3, produced from (*R,S,*)-ABA in cell-free extracts of immature seed of *Pisum sativum*, displayed similar chromatographic properties to those of the 1',4'-*cis* diol of ABA both as the free acid and as its methyl ester (Figure 7.22). However, the identity of the other acidic catabolites remains unknown, although it is tempting to speculate that catabolites 2 and 4 correspond to DPA and PA respectively, while catabolite 5 might correspond to the 1',4'-*trans* diol of ABA.

7.2.4.2.4. Effect of 2-oxoglutarate, Fe^{2+} and ascorbate on the catabolism of (*R,S,*)-ABA in cell-free extracts of *Pisum sativum* L. immature seed.

Analysis of the ethyl acetate-soluble acid fractions from cell-free extracts of immature seed of *Pisum sativum* incubated with 2-oxoglutarate, $FeSO_4$ and ascorbate and (*R,S,*)-[2-^{14}C]-ABA demonstrated only slight increases in ABA catabolism in incubates containing these cofactors (Table 7.29). This suggests that these cofactors are not required for (*R,S,*)-ABA catabolism in this system.

TABLE 7.27: Effect of inhibitors of protease activity on the catabolism of (R,S)-ABA in cell-free extracts of Pisum sativum L. immature seed.

Cell-free extracts were prepared from immature seed of Pisum sativum (10g f.w.) by tissue homogenization in 0.1 M K_2HPO_4/KH_2PO_4 buffer (pH 7.5) containing $MgCl_2$ (2.0 mM) at 0°C. During homogenization either leupeptin (0.002 M), pepstatin (0.01 M), trypsin inhibitor (1.74 mg/ml) or PMSF (0.01 M) were included. Aliquots of the 27 000 g supernatant (equivalent to 20 mg protein) were incubated with (R,S)-[$2-^{14}C$]-ABA (4.2 kBq) and NADPH (0.5 μM) in a total volume of 10 ml. Protease inhibitors were included in the incubation mixtures at the concentrations specified above. Samples were incubated at 28°C for 24h in an orbital shaker under constant illumination (42μmol $m^{-2}s^{-1}$) and terminated by the addition of an equal volume of ice-cold methanol. ABA and its catabolites were extracted and analysed as described in Chapter 2.

Treatment	Radioactivity incorporated into catabolites*				
	1 (Rf 0-0.06)	2 (Rf 0.16-0.2)	3 (RF 0.33-0.4)	4 (Rf 0.46-0.5)	5 (Rf 0.56-0.63)
	Bq (%)				
Control	33.19 (0.79)	9.23 (0.22)	26.69 (0.64)	18.37 (0.44)	40.92 (0.97)
+ Leupeptin	48.39 (1.15)	36.98 (0.88)	54.72 (1.30)	86.89 (2.07)	97.50 (2.32)
+ PMSF	-	8.81 (0.21)	16.68 (0.39)	18.32 (0.44)	37.50 (0.89)
+ Pepstatin	-	7.61 (0.18)	16.28 (0.39)	14.42 (0.34)	-

* Data corrected for heat inactivated control (100°C x 10 min).

TABLE 7:28 Effect of 2-mercaptoethanol on the cell-free catabolism of (R,S)-ABA in extracts of Pisum sativum L. immature seed.

10g fresh weight batches of immature seed of Pisum sativum were homogenized on ice in 0.1M K_2HPO_4/KH_2PO_4 buffer (pH 7.5) containing $MgCl_2$ (2.0 mM), and either leupeptin (0.002 M) or 2-mercaptoethanol (0.02M) or a combination of these. Aliquots of the respective 27 000 g supernatants (equivalent to 20 mg protein) were incubated with (R,S)-[$2-^{14}C$]-ABA (4.2 kBq), NADPH (0.5 μM) and either leupeptin or 2-mercaptoethanol at the concentrations specified above in a total volume of 10 ml. Samples were incubated at 28°C for 24 h in an orbital shaker under constant illumination (42μmol $m^{-2}s^{-1}$). ABA and its catabolites were extracted and analysed as described in Chapter 2.

Treatment	Radioactivity incorporated into catabolites*				
	1 (Rf 0-0.06)	2 (Rf 0.16-0.3)	3 (Rf 0.33-0.4)	4 (Rf 0.46-0.5)	5 (Rf 0.53-0.63)
	Bq (%)				
Control	30.08 (0.72)	-	15.96 (0.38)	-	34.9 (0.83)
Leupeptin	78.18 (1.86)	16.78 (0.39)	47.46 (1.13)	51.20 (1.22)	36.0 (0.86)
Leupeptin + 2-mercaptoethanol	127.68 (3.04)	20.16 (0.48)	226.80 (5.40)	51.24 (1.22)	-

* Data corrected for heat inactivated controls.

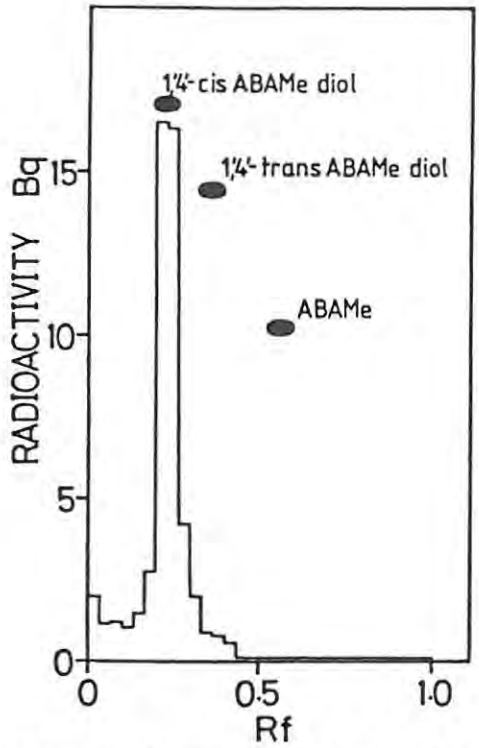

Figure 7.22. TLC analysis of the methyl ester of catabolite 3 (Figure 7.21) generated as the free acid from (*R,S,*)-ABA feeds to cell-free extracts of immature seeds of *Pisum sativum*. Catabolite 3 was eluted from the silica gel following TLC as in Figure 7.15, methylated with ethereal diazomethane and re-chromatographed on thin layers of silica gel GF_{254} in benzene/ethyl acetate/acetic acid (15:3:1, v/v) developed once to 15cm.

TABLE 7.29: Effect of 2-oxoglutarate, $FeSO_4$ and ascorbate on the cell-free catabolism of (R,S)-ABA in extracts of Pisum sativum L. immature seed.

10g fresh weight batches of immature seed of Pisum sativum were homogenized on ice in 0.1M K_2HPO_4/KH_2PO_4 buffer (pH 7.5) containing $MgCl_2$ (2.0 mM), and 9.0 ml aliquots of the 27 000 g supernatants (equivalent to 20 mg protein), incubated with (R,S)-[$2\text{-}^{14}C$]-ABA (4.2 kBq), NADPH (0.5 µM) with or without 2-oxoglutarate (0.5 mM) $FeSO_4$ (0.5 mM) and ascorbate (5.0 mM) in a total volume of 10 ml. Samples were incubated at 28°C for 24 h in an orbital shaker under constant illumination (42µmol $m^{-2}s^{-1}$). Reactions were terminated by the addition of an equal volume of ice-cold methanol. ABA and its catabolites were extracted and analysed as described in Chapter 2.

Treatment	Radioactivity incorporated into catabolites*			
	1 (Rf 0-0.06)	2 (Rf 0.13-0.2)	3 (Rf 0.33-0.4)	4 (Rf 0.46-0.5)
	Bq (%)			
Control	127.68 (3.04)	20.16 (0.48)	226.80 (5.40)	51.24 (1.22)
+ 2-oxoglutarate, $FeSO_4$, ascorbate	130.16 (3.09)	21.73 (0.52)	213.96 (5.09)	62.31 (1.48)

* Data corrected for heat inactivated controls.

7.2.5.Attempts to demonstrate the metabolism of ABA in cell-free extracts of etiolated and light-grown *Hordeum vulgare* cv. Dyan leaves.

7.2.5.1. Attempts to demonstrate the biosynthesis of ABA in cell-free extracts of etiolated and light-grown *Hordeum vulgare* cv. Dyan leaves.

A major factor influencing the activity of the ABA-biosynthesizing enzymes in a cell-free system from vegetative higher plant tissues might be the presence of light-generated inhibitory compounds and plant phenolases (Stafford, 1974; Mayer and Harel, 1979; Sato, 1980a; 1980b; Beart *et al*, 1985). The use of etiolated tissue as a source of enzymes required for ABA biosynthesis, may reduce the influence of such factors. Thus, cell-free extracts were prepared from etiolated and light-grown *Hordeum vulgare* leaves and examined for their ability to synthesize ABA. However, no radioactivity was found to be present in ABA (Table 7.30).

7.2.5.2. Attempts to demonstrate the catabolism of (*R,S,*)-ABA in cell-free extracts of etiolated and light-grown *Hordeum vulgare* cv. Dyan leaves.

Etiolated *Hordeum vulgare* leaves, like their light-grown counterparts, were able to catabolise (*R,S,*)-ABA into the same range of acidic products (see Chapter 6). Cell-free extracts prepared from etiolated and light-grown *Hordeum vulgare* leaves were unable to catabolise (*R,S,*)-[2-^{14}C]-ABA (Table 7.31), probably due to rapid enzyme denaturation during tissue homogenisation and incubation.

TABLE 7.30: Biosynthesis of putative ABA from MVA in cell-free extracts of etiolated and light-grown Hordeum vulgare cv. Dyan leaves.

55g fresh weight of etiolated and light-grown Hordeum vulgare leaves (8 d old) were homogenized in K_2HPO_4/KH_2PO_4 buffer (20 mM, pH 7.5) containing ATP (2.0 mM) and $MgCl_2$ (2.0 mM), with PVP (1 g/10g f.w.) 10 ml of the 27 000 g supernatant (equivalent to 1 mg protein) were incubated with R-[2-^{14}C] -MVA (90kBq) and NADPH (0.5 μM) at 28°C for 24h in a metabolic shaker under constant illumination (42μmol $m^{-2}s^{-1}$) or in total darkness for 20 h. Reactions were initiated by the addition of labelled substrate and terminated by the addition of an equal volume of ice-cold methanol. All manipulations were carried out in total darkness or under a green safety light. ABA was extracted and analysed as described in Chapter 2.

	Radioactivity incorporated into ABA*			
Tissue	ABA	ABAMe	1',4'-cis diol of ABAMe	1',4'-trans diol of ABAMe
	Bq (%)			
Light-grown	0 (0)	- (-)	- (-)	- (-)
Etiolated	0 (0)	- (-)	- (-)	- (-)

*Data corrected for heat inactivated control.

TABLE 7.31: The catabolism of (R,S)-ABA in cell-free extracts from etiolated and light-grown Hordeum vulgare cv. Dyan leaves.

Leaf tissue (100g f.w.) from etiolated and light-grown Hordeum vulgare seedlings (8 d old) was homogenized in 0.1M K_2HPO_4/KH_2PO_4 buffer (pH 7.5) containing $MgCl_2$ (2.0 mM), and PVP (1 g/10g f.w.) in a total volume of 100 ml. Aliquots of the 27 000 g supernatants (equivalent to 6.6 mg protein) were incubated with (R,S)-[2-^{14}C]-ABA (4.2 kBq) and NADPH (0.5 μM) in a total volume of 10.0 ml. Samples were incubated at 28°C for 20 h in total darkness or continuous illumination (14.4μmol $m^{-2}s^{-1}$) and reactions terminated by the addition of an equal volume of ice-cold methanol. ABA was extracted and analysed as described in Chapter 2.

	Radioactivity in ABA and its catabolites		
Tissue System	Aqueous fraction	Acidic products	ABA substrate
	Bq (%)		
Etiolated control	66.7 (1.59)	0 (0)	4133.3 (98.41)
Heat inactivated	0 (0)	0 (0)	4200.0 (100.0)
Light-grown control	216.8 (5.16)	58.1 (1.38)	3925.1 (93.45)
Heat inactivated	0 (0)	0 (0)	4200.0 (100.0)

CHAPTER EIGHT

GENERAL DISCUSSION AND CONCLUSION.

8.1. GENERAL DISCUSSION.

8.1.1. Metabolism of abscisic acid in excised plant tissues.

The biosynthesis of ABA in plants remains a contentious issue. Recently, Zeevaart *et al* (1986) reviewed the arguments for both the postulated C-15 ("direct") and C-40 ("indirect") biosynthetic pathways, which tend to favour the production of ABA from a carotenoid (C-40) origin and these have been recounted in Chapter 1. Thus, the accumulated information appears to suggest that the C-15 compound, xanthoxin, could be a possible intermediate *en route* to ABA particularly for stress-induced increases in levels of this hormone (Creelman and Zeevaart, 1984; Creelman *et al*, 1987; Li and Walton, 1987; Parry *et al*, 1988). However, other, more recent studies have questioned the role of carotenoids as the origin of ABA in plants.

Although Nonhebel and Milborrow (1986) suggested the existence of a large pool of post-cyclisation precursors of ABA, 35 times that of ABA, at present there are no known intermediates between MVA and ABA. The identification of the C-10 dicarboxylic acid, 2,7-dimethyl-2,4-dienoic acid (ODA), and its stucture suggested that it was the residual part of the carotenoid from which ABA was derived (Linforth *et al*, 1987). However, recent work employing $^{2}H_2O$ has shown that ODA and ABA cannot be formed from the same precursor molecule since ABA contained deuterium whereas ODA did not (Milborrow *et al*, 1988b). Similarly, violaxanthin and xanthoxin were unlabelled following treatment of *Lycopersicon esculentum* plants with $^{2}H_2O$ (Nonhebel and Milborrow, 1987). Thus, these results appear to preclude violaxanthin and xanthoxin as precursors of stress-induced ABA production, but do not negate a role for carotenoids in ABA biosynthesis. The characterisation of xanthoxin as an endogenous compound (Feldman *et al*, 1985) coupled with its conversion to ABA in *Lycopersicon esculentum* seedlings (Parry *et al*, 1988) and cell-free extracts of *Phaseolus vulgaris* leaves and roots (Sindhu and Walton, 1987) might suggest that xanthoxin could be a precursor located between FPP and ABA in normal sesquiterpenoid biosynthesis.

The reactions in sesquiterpenoid synthesis occur slowly and the enzymes involved are usually at low

intracellular concentrations (Cane, 1984). Thus, it might be expected that it would be difficult to detect label from a radioactive precursor such as [2-^{14}C]-MVA in either an intermediate like xanthoxin or product ABA, particularly given the competition from other terpenoid biosynthetic pathways. Nevertheless, the detailed studies presented in Chapter 4 showed that in both chloroplast-containing (*Persea americana* mesocarp) and non-chloroplast-containing tissues (*Hordeum vulgare* embryos and endosperms) label from MVA was incorporated into ABA and other acids. These results are in agreement with those from similar studies which utilised *Persea americana* mesocarp tissue and mature seeds of *Triticum aestivum* (Noddle and Robinson, 1969; Milborrow and Robinson, 1973).

The ability of a non-photosynthetic tissue to incorporate label from MVA into ABA might suggest that plastid-localised carotenoids are not involved in ABA biosynthesis. However, root tips of germinating *Zea mays*, another non-photosynthetic tissue, are known to contain high levels of carotenoids including violaxanthin (Maudinas and Lemartre, 1979; Feldman *et al*, 1985). Thus, the results from studies using *Persea americana* mesocarp and mature seeds of *Hordeum vulgare* might not preclude a role for carotenoids, or cyclisation of FPP consistent with that of the β- and ε- rings of higher plant carotenoids (Britton *et al*, 1979; Milborrow, 1984a), in ABA biosynthesis.

In addition to ABA, the results in Chapter 4 showed that label from MVA was also incorporated into two, other acidic products in *Persea americana* fruits. These were tentatively identified as PA and the 1',4'-*trans* diol of ABA by cochromatography and derivatisation (Figure 4.6). In an earlier report, Milborrow (1976) was able to show that label from ABA, synthesized from MVA by fruits of *Persea americana*, was converted to PA in *Lycopersicon esculentum* plants and the 1',4'-*trans* diol of ABA in *Persea americana* but presented no information on the production of these compounds from MVA *per se*. Thus, the above results present for the first time the direct incorporation of label from MVA into the 1',4'-*trans* diol of ABA and PA in plant tissue. However, this does not indicate whether the 1',4'-*trans* diol of ABA is an intermediate or a catabolite of ABA. Nevertheless, some further support for its role as a precursor of ABA might be that when ABA was fed to *Persea americana* fruits it was not converted into the 1',4'-*trans* diol but was transformed into PA (Figure 5.39). The recent characterisation of the 1',4'-*trans* diol of ABA as an endogenous compound in *Persea americana* fruit and *Pisum sativum* seedlings (Okamoto *et al*, 1987; Vaughan and Milborrow, 1987), coupled with the suggestion that it might be a precursor of ABA in the fungus *Botrytis cinerea* (Hirai *et al*, 1986), indicates that a possible role for the 1',4'-*trans* diol as a precursor of ABA in plants cannot be discounted. This is particularly so given the recent

demonstration that the 1',4'-*trans* diol of ABA is converted into ABA in high yield in *Persea gratissima* fruits, *Pisum sativum* seedlings, cultures of *Cercospora rosicola* (Okamoto *et al*, 1987; Neill *et al*, 1987) and *Lycopersicon esculentum* seedlings (Parry *et al*, 1988).

Nevertheless, some controversy surrounds the role of the 1',4'-*trans* diol of ABA as a possible precursor of ABA in higher plants. The 1',4'-*trans* diol was produced as a major acidic catabolite of applied (*R,S,*)-ABA in wilted and rehydrated *Pisum sativum* seedlings (Tietz *et al*, 1979; Milborrow, 1983). Similarly, (*R,S,*)-[2-^{14}C]-ABA was converted to the 1',4'-*trans* diol in *Persea americana* fruit, *Vicia faba* and *Pisum sativum* shoots (Vaughan and Milborrow, 1987). While Nonhebel and Milborrow (1986) have provided evidence for the existence of a large pool of precursors to ABA in plants, Creelman and Zeevaart (1984) have suggested that under conditions of stress, ABA increases might arise from the conversion of a stored precursor which contains oxygen in 1'- and 4'-positions. Tietz *et al* (1979) fed ABA to wilted seedlings of *Pisum sativum* which were being rehydrated. Thus, the endogenous levels of ABA would have been considerably higher than under normal physiological conditions. Nevertheless, ABA was converted predominantly into the 1',4'-*trans* diol (Milborrow, 1983). Thus, it is tempting to speculate that under conditions of stress, precursor 1',4'-*trans* diol is converted into ABA and thereafter, during rehydration, ABA is converted back to the 1',4'-*trans* diol which then assumes its role as a precursor. A similar argument may apply to *Persea americana* fruits which are known to contain high levels of endogenous ABA (Milborrow, 1974). However, the demonstration that the 1',4'-*trans* diol is a precursor of ABA in plants depends largely on the development of a suitable ABA-biosynthesizing cell-free system in which to clarify its role as an intermediate *en route* to ABA.

It is surprising that an ABA-biosynthesizing cell-free system from the ABA-producing fungi, *Cercospora rosicola* and *Botrytis cinerea*, has not been developed, particularly given the advances made on GA biosynthesis using the fungus *Gibberella fujikuroi* (Hedden *et al*, 1978) which assisted in unravelling the entire GA biosynthetic pathway in higher plants (Graebe, 1987). Mycelial cultures of these fungi can incorporate label from MVA, FPP and the 1',4'-*trans* diol of ABA into ABA (Neill *et al*, 1982a; Norman *et al*, 1986; Hirai *et al*, 1986; Neill *et al*, 1987). However, no attempts have been made to demonstrate the transformation of xanthoxin to ABA in these fungal systems. Clearly, this would be useful in further establishing the role of xanthoxin in ABA biosynthesis even though the pathway by which ABA is produced in these fungi may differ from that in higher plants.

The catabolism of ABA in higher plants is a well documented process (Walton, 1980; Milborrow, 1983; Loveys and Milborrow, 1984) and it is generally considered that 8'-hydroxy ABA is the initial acidic product in plants and its conversion to PA appears to be a two-step enzyme-catalysed reaction (Milborrow *et al*, 1988). In the present study (see Chapter 5) 8'-hydroxy ABA was not detected as a product of applied, radiolabelled (*R,S,*)-ABA. Nevertheless, its inferred presence as a catabolite of ABA in a cell-free system from *Echinocystis lobata* liquid endosperm (Gillard and Walton, 1976) and in intact fruits of *Vigna unguiculata* (Adesomoju *et al*, 1980) suggests that further cell-free studies are required in order to confirm this hydroxylation reaction in plants.

As expected, PA and DPA were routinely produced by all the tissues used to study the catabolism of applied (*R,S,*)-ABA and were unequivocally characterised by capillary GC-MS in leaves of *Hordeum vulgare* and seedlings of *Pisum sativum* cv. Black-eyed Susan and *Phaseolus vulgaris* cv. Top-crop. In addition, PA and DPA were identified by micro-chemical analyses and cochromatography in immature seeds of *Pisum sativum* and *Phaseolus vulgaris*, mature seed and aleurone layers of *Hordeum vulgare* cv. Dyan and in *Persea americana* cv. Fuerte mesocarp tissue. In all tissues studied, PA was converted predominantly into DPA.

The conversion of DPA into two further acidic products was demonstrated in aleurone layers of *Hordeum vulgare* by Dashek *et al* (1979). While all attempts to demonstrate the conversion of [^{14}C]-DPA into other acidic products were unsuccessful in this study, DPA was transformed into water-soluble conjugates (see Chapter 5). Zeevaart and Milborrow (1976) showed that *epi*-DPA was produced in amounts ±18% that of DPA in *Phaseolus vulgaris* seedlings. However, no evidence for *epi*-DPA production in *Phaseolus vulgaris* seedlings was obtained. Similarly, *epi*-DPA did not appear to be produced as a catabolite in either leaves of *Hordeum vulgare* or in *Pisum sativum* seedlings. Likewise, no radioactive component corresponding to PISA (Tietz, 1985) was detected in these tissues.

The inability to demonstrate the production of 8'-hydroxy ABA, *epi*-DPA and PISA might have resulted from limitations imposed by the chromatographic procedures used. In the present study, TLC and GLC were routinely used to quantify the acidic products of (*R,S,*)-ABA catabolism. However, it is known that a large number of catabolites are produced by plants fed labelled ABA (Loveys and Milborrow, 1984) and these might be better resolved using HPLC (Loveys and Milborrow, 1984; Vaughan and Milborrow, 1984). This aspect has been well illustrated in a study on the transport and metabolism of (*R,S,*)-ABA in *Vitis vinifera* (Loveys, 1984). However, when a

large number of extracts are involved in studies on ABA metabolism, a TLC procedure which rapidly resolves the major ABA metabolites is desirable. Thus, TLC and the solvent system, toluene/ethyl acetate/acetic acid (50:30:4, v/v) adequately resolved *trans*-ABA, ABA, 1',4'-*trans* ABA diol, PA, 1',4'-*cis* ABA diol, DPA and previously unidentified acidic products and rechromatography of their methyl esters demonstrated their homogeneity. Similarly, bilayer 2D-TLC has been used to resolve cytokinin metabolites and the separation achieved is reported to be unattainable in any one known HPLC system (Jameson *et al*, 1987). Although TLC and GLC enabled the detection of the novel ABA catabolites, nigellic acid (Lehmann *et al*, 1983a) and PISA (Tietz, 1985), the increasing number of ABA catabolites being characterised clearly emphasizes the need to use chromatographic procedures with superior resolving power in future studies on ABA catabolism.

In addition to the aforementioned catabolites, (*R,S,*)-ABA was also converted into a previously unidentified, major acidic catabolite of intermediate polarity between PA and DPA in light-grown and etiolated leaves of *Hordeum vulgare* (Figure 5.1). A similar component was detected following feeds of (*R,S,*)-[2-^{14}C]-ABA to *Pisum sativum* (Figure 5.19A) and *Phaseolus vulgaris* (Figure 5.19B) seedlings which was absent when non-radioactive (*R,S,*)-ABA was used as substrate. In leaves of *Hordeum vulgare* this component which comprised more than 30% of the radioactivity incorporated into the acidic fraction was unequivocally characterised by combined capillary GC-MS as 7'-hydroxy ABA (Figures 5.9 and 5.10) and shown to arise predominantly from the catabolism of (-)-(*R*)-ABA (Figure 5.12). However, confirmation of this must await studies on the catabolism of (+)-(*S*)- and (-)-(*R*)-ABA respectively (Sondheimer *et al*, 1971; 1974; Vaughan and Milborrow, 1984), in *Hordeum vulgare* leaves and efforts to determine its presence as an endogenous compound.

As in previous studies on the catabolism of ABA, where the identity of the products was established by unequivocal spectroscopic techniques (Milborrow, 1970; Zeevaart and Milborrow, 1976; Tietz *et al*, 1979) racemic (*R,S,*)-ABA was used. Although several workers have noted differences in the catabolism of (+)-(*S*) and (-)-(*R*)-ABA (Sondheimer *et al*, 1971; Mertens *et al*, 1982; Vaughan and Milborrow, 1984; Boyer and Zeevaart, 1986), emphasizing that studies using (+)-(*S*)-ABA are essential to our knowledge of ABA catabolism in plants, most studies on the catabolism of applied, radiolabelled ABA still make use of the readily available racemic ABA (Loveys, 1984; Cornish and Zeevaart, 1984; Uknes and Ho, 1984; Tietz, 1985; Grantz *et al*, 1985; Lehmann and Glund, 1986; Radin and Hendrix, 1986; Barthe *et al*, 1986; Barthe and Bulard, 1987; Loveys and Robinson, 1987). Only recently have techniques become available with which to resolve this

enantiomeric mixture (Mertens *et al*, 1982; Vaughan and Milborrow, 1984). However, a routine procedure to achieve this resolution has still to be developed although it now appears that resolution of (*R,S,*)-ABA by chiral-HPLC is the preferred method (Vaughan and Milborrow, 1984; Railton, 1987).

Recently, Vaughan and Milborrow (1988) suggested that interconversion of the diols with inversion at C-1' could provide a mechanism by which (*R*)-ABA might be produced in plants. Thus, the reaction sequence (*S*)-ABA→ (*S*)-*cis*-diol→ (*R*)-*trans*-diol→(*R*)-ABA could occur. Although only low levels of this enantiomer would be present in plants, mostly as ABAGE, the possible existence of endogenous (*R*)-ABA emphasizes that data from studies on the catabolism of racemic ABA in plants cannot be discounted.

(-)-(*R*)-7'-hydroxy ABA was also unequivocally characterised as a product of applied (*R,S,*)-ABA in leaves of *Xanthium strumarium* (Boyer and Zeevaart, 1986). Earlier studies resulted in the identification of a similar compound in cell suspension cultures of *Nigella damascena* supplied with (*R,S,*)-ABA (Lehmann *et al*, 1983a). This product was characterised as nigellic acid ((+)-(*S*)-7'-hydroxy ABA) and it has now been shown to occur naturally in leaves of *Vicia faba* and thus can be produced from both (*R*)-ABA and (*S*)-ABA (Lehmann and Schwenen, 1988), although the chirality of the isolated endogenous material was not determined. Nigellic acid was also been identified as a product of (*R,S,*)-ABA catabolism in cell suspensions of *Lycopersicon esculentum* (Lehmann and Glund, 1986). In addition, 7'-hydroxy ABA was tentatively identified as a product of (*R,S,*)-ABA in embryos and aleurone layers of *Hordeum vulgare*.

The identification of (-)-(*R*)-7'-hydroxy ABA prompted Zeevaart *et al* (1986) to speculate that the oxygenase responsible for the formation of 8'-hydroxy ABA from (*S*)-ABA in the PA pathway might also hydroxylate the 7'-methyl group in the unnatural (*R*)- isomer. This suggests that the 8'-hydroxylating enzyme is non-specific. However, the inferred production of 8'-hydroxy ABA from (*R,S,*)-ABA in a cell-free system from *Echinocystis lobata* (Gillard and Walton, 1976), without the apparent production of 7'-hydroxy ABA, even when (*R*)-ABA was used as substrate, suggests that the 8'-hydroxylating enzyme(s) (Milborrow *et al*, 1988) is substrate specific. Nevertheless, further investigations on the specificity of the 8'-hydroxylating enzyme require the use of an ABA-catabolising cell-free system capable of converting ABA to PA *via* 8'-hydroxy ABA.

The tissues examined in Chapter 5 also had the ability to transform ABA and its acidic products into

water-soluble, base-labile conjugates, a well-documented process in plants. Conjugates of ABA, PA and DPA may represent end products of ABA catabolism in plants (Powell and Seeley, 1974; Zeevaart and Boyer, 1982; Neill *et al*, 1983). However, Rudnicki and Czapski (1974) suggested that CO_2 could be the end product of ABA catabolism, at least in *Pyrus* seeds. Evidence which might support this suggestion was obtained in studies using immature seed of *Pisum sativum* and *Phaseolus vulgaris*. Detailed kinetic analyses on the catabolism of (*R,S,*)-ABA in these tissues (Figure 5.37) demonstrated that with time there was an overall decline in the levels of recoverable radioactivity. This suggested that label was being lost from these tissues during incubation, possibly in the form of CO_2. Studies with inhibitors of prokaryotic protein biosynthesis (see Chapter 6) did not alter ABA catabolism *per se* thereby negating microbial involvement in this process (Milborrow and Vaughan, 1979).

Only one report has been published on the catabolism of ABA by a bacterium. Hasegawa *et al* (1984) demonstrated that a species of *Corynebacterium* was capable of transforming ABA into dehydrovomifoliol ((*R,S,*)-1'-hydroxy-4'-keto-β-ionone), possibly *via* vomifoliol a naturally occurring compound in higher plants (Galbraith and Horn, 1972; Fukui *et al*,1977), a process which did not involve the loss of CO_2.

8.1.2. Modification of abscisic acid metabolism in plants.

In studies on the regulation of PA formation in *Hordeum vulgare* aleurone layers, Uknes and Ho (1984) provided evidence to suggest that ABA induced its own conversion to PA, while the conversion of PA to DPA was unaffected by either ABA or PA. This effect was explained in terms of ABA-induced synthesis of new proteins, some of which were involved in the transformation of ABA to PA. A more reasonable explanation might be that cold ABA was catabolised to PA and increases in cold PA then acted as a "cold-pool trap" during subsequent studies using labelled ABA. In a similar study, pretreatment of aleurone layers with non-radioactive (*R,S,*)-ABA (10^{-3}M) markedly reduced the catabolism of applied, radiolabelled ABA in this tissue (Figure 5.29). This result was indicative of dilution of labelled (*R,S,*)-[2-^{14}C]-ABA by preteatment of aleurone layers with non-labelled ABA. Thus, exogenous applications of (*R,S,*)-ABA to aleurone layers reduced the catabolism of low specific [^{14}C]-ABA by dilution while increases in the PA pool size act as a "cold-pool trap" when high specific activity [^{3}H]-ABA is used.

If ABA could induce its own catabolism to PA it might be expected that wilt-induced increases in

ABA (Davies and Mansfield, 1983) would enhance the conversion of ABA to PA. In response to water stress, endogenous PA levels appear higher which might suggest that stress-induced increases in ABA enhance its own conversion to PA (Harrison and Walton, 1975; Pierce and Raschke, 1981; Zeevaart, 1980; 1983). However, contrasting results have been obtained in studies on the catabolism of radiolabelled ABA in plants following the imposition of water-stress (Harrison and Walton, 1975; Cornish and Zeevaart, 1984; Lehmann and Schütte, 1984; Murphy, 1984). Likewise, the results presented in Chapter 6 (Section 6.2.3) show that whereas water stress did not alter (*R,S,*)-[2-^{14}C]-ABA catabolism in *Phaseolus vulgaris* significantly, it markedly reduced ABA catabolism in *Hordeum vulgare* leaves. Nevertheless, attempts to distinguish the catabolites of applied non-radioactive (*R,S,*)-ABA from the same endogenous compounds in leaves of *Hordeum vulgare* might suggest the possibility of induced catabolism (Chapter 5, section 5.2.1.1.7) since leaves fed ABA contained PA whereas those not fed ABA contained no detectable levels of PA. This implies that either enzyme activity/levels are low and increase following exogenous applications of ABA or enzyme activity/levels are at a maximum under normal physiological conditions. However, when (*R,S,*)-ABA was diluted with (*R,S,*)-[2-^{14}C]-ABA and fed to leaves of *Hordeum vulgare*, kinetic analyses revealed that the increase in PA was associated with a rapid decline in DPA production (Figure 6.11). Thus, in leaves of *Hordeum vulgare* any increase in PA might be attributed to the inhibition of PA reduction to DPA rather than by an increase in the conversion of ABA to PA. A similar result was obtained using stressed leaves and confirmed in studies on the catabolism of [^{14}C]-PA in water-stressed leaves of *Hordeum vulgare* (Figure 6.12).

It has been suggested that any undue increase in the endogenous pool size of a particular phytohormone may alter the physiological status of the tissue and thus the catabolism of that hormone (Hedden *et al*, 1978). Thus many workers now favour the use of high specific activity substrates. This has become evident in several recent studies on ABA catabolism in plants (Zeevaart and Boyer, 1984; Grantz *et al*, 1984; Boyer and Zeevaart, 1986; Loveys and Robinson, 1987). However, Zeevaart and Milborrow (1976) and Tietz *et al* (1979) fed mg amounts of non-radioactive (*R,S,*)-ABA to shoots of *Phaseolus vulgaris* and *Pisum sativum* respectively without any apparent qualitative changes in catabolism. In addition, problems associated with penetration suggest that the amount of original radioactive substrate which eventually reaches the enzyme site within the tissue may be very small. Therefore, it is possible that by increasing the mass of substrate, greater amounts of precursor would become available to the enzyme(s). Nevertheless, without knowledge of the K_m of the enzymes involved, which can only be obtained following the development of an active ABA-catabolising cell-free system, it is difficult to speculate whether or not amounts greater than

physiological levels might be problematic.

It might be assumed that any given tissue accumulates the required amount of a particular endogenous compound and that a certain status quo exists within plant tissues under normal physiological conditions. Therefore it might be argued that if the *in vivo* biochemical mechanisms were sensitive to increases in the pool size of an endogenous compound then any increase would result in changes to the catabolism of ABA. This has not been apparent in studies where both low specific activity [^{14}C]- and high specific activity [^{3}H]-ABA have been used to study ABA catabolism in a single plant tissue. Thus, the incorporation of label from either [^{14}C]-ABA or [^{3}H]-ABA into PA, DPA and polar products was similar in *Hordeum vulgare* aleurone layers (Dashek *et al*, 1979; Uknes and Ho, 1984).

Stress-induced increases in ABA levels could occur as a result of changes in either the activity or the *de novo* synthesis of enzymes catalysing this process (Milborrow and Noddle, 1970). This has in part been confirmed in studies on the effect of inhibitors of transcription and translation on the stress-induced synthesis of ABA (Quarrie and Lister, 1984b; Guerrero and Mullet, 1986; Stewart *et al*, 1986), where most evidence favours changes in enzyme synthesis. The resulting increase in endogenous ABA levels might have been responsible for the reduced catabolism of applied ABA as a result of dilution, provided the applied (*R,S,*)-ABA mixed freely with the endogenous ABA pool. It was envisaged that dilution of applied, radiolabelled (*R,S,*)-ABA by increased endogenous levels might have been responsible for some of the effects of water-stress on (*R,S,*)-ABA catabolism in *Hordeum vulgare* leaves although this aspect was not apparent in similar studies using *Phaseolus vulgaris* leaves (Harrison and Walton, 1975).

When labelled (*R,S,*)-ABA was mixed with 1μg non-radioactive (*R,S,*)-ABA and fed to leaves of *Hordeum vulgare* no significant changes in the catabolism of (*R,S,*)-[2-^{14}C]-ABA were observed (Table 6.6). This implied that increases in endogenous ABA levels in response to stress were not responsible for dilution of applied, radiolabelled substrate. Only in amounts in excess of 100μg of (*R,S,*)-ABA was the catabolism of (*R,S,*)-[2-^{14}C]-ABA significantly altered in leaves of *Hordeum vulgare* (Figure 6.13). Although stress did not alter the catabolism of (*R,S,*)-ABA in leaves of *Triticum aestivum* (Lehmann and Schütte, 1984; Murphy, 1984) the increased incorporation of label from MVA into ABA (Milborrow and Noddle, 1970) in stressed leaves of this species, strongly suggests that ABA levels increase due to an increase in biosynthesis. Whether a similar mechanism operates in leaves of *Hordeum vulgare* is currently unknown since attempts to demonstrate the stress-induced

synthesis of ABA from labelled mevalonate in this tissue were unsuccessful (see Chapter 4). Nevertheless, these results do not negate a role for ABA biosynthesis in this process.

Tissue age was also shown to be an important factor regulating the catabolism of (*R,S,*)-ABA in plants. Several authors have noted that ABA is rapidly catabolised in mature leaves (Zeevaart and Milborrow, 1976; Wareing, 1978; Weiler, 1980). However, ABA is also extensively catabolised in young tissues (King, 1979; Everat-Bourbouloux, 1982). In the present study (see Chapter 6, section 6.2.1), older, turgid leaves of *Hordeum vulgare* catabolised (*R,S,*)-ABA more efficiently than did their younger counterparts. Similarly, older leaves of *Phaseolus vulgaris* catabolised (*R,S,*)-ABA more effectively. In monocotyledons there is an increase in tissue age along the length of the leaves. Thus, this aspect was clarified by examining the catabolism of (*R,S,*)-ABA in excised leaf sections of *Hordeum vulgare* in relation to tissue age. The data obtained confirmed that older leaf tissue was more active at transforming (*R,S,*)-ABA into its catabolites.

It has been suggested that the reduced capacity of immature leaves of *Xanthium strumarium* to catabolise (*R,S,*)-ABA was due to an inability to oxidize ABA to PA (Cornish and Zeevaart, 1984). However, in leaves of *Hordeum vulgare*, studies on the catabolism of PA in relation to leaf age (see Chapter 6, section 6.2.1.3) showed that no such "block" was present in this tissue (Figure 6.3). Clearly then, the overall rate of ABA catabolism is enhanced in older, light-grown leaves of *Hordeum vulgare*. In contrast, older etiolated leaves of this species were less able to catabolise applied (*R,S,*)-ABA than their younger counterparts (Figure 6.2). These differences between ABA catabolism in older, light-grown and etiolated leaves suggested that factors other than photosynthesis might play a role in regulating ABA catabolism in plants and might reflect alterations in the levels of some of the enzymes catalysing these steps.

In plants, phytochrome is well known to stimulate mRNA synthesis (Poulson and Beevers, 1970; Harel and Bogorad, 1973; Schrott and Rau, 1977; Tobin and Silverthorne, 1985) and mediate the expression of both nuclear and plastid genes (Tobin and Silverthorne, 1985). Several specific genes, for example, those encoding the proteins ribulose 1,5-bisphosphate carboxylase-oxygenase (Thompson *et al*, 1983), NADPH-protochlorophyllide reductase (Meyer *et al*, 1983) and phytochrome itself (Colbert *et al*, 1983) are regulated by phytochrome. In addition, it is now well known that the enzymes aminolevulinate dehydrase (Hampp and Ziegler, 1975), cinnamate-4-hydroxylase (Russell, 1971), glutamine synthetase (Lamb and Lawton, 1983), lipoxygenase, nitrate reductase (Roth-Benjaro and Lipps, 1973), malate reductase (Rao *et al*, 1980) and phenylalanine

ammonia lyase (McClure, 1974; Lamb and Lawton, 1983) are positively mediated by photocontrol. Thus, phytochrome may possibly be responsible for regulating the levels of the enzymes involved in ABA catabolism.

In studies using *Persea americana* mesocarp tissue the incorporation of [2-^{14}C]-MVA into ABA was ±30% lower in light (Table 4.8) while the catabolism of [2-^{14}C]-ABA was ±45% greater in the light. This might suggest light does not influence the activity of the enzymes required for ABA biosynthesis. If ABA were derived from a carotenoid origin it might be expected that a similar situation would apply for carotenogenesis. However, in higher plants the biosynthesis of carotenoids is photoregulated and phytochrome mediates the light-induced increases in the rate of carotenogenesis (Harding and Shropshire, 1980; Rau, 1985). In addition, comparisons of carotenogenic enzyme activities of light- and dark-grown cultures of *Aspergillus giganteos* have shown that phytoene synthetase, phytoene dehydrogenase and lycopene cyclase are totally photoinduced (El-Jack *et al*, 1988). Thus, light may act to increase the levels and/or activities of the enzymes required for ABA catabolism in plants. Further support for the light-induced increase of ABA catabolism was obtained from studies using light-grown and etiolated leaves of *Hordeum vulgare* where light increased the rate of ABA and PA catabolism (Chapter 6, section 6.2.2). Similarly, the catabolism of applied ABA was enhanced in immature seeds of *Pisum sativum* and *Phaseolus vulgaris* incubated under conditions of continuous illumination. Thus, light would appear to be necessary for the sustained and efficient catabolism of ABA.

Several reports on the effects of light on levels of endogenous ABA in vegetative tissues have appeared (Barnes and Light, 1972; Tietz, 1974; 1975; Henson, 1983) and phytochrome has been implicated as a regulatory mechanism in this process (Loveys, 1979; Wellburn, 1978). However, no studies on ABA catabolism in light-grown and etiolated tissues have been reported. The demonstration that light stimulated ABA catabolism (see Chapter 6) might imply phytochrome control of this process (Vince-Prue, 1985; Tobin and Siverthorne, 1985). Similarly, the metabolism of GAs was enhanced in light-grown tissues (Musgrave and Kende, 1970; Loveys and Wareing, 1971; Reid *et al*, 1972) and there is now evidence to suggest that this process is regulated by phytochrome (for review see Vince-Prue, 1985). In addition, Gilmour *et al* (1986) have demonstrated the photocontrol of GA metabolism in cell-free extracts of *Spinacia oleracea* leaves where the activities of the oxidizing enzymes were increased in leaves exposed to long-days. However, the low incorporation and the comparison of endogenous amounts of GAs in dark- and light-grown *Pisum sativum* seedlings does not favour the view that overall GA biosynthesis is

photoinduced (Graebe, 1987). With regard to endogenous ABA levels in plants, the situation appears somewhat different. Levels of ABA in intact, unstressed, plants and amounts induced by water-stress are greater in light- than in dark-grown plant tissues (Simpson and Saunders, 1972; Tietz, 1974; 1975; Henson, 1983), and stress-induced ABA accumulation appears to require higher rather than lower light intensities (Rajagopal and Andersen, 1980). Thus, phytochrome may play an indirect role in regulating the biosynthesis of ABA by mediating the enzymes involved in ABA catabolism.

Studies on the metabolism of ABA in a cell-free system from immature seed of *Echinocystis lobata* (Gillard and Walton, 1976) and in excised leaves and roots of *Xanthium strumarium* (Creelman and Zeevaart, 1984; Creelman *et al*, 1987) have intimated that mixed function oxidases are required for this process. Similar enzymes are involved in *ent*-kaurene oxidation in GA biosynthesis (Graebe, 1987) which are inhibited by ancymidol (Coolbaugh, 1984) while AMO1618 and CCC prevent the cyclisation of GGPP to CPP (Fall and West, 1971; Frost and West, 1977) in GA biosynthesis.

The effects of AMO1618, CCC and ancymidol on ABA biosynthesis in *Persea americana* are shown in Table 4.9. Both CCC and ancymidol reduced the incorporation of label from applied MVAL into ABA by ±80% while AMO1618 enhanced this process. The AMO1618 data concur with an earlier study carried out by Milborrow (1976) using this tissue. Similar results for the effect of AMO1618 were reported in studies on the ABA-producing fungus *Botrytis cinerea* (Hirai *et al*, 1986) but which are in contrast to those obtained in studies using another ABA-producing fungus, *Cercospora rosicola* (Norman *et al*, 1983) where no inhibitory effects were observed.

AMO1618 is known to inhibit the cyclisation of GGPP to CPP and kaurene in GA biosynthesis (Shechter and West, 1969; Fall and West, 1971; Coolbaugh *et al*, 1973) and the cyclisation of squalene-2,3-epoxide and hence the synthesis of steroids (Douglas and Paleg, 1972; 1974; 1978). The results obtained in this study therefore suggest that either AMO1618 does not effect the cyclisation of FPP to ABA or that ABA is produced from a carotenoid origin. Although Milborrow (1976) stated that CCC had no effect on ABA biosynthesis in *Persea americana* fruits, the concentration of CCC used is unknown and no data were provided. Nonetheless, when CCC concentrations are high enough, plant growth inhibitory effects may be observed (Douglas and Paleg, 1972). The concentration of inhibitors used in this study, 500μM, may be at "non-physiological" levels but some plants are known to concentrate chemicals to 10M (Mozafar and Goodin, 1970; Staby, 1973). In addition, the many membrane barriers in vascular plants may present penetration problems thus

reducing the amount of inhibitor which eventually reaches the site of the ABA-metabolising enzymes. Although this problem should not be apparent in fungal studies, similar concentrations were used in studies on the effects of these inhibitors of GA biosythesis on ABA biosynthesis in *Cercospora rosicola* (Norman *et al*, 1983) and *Botrytis cinerea* (Hirai *et al*, 1986). Furthermore, it is known that CCC is actively metabolised in plants (Lawrence, 1984) and recently a catabolite of ancymidol was detected in studies on ABA biosynthesis in *Cercospora rosicola* (Norman *et al*, 1986).

CCC inhibits the A-activity of kaurene synthetase, responsible for the cyclisation of GGPP to CPP (Frost and West, 1977). Likewise, CCC also inhibits the production of cyclic carotenoids, resulting in the accumulation of lycopene in *Cucurbita maxima* cotyledons (Knypl, 1969). Thus, CCC inhibition of ABA biosynthesis in *Persea americana* might be due to inhibition of cyclic carotenoid biosynthesis. However, if the cyclisation of FPP, *en route* to ABA, occurred by a similar mechanism to that of β- and ε-ring formation in carotenoid biosynthesis (Milborrow, 1984a), then CCC might be expected to inhibit the production of ABA from FPP. Likewise, AMO1618 does not appear to affect carotenoid biosynthesis (Milborrow, 1976; Norman *et al*, 1983), even though it is a cyclase inhibitor (Fall and West, 1971; Douglas and Paleg, 1972), and thus may be ineffective against the transformation of FPP to ABA, assuming it is consistent with carotenoid cyclisation. The results obtained from feeds of AMO1618 and CCC to *Persea americana* therefore appear to suggest a role for carotenoids in ABA biosynthesis. However, this tentative speculation needs to be clarified by investigating the effects of 2-(4-methylphenoxy) triethylamine (MPTA), a substituted tertiary amine, on ABA biosynthesis in this tissue which readily incorporates label from applied MVAL into ABA. MPTA is known to stimulate the accumulation of lycopene and phytofluene by inhibiting the cyclisation of lycopene to β-carotene in *Citrus limon* (Benedict *et al*, 1985) and other plant species (Britton, 1976). Similarly, the ability of fluridone (Moore *et al*, 1985) and norflurazon (Quarrie and Lister, 1984a; Feldman and Sun, 1986), two inhibitors of carotenoid dehydrogenation (Bartels and Watson, 1978), to inhibit ABA biosynthesis in plants further emphasizes the need to investigate their effect on the incorporation of label from MVAL into ABA in *Persea americana*. However, the expected desaturation steps between FPP and ABA might also be inhibited by fluridone and norflurazon thus, making interpretation of such results very difficult.

Ancymidol, an inhibitor of the cytochrome P450 mixed function oxidase-catalysed oxidation of *ent*-kaurene (Coolbaugh *et al*, 1978; Coolbaugh, 1984), inhibits ABA biosynthesis in the fungus *Cercospora rosicola* (Norman *et al*, 1983). Since similar enzymes have been implicated in the biosynthesis of ABA in intact *Xanthium strumarium* leaves and roots (Creelman and Zeevaart,

1984; Creelman *et al*, 1987) it was considered that ancymidol might also inhibit ABA biosynthesis in *Persea americana* mesocarp tissue. Pretreatment with ancymidol significantly reduced the capacity of *Persea americana* mesocarp to incorporate label from MVAL into ABA (Table 4.9).

Ancymidol bears structural similarities to cytokinins and Coolbaugh (1984) demonstrated that cytokinins, like ancymidol, interact with cytochrome P450-mixed function oxidases in GA biosynthesis to inhibit kaurene oxidation in plants. The cytokinins IPA, BA, zeatin and kinetin all inhibited ABA biosynthesis in *Persea americana* (Table 4.10) when supplied at 500μM, concentrations which inhibited markedly ABA biosynthesis in *Cercospora rosicola* (Norman *et al*, 1982). Of the cytokinins tested, BA appeared to be the most potent, reducing the incorporation of label from MVAL into ABA by 87.81%. This appears to suggest that ancymidol and cytokinins may affect similarly catalysed post-FPP oxidations in ABA biosynthesis by interacting with cytochrome P450 mixed function oxidases. Although it is the meta nitrogen atom of the substituted purine that binds with the iron protohaem group of cytochrome P450 to competitively inhibit oxygenase activity (Coolbaugh *et al*, 1978), the inactivity of adenine suggests that for the meta N to bind, N^6 side-chain substitution is essential. The hydrophylic nature of adenine might suggest that it was unable reach the site of ABA metabolism. However, adenine was also inactive in the *Marah oreganus* cell-free system (Coolbaugh, 1984) where penetration problems would not be apparent and stimulated ABA biosynthesis by 36% in *Cercospora rosicola* (Norman *et al*, 1982). Unfortunately adenine was omitted as a control in similar studies using *Botrytis cinerea* (Hirai *et al*, 1986) and thus its effect in this system remains unknown. Thus, the mechanism of action of cytokinins and ancymidol may be dissimilar.

In *Cercospora rosicola*, BA appears to effect post-FPP reactions (Norman *et al*, 1982) while ancymidol is considered to inhibit reactions in the terpenoid pathway prior to FPP (Norman *et al*, 1986). Likewise, in cell-free extracts of *Marah oreganus* and shoot tips of *Pisum sativum*, ancymidol at higher concentrations than needed to inhibit kaurene oxidation, will also inhibit the conversion of MVA to kaurene which includes several non-oxidative steps common to both ABA and kaurene biosynthesis (Coolbaugh and Hamilton, 1976).

Whether or not the effect of ancymidol and cytokinins is the same in all plant tissues remains to be investigated. It was shown that applications of BA to excised *Hordeum vulgare* leaves did not prevent wilt-induced increases in ABA biosynthesis (Stewart *et al*, 1986), a process believed to require elevated rates of ABA biosynthesis from MVA (Milborrow and Noddle, 1970). Although

it was not possible to demonstrate the synthesis of ABA from MVAL in either turgid or wilted leaves (Section 4.2.2.1), a reduction in the catabolism of (*R,S,*)-ABA in wilted leaves of *Hordeum vulgare* (Section 6.2.3), suggests that wilt-induced increases in ABA levels might occur by mechanisms other than an increase in the rate of synthesis.

Unlike their effect on ABA biosynthesis, inhibitors of GA biosynthesis did not appear to influence significantly the amounts of PA and DPA produced in vegetative tissues although the production of 7'-hydroxy ABA was reduced in leaves of *Hordeum vulgare* (Table 6.9) pretreated with these inhibitors. The significance of this effect from a physiological standpoint appears minimal as 7'-hydroxy ABA seems to be derived predominantly from the unnatural (-)-(*R*)- enantiomer of (*R,S,*)-ABA in this species. In contrast, applications of cytokinins to plant tissues provided evidence to suggest that they could alter the catabolism of applied, radiolabelled (*R,S,*)-ABA.

In leaves of *Hordeum vulgare* IPA appeared to retard the catabolism of ABA to PA and DPA at low and high concentrations. One possible explanation might be that the biotransformation of IPA to zeatin, which enhanced ABA catabolism (Table 6.8), was concentration dependent and that any effect of IPA was associated with the amounts of zeatin being produced. In contrast kinetin enhanced the production of acidic catabolites at low concentrations while enhancing conjugation of applied (*R,S,*)-ABA and its catabolites at higher concentrations. Furthermore, foliar applications of BA to *Phaseolus vulgaris* and *Pisum sativum* similarly enhanced the catabolism of (*R,S,*)-ABA (Figure 6.15 and Table 6.18). Cytokinins also enhance the metabolism of GAs in plants (Reid and Railton, 1974) and BA increases the 13 OH hydroxylation of GA_9 to GA_{20} in *Pisum sativum* seedlings (Railton, 1974) and the conversion of GA_4 in *Lactuca sativa* (Durley *et al*, 1976). Thus, cytokinins appear to enhance hydroxylation reactions in both GA biosynthesis and ABA catabolism although the significance of this is unclear since similar reactions are involved in ABA biosynthesis, a process inhibited by cytokinins. However, cytokinin pretreatment might reduce endogenous ABA levels and thereby increase the availability of labelled substrate for the enzymes involved in catabolism.

The effects of inhibitors of GA biosynthesis and cytokinins on the catabolism of applied, radiolabelled (*R,S,*)-ABA in plants remain unclear. Penetration problems and catabolism of the inhibitors might reduce the amounts which finally reached the subcellular site of ABA catabolism. Thus, further information on the effects of these inhibitors on ABA catabolism will depend largely on studies using a suitable ABA-catabolising cell-free system.

Milborrow (1974b; 1974c; 1976) provided evidence to suggest that ABA was biosynthesized in the chloroplasts of higher plants. The implications from these studies were that plastid-synthesized enzymes could be involved in catalysing some of the steps in ABA biosynthesis. However, when two inhibitors of 70s ribosomal protein biosynthesis, CAP and LINC (Ellis, 1982), were supplied to *Persea americana* mesocarp the biosynthesis of ABA was not markedly affected (Table 4.11). In contrast, CHI an inhibitor of 80s ribosomal translation (Galling, 1982) markedly reduced the incorporation of label from MVAL into ABA in *Persea americana* (Table 4.11). This result suggested that the enzymes required for ABA biosynthesis were rapidly "turned-over" and that cytoplasmically-localised protein biosynthesis was required for this process.

Quarrie and Lister (1984b) reported the effects of various protein synthesis inhibitors on the wilt-induced increase in endogenous ABA in leaves of *Triticum aestivum*. Similarly, Guerrero and Mullet (1986) suggested that dehydration-induced synthesis of ABA in *Pisum sativum* requires nuclear gene transcription. Together, these studies have demonstrated that inhibitors of chloroplast protein biosynthesis are ineffective in preventing such an ABA increase, whereas inhibitors of transcription and cytoplasmic protein synthesis inhibit this process completely. Unfortunately there is only limited, circumstantial evidence to suggest that wilt-induced increases in ABA levels involve changes in the rate of ABA biosynthesis (Milborrow and Noddle, 1970; Zeevaart, 1980; Pierce and Raschke, 1981) and in this study, attempts to demonstrate the wilt-induced synthesis of ABA proved unsuccessful, thus the significance of these findings is not completely clear.

Pretreatment of older leaves of *Hordeum vulgare* (Table 6.10), *Pisum sativum* (Table 6.20) and *Phaseolus vulgaris* (Table 6.17) with CHI curtailed the catabolism of applied (*R,S,*)-ABA. CHI pretreatment also inhibited the catabolism of (*R,S,*)-ABA in immature seeds of *Pisum sativum* and *Phaseolus vulgaris* (Table 6.23) and in *Persea americana* mesocarp (Table 6.26). These results implied that cytoplasmic protein biosynthesis was also required for ABA catabolism and that the enzymes involved are labile. The exception was the enzyme(s) involved in the conjugation of (*R,S,*)-ABA in leaves of *Hordeum vulgare* which was not inhibited by CHI (Table 6.12). This suggested that the ABA-conjugating enzyme(s) was pre-existing and stable within the tissue. A previous study on the effects of CHI on (*R,S,*)-ABA catabolism in *Hordeum vulgare* aleurone layers (Ho and Uknes,

1982; Uknes and Ho, 1984) reported a similar inhibition to that observed in *Hordeum vulgare* leaves. However, no details on the effect of CHI on the production of conjugates were reported.

Inhibitors of chloroplast protein biosynthesis had little or no effect on the catabolism of (*R,S,*)-ABA in plant tissues. However, in light-grown seedlings of *Pisum sativum* (Table 6.20) inhibitors of 70s ribosomal protein synthesis altered significantly the catabolism of (*R,S,*)-ABA. LINC inhibited the incorporation of label from applied, radioactive ABA into PA and DPA while CAP inhibited incorporation into DPA. Although the reasons for these contrasting effects of LINC and CAP on ABA catabolism in *Pisum sativum* seedlings are unclear they might indicate that some DPA is derived in part *via* a pathway other than that of PA reduction, as suggested by Tietz (1985) which requires chloroplast-localised protein biosynthesis. Whether this is so remains to be determined, although some evidence in support of this might be the catabolism of applied (*R,S,*)-ABA into products with similar chromatographic properties to those of DPA and the 1',4'-*trans* diol of ABA in chloroplast lysates prepared from *Pisum sativum* seedlings (Cowan and Railton, 1986).

8.1.3. Cell-free metabolism of abscisic acid in plant tissue extracts.

Although Milborrow (1974b; 1974c; 1976) demonstrated the cell-free biosynthesis of ABA in chloroplasts prepared from *Persea americana* mesocarp and *Phaseolus vulgaris* leaves, recent information suggests that chloroplasts are not a major site of ABA biosynthesis (Hartung *et al*, 1980; Cowan and Railton, 1986; Sossountzov *et al*, 1986). Furthermore, the inhibition of ABA metabolism by CHI suggested that the enzymes involved in this process were cytoplasmically synthesized, although a role for the chloroplast-regulated, stress-induced accumulation of ABA cannot be discounted (Quarrie and Lister, 1984a). Thus, the selection of a non-photosynthetic tissue from which to develop an ABA-metabolising cell-free system limited possible complications arising from an alternative route to ABA such as that involving violaxanthin (Taylor and Burden, 1970). Polyphenol oxidases are also known to occur in plants and plant tissue homogenates (Stafford, 1974; Mayer and Harel, 1979; Sato, 1980a; 1980b) and would result in enzyme denaturation. Thus, it was considered that extracts prepared from etiolated tissue might contain less polyphenol oxidase. However, cell-free extracts from both etiolated and light-grown *Hordeum vulgare* leaves were apparently unable to biosynthesize and catabolise ABA. The inability of these extracts to catabolise ABA was surprising since the intact tissue efficiently transformed ABA to PA, DPA and 7'-hydroxy ABA. However, the enzymes involved in terpenoid metabolism occur at low intracellular

levels (Cane, 1984) and enzyme extracts from vegetative tissues are much less active and often contain inhibitory substances (Graebe, 1987), which might explain the inability of the *Hordeum vulgare* leaf extracts to metabolise ABA.

Nevertheless, Hartung *et al* (1980; 1981) described the incorporation of [^{14}C]-MVA into ABA and demonstrated some catabolism of (*R,S,*)-ABA in cytoplasmic fractions derived from light-grown leaves of *Spinacia oleracea*. In addition, cell-free extracts from leaves of *Phaseolus vulgaris* converted xanthoxin to ABA (Sindhu and Walton, 1987). Thus, cell-free preparations derived from vegetative tissues appear to contain the enzymes required for terpenoid metabolism and some of the enzymes needed for ABA metabolism. The presence of terpenoid metabolising enzymes in cell-free extracts from vegetative tissues has been adequately illustrated in studies on GA biosynthesis and metabolism. Shen-Miller and West (1984) demonstrated the biosynthesis of *ent*-kaurene in extracts prepared from etiolated *Helianthus annuus* seedlings and a cell-free system from shoot tips of *Pisum sativum* seedlings converts MVA to *ent*-kaurene (Chung and Coolbaugh, 1986). In addition, Gilmour *et al* (1986) have shown that cell-free extracts of *Spinacia oleracea* leaves will metabolise GAs.

In comparison to vegetative tissue, immature seeds have proved extremely useful as sources for the enzymes involved in GA metabolism (Graebe, 1987). However, cell-free extracts prepared from immature seed of *Pisum sativum* and *Phaseolus vulgaris* appeared unable to synthesize ABA. In contrast, applied (*R,S,*)-[2-^{14}C]-ABA was catabolised into acidic products (Figure 7.19 and 7.21) with similar chromatographic properties to those of PA, DPA, 1',4'-*cis* diol of ABA and the 1',4'-*trans* diol of ABA. Leupeptin enhanced ABA catabolism only slightly, resulting in a maximum of 2.32% of the total radioactivity being incorporated into any one product. This enhancement allowed for the tentative identification of the 1',4'-*cis* diol as a product of ABA catabolism. However, levels of the other catabolic products were generated in amounts too low for further characterization.

In cell-free extracts prepared from *Phaseolus vulgaris* immature seed (*R,S,*)-ABA was catabolised into products similar to PA, DPA and the 1',4'-*cis* diol of ABA. The biotransformation of (*R,S,*)-ABA was enhanced in the presence of leupeptin and 2-mercaptoethanol. PA and DPA were tentatively identified as catabolites of (*R,S,*)-ABA in cell-free extracts of *Phaseolus vulgaris* by cochromatography of their methyl esters.

Milborrow (1974b) demonstrated that chloroplast lysates of *Persea americana* mesocarp tissue

biosynthesized ABA. However, studies by Cowan and Railton (1986) did not confirm this finding and presented data to suggest that ABA biosynthesis might occur in the cytosol rather than chloroplasts in this tissue. Similar conclusions were reached by Hartung *et al* (1980) in studies utilizing chloroplast lysates obtained from leaves of *Spinacia oleracea*. Tissue homogenates prepared from the mesocarp of *Persea gratissima* fruits were apparently unable to incorporate label from *R*-[2-^{14}C]-MVA into ABA. However, radioactivity was incorporated into a metabolite tentatively identified as the 1',4'-*trans* diol of ABA (Figure 7.15), also detected as a metabolite of applied MVA in intact tissue slices of this species. Recently, Hirai *et al* (1986) suggested that the 1',4'-*trans* diol of ABA might be a precursor of ABA in *Botrytis cinerea*. Thus, it is tempting to assign a similar role for the 1',4'-*trans* diol in the biosynthesis of ABA in higher plants. However, identical cell-free extracts catabolised (*R,S,*)-[2-^{14}C]-ABA into acidic products one of which cochromatographed with the 1',4'-*trans* diol of ABA (Figure 7.17). Therefore, these results do not distinguish between the production of the 1',4'-*trans* diol as a catabolite of ABA or as a precursor of ABA in cell-free extracts of *Persea americana* mesocarp. Nevertheless, the high levels of endogenous ABA in this tissue (Milborrow, 1974c) might have been responsible for label from MVA accumulating as the 1',4'-*trans* diol which was not converted to ABA or which was converted very slowly, in amounts too low to be detected by the techniques used in this study. In addition, (*R,S,*)-ABA was catabolised in low yields into compounds with similar chromatographic properties to those of PA, DPA and the 1',4'-*cis* diol of ABA, some of which were present as water-soluble conjugates. Thus, these results from tissue homogenates of *Persea americana* mesocarp might further support the idea that ABA metabolism occurs in the cytosol rather than in chloroplasts. The low level of incorporation might be due to the high endogenous concentration of ABA (Milborrow, 1974c) which diluted the labelled substrate, while conjugation of ABA might be indicative of the apparent inability of this system to accumulate label from applied MVA in ABA.

Studies using intact tissue and inhibitors of GA biosynthesis have led to the speculation that ABA could arise from FPP if the cyclisation of FPP were consistent with that of carotenoids. Thus, in order to observe ABA biosynthesis in a cell-free system from plant tissue, it was considered essential to use conditions under which phosphorylated terpenoid intermediates were being produced. In addition, employing a non-photosynthetic tissue would reduce the role of chloroplasts (Hartung *et al*, 1980; 1981; Cowan and Railton, 1986) and thus limit the involvement of a carotenoid origin of ABA (Burden and Taylor, 1970).

Intact *Hordeum vulgare* embryos synthesized ABA from MVA (see Chapter 4) and catabolised (*R,S,*)-

ABA to PA, 7'-hydroxy ABA, DPA and conjugates (see Chapter 5). In addition, the sequence from *ent*-kaurene to *ent*-7-hydroxykaurenoic acid had been demonstrated in cell-free extracts of embryos from *Hordeum vulgare* seeds (Murphy and Briggs, 1975), suggesting the presence of hydroxylating activity. Thus, using a crude enzyme preparation from embryos of mature *Hordeum vulgare* seeds a cell-free system capable of metabolising ABA was developed. The cell-free system prepared from *Hordeum vulgare* embryos as described in Chapter 7, synthesized IPP, FPP, GGPP and ABA. The synthesis of IPP, FPP and GGPP was achieved under anaerobic conditions in the presence of AMO1618 while ABA was produced when extracts were incubated in air with NADPH and AMO1618. The production of these terpenyl pyrophosphates in *Hordeum vulgare* embryo cell-free extracts agrees with previous findings (Davies *et al*, 1975).

The percentage incorporation of radioactivity from either MVA or IPP into ABA by *Hordeum vulgare* embryo cell-free extracts agrees with previous published results (Noddle and Robinson, 1969; Robinson and Ryback, 1969; Milborrow and Robinson, 1973; Milborrow, 1974b; Loveys *et al*, 1975) although low (< 1% of total radioactivity applied), incorporation was enhanced when (*R,S,*)-ABA was added as a "cold-pool trap". Product ABA was tentatively identified by derivatization and cochromatography using the criteria proposed for the identification of ABA by Milborrow and Noddle (1970). Although ABA has been characterised as an endogenous compound in embryos of *Hordeum vulgare* (Morris *et al*, 1988), unequivocal identification of the ABA produced in cell-free extracts of *Hordeum vulgare* requires GC-MS analysis. However, low incorporation of label into product ABA made this extremely difficult in the present study.

The reaction itself required ATP and Mg^{2+} which are also required for the initial stages of GA biosynthesis (Moore and Coolbaugh, 1976; Hedden and Graebe, 1982). Since *Hordeum vulgare* embryos are non-photosynthetic and AMO1618 inhibits the synthesis of GAs and steroids (Fall and West, 1971; Douglas *et al*, 1972; 1978) it is tempting to suggest that ABA arises *via* the "direct" route (see Chapter 1) in this tissue. Studies using intact tissues suggested that 80s ribosomal translation was required for ABA biosynthesis and that the enzymes involved were rapidly "turned-over". This proposal was further substantiated using cell-free extracts of *Hordeum vulgare* embryos which showed that active protein biosynthesis, during imbibition, was required for the cell-free biosynthesis of ABA.

A requirement for O_2 and NADPH suggested that some of the enzymes involved in ABA biosynthesis were NADPH-requiring monooxygenases. Similar enzymes are involved in the

hydroxylation of ABA to PA (Gillard and Walton, 1976). A distinguishing feature of these enzymes is the involvement of cytochrome P450 which catalyses *ent*-kaurene oxidation in GA biosynthesis (Graebe, 1987) and many other biological oxidation reactions (Nebert and Gonzalez, 1987). It is now well established that cytochrome P450 mixed function oxidases have a high affinity for NADPH and are inhibited by CO (Russell, 1971; Potts *et al*, 1974; Rich and Bendall, 1975; Hasson and West, 1974; Reichhart *et al*, 1980; Benveniste *et al*, 1982). Thus, the results shown in Table 7.11 strongly suggest that cytochrome P450 mixed function oxidases are involved in the biosynthesis of ABA. The inhibition of ABA biosynthesis in cell-free extracts of *Hordeum vulgare* embryos by cytokinins and the cytokinin analogue, ancymidol (Table 7.13 and 7.14), support a role for cytochrome P450 components in this process, similar to that observed for GA biosynthesis (Coolbaugh, 1984). However, Norman *et al* (1986) showed that ancymidol, in amounts of 100μM, had no effect on the incorporation of label from FPP into ABA in *Cercospora rosicola*. This finding led the authors to conclude that ancymidol inhibited a step prior to FPP formation. If ancymidol inhibits a pre-FPP step in ABA biosynthesis then it should similarly inhibit GA biosynthesis. In cultures of *Gibberella fujikuroi*, at 10μM ancymidol treatment resulted in more *ent*-kaurene and GA_3 accumulating from [^{14}C]-MVA while at 100μM it specifically inhibited the oxidation of *ent*-kaurene in cell-free extracts of this fungus (Coolbaugh *et al*, 1982) and *Marah macrocarpus* (Coolbaugh, 1984). Clearly then, the effect of ancymidol on the incorporation of label from FPP into ABA in *Cercospora rosicola* requires further investigation, particularly as cytochrome P450-catalysed oxidation reactions might be expected to be involved in the transformation of FPP to ABA. Thus, inhibition of ABA biosynthesis by ancymidol does lead to a questioning of the specificity (Coolbaugh *et al*, 1978) of this compound as an inhibitor of mixed function oxidases in GA biosynthesis, particularly in view of its effects on ABA production in the fungus *Cercospora rosicola* (Norman *et al*, 1986).

In addition to NADPH-requiring mixed function oxidase activity, 2-oxoglutarate-Fe^{2+}-ascorbate increased ABA biosynthesis in the *Hordeum vulgare* embryo cell-free system. A requirement for these cofactors might suggest that soluble oxidase activity is necessary for ABA biosynthesis. These enzymes are required in the latter stages of GA biosynthesis (Hedden and Graebe, 1982) and a 2-oxoglutarate-dependent hydroxylase participates in anthocyanin biosynthesis (Forkmann *et al*, 1980). However, further studies employing these cofactors are necessary in order to establish this aspect more fully, particularly since NADPH-dependent monooxygenases and soluble dioxygenases can catalyse similar hydroxylation reactions (Hedden and Graebe, 1982).

Kinetic data on the incorporation of label from MVA into ABA in extracts of *Hordeum vulgare*

embryos showed that ABA biosynthesis was sustained for about 6h after which time the levels of ABA declined (Figure 7.4). This decline in the levels of ABA was assumed to occur as a result of catabolism. In addition, studies on the effect of NADPH concentration on ABA production revealed that when NADPH exceeded 0.5μM the amount of ABA synthesized declined. Since NADPH is required for the conversion of ABA to PA and DPA (Gillard and Walton, 1976) it seemed likely that end-product ABA was being catabolised.

Hordeum vulgare embryo cell-free extracts transformed (*R,S,*)-ABA into 4 radioactive components two of which were tentatively identified as PA and 7'-hydroxy ABA (Figure 7.11 and 7.12). The identity of a third radioactive component, which displayed similar chromatographic properties to that of DPA, remains unknown. The low incorporation of label into these acids (<3% of total label supplied) made it extremely difficult to characterise these products by GC-MS. Although PA appears to be produced as a catabolite of applied, labelled (*R,S,*)-ABA in extracts of *Hordeum vulgare* embryos confirmation of this must await analysis by GC-MS.

The presence of both PA and 7'-hydroxy ABA as products of (*R,S,*)-ABA hydroxylation highlights the need to determine whether the 8'-hydroxylating enzyme is responsible for both reactions. Zeevaart *et al* (1986) have suggested this possibility based on kinetic data obtained in *Xanthium strumarium* leaves. In this tissue PA is always formed rapidly whereas 7'-hydroxy ABA does not accumulate until PA levels off. This aspect could be clarified if unlabelled (*R*)-ABA competed with small amounts of (*S*)-[^{14}C]-ABA and vice versa in this cell-free system (Milborrow, 1986). In the present study, kinetics on ABA catabolism in the *Hordeum vulgare* embryo cell-free system revealed that both PA and 7'-hydroxy ABA were produced at similar rates and that amounts of 7'-hydroxy ABA always exceded those of PA. Thus, it is tempting to suggest that two distinct enzymes are responsible for these hydroxylations in the *Hordeum vulgare* embryo cell-free system.

Data from a single experiment tentatively suggested that the hydroxylation of ABA to PA and 7'-hydroxy ABA requires NADPH, Fe^{2+} and 2-oxoglutarate (Table 7.16). However, further studies are necessary to confirm these cofactor requirements. A requirement for 2-oxoglutarate and Fe^{2+} might be indicative of 7'-hydroxy ABA production by dioxygenase activity while NADPH necessitates PA production *via* 8'-hydroxylation. Similar mechanisms are known to operate in GA metabolism (Hedden and Graebe, 1982) and flavanone biosynthesis (Forkmann *et al*, 1980). In addition, a requirement for NADPH (Table 7.16) in the cell-free catabolism of ABA confirmed that NADPH was a necessary cofactor required by extracts of *Hordeum vulgare*, similar to that demonstrated for

the cell-free catabolism of ABA by *Echinocystis lobata* (Gillard and Walton, 1976).

The presence of an esterase was detected in cell-free extracts of *Hordeum vulgare* embryos. Lehmann and Schütte (1981) also reported the occurrence of an esterase in leaves of *Hordeum vulgare* capable of hydrolysing ABAGE and ABA-tetraacetyl-glucose ester to ABA. The catabolism of ABAMe has also been demonstrated in axes of *Phaseolus vulgaris* (Walton and Sondheimer, 1972b). In this study ABAMe was catabolised more slowly than ABA which was attributed to the slow hydrolysis of ABAMe, based on the amount of free ABA released. However, if free ABA were rapidly catabolised this could have given the appearance of a slow rate of hydrolysis.

In intact leaves of *Hordeum vulgare* ABAMe was efficiently catabolised to ABA, PA, DPA and other acidic products, suggesting esterase activity. Similarly, ABAMe was transformed to ABA, PA and other acids in cell-free extracts of *Hordeum vulgare* embryos. This esterase did not appear to to interfere with the conjugation process since treatment of aqueous residues and polar catabolites with base resulted in the release of free acids which cochromatographed with ABA and some of its catabolites. However, some hydrolysis of the respective glucose esters could have occurred although kinetic analyses demonstrated an increase in incorporation of radioactivity into the aqueous fractions. Thus, the very polar nature of these compounds, coupled with penetration problems leads one to speculate that other factors were responsible for the hydrolysis of conjugates reported by Lehmann and Schütte (1981) to occur in *Hordeum vulgare* leaves.

8.2. CONCLUSION.

In a review on the biochemistry and physiology of ABA, Walton (1980) stated that further advances would depend largely on the isolation and characterisation of novel intermediates and the development of an ABA-metabolising cell-free system.

Several previous reports on the cell-free metabolism of ABA in plant tissues have been inconclusive and until now, no further efforts appear to have been made in this direction. Clearly, chloroplasts do not appear to be a major site of ABA metabolism in plants. The present study has demonstrated the synthesis of ABA from MVA and IPP and its catabolism to PA and 7'-hydroxy ABA in a cell-free system prepared from *Hordeum vulgare* embryos. It is hoped that this cell-free system will assist in elucidating the biosynthetic pathway to ABA in plants and aid in the development of other plant

tissue cell-free systems capable of metabolising ABA. Although cell-free systems are invaluable, this experimental approach does have its limitations, e.g. the loss of compartmentalisation. Thus, it is essential that studies on ABA metabolism be continued using intact plants and plant parts, particularly since an associated area of interest includes a possible role for phytochrome as a regulatory mechanism in ABA metabolism. The advent of chiral-HPLC and immunochemistry as techniques for analysing ABA metabolism in plants should contribute greatly to a better understanding of the biochemistry of this phytohormone. Likewise, further studies with ABA-defficient mutants and studies on the effect of various inhibitors of ABA metabolism should assist with unravelling the ABA biosynthetic pathway in higher plants.

The studies on ABA biosynthesis in the fungi *Cercospora rosicola* and *Botrytis cinerea* have progressed to a stage where it is essential that cell-free systems from these organisms be developed. In these fungi, ABA appears to be produced "directly" from FPP rather than from a carotenoid or higher terpenoid precursor. Evidence has begun to accumulate which favours the 1',4'-*trans* diol of ABA as being a precursor to ABA, both in fungi and in higher plants. Clearly then, the development of a fungal cell-free system would complement similar cell-free studies on ABA metabolism in higher plants. This has been very well illustrated in studies on GA metabolism where the entire biosynthetic pathway has now been delineated (Graebe, 1987).

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