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THE BIOSYNTHESIS OF  
ABSCISIC ACID

by

Richard C. Noddle

A dissertation submitted  
to the University of Warwick for  
admission to the degree of  
Doctor of Philosophy

MEMORANDUM

This dissertation is submitted to the University of Warwick in support of my application for admission to the degree of Doctor of Philosophy. It contains an account of my own work performed at the Milstead Laboratory of Chemical Enzymology in the period September 1967 to July 1970.

The work was under the general supervision of Professor G. Popják initially and subsequently Professor J. W. Cornforth and Dr. B. V. Milborrow.

No part of it has been used previously in a degree thesis submitted to this or any other University. The work described in this thesis is the result of my own independent research except where specifically acknowledged in the text.

Parts of this thesis have already been published with the work of other authors. These publications are:

- (a) Biochemical Journal (1969) Vol.112, p.547-548.
- (b) Biochemical Journal (1970) Vol.119, p.727-734.

Richard C. Noddle

ACKNOWLEDGEMENTS

I wish to thank Professor G. Popják for his advice during the early part of this work and for initially allowing me to carry out the work at Milstead Laboratory. I am also grateful to Professor J. W. Cornforth, Director of this laboratory and Dr. B. V. Milborrow, head of the Plant Physiology section for their advice and encouragement throughout this work.

I gratefully acknowledge the receipt of a Post-Graduate Studentship from Shell International Petroleum Company Limited, London, which provided me with financial support between September 1967 and September 1970.

I wish to thank the staff of this laboratory for the hospitality and assistance which they gave during my stay.

Also I wish to thank those responsible for the preparation of the art work and the typing of this thesis.

This work is dedicated to my parents, my wife and my son.



ABSTRACT

This work is the first investigation into the biosynthesis of abscisic acid.

Green tomato fruit were injected with a solution of either the potassium salt of  $(\pm)$ -[2-<sup>14</sup>C]mevalonic acid in 0.05M phosphate buffer, pH 7.3, containing 10% ethanol, or  $(\pm)$ -[2-<sup>14</sup>C]mevalonic acid in ethanol. Radioactivity was detected in material which co-chromatographed with authentic abscisic acid and the authentic derivatives of abscisic acid. This material exhibited the highly characteristic spectral properties of (+)-abscisic acid. Similar results were obtained when  $(\pm)$ -[2-<sup>14</sup>C]mevalonic acid was supplied to avocado fruit preparations. The results of this work have been published in the Biochemical Journal, Vol.112, p.547-548 (1969).

Many analogues of abscisic acid have been synthesised at Shell Research Limited, Woodstock Agricultural Research Centre, and their growth inhibitory activity assessed. The strong inhibitory activity of  $(\pm)$ -5-(1',2'-epoxy-2',6',6'-trimethylcyclohexyl)-3-methylpenta-cis-2-trans-4-dienoic acid (epoxide) on plant growth was of particular interest since the compound has the same carbon skeleton and similar substitution to abscisic acid. To discover whether this compound was active per se or because it was converted to abscisic acid the  $(\pm)$ -[2-<sup>14</sup>C]labelled material was synthesised and injected, as an ethanol solution, into tomato fruit. Epoxide was converted to abscisic acid in 1.8% yield (3.6% of one enantiomer if only one is utilized) and 15% of the abscisic acid was derived

from the precursor. The 2-trans isomer was not converted to abscisic acid. Neither epoxide nor its 2-trans isomer were converted to abscisic acid in boiled tomato fruit.

Treatment of epoxide with dilute mineral acid produced the 1',2'-dihydroxy derivative. The 1',2'-dihydroxy derivative was not converted to abscisic acid in tomato fruit.

Wright and Hiron (1969) reported that the abscisic acid concentration in wheat leaves increased 40-fold during the first 4 hours of wilting. It was found that this increase was caused by synthesis of abscisic acid rather than by its release from a conjugate or a precursor. This was shown by the increased incorporation of [2-<sup>3</sup>H]mevalonic acid into abscisic acid by wilted wheat shoots in comparison with turgid shoots which had absorbed equal amounts of [2-<sup>3</sup>H]mevalonic acid. When (±)-[2-<sup>14</sup>C] labelled epoxide was supplied, larger amounts of labelled material were again incorporated into abscisic acid by wilted plants.

The incorporation of [<sup>14</sup>C]epoxide into abscisic acid shows that the carbon skeleton of the epoxide becomes the carbon skeleton of abscisic acid. The fate of the epoxy oxygen was also investigated with wilting wheat because any [<sup>18</sup>O] containing abscisic acid formed during the experiment would not be diluted by a large pool of endogenous [<sup>16</sup>O] material. (±)-[1',2'-<sup>18</sup>O] epoxide was converted to abscisic acid, the oxygen of the 1',2'-epoxy group became the tertiary hydroxyl of abscisic acid and the conversion was quantitative. For the [<sup>18</sup>O] to be retained the C-1'-oxygen bond must remain intact and epimerization at C-1' is not possible. It follows that only one enantiomer of the

epoxide is converted into abscisic acid: that one in which the 1',2'-epoxy group is on the same side of the six-membered ring as the hydroxyl group in abscisic acid. The results of this work have been published in the Biochemical Journal, Vol.119, p.727-734 (1970).

The 1',4'-diols of synthetic abscisic acid were found to inhibit the growth of a number of plants (private communication, Mr. R. Leach and Dr. M. Anderson) although they were less potent than abscisic acid.

The demonstration of the conversion of the epoxide into (+)-abscisic acid which entails the introduction of a 4'-keto-group, and which by analogy with similar oxidations (Murphy and West, 1969) probably takes place by a hydroxylation, suggested that plants were able to oxidise the 4'-hydroxy group in the 1',4'-diols of abscisic acid to a 4' ketone. The abscisic acid produced would then account for the observed growth inhibitory activity of the 1',4'-diols.

(±)-[2-<sup>14</sup>C]-cis-1',4' and trans-1',4' diols of abscisic acid are both converted to abscisic acid by wilted and turgid wheat shoots. The abscisic acid recovered showed a preponderance of the unnatural (-) enantiomer. The unreacted cis-1',4'-diol which was recovered from the tissues also showed an excess of the (-) enantiomer, unreacted trans-1',4'-diol which was recovered from the tissue lacked optical activity and is presumed to be a racemic mixture.

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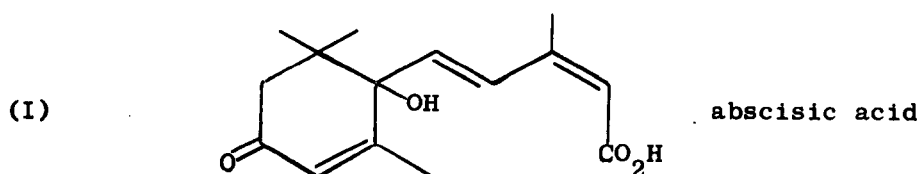
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## CHAPTER I

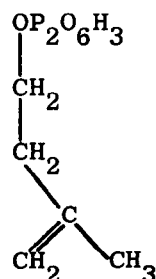
### INTRODUCTION

The structure of abscisic acid (ABA) was first suggested by Ohkuma, Addicott, Smith and Thiessen (1965) and this was confirmed by synthesis by Cornforth, Milborrow and Ryback (1965) as being:



An examination of the structure of ABA and the fact that it contains 15 carbon atoms suggested that it was a terpenoid on the basis of the suggestions made by Ruzicka (1953) about the nature of terpenes. He developed the concept of an "isoprene rule" which stated that "the carbon skeleton of the terpenes is composed of isoprene units linked in regular or irregular arrangement", in an attempt to codify terpene biogenesis. The term "isoprene" was first used by Williams in 1860 to describe the  $C_5H_8$  molecule which was produced when terpentine oil was passed through a red hot tube. It was not until 1956 that mevalonic acid (MVA) (Wolf et al, 1956 and 1957) was found, during the course of an investigation of a mutant bacterium, to be the precursor of the isoprene unit. In 1958, Chaykin et al identified the formation of isopentenyl pyrophosphate (IPP) from 5-phospho-[1- $^{14}C$ ]mevalonate and 5-diphospho-[1- $^{14}C$ ]-mevalonate when either was incubated with a fraction of a

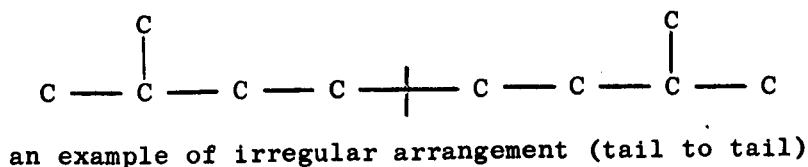
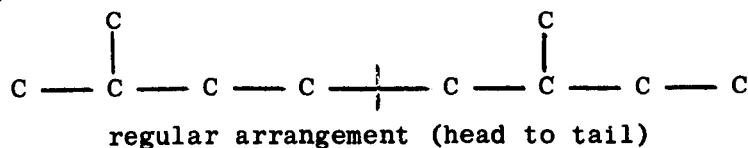
yeast extract. The biological significance of MVA and IPP in



Isopentenyl pyrophosphate  
(abbreviated to IPP)

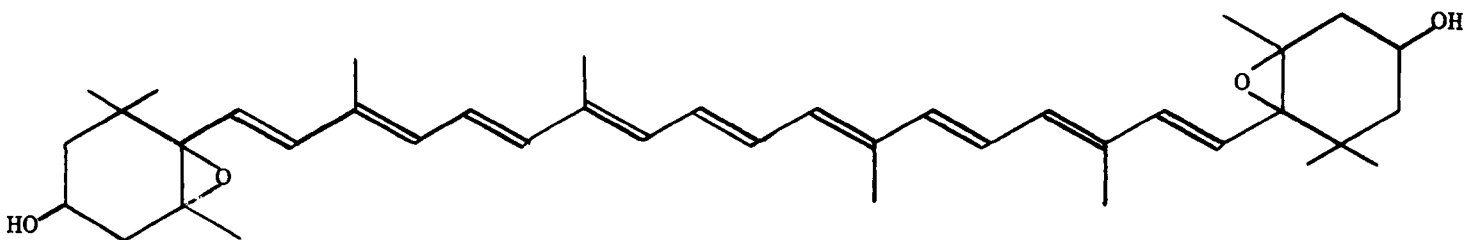
terpene biosynthesis was realized in the study of cholesterol biosynthesis, this has been reviewed by Popják and Cornforth (eg. *Advances in Enzymology* 22, p.281-331, 1960). During the course of the work on cholesterol the sequential addition of the  $\text{C}_5$  unit IPP in the biosynthesis of terpenoids suggested that IPP was the "biological" isoprene unit. An understanding of the enzymes responsible for some of the early transformations, notably between MVA and IPP, was also obtained. Thus farnesyl pyrophosphate is produced by the condensation of three 5-carbon units in a regular head-to-tail addition.

The structure of ABA immediately suggested that it was derived from mevalonic acid by the condensation of three 5-carbon isoprene units as its structure conforms to the "farnesol rule" (Ruzicka, 1953) a special case of the "isoprene rule" which refers to the regular arrangement of (head-to-tail) isoprene units in sesquiterpenes,





The carbon skeleton of ABA was novel although it resembles the terminal parts of the C-40 carotenoids, such as violaxanthin (II):



(II) **Violaxanthin**

The similarity between the structure of the carotenoids and ABA suggested that a carotenoid (Addicott, Ohkuma, Smith and Thiessen, 1966; Taylor and Smith, 1967) could be converted to ABA in the plant. Such a biosynthetic pathway involves the elaboration of 8 isoprene units to a forty-carbon carotene, followed by cleavage of the carbon skeleton and oxidation to a fifteen carbon compound. Taylor and Burden (1970) found that violaxanthin can be cleaved photolytically to give a highly growth-inhibitory compound which appears to be converted to ABA by plants. It has been suggested that the disappearance of chlorophyll from deciduous leaves in early autumn would unmask the xanthophylls and allow them to be photolytically converted to ABA more readily (Taylor and Smith, 1967). However, (Goodwin, 1958) measured the changes in carotenoid and chlorophyll pigments in the leaves of 3 species of deciduous trees during autumn necrosis and found that the metabolism varied for the 3 species he studied. Goodwin states - "The previously accepted general picture was that the chlorophylls first disappeared thus unmasking the carotenoid pigments which then gradually disappeared in the late autumn; as they disappeared the carotenoids were

converted into complex oxidation products, according to Karrer and Walker (1934) or into xanthophyll esters (Kuhn and Brockman, 1932)". Goodwin shows that in sycamore leaves (Acer pseudoplatanus L.) collected between 21st June and 19th November (1956) there was preferential loss of chlorophyll, followed by a subsequent decline in the total carotenoids. Lutein and violaxanthin esters appeared in relatively large amounts in the later stages of leaf necrosis. In oak leaves, (Quercus robur L.) collected during the same period, the chlorophylls and carotenoids disappeared simultaneously whilst in the leaves of the black plum (Prunus nigra L.) there was preferential loss of carotenoids. In the fallen leaves of sycamore the residual carotenoid appeared to be almost entirely violaxanthin whereas in oak leaves and plum leaves the relative amount of violaxanthin in the total carotenoids was comparatively constant during summer and autumn and showed no increase in the fallen leaves. The occurrence of vitamin A in the oil of avocado pear (Persea gratissima Gaerth) (M. T. Francois and M. Gauthier, 1963) indicates that carotenes such as  $\beta$ -carotene may be cleaved and oxidised in the plant.

However, in all other pathways of terpene biosynthesis there is a sequential addition of isoprene units or multiples of isoprene units to give the required number of carbon atoms. The terpene is then modified by cyclization, oxidation or reduction, and, in the conversion of lanosterol to cholesterol, loss of 3 methyl groups, in the conversion of kaurenoic acid to some gibberellic acids, the loss of a carbon atom at C-1 of the A-ring in kaurenoic acid. The potency of ABA in effecting physiological responses

such as the induction of bud dormancy, the inhibition of seed germination the induction of leaf abscission and the closing of stomata would require precise control of its biosynthesis. This would be difficult to operate if ABA were to be formed from a carotenoid by sunlight and would be expected to be more readily achieved if ABA were formed by direct synthesis from MVA.

ABA was initially isolated by several groups of workers who were each following a different physiological property. In the USA a group of workers isolated two substances from plant tissue which would accelerate leaf abscission, these they called abscisin I (Liu and Carns, 1961) and abscisin II (Ohkuma, Lyon, Addicott and Smith, 1963). At the same time, K. Rothwell and R. L. Wain (1964) at Wye College, London University, following the earlier work of R. F. M. van Steveninck in New Zealand (1959), were attempting to identify a substance which accelerates the abscission of fertilized flowers of yellow lupins. Meanwhile, P. F. Wareing and co-workers at the University of Wales, Aberystwyth, were attempting to determine the nature of the bud dormancy inducing factor (which they named "dormin") extracted from the leaves of sycamore (Acer pseudoplatanus, L) which increased in concentration when the plants were subjected to short days (Phillips and Wareing, 1958 and also Eagles and Wareing, 1963). Later the isolation was undertaken at Milstead Laboratory and "Sycamore dormin" was obtained in crystalline form and found to be identical with abscisin II isolated from cotton bolls (Cornforth, Milborrow, Ryback and Wareing, 1965; Cornforth, Milborrow, Ryback and Wareing, 1966). The yellow lupin abscission factor which had

been isolated by Rothwell and Wain (1964) was also found to be abscisin II (Cornforth, Milborrow, Ryback, Rothwell and Wain, 1966).

The chemical synthesis by Cornforth, Milborrow and Ryback (1965) of abscisin II also confirmed its structure as the more likely of the two possibilities proposed by Ohmura, Addicott, Smith and Thiessen, (1965). The compound was renamed "abscisic acid" in 1967 (Addicott et al, 1969) and this is abbreviated to "ABA". The leaf abscission-accelerating activity of the substance has been questioned because ABA, at low concentrations has little or no effect on abscission (in Plant Growth Regulators, Monograph No. 31, Society of Chemical Industry, London, 1968, page 207). In view of recent studies on the level of ABA during the development of tomato fruit from anthesis (Dörffling, 1970) which indicated that the highest concentration of ABA in the fruit occurs when the fruit is still green, the subsequent decrease in the concentration of ABA prior to ripening suggests that abscission does not necessarily occur when the concentration of ABA in the tissue reaches a maximum value. It has been reported that the concentration of ABA in red, ripe strawberry fruit was  $2\frac{1}{2}$  times that in white, unripe berries collected at the same time (Rudnicki, Pieniazek and Pieniazek, 1968), suggesting that the maximum biosynthesis of ABA occurs at a different time during the development of different types of fruit.

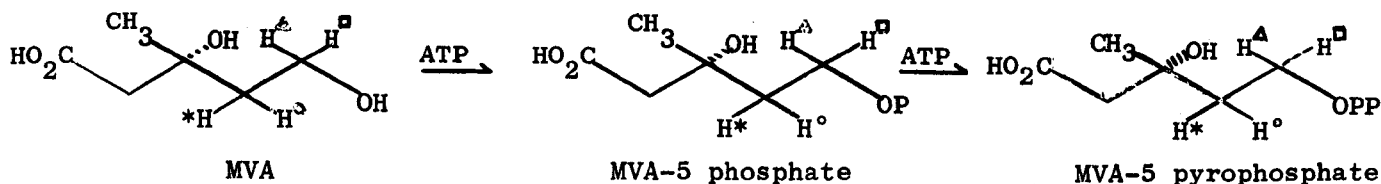
It is conceivable that the developing embryo stimulates the production of ABA by the pericarp in some species (Jackson, 1968) although this is not apparent in fruit such as the banana (Musa sapientum L.) which lack embryos and which synthesise an

appreciable amount of ABA (Noddle and Robinson, 1969) during ripening. In reply to the speculation suggested by Jackson (1968) "that perhaps exports of ABA from the aging flesh (pericarp of Rosa sp.) of this terminal organ contribute to the onset of dormancy in the embryo" it appears that ABA in tomato fruit is biosynthesised from the onset of embryo development. A function of the ABA in the seed may be to overcome the effect of the growth-promoting hormones which are present (Wain, 1968) and to induce dormancy in the embryos (Jackson, 1968) rather than to cause fruit abscission. In tomato fruit the concentration of ABA decreases as carotenoid degradation begins. Although a recent study (Milborrow, 1970(a)) of the metabolism of applied  $(\pm)$ -[2-<sup>14</sup>C]ABA has shown that it is rapidly metabolized to its glucose ester (Koshimitzu, Inui, Fukui and Mitsui, 1968) and to Metabolite C (Milborrow, 1970(a) and Milborrow, 1969(a)) by young tomato fruit there is as yet no data on the fate of the compound during the ripening of fruit.

To investigate the biosynthesis of ABA it was necessary to have a system which would incorporate MVA (presumed to be a natural precursor) into ABA. When the present work began no such system had been found, however, it had been shown that the concentrations of ABA increased during the maturation of leaves and that fruits contained the highest concentrations of ABA. For this reason and because some fruits can be obtained throughout the year they were chosen for the preliminary experiments. Although ABA had been identified in the fruit of many plant species (Milborrow, 1967) the fruit of 4 more species were

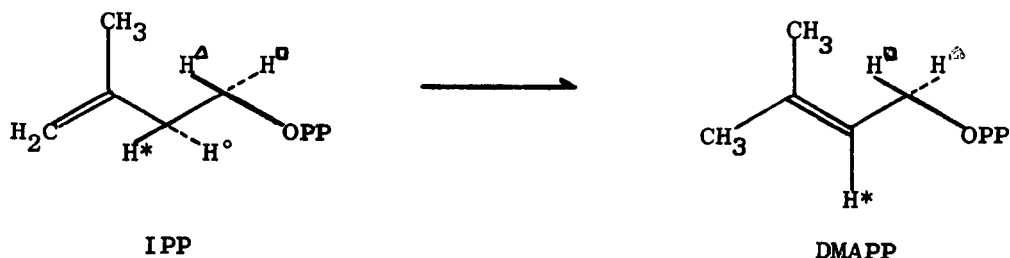
extracted, tomato, banana, mango and grapefruit, and ABA was isolated and identified from all four. Tomato fruit were chosen for the first work on ABA biosynthesis because ABA can be extracted from them relatively easily and also because they are available throughout the year. Purcell, Thompson and Bonner (1959) have reported that tomatoes injected with  $[2-^{14}\text{C}]$ mevalonic acid formed labelled carotenoids, therefore the injected mevalonic acid had been assimilated by the fruit and had entered prenyl metabolism. Therefore, if biosynthesis of ABA occurred in tomato fruit either by the cyclization of a sesquiterpene or by the degradation of a carotenoid then labelled MVA could be expected to be incorporated into ABA.

The conversion of mevalonic acid to farnesyl pyrophosphate has been well documented and will be briefly summarized here. Mevalonic acid is pyrophosphorylated in 2 steps, both requiring ATP, by the enzymes mevalonic kinase and mevalonic phosphokinase, respectively.



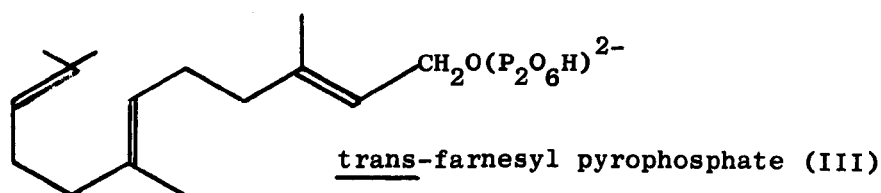
The tertiary hydroxyl group of MVA-5-(P<sub>2</sub>) is phosphorylated and then there follows a trans elimination of CO<sub>2</sub> and phosphate under the action of 5' pyrophosphomevalonic anhydrodecarboxylase to give isopentenyl pyrophosphate (IPP). IPP is isomerized by isopentenyl

pyrophosphate isomerase to dimethallyl pyrophosphate (DMAPP).



IPP and DMAPP are then combined with the formation of a C-C bond to give geranyl pyrophosphate, a precursor of monoterpenes.

Addition of another unit of IPP to geranyl-PP produces the sesquiterpene farnesyl pyrophosphate (FPP). The structure of ABA suggests that it may be made by the cyclization of FPP (III).



If ABA were derived from a carotenoid then farnesyl pyrophosphate would still be an intermediate but would be converted to geranyl-geranyl pyrophosphate by the addition of a further isopentenyl group and this material could then give rise to carotenoids and eventually to ABA.

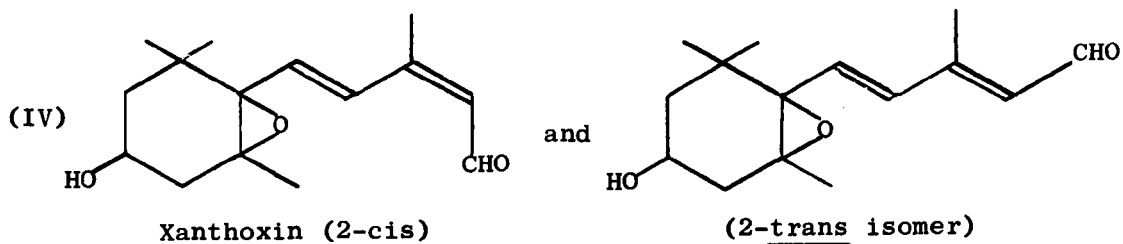
A possible difference between the "direct synthesis" and the "carotenoid pathway" lay in the formation of the 2-cis-double bond of ABA. Previous work (Popják and Cornforth, 1966) has established that during the formation of a cis double bond

the 4-S hydrogen atom of mevalonic acid is retained and during the formation of a trans double bond the 4-R hydrogen atom is retained. If, therefore, the 2-cis-double bond of ABA were formed in the cis configuration then a 4-S hydrogen would be retained on C-2 and this would exclude the carotenoid pathway. However, if the 2-cis bond were formed in the trans configuration in a C<sub>15</sub> precursor and then isomerized during a later stage of biosynthesis then the 4-R hydrogen would be retained at C-2. The same labelling pattern would, of course, occur if a carotenoid were an intermediate. Work by Robinson & Ryback (1969) showed that a 4-R tritium of mevalonic acid was present in ABA (probably at C-2) and no 4-S tritium was retained. This result is ambiguous in that it could occur from either the "direct synthesis" or the "carotenoid pathway".

It has been stated that there is evidence that bright illumination stimulates the production of inhibitor  $\beta$  (Taylor and Smith, 1967), (whose inhibitory activity is attributed to ABA) but the duration of illumination is also important because the concentration of inhibitor  $\beta$ , in leaves, increases when they are subjected to short days. However, the similarity between violaxanthin and ABA suggested the possibility that photo-oxidation of violaxanthin gives rise to ABA and that this may be the way in which ABA is formed in the plant (Taylor and Smith, 1967). Violaxanthin exposed to light produced neutral materials which inhibited the germination of cress seeds (Lepidium sativum L.) kept in darkness. Recent work has shown the presence of two neutral inhibitors, (Xanthoxin, IV) in the extracts of seedlings of dwarf bean (Phaseolus vulgaris, cultivar "Canadian Wonder") (Taylor and Burden, 1970). It is reported that these compounds are also

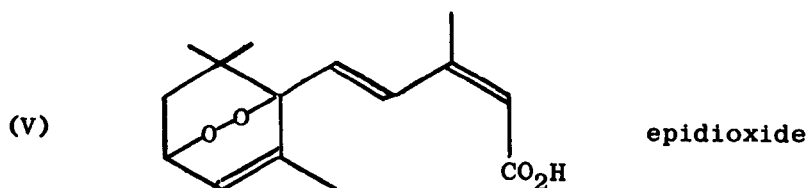


produced on photo-oxidation of violaxanthin and that they can be chemically converted into ABA.



As with the experiments with stereospecifically tritiated MVA the occurrence of xanthoxin is not a conclusive proof that ABA is derived from a carotenoid, xanthoxin may be one of the final C-15 precursors of ABA derived directly from FPP.

The chemical synthesis of ABA first employed by Cornforth, Milborrow and Ryback (1965) involved the rearrangement of an epidioxide (V),

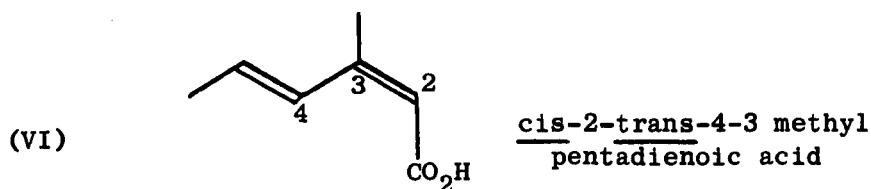


This was the first analogue of ABA to be synthesized. In a biological assay it was found to inhibit the germination of seeds and its biological activity was reported as 1/6 that of natural ABA. This was interpreted as reflecting the possibility that the epidioxide was a biosynthetic precursor of ABA.

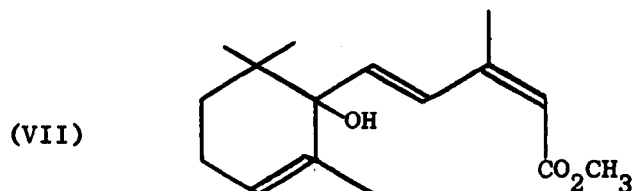
Ohkuma (1965) described the chemical synthesis of some analogues of ABA, closely resembling the structure of ABA, but gave little indication of their biological activities.

Cis-trans and trans-trans  $\alpha$ -ionylidene acetic acids were synthesised by Asmundson, Ingersoll, Smith and Kumamoto, (1968) and in view of their abscission-accelerating activity in a cotton explant bioassay and inhibitory activity in other unspecified bioassays it was suggested that the cis-trans  $\alpha$ -ionylidene acetic acid was a possible biosynthetic precursor of ABA. This suggestion was followed up in 1970 (Kumamoto, Smith, Asmundson, Ingersoll and Sadri, 1970) by more detailed information which confirmed the earlier report about the physiological activity of  $\alpha$ -ionylidene acetic acid.

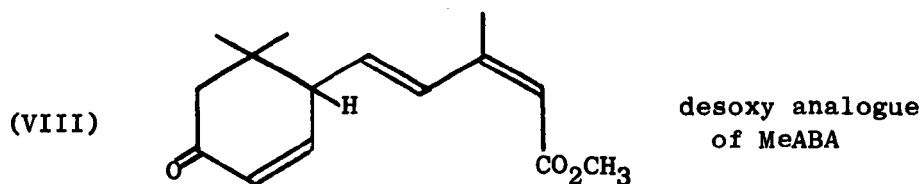
The chemical synthesis and biological activities of the cis-trans and trans-trans  $\beta$ -ionylidene acetic acid has been reported by Tamura and Nagao (1969(a) and 1969(b)). The inhibition produced by the 5'-6' epoxide of cis-2-trans-4  $\beta$ -ionylidene acetic acid (1',2'-epoxide by ABA numbering) on the growth of the 2nd leaf sheath of rice seedlings (c.v. "Koshiziwase") was approaching that of ABA. The methyl and ethyl esters of the epoxide had greater activity than ABA but they neglected to make the critical comparison with the respective esters of ABA. Some of the data reported by these workers has been used in the present work. Tamura and Nagao concluded that only molecules with a cis-2-trans-4-3 methyl-pentadienoic acid side chain (VI), were biologically active and they confined their subsequent work (Tamura and Nagao, 1969(c))



to the chemical synthesis of molecules possessing this side chain. The carboxyl group of the side chain in these analogues was modified in several ways, such as reduction to give an alcohol, esterified to the methyl and ethyl esters and replaced by  $C\equiv N$ . The ring of these analogues was variously substituted with epoxide at the 1'2' position and separately with hydroxyl at the C-1' position. Unsaturation was introduced into the ring, eg. a double bond at the C-2',3' position and a methylene group attached to C-2' position. None of these analogues possessed significant biological activity. Tamura and Nagao (1969(c)) concluded that the ketone group of ABA may not be required for biological activity as the biological activity of compound (VII) was slightly

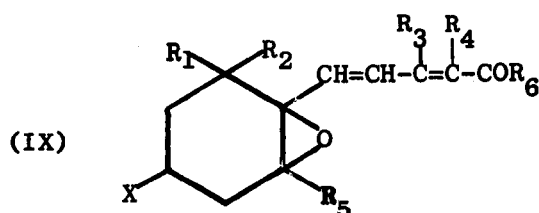


greater than was recorded for ABA. They suggested that the C-2',3' double bond may play an important role in causing the observed physiological effects of the analogues. It had already been suggested (Milborrow, 1968) that the tertiary hydroxyl group of ABA may not be required in view of the observed biological activity of the desoxy analogue of MeABA (VIII);



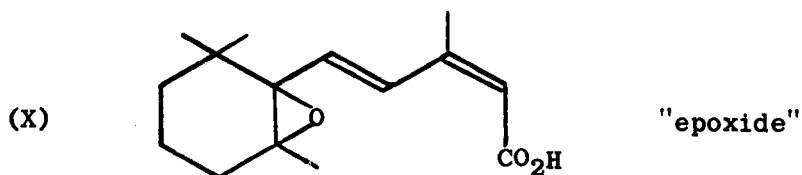
which is half as active as MeABA as a growth inhibitor. Therefore, if the ketone group is removed (Tamura and Nagao, 1969(c)) with the hydroxyl group (Milborrow, 1968) from the carbon skeleton of ABA, cis-trans  $\alpha$ -ionylidene acetic acid remains, and this has been found to have high biological activity.

Many analogues of ABA were synthesised at Shell Research Ltd., Woodstock Agricultural Research Centre and their growth inhibitory activity assessed. As a result of this testing, compounds having the general structure (IX):



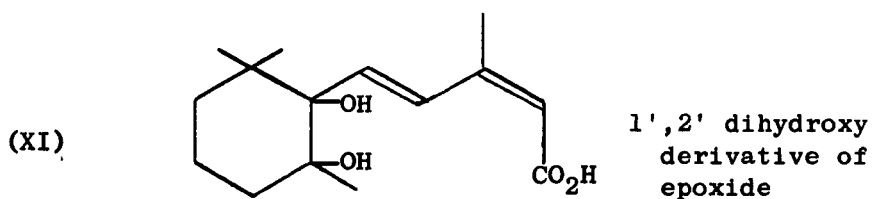
Where  $R_1$  to  $R_6$  and X and either H or specified alternative groups.

were patented (Anderson, 1969). Their activity, as inhibitors of seed germination and as inhibitors of the growth of germinating seedlings, was similar to ABA. In particular the epoxide (X),

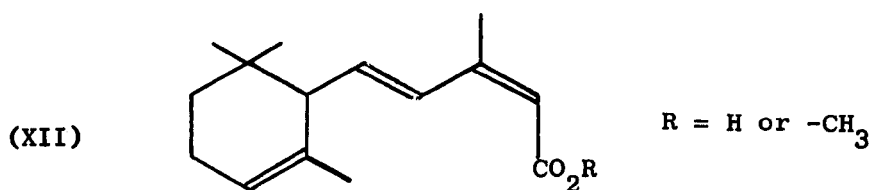


exhibited substantial inhibition of growth and in view of the similarity of its carbon skeleton and substitution to ABA it was of interest to find out whether the epoxide was active per se or by virtue of its conversion to ABA within the plant.

To study the metabolism of epoxide by plants the  $[2-^{14}\text{C}]$ labelled epoxide and its 2-trans-isomer were synthesised at Milstead Laboratory by Dr. G. Ryback and Mr. R. Mallaby. The metabolism of these compounds and their 1',2' dihydroxy derivatives (XI), in tomato fruit and cut wheat shoots is reported in this thesis.

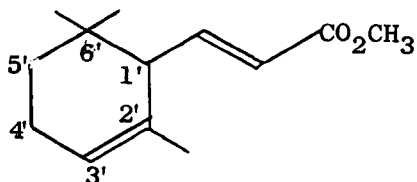


Synthesis of ketone derivatives of both  $\alpha$ - and  $\beta$ -ionylidene acetic acids (the cis-2-trans-4 and trans-2-trans-4-3 methyl-pentadienoic acid, side chain, isomers in both substances) was reported by Oritani and Yamashita (1970(a)) and their growth inhibitory activity on the 2nd leaf sheath of germinating rice seedlings measured. They concluded that the carbon structure (XII),

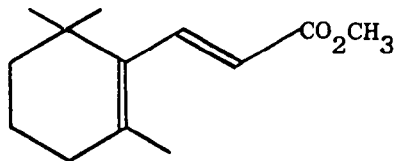


was fundamental for the appearance of physiological activities similar to those of ABA.

Subsequent work by Oritani and Yamashita (1970(b)) dealt with the synthesis of the oxidation products of methyl  $\alpha$ - and  $\beta$ -cyclocitrylidene acetates (XIII) and of methyl  $\alpha$ -cyclogeranate,



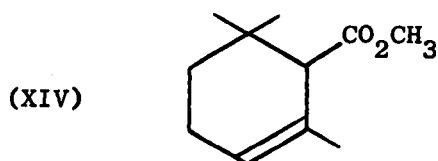
(XIII)  $\alpha$ -cyclocitrylidene acetic acid, methyl ester



$\beta$ -cyclocitrylidene acetic acid, methyl ester

Under the conditions they employed oxidation of the  $\beta$ -cyclocitrylidene acetic acid methyl ester resulted in the formation of a mixture containing 4 main products, the (a) 1',hydroxyl, (b) 4',keto and (c) the 1',hydroxy-4',keto-derivatives of  $\alpha$ -cyclocitrylidene acetic acid, methyl ester and (d) the d'-keto derivative of  $\beta$ -cyclocitrylidene acetic acid, methyl ester. None of these compounds were biologically active in the rice bioassay.

Oxidation of  $\alpha$ -cyclogeranic acid methyl ester (XIV),

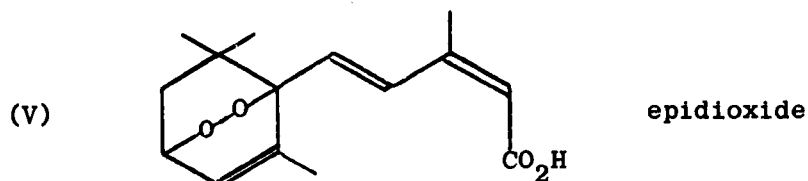


methyl  $\alpha$ -cyclogeranic acid

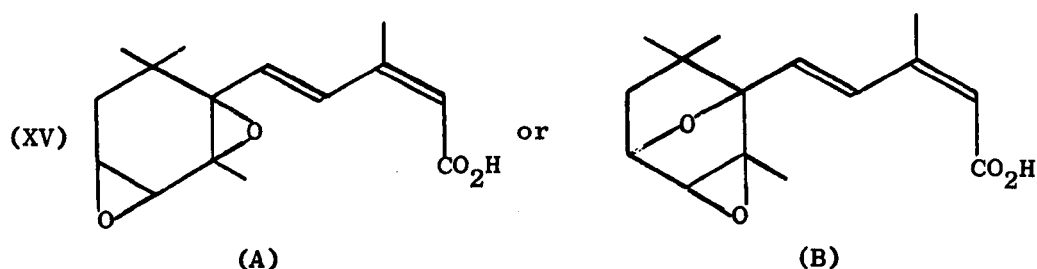
under conditions similar to those used for the previous esters, yielded 3 major products, (a) the 3'-keto derivative of  $\beta$ -cyclogeranic acid methyl ester, (b) the 4'-keto and (c) the 1' hydroxyl, 4'-keto derivatives of  $\alpha$ -cyclogeranic acid methyl ester. Again, none of these compounds were biologically active in the rice bioassay. Elaboration of the 3-methyl pentadienoic acid side chain (VI), as found in ABA, presumably does not occur with these analogues, nor were they sufficiently similar to ABA to

exhibit any physiological activity. Thus the pentadienoic acid side chain appears to be obligatory for an analogue of ABA to be a growth inhibitor. Unsaturation in the ring, as shown by  $\alpha$ -ionone, or the possession by the ring of substituents which can produce this unsaturation on rearrangement, such as a 1',2'-epoxy group, would also appear necessary for biological activity of an analogue.

Sondheimer, Michniewicz and Powell (1969) reported the inhibitory activity of the epidioxide (V),



(previously reported by Cornforth, Milborrow and Ryback, 1965) and a photolytic rearrangement product, (XV).

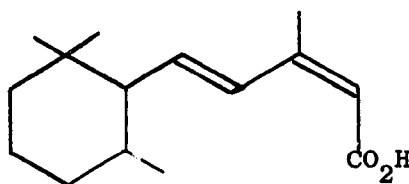


[Note: the authors were not certain of the absolute structure, but favoured structure (B)].

The rearrangement product had only slight biological activity. The inhibitory activity of the epidioxide was assayed in two ways; firstly by the increase in length of wheat coleoptiles sections after 24 hours incubation, 50% inhibition of growth was produced by 0.06 $\mu$ g/ml ABA and 0.5 $\mu$ g/ml epidioxide, the diepoxy rearrangement product (XV) was not active. Secondly, by a bean stem assay, after

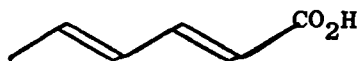
20 hours incubation the epidioxide showed  $\frac{1}{3}$  to  $\frac{1}{4}$  of the activity of ABA whilst the rearrangement product had at  $\frac{1}{10}$ th or less of the activity of ABA. The method of calculating the results were not clearly stated by the authors. In view of the observed physiological response to epidioxide in the two assay systems and because the epidioxide could be chemically rearranged to produce ABA the authors concluded that epidioxide was biologically active by virtue of its non-enzymic conversion to ABA in about 20% yield. No attempt was made to detect ABA either in the incubation mixture or a rise in the level of ABA in the assay tissue, nor was consideration given to the suggestion that the epidioxide could be active per se or by its having a sparing action on ABA. Recently Sondheimer and Walton (1970) reported the growth inhibitory activity of many compounds, some analogues of ABA, such as the cis -2-trans-4-dihydro-ionylidene acetic acid, (XVI),

(XVI)



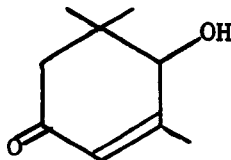
others such as trans-2-trans-4 sorbic acid, (XVII)

(XVII)



and (R,S) 4-hydroxy isophorone (XVIII) which resemble the side chain and ring moieties respectively in ABA.

(XVIII)

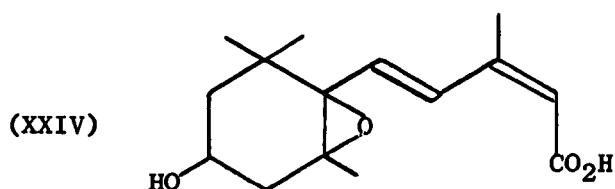




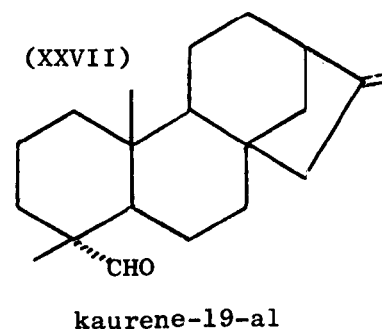
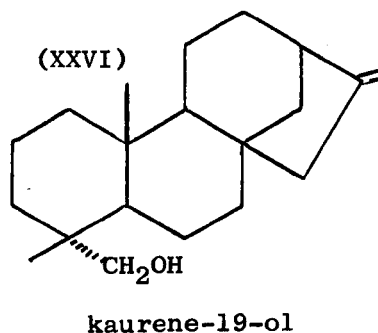
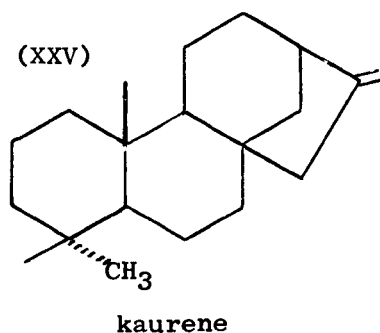
Growth activity of these and other compounds were assayed, using the excised axes from the dry seed of Phaseolus vulgaris L. (var. White Marrowfat) to give a measure of their effects on fresh weight increase of the embryonic axes. The axes were incubated in 2ml of a solution containing 0.01M potassium phosphate buffer, pH 7.0 containing 20 $\mu$ g/ml chloramphenicol and the appropriate concentration of test compound. Of the compounds tested cis-2-trans-4  $\beta$ -ionylidene acetic acid was the most active and had approximately  $\frac{1}{6}$  the activity of ABA in the above assay system. In a separate assay,  $\beta$ -ionylidene acetic acid failed to inhibit the germination of excised embryos from Fraxinus ornus seeds whose germination was inhibited 90% by a 10 $\mu$ M solution of ( $\pm$ )-ABA assayed at 22° over a 10 day period. In conclusion, Sondheimer and Walton (1970) suggested that the physiological responses produced in the assay tissue, inhibition of growth and of germination, due to analogues and compounds related to ABA was unlikely to be accounted for by their biochemical conversion to ABA in the plant. However, their evidence that these compounds are operating on the same site or sites as ABA is inconclusive.

The biological activity of the methyl ester of ABA (XIX), the cis-1',4' (XX) and trans-1',4' (XXI) diols of MeABA and of ABA (XXII and XXIII respectively) have not yet been published (Cornforth, Draber, Milborrow and Ryback, 1967). However, early work on the identification of the active principle in the inhibitor- $\beta$  fraction of sycamore leaves suggested that the inhibitor- $\beta$  fraction was inactive after reduction with an aqueous methanolic sodium borohydride solution (Robinson and Wareing, 1964). With

the identification of ABA as the active component of the inhibitor- $\beta$  fraction from sycamore leaves the products of  $\text{NaBH}_4$  reduction (performed at  $0^\circ\text{C}$ ) were identified as the cis-1',4'-diol of ABA (XXII) and the trans-1',4'-diol of ABA (XXIII). Unpublished work at Woodstock Agricultural Research Centre, Shell Research Ltd., indicated that the 1',4' diols of ABA were inhibitory in a wheat germination bioassay but less inhibitory than ABA. It was of interest to discover whether the reported activity of the 1',4'-diols could be attributed to their conversion into ABA or whether they were active per se. With the suggestion that a compound such as (XXIV),



may be converted to ABA, (Taylor and Burden, 1970; Milborrow and Noddle, 1970) it was also of interest to find out if a hydroxyl group at C-4' of the ring, such as is found in the ring of the 1',4'-diols of ABA, could be oxidized to a C-4' keto group; and in the event of such a conversion being enzymic, whether the enzyme was stereospecific for a particular configuration about C-4'. The addition of 4'-keto oxygen to the carbon skeleton of ABA may be brought about by hydroxylation at C-4' followed by oxidation. There are many examples of such transformations, eg. kaurene to kaurene-19-ol and then kaurene-19-al. The initial hydroxylation in this example is catalysed by a "mixed-function oxidase" system (Murphy and West, 1969).



Hitherto the work on ABA analogues had been restricted to observation of their physiological responses and interpretation of this data in terms of simple structure/activity relationships without investigating the possibility that activity depended on their metabolism to ABA. It has been suggested that the analogues are active due to their conversion to another and biologically active analogue (Sondheimer and Walton, 1970). Sondheimer and Walton suggested that the metabolism to ABA could not account for the observed results and whilst this may be true for some of the compounds they studied, the present work suggests that the growth-inhibitory activity of certain analogues can be attributed to the ABA to which they are metabolized.

A detailed conspectus of the physiological properties of ABA will not be presented here, as a number of reviews have dealt with this recently (i.e. in Biochemistry and physiology of plant growth substances, ed. F. Wightman and G. Setterfield, Runge Press Ltd., Ottawa, 1968 (Proceedings of the 6th International Conference on plant growth substances, Ottawa 1967) and B. V. Milborrow, Sci.Prog., (1969(b))57, 533-558, "The Occurrence and Function of Absciscic Acid in Plants"). However a brief mention will be made of the action of ABA in plants because of its crucial importance in plant physiology

in inhibiting the growth promoting effects of the other three groups of plant hormones, the auxins, the gibberellins and the cytokinins.

A material which was later identified as abscisic acid (Cornforth, Milborrow, Ryback, Rothwell and Wain, 1966) inhibited the growth promoting action of indole acetic acid (IAA) (Rothwell and Wain, 1964) in a wheat coleoptile bioassay. Growth of excised oat mesocotyls, as measured by an increase in length, was promoted by IAA and by gibberellic acid (GA) and by a combination of IAA and GA. ABA counteracted the effect of IAA and GA singly and in combination it also inhibited the small amount of growth that occurred in the absence of IAA and GA. Kinetin did not affect oat mesocotyl growth nor did it interact (Milborrow, 1966) with the inhibition of IAA or GA stimulated growth. It was concluded that ABA inhibited the growth promoted by IAA and GA. The failure of GA to relieve this inhibition suggested that ABA cannot act as an inhibitor of GA synthesis in this tissue.

Synthetic abscisic acid has been found to reproduce all the effects attributed to the active constituent of inhibitor- $\beta$ . It has been found to induce dormancy in sycamore and birch where it causes the growing buds of plants kept in long days to stop growing and to form typical resting buds covered with bud scales (El-Antably, Wareing and Hillman, 1967).

It prevents the germination of seeds, but is non-toxic and it can be leached out with water whereupon the seeds begin to germinate. Its effect in preventing the germination of seeds can be overcome, at least partially, by GA or kinetin.

IAA, GA and ABA have been implicated in the control of DNA and RNA synthesis. ABA has been implicated in the prevention of the formation of RNA in germinating ash embryos (Villiers, 1968) and the antagonism between IAA and ABA in relation to RNA production by the root apices of the seedlings of Lens culinaris (var. Vilmorin) has been reported by Pilet (1970). He found that while ABA strongly inhibited the accumulation of RNA, and accelerated the RNA-ase activity, IAA had the opposite effect; with ABA and IAA together there was an increase of RNA content but not as marked as for IAA alone.

Knowledge of the precise reaction affected by ABA is still unknown, as it is for the other groups of hormones, and even interpretations of the physiological roles of the compound are confused by the dramatic changes of endogenous concentrations which can occur in response to some changes in environmental conditions. The work described here attempts to clarify some of the factors involved in the biosynthesis of abscisic acid and to help give an understanding of the means by which it regulates plant growth.

## CHAPTER II

### MATERIALS

Samples of the following compounds are available in Milstead Laboratory:-

(±)-5-(1'-hydroxy-2',6,6'-trimethyl-4'-oxocyclohex-2'-enyl)-3-methylpenta-cis-2-trans-4-dienoic acid (I) (referred to as abscisic acid or ABA), (Cornforth, Milborrow and Ryback, 1965) and its 2-trans isomer (XXVIII) (referred to as trans abscisic acid or t-ABA); the methyl ester of ABA (XIX) (referred to as methyl abscisate or MeABA, the methyl ester of t-ABA (XXIX) (referred to as methyl trans abscisate or t-MeABA), and the cis-1',4'-diol (XX) and trans-1',4'-diol (XXI) of methyl abscisate (Cornforth, Draber, Milborrow and Ryback, 1967).

Samples of the following compounds, synthesised by Dr. M. Anderson, Woodstock Agricultural Research Centre, Shell Research Limited, Sittingbourne, Kent, were generously made available for the present work:-

(±)-5-(1',2'-epoxy-2',6',6'-trimethylcyclohexyl)-3-methylpenta-cis-2-trans-4-dienoic acid (X) (referred to as epoxide, Anderson, 1969) and its 2-trans isomer (XXX) (referred to as 2-trans epoxide). The (±)-1',2'-dihydroxy derivatives of epoxide (XI) and of its 2-trans isomer (XXXI). (±)-3-methyl-5-(2',6',6'-trimethylcyclohex-1-enyl)-penta-cis-2-trans-4-dienoic acid (XXXII) (referred to as β-ionylidene acetic acid).

(±)-[2-<sup>14</sup>C]epoxide (1.3mCi/mmole) and its 2-trans-isomer were synthesised in Milstead Laboratory by Dr. G. Ryback and Mr. R. Mallaby.

( $\pm$ )-[2- $^{14}\text{C}$ ]1',2'-dihydroxy derivatives of epoxide (1.3mCi/mmole) and its 2-trans isomer were prepared by treatment of ( $\pm$ )-[2- $^{14}\text{C}$ ]epoxide (1.3mCi/mmole) and its 2-trans-isomer with dilute sulphuric acid (Methods, section (X), (b)).

( $\pm$ )-[1',2'- $^{18}\text{O}$ ]epoxide was prepared from the methyl ester of  $\beta$ -ionylidene acetic acid and  $^{18}\text{O}_2$  (98.3 atoms %  $^{18}\text{O}$ ) by a method described by Tamura and Nagao (1969(a)).  $^{18}\text{O}_2$  was obtained from YEDA Research and Development Co. Ltd., at the Weizman Institute of Science, Rehovoth, Israel (98.3 atoms per cent  $^{18}\text{O}$ ), (Methods, section (X), (a)).

( $\pm$ )-[2- $^{14}\text{C}$ ]ABA (1.6mCi/mmole) was synthesised in Milstead Laboratory (Cornforth, Mallaby and Ryback, 1968). ( $\pm$ )-[2- $^{14}\text{C}$ ]cis-1',4'-diol (21.6 $\mu\text{Ci}$ /mmole) and trans-1',4'-diol of ABA were prepared from ( $\pm$ )-[2- $^{14}\text{C}$ ]ABA (21.6 $\mu\text{Ci}$ /mmole) by reduction with an aqueous methanolic sodium borohydride solution (Methods, section (X), (c)).

( $\pm$ )-[2- $^{14}\text{C}$ ]mevalonic acid (4.82mCi/mmole) (referred to as MVA) was purchased from The Radiochemical Centre, Amersham, England.

2,5-bis[5'-tertiarybutylbenzoxazolyl (2')]thiophene (referred to as BBOT) was purchased from the CIBA (A.R.L.) Chemical Co. Ltd., England.

2,6-di-tert.butyl-p-cresol (referred to as BHT) was purchased from B.D.H. Chemicals, Poole, England. Analar grade chemicals were obtained from B.D.H. Chemicals, Poole, England; Hopkin and Williams, Chadwell Heath, England and CIBA Chemical Company Ltd., Duxford, England. Redistilled solvents were used for spectroscopy and elution of compounds from chromatograms.

Table 1

	Structure	Abbreviation used in test
(I)		(+)-ABA
(XXXVIII)		(+)-t-ABA
(XIX)		(+)-MeABA
(XXIX)		(+)-t-MeABA
(XX)		(+)-cis-1,4'-diol of MeABA
(XXI)		(+)-trans-1,4'-diol of MeABA
(X)		epoxide
(XXX)		2-trans-epoxide
(XI)		1,2'-dihydroxy derivative of epoxide
(XXXI)		1,2'-dihydroxy derivative of 2-trans-epoxide
(XXXII)		$\beta$ -ionylidene acetic acid



Wheat seedlings (Triticum vulgare, c.v. Capelle) were harvested 14 days after sowing when the shoots, cut at ground level, weighed an average of 0.37g and measured 120mm in height.

Green tomato fruit (Lycopersicon esculentum, var. F<sub>1</sub> Supercross) were used when their diameter was greater than 30mm and before they showed signs of yellowing.

Kodak Kodirex X-ray film was obtained from Kodak Ltd., England. The exposed film was developed using D11 developer and May and Baker Amfix fixative.

Avocado fruit were purchased locally.

## CHAPTER III

### METHODS

#### (i) Growth and harvesting of plant tissue

(a) Tomato fruit were grown in a greenhouse at 23°C, with supplementary lighting provided by mercury vapour lamps where necessary. Immediately prior to an experiment the fruit were removed from the plant; fruit greater than 30mm in diameter but showing no signs of ripening were harvested and selected randomly for each individual experiment.

(b) Wheat shoots were between 10-14 days old at the time of harvesting. They were cut off at soil level and trimmed to produce a flat surface at the cut ends so that small volumes of liquid could be absorbed equally by all shoots.

(c) Avocado pears were fully ripe.

#### (ii) Application of labelled compounds to plant tissue

(a) Injection of [ $^{14}\text{C}$ ]labelled compounds, which were dissolved in either 100% ethanol or 0.01M potassium phosphate buffer, pH 7.3, containing 10% ethanol (v/v), was carried out with a Hamilton syringe. In later experiments disposable, sterile, 1.0ml syringes fitted with a fine needle were used. Multiple injections were made round the circumference of the fruit, occasionally a drop of liquid would be exuded from an injection pore - this was left to dry on the surface of the fruit. No precautions were taken to sterilize or block the pore after injection.

(b) The cut ends of freshly harvested wheat shoots were placed in a solution of 0.01M potassium phosphate buffer, pH 7.3 containing 10% ethanol (v/v) in which the labelled compounds were

dissolved. The shoots were left in the solution for between 18-23 hours at between 4°-23°C, in light. During this time they look up approximately 50% of the solution.

(c) Avocado fruit were split lengthways and the large single seed removed. [ $^{14}\text{C}$ ]labelled compound, dissolved in 0.01M potassium phosphate buffer, pH 7.3, containing 90% ethanol (v/v) was applied to the cut surfaces and placed in the cavity of each half of the fruit. The [ $^{14}\text{C}$ ]labelled compound was applied over a period of 20 hours as three equal portions.

(iii) Treatment of plant tissue which had been supplied with labelled compound

(a) Tomato fruit were placed in a sterilized glass beaker and incubated in darkness at 20°C. On one occasion tomato fruit were incubated under laboratory light. Incubation was concluded after 48-64 hours when the fruit were macerated in methanol.

(b) When the wheat shoots had taken up labelled solution the submerged parts were rinsed in distilled water and blotted. Shoots were subjected to one of two treatments, half the batch was placed with their cut ends in distilled water and kept in a damp atmosphere at 20°C (this is referred to as water treatment). The other half were spread out on dry paper and exposed to a draught of air for between 30-50 minutes, during which they they lost between 25-50% of their weight (this is referred to as wilt treatment), they were then placed between transparent polythene sheets and left in laboratory light. Both batches of plants were harvested after 3-5 hours.

(c) Avocado halves were placed in a glass vessel, through which CO<sub>2</sub> free air was drawn, at 20°C, under normal laboratory light. The fruit were harvested after 5 days.

(iv) Extraction of abscisic acid from the plant tissue

(a) Whole tomato fruit and avocado fruit were macerated in a basedrive "MSE ATO-MIX" for 3-5 minutes in methanol containing a known amount of synthetic (±)-abscisic acid (in the preliminary tomato experiments the (±)-abscisic acid was omitted). The slurry produced was filtered through grade 1 Whatman cellulose powder over a Whatman No. 1 filter paper. The residue was washed with methanol and then resuspended in aqueous methanol (1:4 v/v) and stored in darkness at 20°C for 24 hours. This mixture was filtered and the filtrate added to the original methanol extract.

(b) The wheat shoots from both treatments were submerged in methanol (containing 10mg BHT/litre, and a precisely determined amount of synthetic (±)-abscisic acid where necessary) and cut into short lengths (10mm). The material was stored in darkness for 24 hours and then filtered. The residue was washed with methanol and then resuspended in aqueous methanol (1:4 v/v), containing 10mg BHT/litre, and left in darkness at 20°C for 48 hours. After 24 hours the mixture was filtered and the residue resuspended in aqueous methanol (1:4 v/v) (containing 10mg BHT/litre). All the filtrates were combined.

(c) Filtrates were concentrated to a small volume using a rotary evaporator. Filtrates were poured into a 2 litre evaporating flask containing 100ml of saturated sodium bicarbonate solution, kept at a temperature between 30°-40°C. At this

temperature all the methanol and most of the water were removed from the flask under vacuum. The final volume of the filtrate was approximately 50ml.

(d) Partition of the ether soluble acid fraction of the filtrate was carried out according to Milborrow (1967). The filtrate, pH 8.5-9.0 was extracted with diethyl ether. Gentle shaking was sufficient to remove much of the neutral material from the aqueous phase, into the ether phase without forming a stable emulsion. The pH of the aqueous phase was adjusted to pH 3.0-3.5, using  $4N\ H_2SO_4$  and monitored with pH indicator papers, and extracted several times with ether. The abscisic acid was extracted from the ether into aqueous sodium bicarbonate by repeated washings. Acidification of the bicarbonate solution, with  $4N\ H_2SO_4$ , to pH 3.0-3.5 and extraction with ether gave a yellow-green solution which on evaporation yielded a rancid-smelling gum. In later experiments the final ether solution was washed with 10ml of water or saturated sodium chloride solution (pH 3.0) to reduce the acidity of the ether solution prior to evaporation.

(v) Thin layer chromatography

(a) Type of plate. In the initial experiments, thin layer chromatography was performed using hand-made plates. Silica gel  $F_{254}$  was mixed with boiling distilled water and shaken until a homogeneous slurry was obtained. The slurry was spread on 5 x 20cm and 20 x 20cm, acetone washed, glass plates using a Shandon applicator, giving a gel thickness of 250 $\mu$ . The plates were allowed to set for 10 minutes after which they were activated by heating at 110°C for 30 minutes. Plates were stored in a

desiccator at room temperature until used. For most experiments Merck silica gel F<sub>254</sub> precoated thin layer plates, gel thickness 250 $\mu$  and 2mm, were used because of their convenience and greater reproducibility. They were not stored in a desiccator and activation at 100°C did not change their absorption properties. When necessary the plates were developed in redistilled ethanol several times, with drying between each development; this removed contaminating material which interfered with ultraviolet absorption and mass spectrometry of compounds eluted from the gel.

(b) Procedure: T.l.c. plates were developed in "Shandon", glass, chromatography tanks. The atmosphere was kept near saturation by placing a layer of absorbent paper around the inner surfaces of the chamber. The absence of this paper and the consequent loss of solvent vapour during insertion of the plates led to differential over-development and less reproducible and higher R<sub>f</sub> values for a given compound. Material to be chromatographed was applied to the plates in 0.1ml of redistilled ethanol. The walls of the tube were then washed down with a few drops of solvent. The sample was applied to the absorbent layer, by means of a fine Pasteur pipette, as a narrow band at a point 2cm from one end of the plate. The plate was then developed to a line cut through the absorbent layer 15cm from the origin.

(c) Location of radioactive components in thin layers; in early experiments the location of radioactive components in thin layers was determined solely by a Packard strip-scanner. Because of its low sensitivity (approximately 10% of the disintegrations

per minute were recorded) this method was limited to the detection of comparatively high levels of radioactivity. In all other experiments radioactivity in terms of counts per minute (c.p.m.) was obtained by scraping the appropriate regions of gel into 10ml of toluene/methanol based scintillation solution followed by counting with a Packard liquid scintillation spectrometer. Absorbed material could be eluted from the gel with a few millilitres (ml) of a suitable solvent. Redistilled ethanol, ethyl acetate and diethylether (ether) either alone or in mixtures were used for elution. The eluting solvent of each sample counted was evaporated in the counting vial prior to the addition of scintillation solution, this minimized quenching.

(d) Identification by co-chromatography. An important and simple procedure in the establishment of the identity of a compound is the demonstration that its mobility in different chromatographic systems is identical to that of an authentic specimen. A sample of 10-30 $\mu$ g of the authentic compound was applied to the absorbent as a narrow 1cm long band. An aliquot of the radioactive sample to be tested was applied to the absorbent as a spot in the centre of the band of authentic compound. The remainder of the radioactive compound was then applied to the absorbent as a narrow band adjacent to the position of the authentic marker. A second application of the authentic compound alone was then made to the absorbent at the other side of the radioactive test compound. The plate was then developed in an appropriate solvent system. The position of the authentic compound alone and with radioactive material present was located under ultraviolet light and marked. Material from the

radioactive sample area alone which co-chromatographed with the authentic compound was eluted from the gel and an aliquot counted. The gel was divided up into 2mm segments in the vicinity of the authentic compound, outside this area larger segments up to 20mm were taken. A histogram was constructed using the counts per minute per 2mm distance along the plate (c.p.m. per 10mm in some instances) and the position of the authentic compounds superimposed upon it.

Identification of one of the radioactive acids as abscisic acid was made by precise co-chromatography of the acid with authentic abscisic acid in propan-1-ol-butan-1-ol-aq. ammonia (sp.gr.0.88) - water (6:2:2:1, by vol) and with authentic abscisic acid in toluene-ethyl acetate-acetic acid (15:3:1, by vol). After methylation the material migrated with authentic methyl ester of abscisic acid when developed in hexane-ethyl acetate (1:1, v/v). Treatment of the material co-chromatographing with authentic methyl abscisate with excess sodium borohydride in aqueous methanol (1:4, v/v) produced two radioactive entities which migrated (when developed in ethyl acetate-hexane, (1:1, v/v)) with the authentic epimeric 1',4'-diol esters (produced by the like treatment of authentic methyl abscisate, Cornforth et al., 1967). Both radioactive entities were oxidized by manganese dioxide ( $MnO_2$ ), in dry chloroform, for 25-90 hours in darkness at room temperature (ca. 20°C). In both cases the product migrated with methyl abscisate when developed in hexane-ethyl acetate (1:1, v/v).

Co-chromatography was employed in the purification and identification of epoxide and its 1',2'-dihydroxy derivative and



also the epimeric 1',4'-diols of abscisic acid. The solvent systems used were the same as those used for abscisic acid unless stated otherwise.

(vi) Gas liquid chromatography

Gas liquid chromatography was carried out using a Varian aerograph (Series 1520) gas chromatograph. The output of the flame ionization detector was monitored by a Honeywell recorder. Materials to be chromatographed were first converted to their methyl esters (using an ethereal solution containing diazomethane). The stationary phase was 5% SE 30 on a support phase of Chromosorb W (60-80 mesh) held in a 1/8" i.d. x 5' stainless steel tube. The carrier gas was helium, the flame ionization detector was supplied with hydrogen 25ml/minute and air 300ml/minute. With the column temperature at 195°C Isothermal and a carrier gas flow rate of 15ml/minute the following retention times were recorded:

<u>Compound</u>	<u>Retention time (minutes)</u>
(1) methyl epoxide	8.25
(2) methyl 2- <u>trans</u> epoxide	9.75
(3) methyl ester of 2- <u>trans</u> $\beta$ -ionylidene acetic acid	1.75

With the column temperature at 225°C Isothermal and a carrier gas flow rate of 30ml/minute the retention time of methyl abscisate was 8.25 minutes.

Samples to be chromatographed were dissolved in a known volume of redistilled ethanol and a measured volume of this solution injected into the column using a 10 $\mu$ l Hamilton syringe. Baseline separation of methyl epoxide and of methyl 2-trans-

epoxide was achieved and the peaks were symmetrical. The methyl ester of abscisic acid gave a sharp peak with slight tailing.

[<sup>18</sup>O]epoxide and the [<sup>18</sup>O]abscisic acid formed from it were chromatographed separately under the appropriate conditions to determine the purity of the material prior to [<sup>18</sup>O] assay.

(vii) Assay of [<sup>18</sup>O] using a combined Gas Chromatograph-Mass Spectrometer

The ratio of [<sup>18</sup>O] to [<sup>16</sup>O] in epoxide and abscisic acid formed from it was determined by mass spectrometry of their methyl esters. This was carried out with a Varian Gas Chromatograph (Series 1200) coupled to an AEI MS9 (mass spectrometer) via a Llewellyn-type separator (Hawes, Mallaby and Williams, 1969). Separation of the methyl esters was achieved using a 1/8" i.d. x 3' glass column containing 5% SE 30 stationary phase on a support phase of Chromosorb W (60-80 mesh). The carrier gas was helium. Prior to injection of the methyl esters 40μl of N,O-bis(trimethylsilyl)-acetamide were injected into the column, this improved the separation of the esters by the column. With the column temperature at 180°C Isothermal and a carrier gas flow rate of 12ml/minute the following retention times were recorded:

<u>Compound</u>	<u>Retention time (minutes)</u>
(1) methyl epoxide	7.4
(2) methyl 2- <u>trans</u> epoxide	8.7
(3) methyl ester of 2- <u>trans</u> β-ionylidene acetic acid	2.4

A mass spectrum was recorded for each of the above [<sup>16</sup>O] compounds. A sample of the synthesised [<sup>18</sup>O]methyl epoxide was

injected into the chromatograph. An impurity, noticed in the earlier g.l.c. of this material had a retention time of 3.4 minutes and represented approximately 7% of the total sample. A mass spectrum was recorded for material having the same retention time on the gas chromatograph as authentic methyl epoxide. A mass spectrum was recorded for the [ $^{18}\text{O}$ ]abscisic acid, biosynthesised from the [ $^{18}\text{O}$ ]epoxide, following gas chromatography of the methylated abscisic acid extracted from the plant tissue (Experimental, section (ii) (j)). Full mass spectral data are given in Appendix 1.

(viii) Estimation of Radioactivity

(a) Liquid scintillation counting - samples containing [ $^{14}\text{C}$ ] were dissolved in a known volume of solvent, usually ethanol, and a known aliquot transferred to a counting vial. The solution was blown to near dryness in the vial, with nitrogen, and 10ml of scintillation solution containing 2,5-bis-[5'-tertiarybutylbenz-oxazolyl (2')]-thiophene (referred to as BBOT), 4g per litre, in redistilled methanol-toluene (1:1, v/v), added. Samples were measured in either a Packard Tri-Carb scintillation spectrometer (series 314E) which gave approximately 50% efficiency of counting, or a Packard Tri-Carb Model 3375 which gave approximately 78% efficiency of counting.

Simultaneous measurements of [ $^3\text{H}$ ] and [ $^{14}\text{C}$ ] were carried out in a BBOT (6g/litre), naphthalene (80g/litre) methoxyethanol-toluene (400:600, v/v) solution in a Packard Tri-Carb Model 3375. This gave 56.6% counting efficiency for [ $^{14}\text{C}$ ], and 0.09% for [ $^3\text{H}$ ] in one channel and 18% [ $^{14}\text{C}$ ], 24% [ $^3\text{H}$ ] in the other.

(b) Radioactive spots on thin layer chromatography plates were located either with a Packard radiochromatogram scanner (Model 7200, ratemeter Model 385) and Honeywell Elektronik 18 recorder or by covering the plates with Kodak "Kodirex" X-ray film. The autoradiograms were prepared with D11 developer and May and Baker "Amfix" fixative.

(ix) Ultraviolet absorption (u.v.) and optical rotatory dispersion (o.r.d.) measurement

(a) Ultraviolet absorption spectra were measured on one of the following spectrophotometers: Unicam SP 800, Cary 14 or Perkin-Elmer model 137 u.v. Samples were dissolved in redistilled ethanol, or redistilled ethanol containing  $0.005N \text{ H}_2\text{SO}_4$  when free acids were present, and spectra measured using a quartz silica cell (path length 10.00mm). In a highly purified state the strong and characteristic ultraviolet absorption spectra of abscisic acid, epoxide and its 1',2'-dihydroxy derivatives and the 1',4'-diols of abscisic acid methyl ester provided further proof of their identity (Table 2).

(b) Optical rotatory dispersion - the quantity of (+) or (-) abscisic acid in a purified extract was measured by its rotation of plane polarized light at 289nm. The highly characteristic optical rotatory dispersion curve exhibited by both (+) and (-) abscisic acid (the curves are identical for the methyl esters - B. V. Milborrow, personal communication) provided a qualitative and quantitative assessment of the abscisic acid present. Abscisic acid was dissolved in redistilled ethanol containing  $0.005N \text{ H}_2\text{SO}_4$  and the solution measured in a quartz silica cell having a path

Table 2. The ultraviolet absorption and optical rotatory dispersion parameters for abscisic acid and its derivatives, and the ultraviolet absorption parameters for epoxide and its derivatives used in the present work.

Compound	Ultraviolet absorption $\lambda_{\max}$ (nm) $\epsilon$		Optical rotatory dispersion curve parameters			
			1st $\lambda$ (nm)	Extremum $\alpha$ (degrees)	2nd $\lambda$ (nm)	Extremum $\alpha$ (degrees)
(+)-ABA <sup>x</sup>	260.5	21,400	289	+24,000	246	-69,000
(-)-ABA <sup>x</sup>	260.5	21,400	289	-24,000	246	+69,000
(±)-MeABA <sup>x</sup>	265.5	20,900				
(+)- <u>trans</u> -1',4'-diol MeABA <sup>x</sup>	267	22,200	287	+12,200	243	-17,560
(+)- <u>cis</u> -1',4'-diol MeABA <sup>x</sup>	265.5	20,900	286	+ 4,300	242	- 4,300
(±)- <u>trans</u> -1',4'-diol ABA <sup>+</sup>	268 (265 <sup>Δ</sup> )	-				
(±)- <u>cis</u> -1',4'-diol ABA <sup>+</sup>	268 (265 <sup>Δ</sup> )	-				
(±)-epoxide*	265	20,500				
(±)-2- <u>trans</u> -epoxide*	264	26,240				
(±)-methyl epoxide*	267	18,600				
(±)-methyl-2- <u>trans</u> epoxide*	267	25,670				
(±)-1',2'-dihydroxy derivative of epoxide*	269	18,560				
(±)-1',2'-dihydroxy derivative of 2- <u>trans</u> epoxide*	268	25,600				
(±)-1',2'-dihydroxy derivative of epoxide methyl ester*	272	19,400				
(±)-1',2'-dihydroxy derivative of 2- <u>trans</u> epoxide methyl ester*	271	25,400				

<sup>x</sup>Cornforth, Milborrow and Ryback (1967)

<sup>+</sup>Cornforth, Milborrow and Ryback (1965)

\*Tamura and Nagao (1969)

Δ Observed values in present work. (See Methods, section (x) c.)

λ = Wavelength; ε = molar extinction co-efficient; α = specific rotation.

length of 10.00mm. The spectropolarimeter used to measure the o.r.d. curves was a Bellingham-Stanley (Bendix) "Polarmatic 62" coupled to a Bryans x-y plotter (Model 20180/S). (Cornforth, Milborrow and Ryback, 1966). This produced a deflection of 1.0cm (289nm) for 1.0 $\mu$ g (+) or (-) abscisic acid/ml/1.0cm path length in acidic ethanol. Abscisic acid methyl ester and the 1',4'-diols derived from it were measured in ethanol as their o.r.d. curves are unaffected by pH. Amounts as small as 0.3 $\mu$ g optically active abscisic acid could be detected by this apparatus.

(c) The circular dichroism of solutions containing (+)-abscisic acid was occasionally measured providing further qualitative evidence as to the identity of the material, from a comparison of its curve with the curve obtained with authentic (+)-abscisic acid (Milborrow, 1967).

(x) Synthesis of labelled materials

(a) Synthesis of ( $\pm$ )-[1',2'-<sup>18</sup>O]epoxide (33.2 atoms % [<sup>18</sup>O])

Experiment commenced on 12th December, 1969.

Racemic [1',2'-<sup>18</sup>O]epoxide was synthesised from [<sup>18</sup>O] enriched oxygen and the methyl ester of  $\beta$ -ionylidene acetic acid by a method similar to that described by Tamura and Nagao (1969(a)).

A preliminary synthesis of [1',2'-<sup>16</sup>O]epoxide using [<sup>16</sup>O] oxygen and the methyl ester of 2-trans  $\beta$ -ionylidene acetic acid was carried out to ascertain the yield of epoxide by this method. 48.7mg of the methyl ester were dissolved in ethyl acetate (50ml) and an equal weight of Kieselguhr added. The ester was absorbed onto the Kieselguhr by evaporation of the solvent under vacuum. The mixture was then held, in an atmosphere of pure oxygen, in darkness, at room temperature (20-23°C) for 5 days.

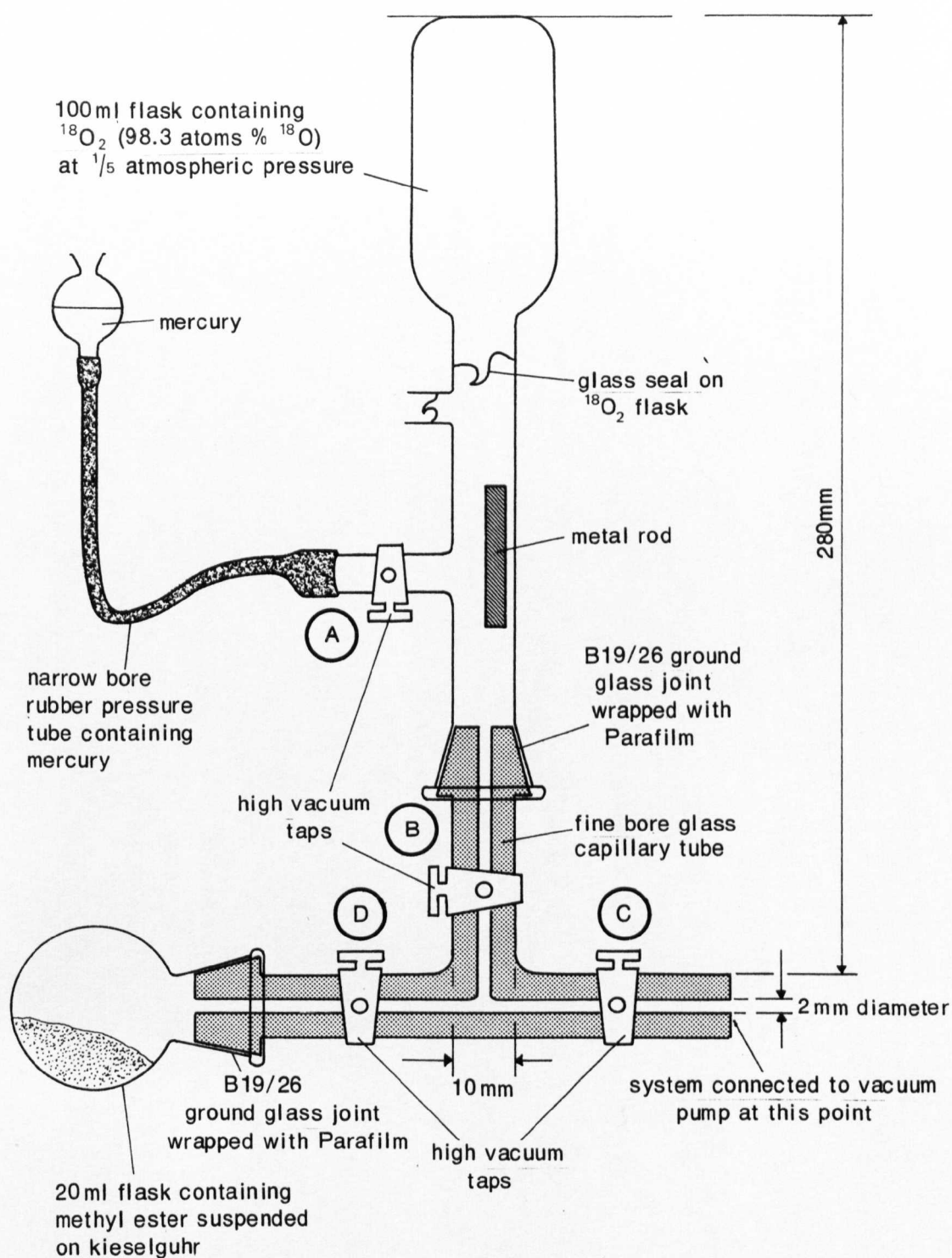
A subsample removed from the flask and chromatographed on a t.l.c. plate in a chloroform-benzene-acetic acid system (3:2:0.1, by vol) showed that most of the material chromatographed with marker methyl ester of 2-trans- $\beta$ -ionylidene acetic acid; only a very small amount chromatographed with marker methyl 2-trans epoxide and methyl epoxide as indicated by the intensity of quenching of the phosphor when the t.l.c. plate was viewed under u.v. light. The flask was left for a further 7 days in laboratory light. A sub-sample taken at that time and examined in the same way showed that 33.6% of the methyl 2-trans- $\beta$ -ionylidene acetic acid had been converted to methyl-2-trans epoxide and 2.8% converted to methyl epoxide, as measured by ultraviolet absorption of the eluted materials.

Saponification of methyl-2-trans epoxide was carried out in 10% KOH solution, ethanol/water (1:1, v/v). 68.0mg of methyl-2-trans epoxide were dissolved in 2.0ml of the KOH solution and stirred for 66 hours at room temperature (20-23°C) in darkness. The pH of the solution was adjusted to pH 2.5-3.0 with 4N H<sub>2</sub>SO<sub>4</sub> and extracted several times with diethyl ether. The ether extract was chromatographed in benzene-ethyl acetate-acetic acid (75:10:1, by vol). Material chromatographing with marker epoxide and with marker 2-trans epoxide were eluted separately from the gel. The ultraviolet absorption spectra of these eluates indicated that 0.50mg epoxide and 49.0mg of 2-trans epoxide had been recovered as free acid. These represented 0.7% and 72% yields respectively.

Synthesis of ( $\pm$ )-[1',2'-<sup>18</sup>O]epoxide. 200mg of the methyl ester of 2-trans- $\beta$ -ionylidene acetic acid were dissolved in ethyl acetate and 200mg of Kieselguhr (acid/alkali washed) added. After

removal of the solvent under vacuum the 20ml flask containing the mixture was evacuated using a vacuum diffusion pump. The evacuated flask (at  $10^{-2}$  mm Hg) was connected to a 100ml vial containing  $^{18}\text{O}_2$  (98.3 atoms % [ $^{18}\text{O}$ ]) at  $\frac{1}{5}$  atmospheric pressure (Fig. 1). Tap (C) was closed and the  $^{18}\text{O}_2$  was slowly introduced into the evacuated flask; when the pressure in the two flasks was equal the  $^{18}\text{O}_2$  flask was filled with mercury through a side arm (tap (A) was opened) thereby raising the partial pressure of  $^{18}\text{O}_2$  in the 20ml reaction flask to atmospheric pressure. Tap (D) was closed and the joint between the reaction flask and the rest of the apparatus was wrapped with "Parafilm", the reaction flask was left in normal light for 8 days and shaken at intervals. The product was eluted with ethyl acetate and ethanol over a grade 4 glass sinter funnel. Evaporation of the solvent gave an oily residue which was hydrolysed in a 10% KOH solution, ethanol/water (1:1, v/v), and stirred for 24 hours at room temperature (20-23°C) in darkness. Ethanol was removed from the saponification mixture by vacuum distillation and the pH of the remaining aqueous solution adjusted to pH 2.5-3.0 with  $4\text{N H}_2\text{SO}_4$ . The acidic solution was extracted several times with diethyl ether; the ether extract was applied to an "ethanol washed" t.l.c. plate and developed in chloroform-benzene-acetic acid (150:100:0.5, by vol). Material chromatographing with authentic epoxide was eluted and weighed 49.1mg. This material was developed on a second thin layer plate, in toluene-ethyl acetate-acetic acid (75:10:1, by vol) and material chromatographing with marker epoxide was eluted and chromatographed, on another plate, in the chloroform-benzene-acetic acid (150:100:0.5, by vol) system. Material chromatographing with marker epoxide was eluted





**Fig 1. Apparatus used in the preparation of (+)-[1,2'- $^{18}\text{O}$ ]epoxide from the methyl ester of  $\beta$ -ionylidene acetic acid and  $^{18}\text{O}_2$  (98.3 atoms %  $^{18}\text{O}$ ). See text for procedure.**

and applied to a 4th t.l.c. plate which was developed in toluene-ethyl acetate-acetic acid (75:20:0.5, by vol). Material chromatographing with marker epoxide was eluted with ethanol-ethyl acetate (1:1, v/v). Gas chromatography of an aliquot of this material, which had been methylated with ethereal diazomethane, showed that 10.0mg of epoxide had been obtained. The  $^{18}\text{O}/^{16}\text{O}$  ratio of the epoxide was determined by mass spectrometry of the methyl ester on a combined gas chromatograph - mass spectrometer. The fragmentation pattern was identical with that of authentic epoxide methyl ester (Appendix 1) except that some peaks, which contained the  $[1',2'-^{18}\text{O}]$ , were two mass units heavier. The  $[1',2'-^{18}\text{O}]$ epoxide contained 33.2 ( $\pm 1.2\%$  S.E.) atoms %  $^{18}\text{O}$  as measured by the ratio of the parent ions  $\frac{m}{e} \frac{266}{264 + 266}$ .

Relative intensity of the parent ions from the mass spectra  
of authentic  $^{16}\text{O}$  and experimentally produced  
 $^{18}\text{O}$ epoxide, methyl esters

	$\frac{m}{e}$	$[1',2'-^{16}\text{O}]$ epoxide methyl ester, Relative intensity	$[1',2'-^{18}\text{O}]$ epoxide methyl ester, Relative intensity
Parent (P)	264	100	100
P + 1	265	17.8	22.1
P + 2	266	0.0	51.9
P + 3	267	0.0	11.7

(b) Synthesis of the  $(\pm)$ -[2- $^{14}\text{C}$ ] 1',2'-dihydroxy derivatives of epoxide (1.3mCi/mmole) and of 2-trans epoxide.

Experiment commenced on 19th February, 1970.

Unreacted  $(\pm)$ -[2- $^{14}\text{C}$ ]epoxide and 2-trans epoxide (1.3mCi/mmole) were recovered from the experiments in which epoxide and 2-trans epoxide had been incubated with living and with boiled tomato fruit (Experimental, section (ii), (b)-(e)), as their methyl esters. The  $(\pm)$ -[2- $^{14}\text{C}$ ] 1',2'-dihydroxy derivative of epoxide and of 2-trans epoxide formed during certain of these experiments were also recovered as their methyl esters. The total weight of the recovered material was 3.9mg. This mixture was saponified by dissolving it in 20ml of a 10% KOH solution, ethanol-water (1:1, v/v) and stirring for 21 hours at 20°C in light. Ethanol was removed from the reaction mixture by vacuum distillation and the epoxide and 2-trans epoxide were hydrolysed to their respective 1',2'-dihydroxy derivatives when the solution was acidified to pH 2.5 with 4N  $\text{H}_2\text{SO}_4$ . The acidified solution was stirred at 20°C in darkness for 3 hours and then extracted with diethyl ether, Tamura and Nagao (1969(a)). The extract was evaporated under nitrogen and the residue applied to a precoated t.l.c. plate. The following marker compounds were applied, as separate spots, to the plate adjacent to the ether extract;

- (1) methyl ester of 1',2'-dihydroxy derivatives of epoxide and 2-trans epoxide
- (2) methyl esters of epoxide and 2-trans epoxide
- (3) epoxide and 2-trans epoxide
- (4) 1',2'-dihydroxy derivative of epoxide and of 2-trans epoxide.

Multiple development of the t.l.c. plate in toluene-ethyl acetate-acetic acid (75:10:1, by vol) separated the 1',2'-dihydroxy derivatives of epoxide and 2-trans epoxide. Sample material chromatographing with marker 1',2'-dihydroxy derivative of epoxide and with marker 1',2'-dihydroxy derivative of 2-trans epoxide were eluted with methanol (Table 3) and their u.v. absorption spectra were identical with the u.v. absorption spectra of the respective authentic materials. The samples were assayed for [ $^{14}\text{C}$ ] (Table 4).

(c) Synthesis of ( $\pm$ )-[2- $^{14}\text{C}$ ]cis-1',4'-diol and ( $\pm$ )-[2- $^{14}\text{C}$ ]trans-1',4'-diol of abscisic acid by reduction of ( $\pm$ )-[2- $^{14}\text{C}$ ] abscisic acid (21.6 $\mu\text{Ci/mmole}$ ) in aqueous methanolic sodium borohydride solution

Experiment commenced on 27th April, 1970.

Synthesis of the cis-1',4'-diol and trans-1',4'-diol of MeABA by reduction of MeABA in aqueous methanolic sodium borohydride solution was reported by Cornforth, Draber, Milborrow and Ryback (1967) and Noddle and Robinson (1969).

A preliminary experiment, using unlabelled ( $\pm$ )-abscisic acid, was performed to determine the % yield of each diol. The 1',4'-diols were prepared in two ways:

Method 1. Reduction of abscisic acid by sodium borohydride in aqueous methanol solution (1:4, v/v).

Method 2. Saponification of cis-1',4'-diol of MeABA in 10% KOH solution, ethanol-water (1:1, v/v).

The former method was subsequently used to prepare the [ $^{14}\text{C}$ ]labelled samples which were supplied to the wheat shoots. The latter method was used to obtain the cis-1',4'-diol acid from authentic cis-1',4'-diol methyl ester for use as a chromatographic marker.

Table 3. Relative  $R_f$ s of epoxide and its derivatives after t.l.c. on a Merck precoated  $F_{254}$  plate developed in toluene-ethylacetate-acetic acid (75:10:1, by vol.)

Compound	$R_f$
1',2'-dihydroxy derivative of epoxide	0.22
1',2'-dihydroxy derivative of 2- <u>trans</u> epoxide	0.30
epoxide	0.53
2- <u>trans</u> epoxide	0.67
Methyl esters of all four previous compounds	0.59

Table 4. Synthesis of  $(\pm)$ -[2- $^{14}\text{C}$ ]1',2'-dihydroxy derivatives of epoxide and 2-trans epoxide by hydrolysis of  $(\pm)$ -[2- $^{14}\text{C}$ ]epoxide and 2-trans epoxide (1.3mCi/mmole) with dilute sulphuric acid (pH 2.5) for 3 hours at room temperature (20°C). The ether extract of this solution was chromatographed in toluene-ethyl acetate-acetic acid (75:10:1, by vol.) and material co-chromatographing with marker 1',2'-dihydroxy derivative of epoxide and with marker 1',2'-dihydroxy derivative of 2-trans epoxide were eluted and assayed for [ $^{14}\text{C}$ ]. The efficiency of counting was 78.7% and the background was 20.2 c.p.m.

Source of material	Chromatographic solvent used	c.p.m. in subsample	d.p.m. in total sample
Zone on t.l.c. plate adjacent to marker 1',2'-dihydroxy derivative of epoxide	toluene-ethyl acetate-acetic acid, (75:10:1, by vol.)	1746	199,660
Zone on t.l.c. plate adjacent to marker 1',2'-dihydroxy derivative of 2-trans epoxide		9247	1,119,000

Method 1. 3.32mg ( $\pm$ )-ABA were dissolved in 0.5ml methanol and 0.15ml water added. 20.0mg solid sodium borohydride were added to the solution and the mixture kept at 0°C for 60 minutes with occasional shaking. The solution was adjusted to pH 3.0 with 4N  $\text{H}_2\text{SO}_4$  and thoroughly extracted with diethyl ether. The ether solution was blown down to 5ml and stored at -10°C.

Method 2. A sample of authentic cis-1',4'-diol of methyl abscisate (approximately 1mg) was dissolved in 20ml of 10% KOH solution, ethanol-water (1:1, v/v) and stirred for 24 hours at 20°C in darkness. Ethanol was removed by vacuum distillation and the solution was adjusted to pH 3.0 with 4N  $\text{H}_2\text{SO}_4$  and extracted thoroughly with diethyl ether. The ether solution was blown down to approximately 5ml and stored at -10°C.

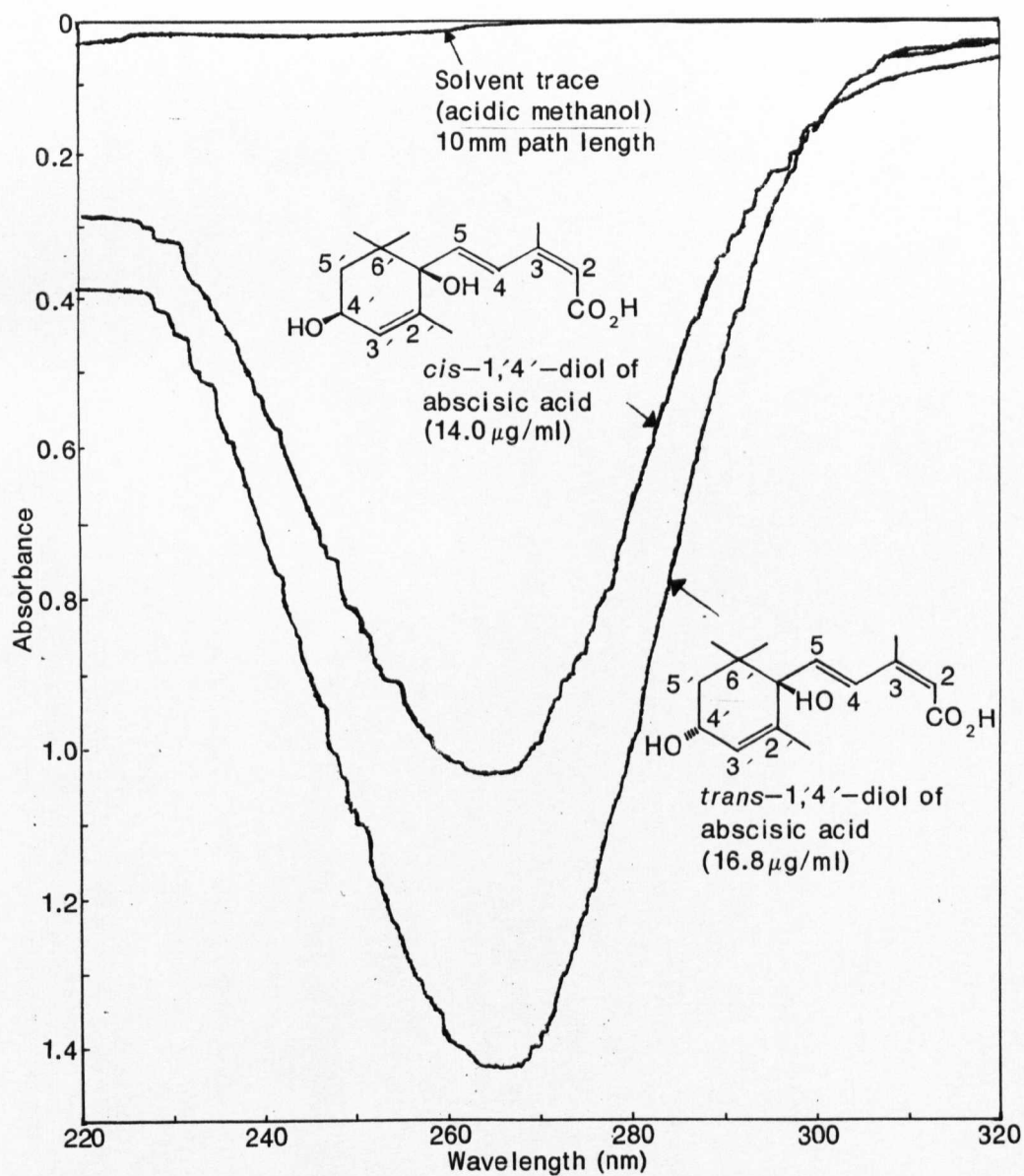
An aliquot of the ether solution from Methods 1 and 2 was applied at adjacent positions, to a t.l.c. plate which was subjected to multiple development in a toluene-ethyl acetate-acetic acid solvent system (75:40:2, by vol). The plate was viewed under u.v. light and the positions of the authentic compounds marked, the  $R_f$ 's of the major bands chromatographing with authentic material are given in Table 5.

Material which chromatographed at  $R_f$  0.17 and  $R_f$  0.36 (produced according to Method 1) was eluted from the gel with methanol and the solvent evaporated under nitrogen. In each case the sample was dissolved in 1.0ml methanol, a 0.03ml aliquot was removed and diluted with 2.5ml of methanol containing 0.005N  $\text{H}_2\text{SO}_4$  and the u.v. spectrum recorded. For both materials  $\lambda_{\text{max}}$  was found to be 265nm (Fig. 2).

Table 5.  $R_f$ s of materials (i) produced by treatment of ( $\pm$ )-ABA with aqueous methanolic  $\text{NaBH}_4$  solution, (ii) produced by hydrolysis of cis-1',4'-diol of ( $\pm$ )-MeABA in 10% KOH solution, ethanol-water (1:1, v/v); after t.l.c. in a toluene-ethyl acetate-acetic acid (75:40:2, by vol) solvent system. The  $R_f$  of authentic ( $\pm$ )-ABA is also given.

Compound	$R_f$ s		
	(i)	(ii)	Authentic ( $\pm$ )-ABA marker
<u>cis</u> -1',4'-diol of ABA	0.17	0.17	0.49
<u>trans</u> -1',4'-diol of ABA	0.36		
( $\pm$ )-ABA	0.48		





**Fig 2** Ultraviolet absorption spectra of *cis*-1,4'- and *trans*-1,4'-diols of abscisic acid, in ethanol containing 0.005 *N* H<sub>2</sub>SO<sub>4</sub>. The diols were produced by reduction of ABA with aqueous methanolic sodium borohydride solution at 0°C.

Material having an  $R_f$  of 0.17 (Table 5) was identified as the cis-1',4'-diol of abscisic acid by co-chromatography of the methyl derivative with authentic cis-1',4'-diol of MeABA in a hexane-ethyl acetate (1:1, v/v) solvent system. No other products were formed on methylation. Material having an  $R_f$  of 0.36 (Table 5) was identified as the trans-1',4'-diol of abscisic acid by co-chromatography of its methyl derivative with authentic trans-1',4'-diol of MeABA in the hexane-ethyl acetate (1:1, v/v) solvent system.

In the absence of any published values for  $\lambda_{\max}$  and  $\epsilon$  for the cis-1',4'-diol and for the trans-1',4'-diol of abscisic acid the observed  $\lambda_{\max}$ , 265nm in acidic methanol (Fig. 2) was used in the present work. The  $\epsilon$  value of the 1',4'-diol acids was taken as 22,000; this being close to the  $\epsilon$  value of the methyl ester of the trans-1',4'-diol of abscisic acid (Cornforth, Draber, Milborrow and Ryback, 1967) and there being no published data for the free acids.

Calculations based on the spectra given in Fig. 2 indicated that 1177 $\mu$ g cis-1',4'-diol abscisic acid and 1420 $\mu$ g trans-1',4'-diol abscisic acid had been formed, yields of 35.5% and 42.8% respectively.

(d) Synthesis of ( $\pm$ )-[2- $^{14}$ C]cis-1',4'-diol and ( $\pm$ )-[2- $^{14}$ C]trans-1',4'-diol of abscisic acid (21.6 $\mu$ Ci/mmole; 181.7dpm/ $\mu$ g)  
( $\pm$ )-[2- $^{14}$ C]abscisic acid (1.6mCi/mmole) was synthesised in Milstead Laboratory (Cornforth, Mallaby and Ryback, 1968). The experiment for which the two diol acids were intended did not require that they should have a specific activity as high as 1.6mCi/mmole.

49.8  $\mu\text{g}$  ( $\pm$ )-[2- $^{14}\text{C}$ ]ABA (1.6mCi/mmole;  $6.7 \times 10^5$  dpm) were added to 3640  $\mu\text{g}$  unlabelled ( $\pm$ )-ABA. The diluted ( $\pm$ )-[2- $^{14}\text{C}$ ]ABA (21.6  $\mu\text{Ci}$ /mmole, 181.6dpm/ $\mu\text{g}$ ) was treated with an aqueous methanolic sodium borohydride solution and the ( $\pm$ )-[2- $^{14}\text{C}$ ]cis-1',4'-diol and ( $\pm$ )-[2- $^{14}\text{C}$ ]trans-1',4'-diol of abscisic acid were isolated by the methods described earlier. The products of sodium borohydride treatment were chromatographed in a toluene-ethyl acetate-acetic acid (75:15:1, by vol) solvent system and material co-chromatographing with authentic cis-1',4'-diol and authentic trans-1',4'-diol of abscisic acid were eluted separately with methanol. The relative  $R_f$ 's are given in Table 6.

Table 6.  $R_f$ 's of the products of  $\text{NaBH}_4$  treatment of ( $\pm$ )-[2- $^{14}\text{C}$ ]ABA co-chromatographing with authentic cis-1',4'-diol and trans-1',4'-diol of ABA in toluene-ethyl acetate-acetic acid (75:15:1, by vol). The  $R_f$  of ABA is also given.

Compound	$R_f$
<u>cis</u> -1',4'-diol of ABA	0.13
<u>trans</u> -1',4'-diol of ABA	0.29
ABA	0.37

Each ( $\pm$ )-[2- $^{14}\text{C}$ ] 1',4'-diol was then chromatographed on a separate t.l.c. plate in a toluene-ethyl acetate-acetic acid (75:25:1.5, by vol) solvent system. The only material visible on the plates when viewed under u.v. light had chromatographed at the

same  $R_f$  as the respective authentic 1',4'-diol (Table 7). These labelled materials were eluted with methanol.

Table 7. Chromatography of (a)  $(\pm)$ -[2- $^{14}$ C]cis-1',4'-diol of ABA and (b)  $(\pm)$ -[2- $^{14}$ C]trans-1',4'-diol of ABA in toluene-ethyl acetate-acetic acid (75:25:1.5, by vol). The  $R_f$  of material co-chromatographing with the respective authentic diol. In each case the alternate diol isomer and the  $R_f$  of ABA is given.

Compound	(a)			(b)		
	<u>cis</u> -1',4'-diol	<u>trans</u> -1',4'-diol	ABA	<u>cis</u> -1',4'-diol	<u>trans</u> -1',4'-diol	ABA
$R_f$	0.13	0.32	0.42	0.13	0.29	0.39

To ensure that the  $(\pm)$ -[2- $^{14}$ C]trans-1',4'-diol of ABA was not contaminated with  $(\pm)$ -[2- $^{14}$ C]ABA, 335 $\mu$ g  $(\pm)$ -ABA were added to the  $(\pm)$ -[2- $^{14}$ C]trans-1',4'-diol of ABA (21.6 $\mu$ Ci/mmol) and the mixture chromatographed in a toluene-ethyl acetate-acetic acid (75:40:2, by vol) solvent system. After multiple development of the plate separation of the two compounds was obtained and material co-chromatographing with authentic trans-1',4'-diol of ABA was eluted with methanol (Table 8).

Table 8. Separation of a mixture containing  $(\pm)$ -[2- $^{14}$ C]trans-1',4'-diol of ABA and  $(\pm)$ -ABA by chromatography in toluene-ethyl acetate-acetic acid (75:40:2, by vol).  $R_f$  of material co-chromatographing with authentic trans-1',4'-diol of  $(\pm)$ -ABA and with  $(\pm)$ -ABA.

Compound	$R_f$
<u>trans</u> -1',4'-diol ABA	0.33
ABA	0.40

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## CHAPTER IV

### EXPERIMENTAL

#### (i) Incorporation of [ $^{14}\text{C}$ ] and [ $^3\text{H}$ ]mevalonic acid into ABA by intact tissue

Tomato fruits were chosen for the initial investigation into ABA biosynthesis because they had been shown to contain (+)-ABA and were available throughout the year. The carbon skeleton of ABA suggested that mevalonic acid (MVA) could be a precursor. Furthermore, tomato fruit had been shown to biosynthesise carotenoids from [ $2\text{-}^{14}\text{C}$ ]mevalonic acid (Purcell, Thompson and Bonner, 1959) injected into the fruit and one possible biosynthetic pathway to (+)-ABA involves the splitting and further metabolism of a carotenoid. Mevalonic acid was injected into tomato fruit, in a solution of either 100% ethanol or as the potassium salt in 0.05M potassium phosphate buffer, pH 7.3 containing 10% ethanol, using a hypodermic syringe.

Avocado fruit were used as they had been shown to contain large amounts of (+)-ABA (Milborrow, 1967) and it had been reported that the concentration of (+)-ABA can increase during the maturation of fruit (Rudnicki, Machnik and Pieniazek, 1968). It has been reported (Wright and Hiron, 1969) that the concentration of (+)-ABA in freshly harvested wheat leaves increased 40-fold during 4 hours of wilting. To investigate whether this increase were due to de novo synthesis from MVA or to release from a conjugate a solution of [ $2\text{-}^3\text{H}$ ]MVA in phosphate buffer was supplied to cut wheat shoots. The wheat shoots were allowed to take up the [ $2\text{-}^3\text{H}$ ]MVA solution

for 10-15 hours. The shoots were then divided into two batches, one was wilted, the other kept turgid. Both batches were harvested after 4 hours. If de novo synthesis of (+)-ABA occurred in the wilted wheat shoots then a greater incorporation of  $[2-^3\text{H}]\text{MVA}$  into (+)-ABA would be expected than in the wheat shoots which had been kept turgid.

(a) Incorporation of  $(\pm)$ - $[2-^{14}\text{C}]$ mevalonic acid into abscisic acid in tomato fruit incubated in darkness and in light at  $20^\circ\text{C}$ .

Experiment commenced on 6th October, 1968.

Ten green tomato fruits (each weighing about 30g) were injected twice with  $25\mu\text{l}$  of a solution of the potassium salt of  $[2-^{14}\text{C}]$ mevalonic acid ( $4.82\text{mCi}/\text{mmole}$ ;  $7.41\mu\text{mole}$ ) in  $0.05\text{M}$  potassium phosphate buffer, pH 7.3, which contained 10% ethanol (v/v). The tomatoes were incubated for 29 hours in darkness then in laboratory light for a further 29 hours. During this time they began to turn yellow as they ripened. The tomatoes were macerated and thoroughly extracted with methanol. No racemic ABA was added to the extraction medium in this experiment. Identification of one of the radioactive acids as abscisic acid was made by the methods described in Methods (sections (iv), (v), (viii) and (ix)). The radioactivity which co-chromatographed with authentic abscisic acid and with the authentic derivatives of abscisic acid in several solvent systems is shown in Table 9.

$7.41\mu\text{moles}$  of  $[2-^{14}\text{C}]$ mevalonic acid ( $7.93 \times 10^7$  d.p.m.) incubated with 300g of tomato fruit gave  $0.113\text{nmole}$  (3639 dpm) of abscisic acid. (These values are calculated from measurement of radioactivity assuming that  $1\text{mole}$  of abscisic acid is formed from

3moles of mevalonic acid.) The weight of abscisic acid formed from labelled mevalonic acid represented 0.53% of the total abscisic acid extracted, (determined by spectropolarimetry).

(b) Incorporation of ( $\pm$ )-[2-<sup>14</sup>C]mevalonic acid into abscisic acid in avocado pear kept at 20°C in light

Experiment commenced on 26th November, 1968.

A ripe avocado fruit (177g without seed) was split lengthways and the seed removed. A solution of the potassium salt of [2-<sup>14</sup>C]mevalonic acid (a total of 4.0ml containing 2.02 $\mu$ mole, 4.82mCi/mmole) in 0.05M potassium phosphate buffer, pH 7.3, containing 90% ethanol (v/v) was placed in the recess of both halves. The first application (0.5ml) to each half, was absorbed into the fruit during the first five hours of incubation in a glass vessel through which CO<sub>2</sub>-free air was drawn. The air was bubbled through a 0.05M solution of KOH. After 5 hours each half received a further 0.5ml of labelled solution and the remainder was applied after a further 15 hours. The experiment, which was carried out at room temperature (20°C) under laboratory light was terminated 5 days after the initial application of mevalonic acid solution. The fruit was macerated in 1 litre of methanol containing 389 $\mu$ g of racemic abscisic acid and thoroughly extracted with a further 1.5 litres of a methanol:water mixture (4:1, v/v).

Some of the radioactivity in the ether-soluble acid extract chromatographed with authentic ABA in a propanol/butanol/ammonia/water solvent system. A small sub-sample (1/20) of the acid extract (Methods, section (v)) was applied to the t.l.c. plate



Table 9 Incorporation of  $(\pm)$ -[2- $^{14}$ C]mevalonic acid ( $4.82\text{mCi}/\text{mmole}$ ,  $7.93 \times 10^7$  d.p.m.) into ABA by 10 ripening tomato fruits, (total weight 300g), incubated at  $20^\circ\text{C}$  for 58 hours. Radioactivity chromatographing with marker ABA and its authentic derivatives. The efficiency of counting was 46.8% and the background was 48.0 c.p.m.

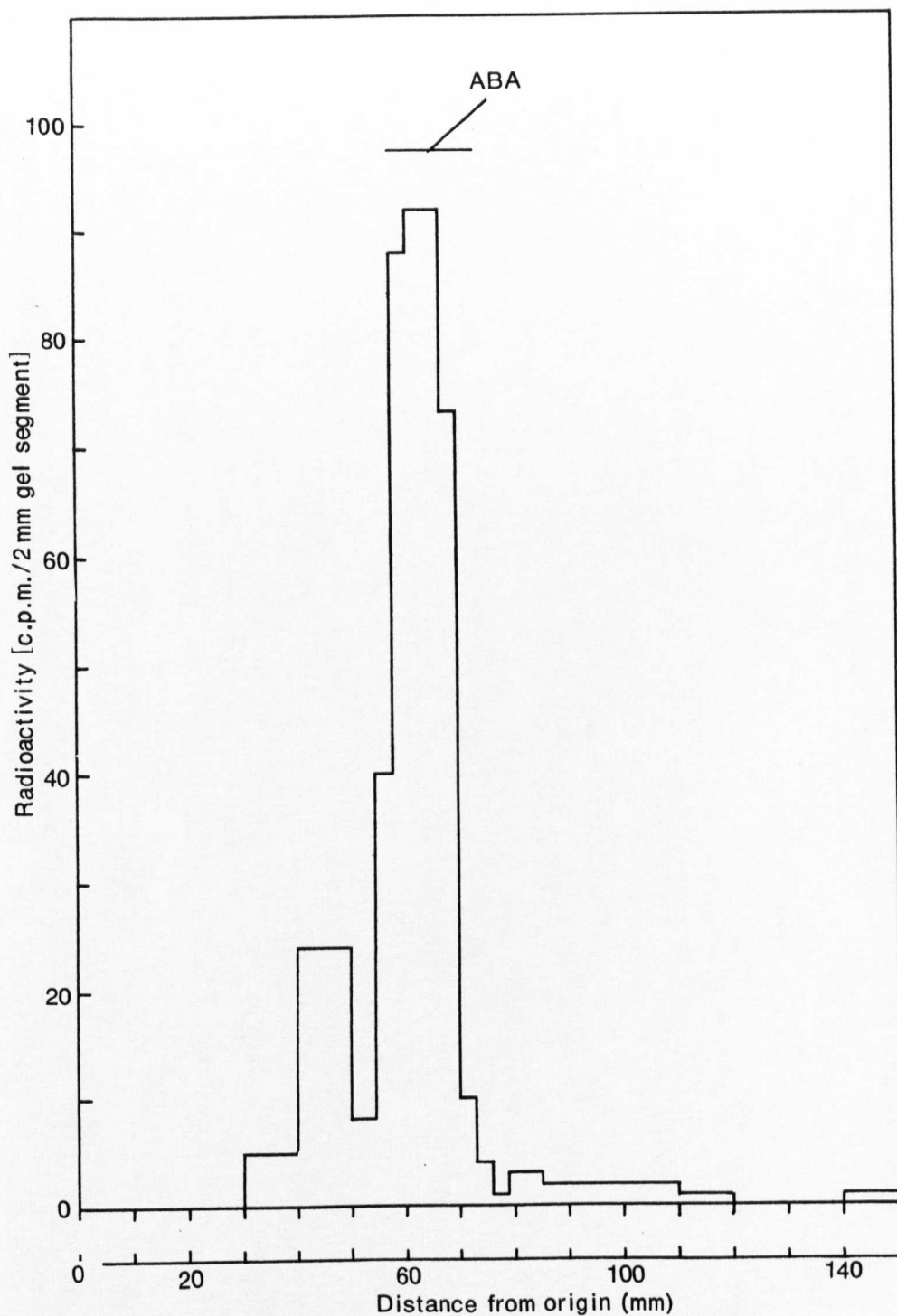
Source of material	Chromatographic solvents used	c.p.m. in subsample	d.p.m. in total sample	Weight of ABA by o.r.d., $\mu\text{g}$	Specific activity of ABA d.p.m./ $\mu\text{g}$
Acid fraction-zone on t.l.c. plate adjacent to marker ABA	Propan-1-ol-butan-ol-aq. ammonia (sp.gr. 0.88) water (6:2:1:2, v/v)	3329	70,530		
Sample from 1st t.l.c. adjacent to marker ABA	toluene-ethyl acetate-acetic acid (75:15:3, v/v)	614.2	26,248		
Sample from 2nd t.l.c. adjacent to marker ABA	Propan-1-ol-butan-1-ol-aq. ammonia (sp.gr. 0.88) water (6:2:1:2, v/v)	521.4	22,282		
Methylated sample from 3rd t.l.c. adjacent to marker MeABA.	hexane-ethyl acetate (1:1, v/v)	85.1	3,637	5.61	648.3
Sample from 4th t.l.c. treated with aq. methanolic $\text{NaBH}_4$ solution. Zone on t.l.c. plate adjacent to:- a) <u>cis</u> -1',4'-diol MeABA b) <u>trans</u> -1',4'-diol MeABA	hexane-ethyl acetate (1:1, v/v)	26.1 35.3	557.7 754.3		

gel as a small spot (approximately 1.0mm diameter) and authentic material was superimposed on it as a narrow 10mm band. This mixed marker was applied adjacent to the main sample band but separated from it by a 10mm space. After development of the chromatogram the authentic material was detected by its quenching of the u.v. stimulated fluorescence of the phosphor in the gel. The gel was divided into segments which were scraped from the glass back plate into vials containing 10ml of scintillation solution and counted directly. A histogram of c.p.m./2mm along the plate was drawn and the position of the authentic material indicated by a horizontal line above the histogram. Figure 4 shows the distribution of radioactivity, from the crude extract, along the t.l.c. plate and the position of authentic ABA. Material co-chromatographing with authentic ABA on the first plate was applied to a second t.l.c. plate, 1/20 was applied together with authentic ABA as a separate band adjacent to the main sample band and the plate was developed in an acidic system. Figure 5 shows the distribution of radioactivity along the plate and the position of authentic ABA is denoted by a horizontal bar. Sample material co-chromatographing with authentic ABA was eluted with ethanol. Treatment of the material co-chromatographing with authentic ABA with a solution of diazomethane ( $\text{CH}_2\text{N}_2$ ) in ether produced radioactive material which co-chromatographed with authentic MeABA. The material was eluted from the gel with ethanol and the u.v. and o.r.d. spectra of the eluate recorded. In the present series of experiments (4(i) a, b, c and d) the amount of ABA extracted from the fruit and its specific activity

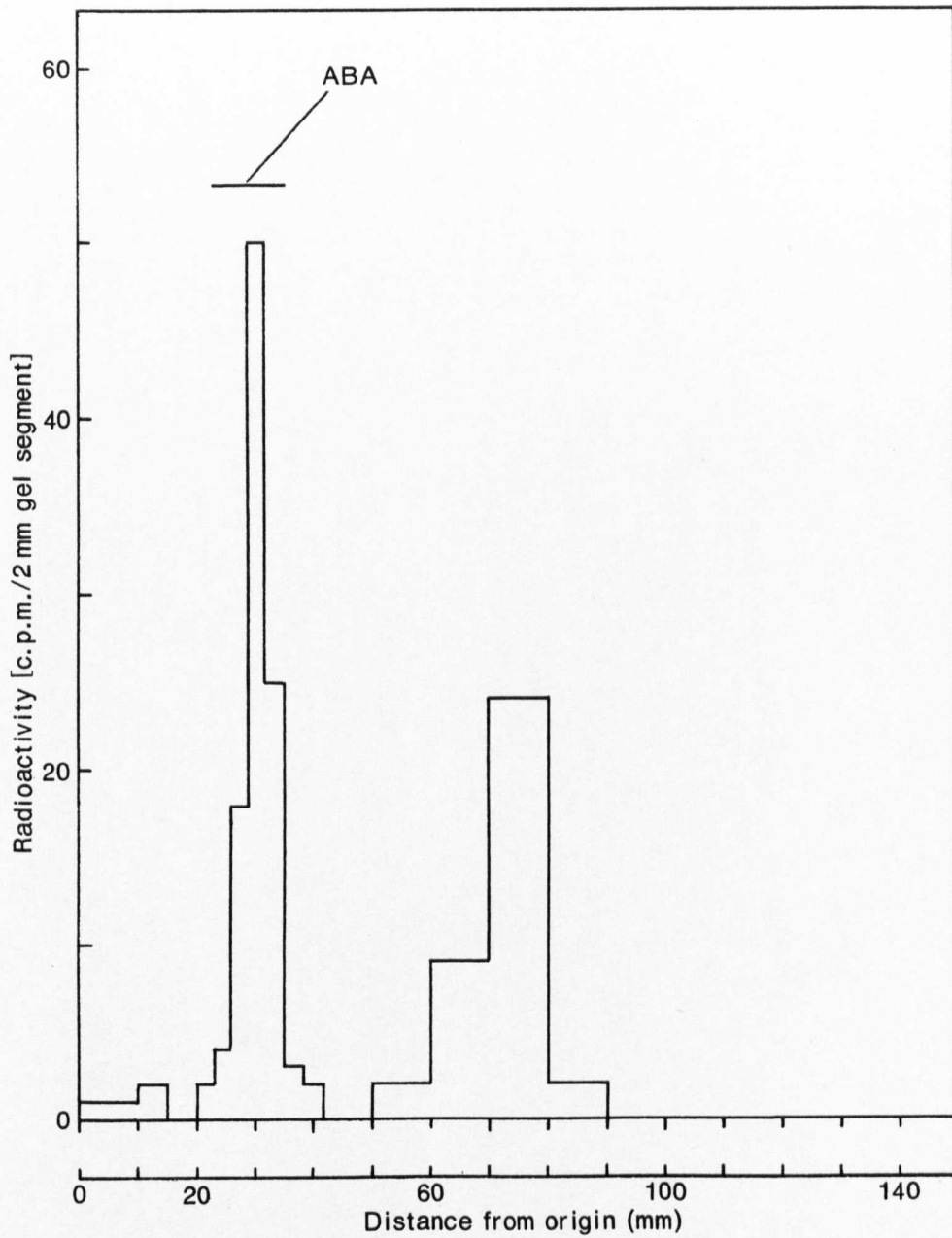
were calculated from the u.v. and o.r.d. spectra and radioactivity data obtained for the methylated abscisic acid. The distribution of radioactivity along the plate is shown in Figure 6, the position of authentic ABA and of authentic MeABA are denoted by a horizontal bar.

Treatment of the material co-chromatographing with authentic MeABA with an excess of sodium borohydride in aqueous methanol (1:4, v/v) produced almost equal amounts of two radioactive materials which co-chromatographed with the authentic cis-1',4'-diol and trans-1',4'-diol of MeABA. The distribution of radioactivity along the plate is shown in Figure 7, the position of authentic materials are shown as horizontal bars above the histogram. The counts per minute in the eluted material co-chromatographing with authentic ABA and with authentic derivatives of ABA are shown in Table 10.

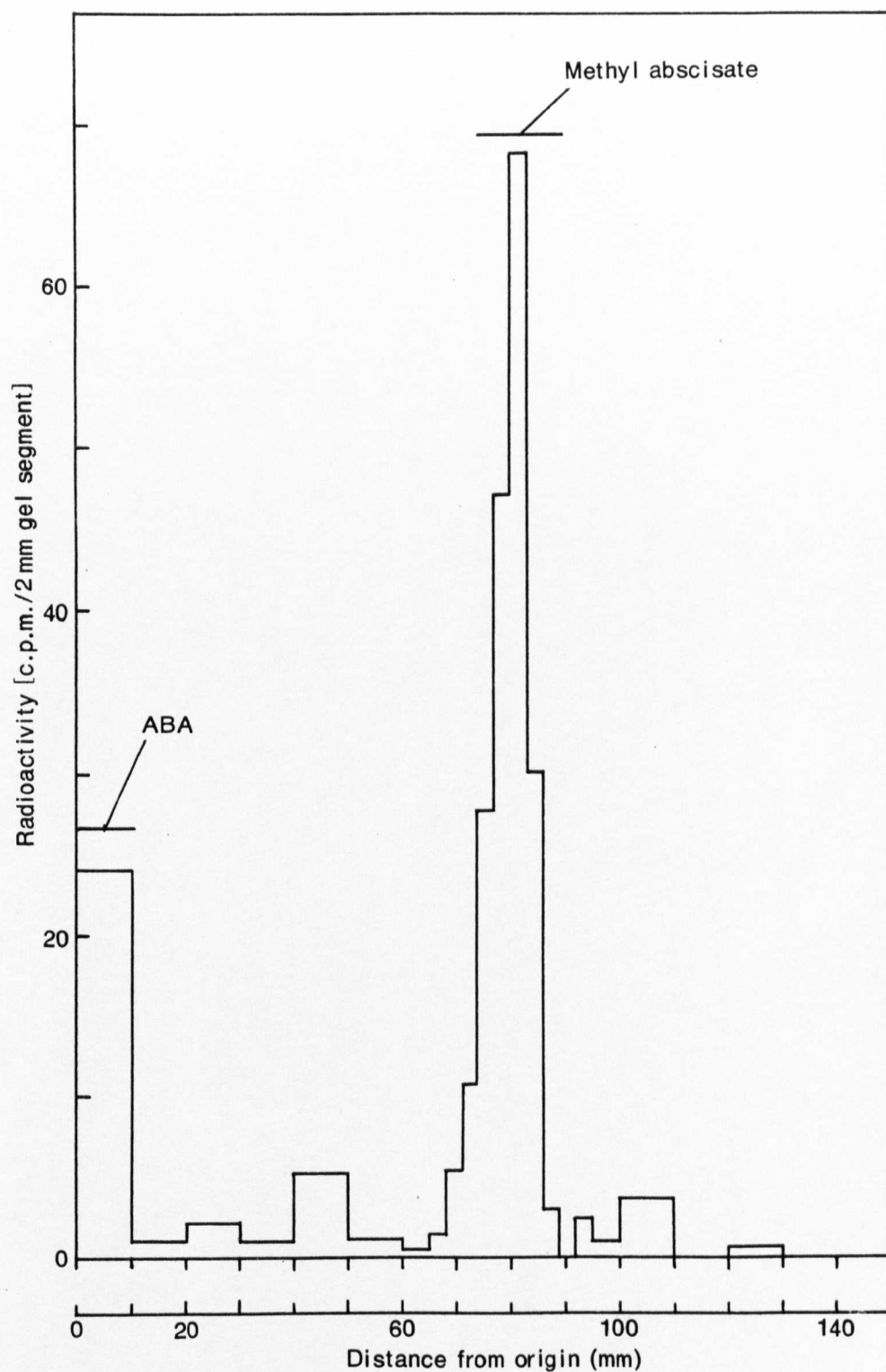
The racemate dilution method. The racemate dilution method for the measurement of the amount of free (+)-ABA present in plant tissue has been described by Milborrow (1969). A known amount of racemate is added to the methanol in which the tissue is macerated. The ABA is purified so that the profile of the u.v. absorption spectrum is identical with that of authentic material. The optical density can then be used to measure the ABA in a sample, this is most conveniently done at the absorption maximum at 260.5nm for ABA and 265.5nm for MeABA. The amount of optically active ABA in excess of the racemate, in the same solution, is then calculated from the optical rotatory dispersion curve.



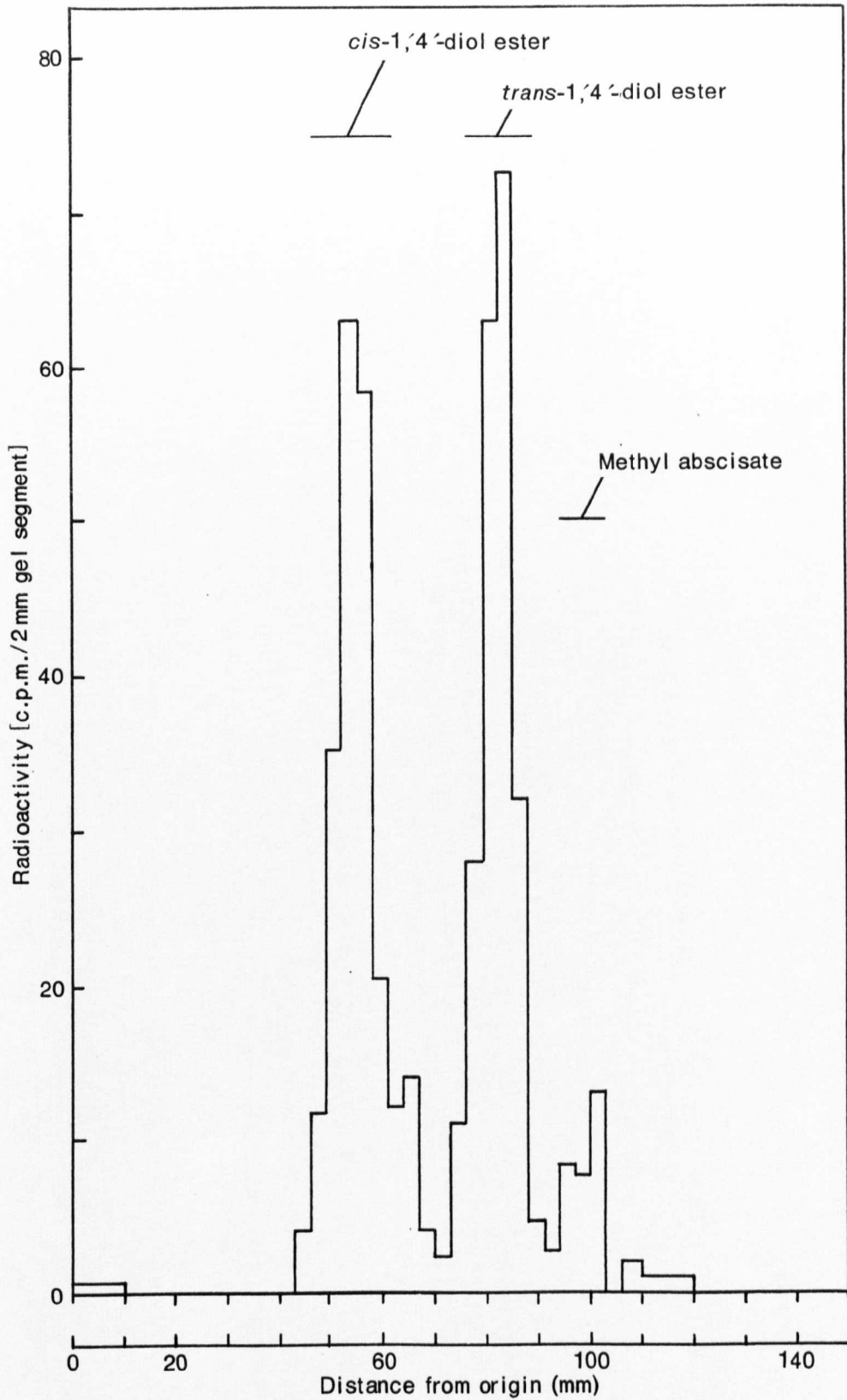
**Fig 4. Histogram of the distribution of [ $^{14}\text{C}$ ] from (+)-[2- $^{14}\text{C}$ ] mevalonic acid (4.82 mCi/m mole) in the ether soluble acid fraction from avocado fruit. The extract was chromatographed in propan-1-ol-butan-1-ol-aq. ammonia (Sp. gr. 0.88)-water (6:2:2:1, by vol) on a precoated silica gel  $\text{F}_{254}$  t.l.c. plate. The silica gel was put in the scintillation solution and counted.**



**Fig 5. Incorporation of [ $^{14}\text{C}$ ] from (+)-[2- $^{14}\text{C}$ ] mevalonic acid into ABA in avocado fruit. The zone of the t.l.c. plate in Fig 4 corresponding to ABA was eluted with ethanol and the material was chromatographed in toluene-ethyl acetate-acetic acid (15:3:1, by vol).**



**Fig 6. Incorporation of  $[^{14}\text{C}]$  from  $(+)\text{-}[2\text{-}^{14}\text{C}]$  mevalonic acid into ABA in avocado fruit. The zone on the t.l.c. plate in Fig 5 corresponding to ABA was eluted with ethanol and the acids were methylated and chromatographed in hexane-ethyl acetate (1:1, by vol).**



**Fig 7.** In corporation of [ $^{14}\text{C}$ ] from (+)-[2- $^{14}\text{C}$ ] mevalonic acid into ABA in avocado fruit. The zone on the t.l.c. plate in Fig 6 corresponding to MeABA was eluted and reduced with sodium borohydride. The [ $^{14}\text{C}$ ] incorporated into ABA co-chromatographed with marker *cis*-1,4' and *trans*-1,4'-diols of MeABA in hexane-ethyl acetate (1:1, by vol).

Table 10. Incorporation of ( $\pm$ )-[2- $^{14}$ C]mevalonic acid (4.82mCi/mole,  $21.59 \times 10^6$  d.p.m.) by an avocado fruit (weight 177g) split lengthways. The ( $\pm$ )-[2- $^{14}$ C]mevalonic acid was applied to the cut surfaces and the cavity of each half and the preparation was kept at 20°C, in normal laboratory light for 5 days. Radioactivity chromatographing with marker ABA and its authentic derivatives. The efficiency of counting was 46.8% and the background was 44.3 c.p.m.

Source of material	Chromatographic solvents used	c.p.m. in subsample	d.p.m. in total sample	(+) ABA by R.D. method, $\mu$ g	Specific activity of ABA d.p.m./ $\mu$ g
Acid fraction-zone on t.l.c. plate adjacent to marker ABA	Propan-1-ol-butan-1-ol-aq. ammonia (sp.gr. 0.88) water (6:2:2:1, v/v)	535.3	50,317		
Sample from 1st t.l.c. adjacent to marker ABA	Toluene-ethylacetate-acetic acid (75:15:5, v/v)	989.4	29,616		
Methylated sample from 2nd t.l.c. adjacent to marker MeABA	Hexane-ethyl acetate (1:1, v/v)	165.0	14,720 (73,900 by R.D.)	3,720	19.9
Sample from 3rd t.l.c. treated with aq. methanolic NaBH <sub>4</sub> solution. Zone on t.l.c. plate adjacent to: a) <u>cis</u> -1',4'-diol MeABA b) <u>trans</u> -1',4'-diol MeABA	Hexane-ethyl acetate (1:1, v/v)	55.6 56.3	4,755 4,815		



Total free (+) ABA in tissue =

$$\frac{(\text{Optically active ABA}) \times (\text{Racemic ABA added})}{(\text{Total ABA by ultraviolet absorption}) - (\text{Optically active ABA})}$$

Values obtained in the avocado experiment will be used to illustrate the application of this method to the experiments described in this chapter. Values obtained by this method are shown as "R.D. corrected" to differentiate them from values obtained by direct extraction and measurement.

389 $\mu$ g ( $\pm$ )ABA were added to the methanol prior to maceration of the avocado halves. The ultraviolet absorption (Figure 8) and optical rotatory dispersion (Figure 9) spectra of the methylated abscisic acid extracted were measured

u.v. data:- Cell = 5.00mm; solvent = redistilled ethanol

$$\lambda_{\max} = 265\text{nm}; \quad \epsilon = 21,400; \quad \text{MW} = 278$$

Volume of solution in cell = 1.7ml, this contained 1/30 of total sample. The optical density (O.D.) of this solution ( $\lambda = 265\text{nm}$ ) was 0.62 units.

$$c = \frac{\text{O.D.} \times \text{M.W.} \times 1000}{\epsilon \times l}$$

where c = concentration in  $\mu\text{g/ml}$

O.D. = optical density

M.W. = molecular weight

$\epsilon$  = molar extinction co-efficient at  $\lambda_{\max}$

$l$  = pathlength in cm.

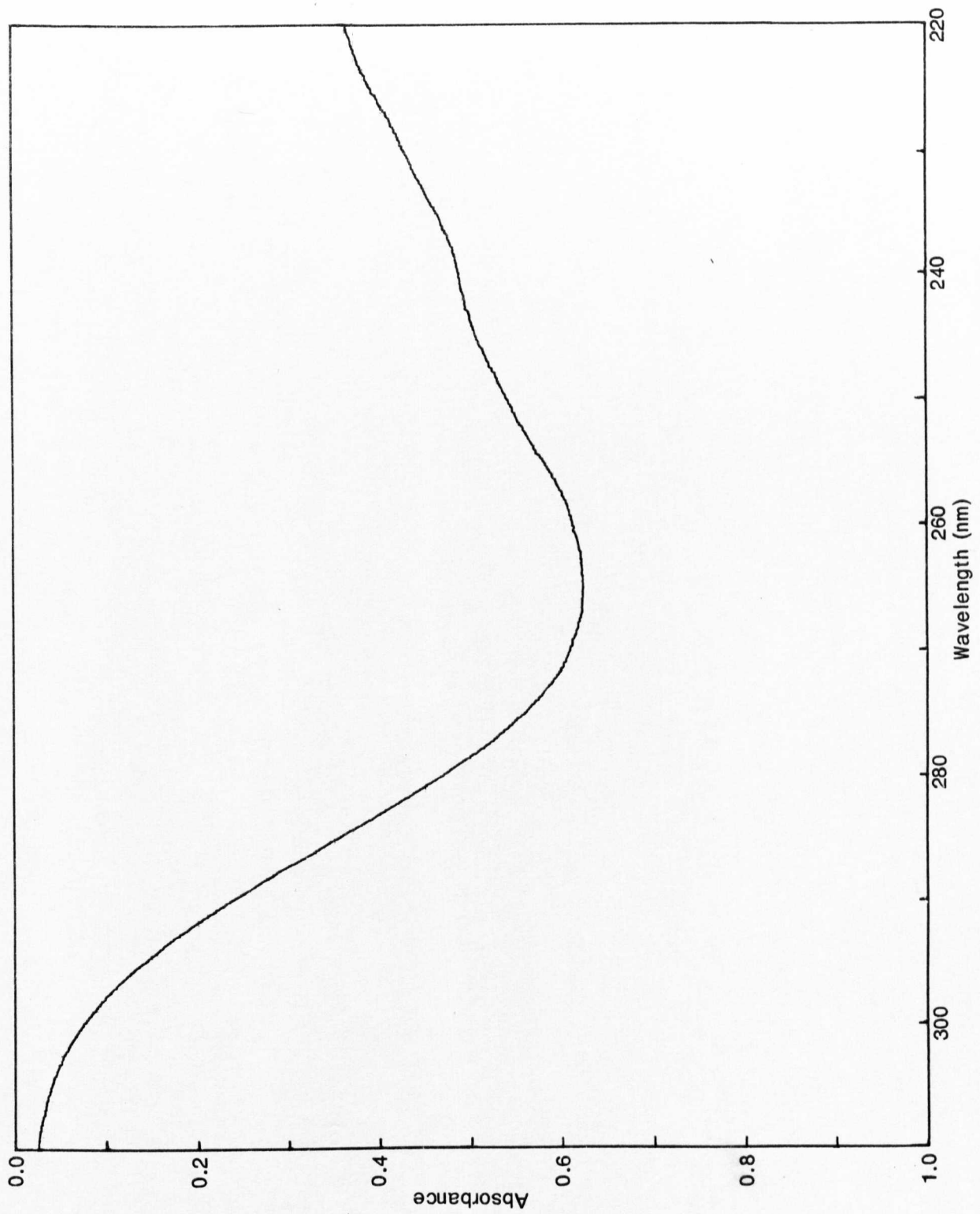


Fig 8. Ultraviolet absorption spectrum of methylated ABA recovered from avocado fruit, measured in ethanol, (see text for details). The o.r.d. curve of this material is shown in Fig 9.

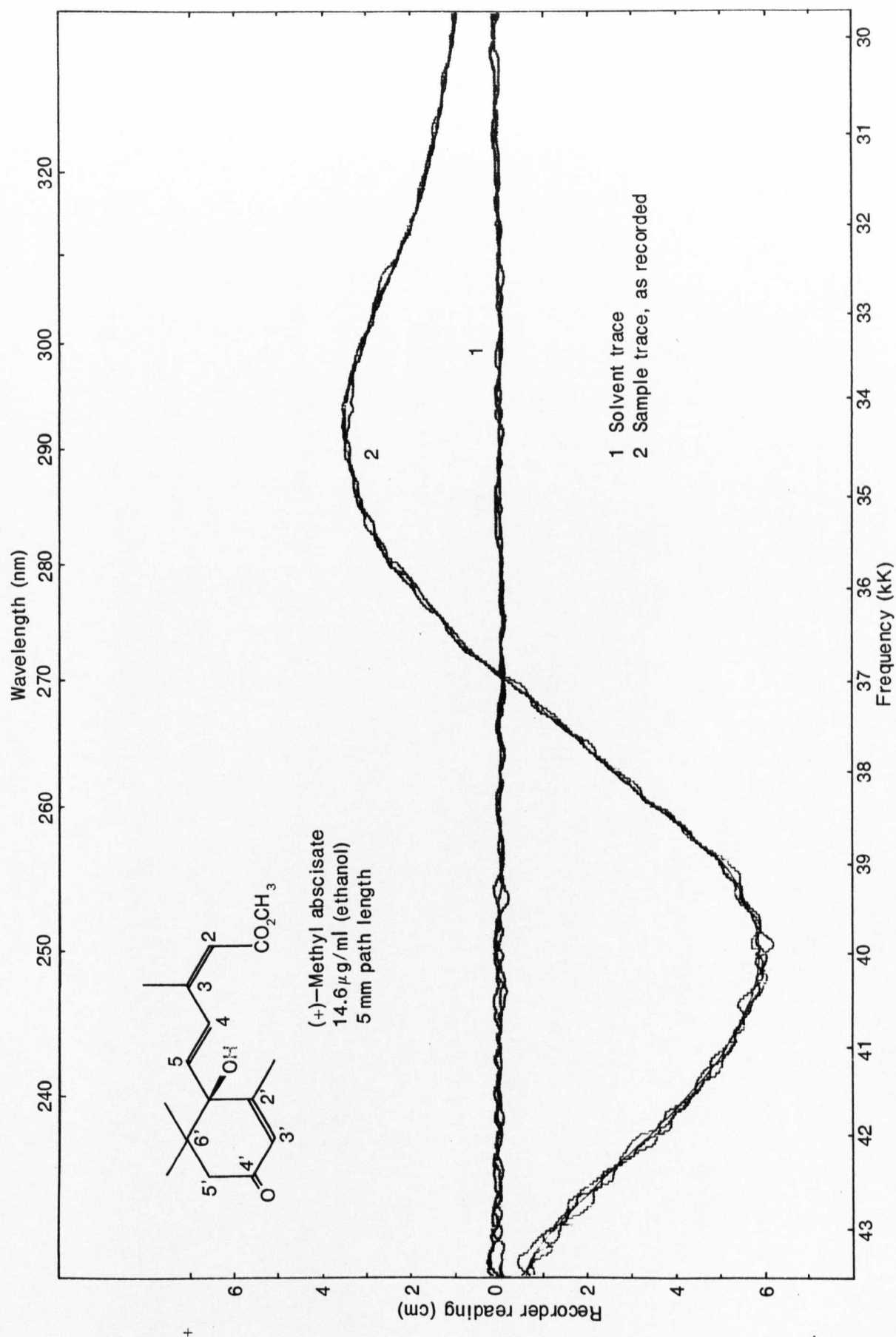


Fig 9. The optical rotatory dispersion curve of methylated ABA recovered from avocado fruit, measured in ethanol, (see text for details). The ultraviolet absorption spectrum is shown in Fig 8.

$$\therefore c = \frac{0.62 \times 278 \times 1000}{21,400 \times 0.50} \mu\text{g ABA/ml}$$

$$= 16.11 \mu\text{g ABA/ml}$$

$$\therefore \text{total volume of solution in cell} = 27.39 \mu\text{g ABA}$$

$$\therefore \text{ABA in total sample} = 30 \times 27.39 = 821.7 \mu\text{g ABA}$$

o.r.d. data:-

The optical rotatory dispersion spectrum of a solution was measured immediately after the u.v. absorption spectrum had been recorded. The spectropolarimeter was adjusted so that a 10mm deflection of the recorder, measured at 285.7nm in a 10.00mm cell, was produced by 1 $\mu$ g (+)-ABA/ml of solution.

The MeABA solution was measured at one half of the normal sensitivity, and in a 5mm cell; i.e. 10mm deflection = 4 $\mu$ g (+)-ABA/ml and the observed deflection was 36.5mm at 285.7nm.

The concentration of (+)-MeABA was,

$$\therefore 4 \times 3.65 \mu\text{g/ml}$$

The volume of solution in the cell was 1.7ml,

$$\therefore \text{weight of } (\pm)\text{-MeABA in cell} = 24.82 \mu\text{g},$$

$$\therefore (+)\text{-MeABA in total sample} = 30 \times 24.82 = \underline{744.6 \mu\text{g}}.$$

Total ABA by u.v. absorption 822 $\mu$ g [( $\pm$ ) + (+)-ABA]

Total (+)-ABA by o.r.d. 744 $\mu$ g [(+)-ABA]

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by difference 78 $\mu$ g ( $\pm$ )-ABA recovered

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389 $\mu$ g ( $\pm$ )-ABA were added to the extraction medium

$$\therefore \frac{78}{389} \times 100\% \text{ recovery of racemate}$$

$$= 20.1\%$$

$\therefore$  actual amount of (+)-ABA in avocado (177g fresh weight)

$$\frac{100}{20.1} \times 744 \mu\text{g} = 3720 \mu\text{g } (+)\text{-ABA}$$

This represents 21.0mg (+)-ABA/kg fresh weight avocado

∴ actual d.p.m. in MeABA (Table 10)

$$14,720 \times \frac{100}{20.1} = 73,900 \text{ d.p.m.}$$

2.02μmole [2-<sup>14</sup>C]mevalonic acid (4.82mCi/mmole, 21.59 x 10<sup>6</sup> d.p.m.) applied to 177g of avocado pear tissue for 5 days gave 2.3nmole (73,900 d.p.m. - R.D. corrected value) of ABA. The yield of biosynthesised ABA represents 0.016% of the total ABA extracted. Using the racemate dilution method the extraction of ABA was 20.1% efficient at the methyl abscisate stage of purification. The total amount of ABA was 21.0mg/kg (fresh weight). This was an exceptionally high value for avocado fruit and the highest ABA content recorded. In most experiments the amount of ABA was not greater than 10.0mg/kg (fresh weight). The high concentration of ABA may indicate that the main phase of biosynthesis was over in the ripe fruit used in this experiment. Thus the large amount of ABA already formed and the small amount of synthesis taking place would explain the low percentage of ABA formed from the labelled MVA.

(c) Incorporation of [2-<sup>14</sup>C]mevalonic acid into abscisic acid by green tomato fruit incubated in darkness at 20°C for 72 hours.

Experiment commenced on 26th November, 1968.

Ten green tomato fruit (each weighing 23g) were each injected with 10μl from a 0.40ml solution of the potassium salt of [2-<sup>14</sup>C]mevalonic acid (4.82mCi/mmole, 2.02μmole) in 0.05M potassium phosphate buffer, pH 7.3, containing 10% ethanol (v/v)

with a Hamilton syringe. Each tomato received a further 10 $\mu$ l from the 0.40ml solution after 24 hours and 20 $\mu$ l after 48 hours; they were incubated in darkness at room temperature for a total of 72 hours and then macerated in 1 litre of methanol containing 178 $\mu$ g racemic abscisic acid and thoroughly extracted with a further 1.5 litres of a methanol:water mixture (4:1, v/v).

Identification of one of the radioactive acids as abscisic acid was made by the methods previously described. Radioactivity co-chromatographing with authentic abscisic acid and authentic derivatives of abscisic acid in several solvent systems is given in Table 11.

2.02 $\mu$ mole [2-<sup>14</sup>C]mevalonic acid (4.82mCi/mmole, 21.59 x 10<sup>6</sup> d.p.m.) applied to 230g tomato fruits for 72 hours gave 0.30nmole (9176 d.p.m. - R.D. corrected value) of ABA. The yield of biosynthesised ABA represents 0.067% of the total ABA extracted. Using the racemate dilution method the extraction of ABA was 67% efficient at the methyl abscisate stage of purification 78.1 $\mu$ g (+)-ABA were extracted, measured as MeABA (116.6 $\mu$ g (+)-ABA - R.D. corrected value) and the concentration of (+)-ABA in the fruits was 534.8 $\mu$ g/kg (fresh weight). As in the experiment with avocado fruit the concentration of (+)-ABA in the tissue is higher than the values reported previously (Milborrow, 1967; Noddle and Robinson, 1969).

Table 11. Incorporation of [ $^{14}\text{C}$ ]mevalonic acid ( $4.82\text{mCi/mmole}$ ,  $21.59 \times 10^6$  d.p.m.) into ABA by ten green tomato fruit, (total weight 230g), incubated at  $20^\circ\text{C}$  for 72 hours in darkness. Radioactivity chromatographing with marker ABA and its authentic derivatives. The efficiency of counting was 46.8% and the background was 40.5 c.p.m.

Source of material	Chromatographic solvents used	c.p.m. in subsample	d.p.m. in total sample	(+) ABA by R.D. method, $\mu\text{g}$	Specific activity of ABA d.p.m./ $\mu\text{g}$
Acid fraction, zone on t.l.c. plate adjacent to marker ABA	propan-1-ol-butan-1-ol aq. ammonia (sp.gr. 0.88) water (6:2:2:1, v/v)	335.1	37,970		
Sample from 1st t.l.c. adjacent to marker ABA	toluene-ethylacetate-acetic acid (75:15:3, v/v)	120.3	7,199		
Methylated sample from 2nd t.l.c. adjacent to marker MeABA	hexane-ethylacetate (1:1, v/v)	151.1	6,148 9,176 (by R.D.)	116.6	7.87
Sample from 3rd t.l.c. treated with aq. methanolic $\text{NaBH}_4$ solution. Zone on t.l.c. plate adjacent to marker a) <u>cis</u> -1',4'-diol MeABA b) <u>trans</u> -1',4'-diol MeABA	hexane ethylacetate (1:1, v/v)	19.8 18.5	1,375 2,571	3.74 (not R.D. corrected)	
Zones (a) and (b) from 4th t.l.c. plate treated with $\text{MnO}_2$ in dry chloroform. Zone on t.l.c. plate adjacent to marker MeABA a) <u>cis</u> -1',4'-diol MeABA b) <u>trans</u> -1',4'-diol MeABA	hexane ethylacetate (1:1, v/v)	53.0 31.0	565 1,175		

(d) Incorporation of  $(\pm)$ -[2-<sup>3</sup>H]mevalonic acid (93mCi/mmole)  
into (+)-abscisic acid by cut wheat leaves.

Experiment commenced 10th September, 1969.

(This experiment was performed in collaboration with  
Dr. B. V. Milborrow, Milstead Laboratory, Shell Research Limited,  
Sittingbourne, Kent.)

145g of cut wheat shoots were placed in a solution containing 47.5 $\mu$ g  $(\pm)$ -[2-<sup>3</sup>H]mevalonic acid (93mCi/mmole, 66.27 x 10<sup>6</sup> d.p.m.) dissolved in 50ml of 0.01M-potassium phosphate buffer, pH 7.3, containing 2% of ethanol (v/v). The shoots were left for 2 hours at room temperature (20-22°C) during which time they took up 19 $\mu$ g  $(\pm)$ -[2-<sup>3</sup>H]mevalonic acid (26.52 x 10<sup>6</sup> d.p.m.) in 20ml of solution. The shoots were removed from the solution, the submerged parts rinsed in water and blotted. Half of the batch (75g fresh weight) was placed with the cut ends in water (water treatment) and kept in a damp atmosphere at 20°C. The other half 70g (fresh weight) was spread out on filter paper and exposed to a draught of air for 30 minutes (wilt treatment) by which time the plants had lost 20% of their weight; they were covered with a sheet of transparent polythene for 5½ hours. Both treatments were illuminated until harvested.

The wheat shoots from both treatments were placed in 500ml methanol which contained 20 $\mu$ g racemic abscisic acid for the wet treatment and 50 $\mu$ g for the wilt (and 10mg B.H.T./litre) and chopped up into 1cm lengths. After 24 hours the mixture was filtered and thoroughly extracted with a further 1 litre of a methanol:water (4:1, v/v) mixture (containing 10mg B.H.T./litre).



The ether-soluble acid fraction from the water and the wilt treatments were chromatographed separately. In each case one of the radioactive acids in the ether-soluble acid fraction was found to chromatograph with authentic ABA in a propanol/butanol/ammonia/water (6:2:2:1, v/v) solvent system and also in a toluene/ethyl acetate/acetic acid (15:3:1, v/v) solvent system. On methylation the labelled material co-chromatographed with authentic MeABA in a hexane-ethyl acetate (1:1, v/v) solvent system on a Merck F<sub>254</sub> silica gel, precoated plate. Material co-chromatographing with authentic MeABA was eluted with methanol and aliquots were taken for [<sup>3</sup>H]assay, Table 12. The amount of methylated ABA extracted was calculated using the u.v. absorption and o.r.d. spectra of this material (Table 12). The profiles of the u.v. absorption spectra and optical rotatory dispersion spectra were identical with those of authentic abscisic acid methyl ester and (+)-abscisic acid methyl ester respectively.

Treatment of the labelled material co-chromatographing with authentic MeABA, with an aqueous methanolic sodium borohydride solution produced two labelled entities which migrated equally with the authentic cis-1',4'- and trans-1',4'-diols of MeABA.

The efficiency of extraction of ABA, as measured by the racemate dilution method, at the MeABA stage in purification was 60.6% from the wheat plants subjected water treatment and 50.0% from the wheat plants subjected to the wilt treatments. The amount of (+)-ABA extracted from the wheat plants subjected to water treatment was insufficient to provide an accurate quantitative value although the profile of the o.r.d. spectra was characteristic

Table 12. Incorporation of (+)-[2-<sup>3</sup>H]mevalonic acid (93mCi/mmole) into ABA by 145g of cut wheat shoots. The wheat shoots absorbed 19 $\mu$ g of mevalonic acid (26.52 x 10<sup>6</sup> d.p.m.) in 20ml of 0.01M-potassium phosphate buffer, pH 7.3, 2% ethanol (v/v) during 2 hours. The batch was then divided and one half was allowed to wilt so that it lost 20% of its weight; the other half was placed with the cut ends in water for 5 hours. Radioactivity chromatographing with marker MeABA. The efficiency of counting was 24% and the background was 53 c.p.m.

Treatment	c.p.m. in subsample	d.p.m. in total sample	(+)-ABA extracted, $\mu$ g	(+)-ABA extracted R.D. corrected values, $\mu$ g	% ( $\pm$ )-MVA incorporated	% ABA formed from MVA
Water	86	173.4 286-RD corrected	<0.3	<0.5	0.0013	-
Wilt	389	1300 2600-RD corrected	4.2	8.4	0.012	0.012

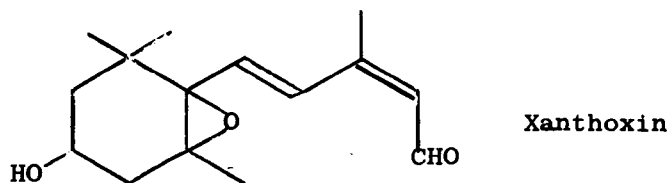
of (+)-ABA, the value for (+)-ABA given in Table 12 represents a maximum value. For the water and wilt treatments the concentration of (+)-ABA was  $\leq 6.6\mu\text{g}$  and  $120\mu\text{g/kg}$  (fresh weight) wheat shoots as measured by the racemate dilution method.

Discussion of results. In the preceding experiments tomato fruit and avocado fruit converted  $[2-^{14}\text{C}]$ mevalonic acid into  $[^{14}\text{C}]$ labelled ABA. The conversion occurred in light and in darkness at room temperature. The proportion of  $[^{14}\text{C}]$ mevalonic acid recovered as ABA varied for reasons yet unknown. The rapid metabolism of (+)-ABA that occurs under certain conditions (Milborrow, 1970) may be a contributory factor.

Rudnicki, Pieniazek and Pieniazek (1968) suggested that ABA found in strawberry fruit, attached to the mother plant, was synthesised in the fruit and not synthesised in the leaves and then transported to the fruit. Rudnicki, Machnik and Pieniazek (1968) subsequently reported that the level of (+)-ABA in ripening pears increased from the time they were harvested. The rate of increase in the concentration of (+)-ABA was lower when the fruit were stored at low temperature. The increase in (+)-ABA concentration took place both in darkness and in light at  $18^\circ\text{C}$ .

The present results show that de novo synthesis of (+)-ABA occurs in detached fruit and that the observed increase in the amount of (+)-ABA in detached fruit is caused by synthesis of (+)-ABA rather than by its release from a conjugate or a precursor. Synthesis of ABA from MVA occurred in light and darkness. The experiment with tomato fruit was repeated with the fruits incubated in darkness because Taylor and Smith (1967) had

shown that photolysis of violoxanthin (absorbed on filter paper) produced an inhibitory compound, later identified as xanthoxin,



which might be a natural precursor of ABA. The incorporation of [ $^{14}\text{C}$ ]mevalonic acid into (+)-ABA by tomato fruit in darkness excluded the necessity of a photolytic step in the biosynthesis although it did not exclude the involvement of a carotenoid in the biosynthesis. In these experiments it was found that wilted wheat plants incorporated more [ $2\text{-}^3\text{H}$ ]MVA into (+)-ABA than did turgid plants. The concentration of (+)-ABA in the wilting wheat shoots was higher than in the wheat shoots kept turgid. The major part, if not the whole increase in concentration of (+)-ABA which occurs during/wilting can, therefore, be attributed to de novo synthesis rather than to release of (+)-ABA from a conjugate or a preformed precursor.

(ii) Conversion of epoxide and its 2-trans isomer into (+)-ABA

The growth inhibitory activity of some analogues of ABA has been reported (Anderson 1969, Tamura and Nagao 1969(b) and 1969(c)) but there has been no indication whether these compounds are active per se or because they give rise to ABA. The strong inhibitory activity of epoxide (X) was of particular interest since the compound has the same carbon skeleton and similar substitution to ABA and it seemed likely that its inhibitory activity resulted from its conversion to ABA by the plant. The metabolism of epoxide

and its 2-trans isomer (XXX) was studied using  $[2-^{14}\text{C}]$  labelled material which was incubated with green tomato fruit. At the conclusion of incubation ABA and unreacted precursor were identified and assayed for  $[^{14}\text{C}]$  activity. To confirm that the metabolism of epoxide and its 2-trans isomer was brought about by enzyme action,  $[2-^{14}\text{C}]$ epoxide and its 2-trans isomer were incubated with tomatoes which had been denatured at  $100^{\circ}\text{C}$ . During the course of these studies the formation of the  $(\pm)-[2-^{14}\text{C}]1',2'$ -dihydroxy derivatives of epoxide and 2-trans epoxide was observed in only the denatured tomato fruit. The metabolism of these materials was studied by re-incubating them with living green tomato fruit.

During the course of this work, Wright and Hiron (1969) reported that the (+)-ABA concentration in freshly harvested wheat leaves increased 40-fold during 4 hours of wilting. This increase has now been attributed to de novo synthesis of  $[2-^3\text{H}]$ MVA into (+)-ABA because wilted wheat plants incorporate more  $[2-^3\text{H}]$  than do turgid plants (Milborrow and Noddle, 1970).

These results suggested a novel way of investigating whether the conversion of epoxide to ABA were regulated in the same way as the pathway by which ABA is made in vivo. Wilted wheat shoots previously supplied with  $(\pm)-[2-^{14}\text{C}]$ epoxide would be expected to convert a greater proportion of epoxide into ABA than unwilted plants if the conversion were regulated in the same way as the endogenous biosynthesis of (+)-ABA. If the conversion were not regulated by the mechanism responsible for the increased biosynthesis during wilting then the amounts of epoxide incorporated into (+)-ABA would be expected to be similar under wet and dry conditions.

The fate of the epoxy-oxygen of epoxide was investigated using  $[1',2'-^{18}\text{O}]$  labelled epoxide. This compound was fed together with  $[2-^{14}\text{C}]$  labelled epoxide to wheat plants which were subsequently wilted. From previous experiments it was known that any  $[^{18}\text{O}]$ -containing (+)-ABA formed during the experiment would not be diluted by a large pool of endogenous  $[^{16}\text{O}]$  material. For the epoxy-oxygen to be retained in (+)-ABA the C-1'-oxygen bond must remain intact, and epimerization at C-1'- is not possible. It therefore follows that only one enantiomer of epoxide can be converted to (+)-ABA: that one in which the epoxide oxygen is on the same side of the six-membered ring as the hydroxyl group of (+)-ABA.

(a) Conversion of ( $\pm$ )-5-(1',2'-epoxy-2',6',6'-trimethylcyclohexyl)-3-methyl-[2- $^{14}\text{C}$ ]-penta-cis-2-trans-4-dienoic acid (epoxide) to abscisic acid by ripening tomato fruit

Experiment commenced on 12th February, 1969.

A preliminary experiment using ripening tomato fruit was performed to determine the extent of  $[^{14}\text{C}]$  appearing in ABA when ( $\pm$ )-[2- $^{14}\text{C}$ ]epoxide was injected into the fruit.

A sample of ( $\pm$ )-[2- $^{14}\text{C}$ ]epoxide, (1.3mCi/mmole,  $8.06 \times 10^6$  d.p.m.), was dissolved in 0.05ml ethanol and applied to a t.l.c. plate as a narrow band, adjacent to it was marker epoxide and marker 2-trans epoxide. The plate was developed in a toluene-ethyl acetate-acetic acid (15:3:1, by vol.) solvent system and the position of the materials on the plate detected with u.v. light. Material co-chromatographing with authentic epoxide was eluted from the gel, with ethanol, and an aliquot taken for counting (Table 13).

Table 13. Purification of (±)-[2-<sup>14</sup>C]epoxide. Radioactivity co-chromatographing with marker epoxide on Merck F254 t.l.c. plate. The efficiency of counting was 50% and the background was 47.7 c.p.m.

Source of material	Chromatographic solvent used	c.p.m. in subsample	d.p.m. in total sample
Zone on t.l.c. plate adjacent to marker epoxide	toluene-ethyl acetate-acetic acid, (15:3:1, v/v)	5170	8,060,000

Nine small ripening tomatoes, yellow at the commencement of the experiment (total fresh weight 67.2g) were injected with a total of 0.10ml ethanol containing 2.93 $\mu$ mole ( $\pm$ )-[2-<sup>14</sup>C]epoxide (1.3mCi/mmole, 8.06 x 10<sup>6</sup> d.p.m.). The tomatoes were incubated at 20°C for 48 hours in darkness and then 24 hours in light during which time they became red. The tomatoes were macerated in 1 litre methanol containing 93.2 $\mu$ g ( $\pm$ )-ABA, and thoroughly extracted with a total of 2 litres of a methanol:water mixture (4:1, v/v). Identification of one of the radioactive acids as ABA was made by the previously described methods. The amounts of radioactive material co-chromatographing with authentic ABA and its authentic derivatives is given in Table 14.

2.93 $\mu$ mole ( $\pm$ )-[2-<sup>14</sup>C]epoxide (1.3mCi/mmole), 8.06 x 10<sup>6</sup> d.p.m. gave 39.02nmole (107,303 d.p.m. - R.D. corrected) of ABA which represents 1.33% incorporation of epoxide into ABA. The yield of biosynthesised ABA represents 4.6% of the total ABA extracted. Using the racemate dilution method the extraction of ABA was 30.4% efficient at the methyl abscisate stage of purification. 66.6 $\mu$ g (+)-ABA were extracted, measured as MeABA (222 $\mu$ g (+)-ABA - R.D. corrected value) and the concentration of (+)-ABA in the fruit was 3.3mg/kg (fresh weight).

(b) Conversion of ( $\pm$ )-[2-<sup>14</sup>C]epoxide to abscisic acid by tomato fruit incubated for 48 hours in darkness at 20°C

Experiment commenced on 2nd April, 1969.

( $\pm$ )-[2-<sup>14</sup>C]labelled epoxide was freed of contamination with its 2-trans isomer by an addition of 10% of the unlabelled impurity; this was followed by a chromatographic separation.



Table 14. Conversion of  $(\pm)$ -[2- $^{14}$ C]epoxide (1.3mCi/mmole,  $8.06 \times 10^6$  d.p.m.) into ABA by 9 ripening tomato fruit (total weight 67.2g) incubated at 20°C, in darkness and in light, for a total of 72 hours. Radioactivity chromatographing with marker ABA and its authentic derivatives. The efficiency of counting was 50.0% and the background was 44.6 c.p.m.)

Source of material	Chromatographic solvents used	c.p.m. in subsample	d.p.m. in total sample	(+)-ABA by R.D. method, $\mu$ g	Specific activity of ABA, d.p.m./ $\mu$ g
Acid fraction, zone on t.l.c. plate adjacent to marker ABA	propan-1-ol-butan-1-ol aq. ammonia (sp.gr. 0.88) water (6:2:2:1, v/v)	1025.8	378,600		
Sample from 1st t.l.c. adjacent to marker ABA	toluene-ethylacetate-acetic acid (15:3:1, v/v)	178.8	53,660		
Methylated sample from 2nd t.l.c., zone adjacent to marker MeABA	hexane-ethylacetate (1:1, v/v)	142.3	32,720 (107,303 - R.D. value)	222.0	483.3
Sample from 3rd t.l.c. treated with aq. methanolic $\text{NaBH}_4$ solution, zone on t.l.c. plate adjacent to: a) <u>cis</u> -1',4'-diol MeABA b) <u>trans</u> -1',4'-diol MeABA	hexane-ethylacetate (1:1, v/v)	47.8 62.3	3,285 3,890		

1,600 $\mu$ g ( $\pm$ )-[2-<sup>14</sup>C]epoxide were dissolved in 1.0ml of ethanol containing 200 $\mu$ g of the unlabelled 2-trans isomer. The mixture was chromatographed in a toluene-ethyl acetate-acetic acid (15:3:1, by vol) solvent system, with epoxide and 2-trans epoxide markers. After multiple development of the plate a 10mm separation of the two isomers was obtained and the materials co-chromatographing with marker epoxide and with marker 2-trans epoxide were eluted with ethanol separately. An aliquot of each eluate was assayed for radioactivity (Table 15).

Ten green tomato fruit (total 125.2g fresh weight) were each injected with 10 $\mu$ l of an 0.1ml ethanol solution containing 4.25 $\mu$ mole ( $\pm$ )-[2-<sup>14</sup>C]epoxide, (1.3mCi/mmole, 11.69 x 10<sup>6</sup> d.p.m.). The tomatoes were incubated in darkness for 48 hours at 20°C and showed no signs of ripening at the end of this time; they were macerated in 1 litre methanol containing 156 $\mu$ g racemic ABA, and thoroughly extracted with 2.5 litres of a methanol:water (4:1, v/v) mixture. Identification of one of the radioactive acids as abscisic acid was made by previously described methods, radioactivity co-chromatographing with authentic ABA and its authentic derivatives is given in Table 16.

The distribution of radioactivity along the t.l.c. plates mentioned in Table 16 are shown in Figures 10-15. Unreacted materials co-chromatographing with marker epoxide and with 2-trans epoxide were eluted with ethanol separately from the first t.l.c. plate mentioned in Table 16 (Fig. 10). 61.0 $\mu$ g [2-<sup>14</sup>C]epoxide (1.3mCi/mmole, 671,155 d.p.m.) were recovered and 0.4 $\mu$ g [2-<sup>14</sup>C] 2-trans epoxide (1.3mCi/mmole, 4347 d.p.m.) were recovered. The

Table 15. Purification of ( $\pm$ )-[2-<sup>14</sup>C]epoxide (1.3mCi/mmole) by addition of its unlabelled 2-trans isomer and subsequent t.l.c. separation of the isomers. Radioactivity chromatographed with marker epoxide and its 2-trans isomer. The efficiency of counting was 66.3% and the background was 22.0 c.p.m.

Source of material	Chromatographic solvent used	c.p.m. in subsample	d.p.m. in total sample
Zone on t.l.c. plate adjacent to marker epoxide	toluene-ethyl acetate-acetic acid, (15:3:1, v/v)	9998	11,687,000
Zone on t.l.c. plate adjacent to marker 2-trans epoxide		2117	319,547

Table 16. Conversion of  $(\pm)$ -[2- $^{14}\text{C}$ ]epoxide (1.3mCi/mmole, 11.69 x 10<sup>6</sup> d.p.m.) into ABA by 10 green tomato fruit (total weight 125g) incubated at 20°C in darkness for 48 hours. Radioactivity chromatographed with marker ABA and its authentic derivatives. The efficiency of counting was 69.6% and the background was 22.0 c.p.m.

Source of material	Chromatographic solvents used	c.p.m. in subsample	d.p.m. in total sample	(+)-ABA by R.D. method, $\mu\text{g}$	Specific activity of ABA d.p.m./ $\mu\text{g}$
Acid fraction-zone on t.l.c. plate adjacent to marker ABA	benzene-ethylacetate acetic acid (75:15:5, v/v)	1419	295,625		
Sample from 1st t.l.c. adjacent to marker ABA	propan-1-ol-butan-1-ol-aq. ammonia (sp.gr. 0.88) water (6:2:2:1, v/v)	915.0	256,358		
Methylated sample from 2nd t.l.c., zone adjacent to marker MeABA		836.9	84,171 (206,302 - R.D. value)	129	1600
Sample from 3rd t.l.c. treated with aq. methanolic $\text{NaBH}_4$ solution, zone on t.l.c. plate adjacent to marker: a) <u>cis</u> -1',4'-diol MeABA b) <u>trans</u> -1',4'-diol MeABA	hexane-ethylacetate (1:1, v/v)	3905 9658	14,820 36,652		
Zones a) and b) from 4th t.l.c. plate treated with $\text{MnO}_2$ in dry chloroform. Zone on t.l.c. plate adjacent to marker MeABA from: a) <u>cis</u> -1',4'-diol MeABA b) <u>trans</u> -1',4'-diol MeABA	hexane-ethylacetate (1:1, v/v)	140.2 311.0	3,594 7,974	1.78 3.8	2019 2098

$R_f$  values of ABA and related compounds on Merck precoated F<sub>254</sub> silica gel plates in the three solvent systems used in this experiment are given in Table 17.

Table 17.  $R_f$ s of ABA and related compounds in three solvent systems on Merck precoated F<sub>254</sub> silica gel t.l.c. plates

compound	SOLVENT SYSTEM		
	benzene-ethyl acetate-acetic acid (75:15:5, v/v)	propan-1-ol-butan-1-ol-aq. ammonia (sp.gr. 0.88)-water, (6:2:2:1, v/v)	hexane-ethyl acetate (1:1, v/v)
	$R_f$	$R_f$	$R_f$
ABA	0.06	0.58	0.1
<u>t</u> -ABA	0.10	0.58	
epoxide	0.21	0.58	
2- <u>trans</u> epoxide	0.26	0.58	
MeABA	0.60		0.76
Methyl epoxide			0.95
<u>cis</u> -1',4'-diol of MeABA	0.38		0.42
<u>trans</u> -1',4'-diol of MeABA	0.54		0.60

4.25 $\mu$ mole ( $\pm$ )-[2-<sup>14</sup>C]epoxide (1.3mCi/mmole, 11.69 x 10<sup>6</sup> d.p.m.) gave 75.04nmole (206,300 d.p.m. - R.D. corrected) ABA which represents 1.77% conversion of epoxide into ABA. 15.4% of the total (+)-ABA extracted (measured as MeABA) was derived from the precursor. 52.56 $\mu$ g (+)-ABA were extracted and the extraction was 40.8% efficient, as measured by the racemate dilution method, at the MeABA stage of purification. This represents 1016 $\mu$ g (+)-ABA/kg fresh weight tomato fruit.

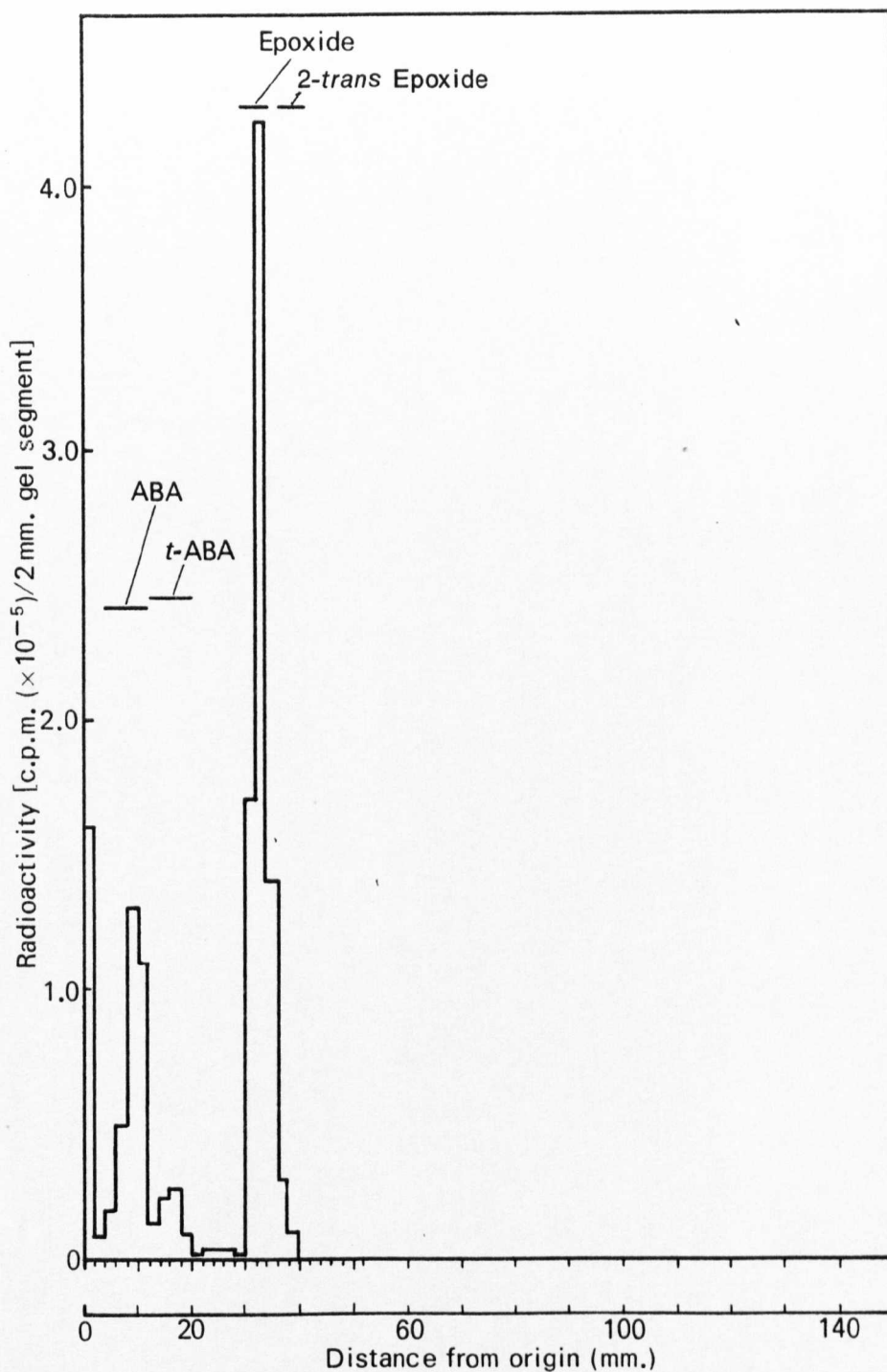


Fig 10. Histogram of the distribution of [ $^{14}\text{C}$ ] from (+)-[2- $^{14}\text{C}$ ] epoxide (1.3 mCi/m mole) in the ether-soluble acid fraction from tomato fruit. The extract was chromatographed in benzene-ethyl acetate-acetic acid (15:3:1, by vol) on a precoated silica-gel F<sub>254</sub> t.l.c. plate. The silica-gel was put in the scintillation solution and counted.

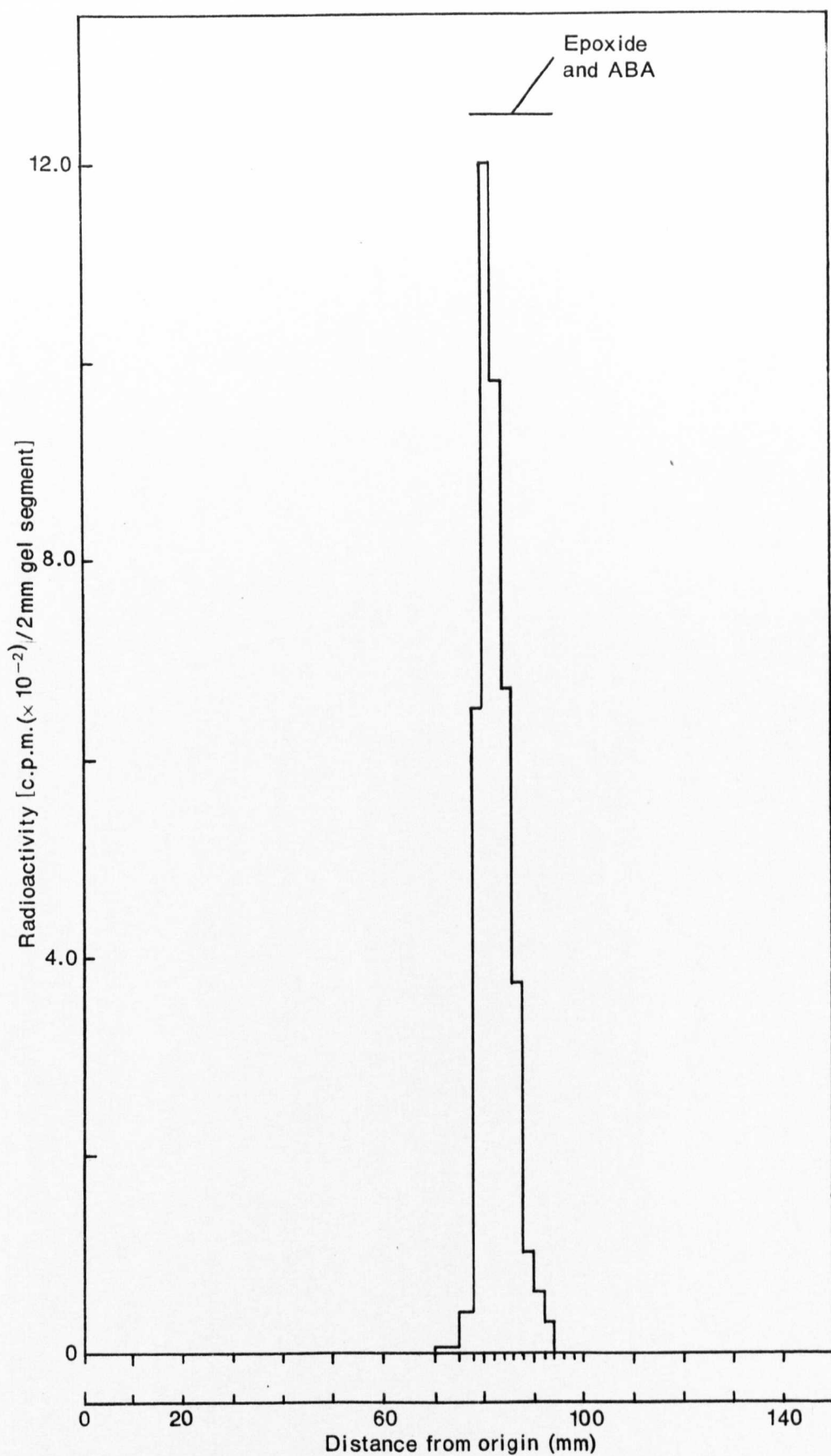
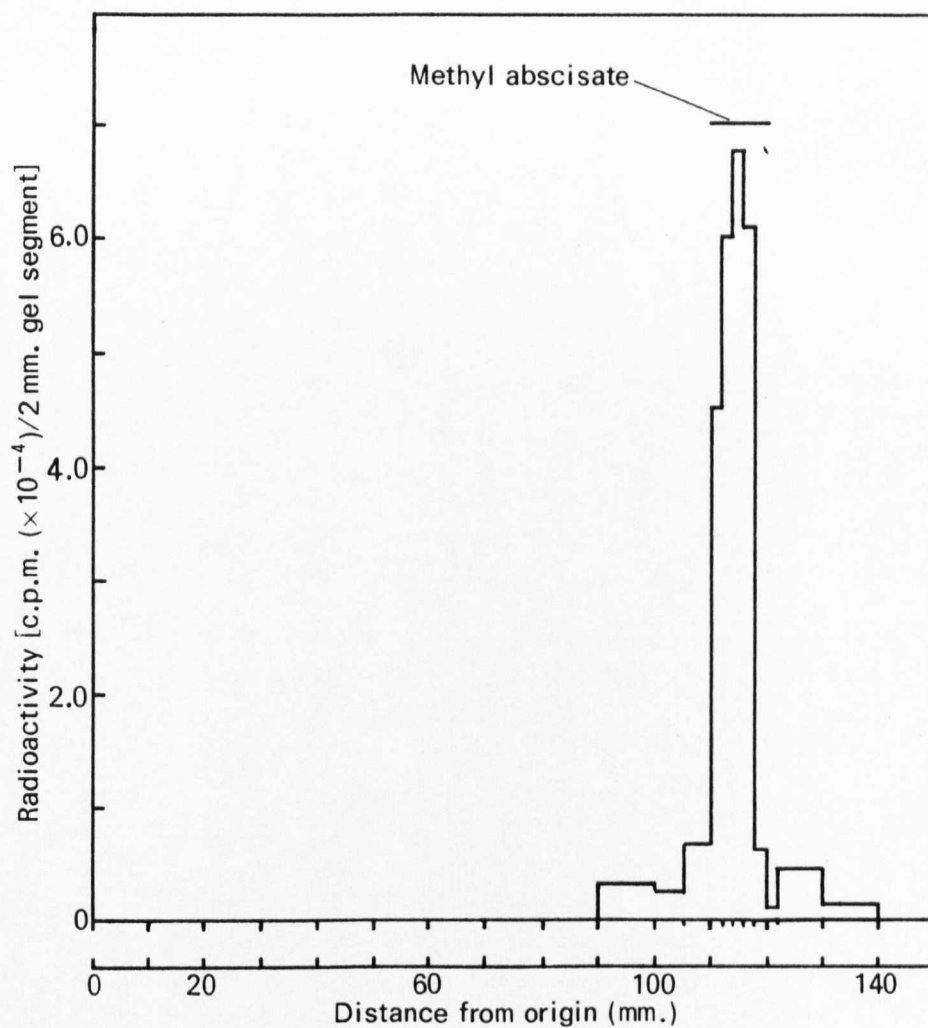


Fig 11. Incorporation of [ $^{14}\text{C}$ ] from (+)-[2- $^{14}\text{C}$ ] epoxide into ABA. The zone on the t.l.c. plate in Fig 10 corresponding with ABA was eluted with methanol. The eluate was chromatographed in propan-1-ol-butan-1-ol-aq. ammonia (sp. gr. 0.88) -water (6:2:2:1, by vol).



**Fig 12.** Incorporation of [ $^{14}\text{C}$ ] from (+)-[2- $^{14}\text{C}$ ] epoxide into ABA. The zone on the t.l.c. plate in Fig 11, corresponding with ABA was eluted with methanol and the acids were methylated and chromatographed in hexane-ethyl acetate (1 : 1, by vol).



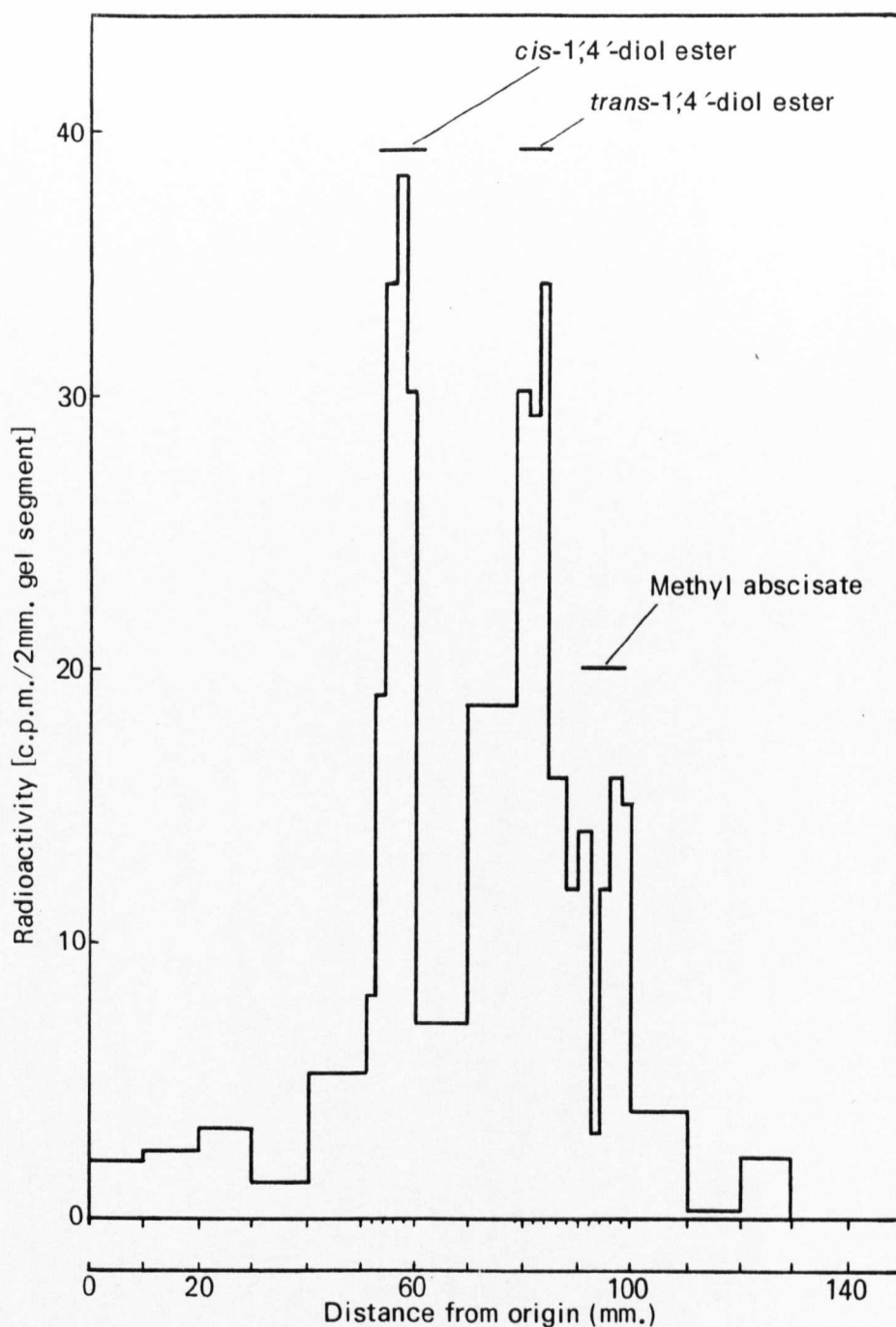
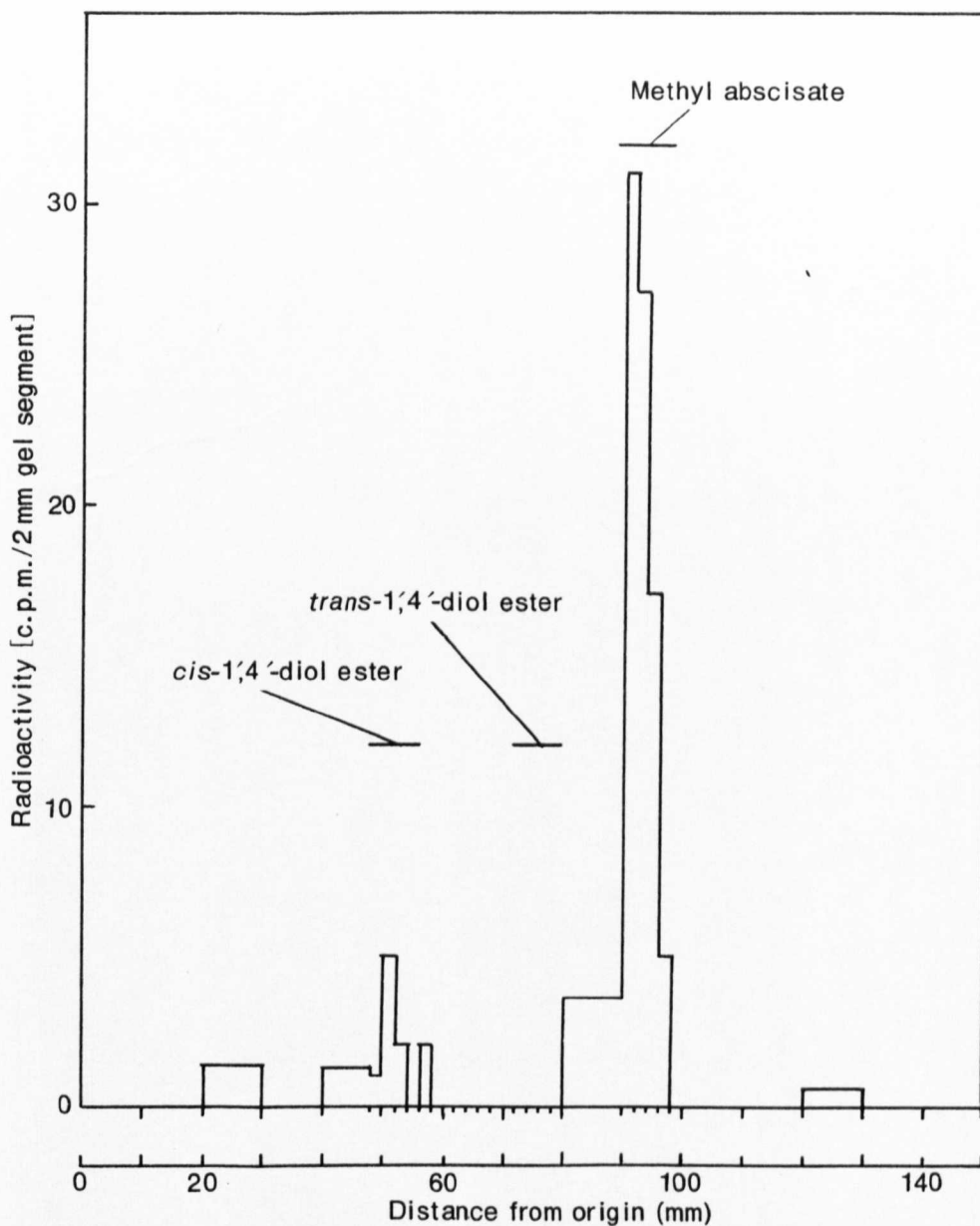
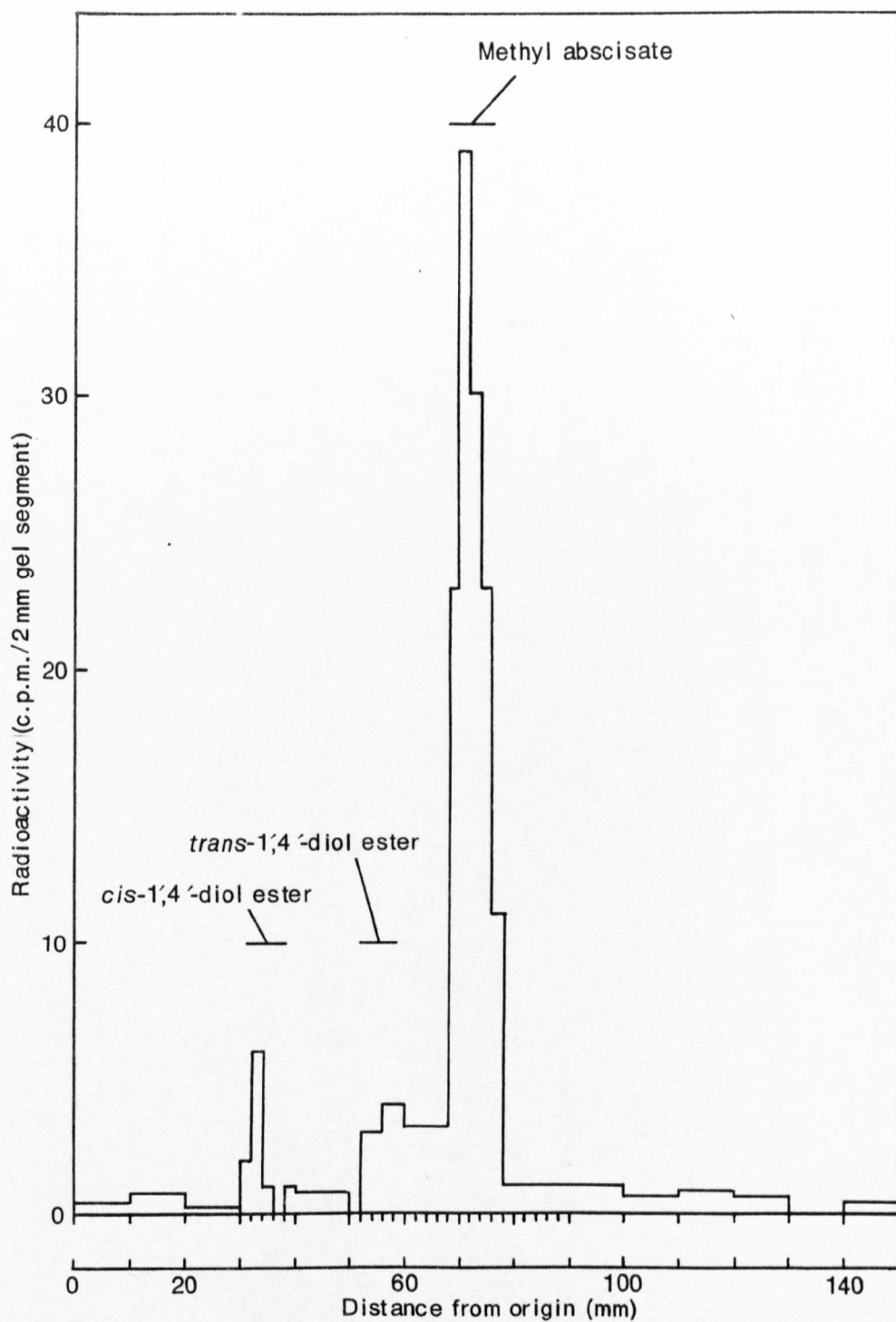


Fig 13. Incorporation of  $[^{14}\text{C}]$  from  $(\pm)\text{-}[2\text{-}^{14}\text{C}]$  epoxide into ABA. The zone on the t.l.c. plate in Fig 12 corresponding to MeABA was eluted with methanol and reduced with sodium borohydride. The  $[^{14}\text{C}]$  incorporated into ABA from the epoxide co-chromatographed with marker *cis*-1,4'- and *trans*-1,4'-diols of MeABA in benzene-ethyl acetate-acetic acid (15:3:1, by vol).



**Fig 14.** Incorporation of [ $^{14}\text{C}$ ] from (+)-[2- $^{14}\text{C}$ ] epoxide into ABA. The zone of the t.l.c. plate in Fig 13 corresponding to *cis*-1,4'-diol of MeABA was eluted with methanol and oxidized with manganese dioxide. The [ $^{14}\text{C}$ ] incorporated into ABA from the epoxide co-chromatographed with authentic MeABA marker in hexane-ethyl acetate (1:1, by vol).



**Fig 15.** Incorporation of  $[^{14}\text{C}]$  from  $(+)-[2-^{14}\text{C}]$  epoxide into ABA. The zone of the t.l.c. plate in Fig 13 corresponding to *trans*-1,4'-diol of MeABA was eluted with methanol and oxidised with manganese dioxide. The  $[^{14}\text{C}]$  incorporated into ABA from the epoxide co-chromatographed with authentic MeABA marker in hexane-ethyl acetate (1 : 1, by vol).

(c) Conversion of  $(\pm)$ -[2-<sup>14</sup>C] 2-trans epoxide into abscisic acid by tomato fruit incubated in darkness for 48 hours at 20°C.

Experiment commenced on 2nd April, 1969.

Labelled 2-trans epoxide was freed of contamination with epoxide by an addition of 10% of the unlabelled impurity followed by a chromatographic separation.

3,100 $\mu$ g  $(\pm)$ -[2-<sup>14</sup>C] 2-trans epoxide were dissolved in 1.0ml ethanol containing 400 $\mu$ g of the unlabelled epoxide. The mixture was applied to a t.l.c. plate and chromatographed in a benzene-ethyl acetate-acetic acid (75:10:1, by vol.) solvent system with epoxide and 2-trans epoxide markers. After multiple development of the plate a 10mm separation of the two isomers was obtained and material co-chromatographing with epoxide and 2-trans epoxide was eluted separately with ethanol. An aliquot of each eluate was counted, the radioactivity is given in Table 18.

Ten green tomato fruit (total fresh weight, 124g) were each injected with 10 $\mu$ l of an 0.1ml ethanol solution containing 4.43 $\mu$ mole  $(\pm)$ -[2-<sup>14</sup>C] 2-trans epoxide (1.3mCi/mmole;  $12.19 \times 10^6$  d.p.m.). The tomatoes were incubated in darkness at 20°C for 48 hours and then macerated in 1 litre methanol containing 156 $\mu$ g  $(\pm)$ -ABA, and thoroughly extracted with 2.5 litres of a methanol:water (4:1, v/v) mixture.

Identification of one of the radioactive acids as abscisic acid was made according to the criteria described earlier. Radioactivity co-chromatographing with authentic ABA and its authentic derivatives is shown in Table 19.

Table 18. Purification of ( $\pm$ )-[2- $^{14}$ C] 2-trans epoxide (1.3mCi/mmole) by addition of unlabelled epoxide and t.l.c. separation of the isomers. Radioactivity chromatographing with marker epoxide and its 2-trans-isomer. The efficiency of counting was 66.3% and the background was 22.0 c.p.m.

Source of material	Chromatographic solvent used	c.p.m. in subsample	d.p.m. in total sample
Zone on t.l.c. plate adjacent to marker epoxide	benzene-ethyl acetate-acetic acid;	2,117	886,630
Zone on t.l.c. plate adjacent to marker 2- <u>trans</u> -epoxide	(75:10:1, v/v)	28,540	37,832,450

Table 19. Conversion of  $(\pm)$ -[2- $^{14}\text{C}$ ]2-trans epoxide (1.3mCi/mmole, 12.19 x 10<sup>6</sup> d.p.m.) by 10 green tomato fruit (total weight 124g) incubated at 20°C, in darkness for 48 hours. Radioactivity chromatographing with ABA and its authentic derivatives. The efficiency of counting was 69.6% and the background was 23.1 c.p.m.

Source of material	Chromatographic solvents used	c.p.m. in subsample	d.p.m. in total sample	(+)-ABA by R.D. method, $\mu\text{g}$	Specific activity of ABA d.p.m./ $\mu\text{g}$
Acid fraction, zone on t.l.c. plate adjacent to marker ABA	benzene-ethyl acetate-acetic acid (75:10:1, v/v)	259.0	62,551		
Sample from 1st t.l.c. adjacent to marker ABA	propan-1-ol-butan-1-ol-aq. ammonia (sp.gr.0.88)-water (6:2:2:1, v/v)	174.0	47,800		
Methylated sample from 2nd t.l.c., zone adjacent to marker MeABA	hexane-ethyl acetate (1:1, v/v)	22.4	1,368 (5124 - R.D. value)	97.1	52.8
Sample from 3rd t.l.c. treated with aq. methanolic $\text{NaBH}_4$ solution, zone on t.l.c. plate adjacent to marker:- a) <u>cis</u> -1',4'-diol MeABA b) <u>trans</u> -1',4'-diol MeABA	hexane-ethyl acetate (1:1, v/v)	58.3 78.4	167.4 225.4		

Materials from the ether-soluble-acid fraction which had co-chromatographed with authentic epoxide and with 2-trans epoxide in a benzene-ethyl acetate-acetic acid (75:10:1, by vol.) solvent system (Table 19) were eluted from the gel with ethanol. The profiles of the u.v. absorption spectra of the eluates corresponded with the profiles of the u.v. absorption spectra of authentic epoxide and authentic 2-trans epoxide respectively; aliquots of the eluates were assayed for [ $^{14}\text{C}$ ] activity, the data is given in Table 20. Methylation of these materials and t.l.c. in a hexane-ethyl acetate (1:1, v/v) solvent system, produced radioactive material which co-chromatographed with the respective authentic methyl esters of epoxide and 2-trans epoxide.

4.43 $\mu\text{mole}$  ( $\pm$ )-[2- $^{14}\text{C}$ ] 2-trans epoxide (1.3mCi/mmole,  $12.19 \times 10^6$  d.p.m.) incubated with 124g of tomatoes for 48 hours at 20°C in darkness gave 1.9nmole (5,124 d.p.m.) ABA which represents 0.042% incorporation. 0.5% of the total (+)-ABA extracted was derived from the precursor, 25.92 $\mu\text{g}$  (+)-ABA were extracted and the extraction was 26.7% efficient, as measured by the racemate dilution method, at the MeABA stage of purification. This represents 776.8 $\mu\text{g}$  (+)-ABA/kg fresh weight tomato fruit.

To confirm that the conversion of epoxide to ABA in green tomato fruit was brought about by enzymes and could not occur in denatured tissues ( $\pm$ )-[2- $^{14}\text{C}$ ]epoxide (1.3mCi/mmole) and ( $\pm$ )-[2- $^{14}\text{C}$ ] 2-trans epoxide (1.3mCi/mmole) were incubated with tomato fruit which had been boiled.

Table 20. Radioactivity chromatographing with epoxide and with 2-trans epoxide after incubation of tomato fruit (total weight 124g) with  $(\pm)$ -[2- $^{14}\text{C}$ ] trans epoxide (1.3mCi/mmole,  $12.19 \times 10^6$  d.p.m.). The acid fraction was chromatographed on a Merck F254 precoated plate (see Table 19). The efficiency of counting was 66.3% and the background was 23.0 c.p.m.

Source of material	Chromatographic solvents used	c.p.m. in subsample	d.p.m. in total sample
Acid fraction, zone on t.l.c. plate adjacent to marker epoxide	benzene-ethyl acetate-acetic acid (75:10:1, v/v)	101.0	22,106
Acid fraction, zone on t.l.c. plate adjacent to marker 2-trans-epoxide		601.0	128,906



- (d) Incubation of boiled tomato fruit (97.3g fresh weight)  
with  $(\pm)$ -[2- $^{14}$ C]epoxide (1.3mCi/mmole) in darkness for  
48 hours at 20°C.

Experiment commenced on 28th April, 1969.

Tomato fruit were boiled in a steam bath for 5 hours and then allowed to cool to room temperature, the cuticles of the tomatoes remained intact.

Ten tomatoes (total fresh weight 97.3g) were each injected with 10 $\mu$ l of an ethanol solution containing 20.9 $\mu$ mole/ml  $(\pm)$ -[2- $^{14}$ C] epoxide (1.3mCi/mmole,  $5.75 \times 10^6$  d.p.m.). The  $(\pm)$ -[2- $^{14}$ C]epoxide was freed of contamination with 2-trans epoxide according to methods described in experiment (ii)(b). The tomatoes remained intact after injection and were incubated in darkness at 20°C for 48 hours. The tomatoes were macerated in 1 litre methanol containing 156 $\mu$ g  $(\pm)$ -ABA and thoroughly extracted with 2 litres of a methanol:water (4:1, v/v) mixture. Radioactivity co-chromatographing with authentic ABA and its authentic derivatives is shown in Table 21.

The distribution of radioactivity on the first chromatogram of the ether-soluble-acid fraction (Table 21) indicated than an appreciable amount of radioactivity had co-chromatographed with marker epoxide and with marker 2-trans epoxide. The labelled materials co-chromatographing with marker epoxide and with 2-trans epoxide were eluted from the gel with ethanol, the profiles of the u.v. absorption spectra of the eluates corresponded with the profiles of the u.v. absorption spectra of epoxide and 2-trans epoxide, respectively. An aliquot of each eluate was taken for counting (Table 22).

Table 21. Incubation of 10 boiled green tomato fruit (total fresh weight 97.3g) with  $(\pm)$ -[2- $^{14}$ C]epoxide (1.3mCi/mole,  $5.75 \times 10^6$  d.p.m.) at 20°C in darkness for 48 hours. Radioactivity chromatographing with ABA and its authentic derivatives. The efficiency of counting was 52.7% and the background was 53.8 c.p.m.

Source of material	Chromatographic solvents used	c.p.m. in subsample	d.p.m. in total sample	(+)-ABA by R.D. method, $\mu$ g	Specific activity of ABA d.p.m./ $\mu$ g
Acid fraction, zone on t.l.c. plate adjacent to marker ABA	benzene-ethyl acetate-acetic acid (75:10:1, v/v)	1998	303,300		
Sample from 1st t.l.c. adjacent to marker ABA	propan-1-ol-butanol-1-ol-aq. ammonia (sp.gr.0.88)-water (6:2:2:1, v/v)	1565	259,844		
Methylated sample from 2nd t.l.c., zone adjacent to marker MeABA	hexane-ethyl acetate 1:1, v/v	15.8	449.7 (2196 - R.D. value)	24.6	88.74
Sample from 3rd t.l.c. treated with aq. methanolic $\text{NaBH}_4$ solution. Zone on t.l.c. plate adjacent to marker:- a) <u>cis</u> -1',4'-diol MeABA b) <u>trans</u> -1',4'-diol MeABA	hexane-ethylacetate (1:1, v/v)	16.8 22.3	63.8 84.6		

Table 22. Radioactivity chromatographing with, epoxide, 2-trans-epoxide and the 1',2'-dihydroxy derivative of epoxide after incubation of boiled tomato fruit with (±)-[2-<sup>14</sup>C]-epoxide (1.3mCi/mmole, 5.75 x 10<sup>6</sup> d.p.m.). The acid fraction was chromatographed on a Merck F<sub>254</sub> precoated plate. The efficiency of counting was 52.7% and the background was 53.8 c.p.m.

Source of material	Chromatographic solvent used	c.p.m. in subsample	d.p.m. in total sample
Acid fraction, zone on t.l.c. plate adjacent to marker epoxide	benzene-ethyl acetate-acetic acid; (75:10:1, v/v)	10,760	2,367,670
Acid fraction, zone on t.l.c. plate adjacent to marker 2-trans-epoxide		98.0	28,270
Acid fraction, zone on t.l.c. plate adjacent to marker 1',2'-dihydroxy derivative of epoxide		1980	200,000

To confirm their identity, a sample of each compound which had been eluted from the plate was methylated. The radioactivity co-chromatographed with the respective authentic methyl ester and the profiles of their u.v. spectra corresponded with the profiles of the u.v. spectra of authentic methyl esters.

When the t.l.c. plate on which the ether-soluble-acid fraction was separated was viewed under u.v. light a faint band was detected between ABA and the epoxide (Table 23).

Table 23.  $R_f$  of ABA and related compounds from the ether-soluble acid fraction, isolated from boiled tomatoes, on Merck precoated F<sub>254</sub> plate developed in benzene-ethyl acetate-acetic acid (75:10:1, by vol.).

Compound	$R_f$
ABA	0.13
<u>t</u> -ABA	0.19
1',2'-dihydroxy derivative of epoxide	0.24
epoxide	0.45
2- <u>trans</u> epoxide	0.55

This band was eluted, and the u.v. spectrum of the eluate suggested that the material was the 1',2'-dihydroxy derivative of epoxide (Table 22). On methylation the labelled material co-chromatographed with authentic methyl ester of 1',2'-dihydroxy derivative of epoxide in a hexane-ethyl acetate (1:1, v/v) solvent system. Autoradiography of the t.l.c. plate on which the methyl esters had been separated

showed that the majority of radioactivity was located in the material co-chromatographing with the authentic methyl ester, Fig. 16.

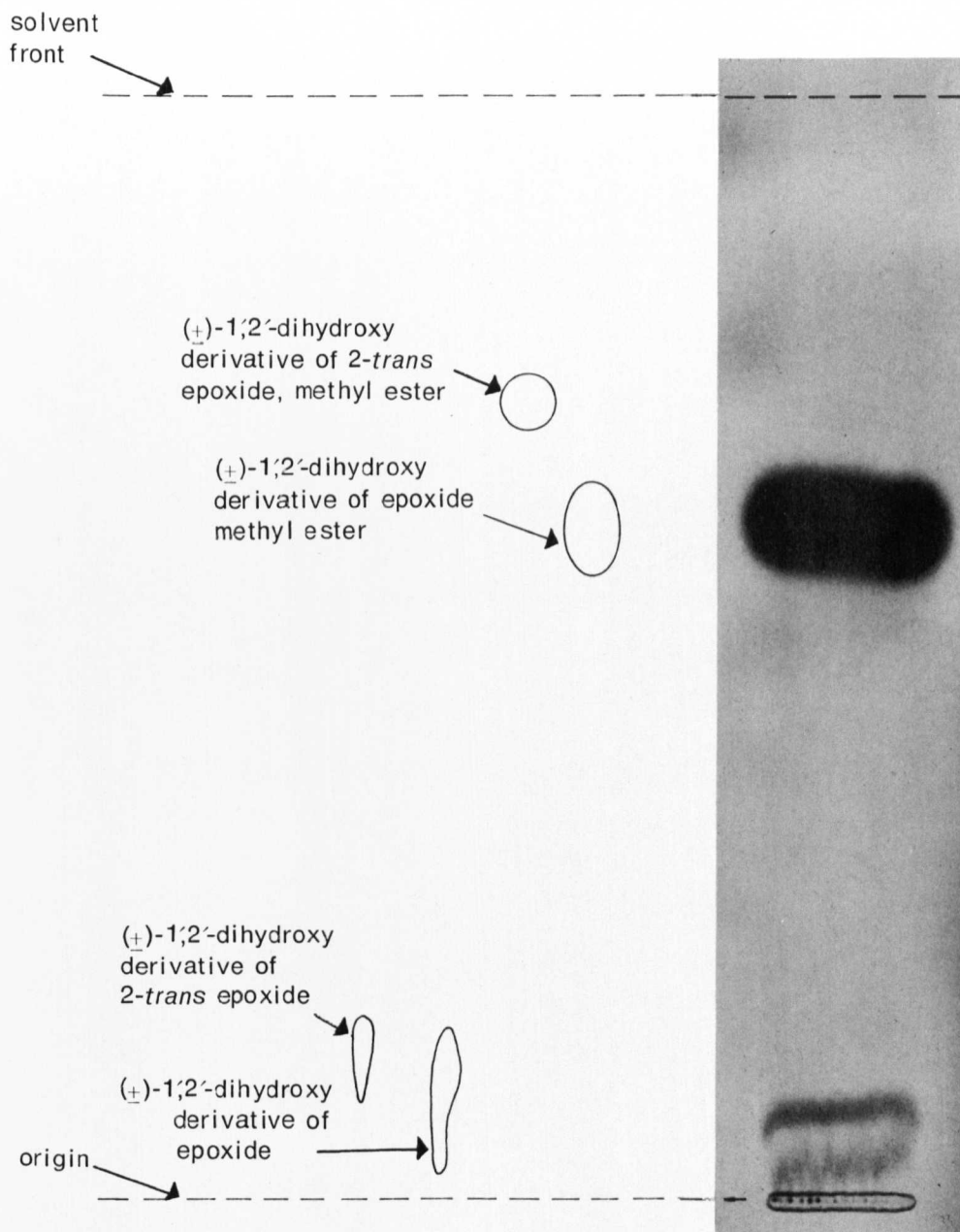
2.09  $\mu$ mole ( $\pm$ )-[2- $^{14}$ C]epoxide (1.3mCi/mmole,  $5.75 \times 10^6$  d.p.m.) incubated with 97.3g (fresh weight) of boiled tomato fruit for 48 hours in darkness at 20°C gave 0.76nmole (2196 d.p.m.) ABA which represents 0.038% incorporation. 3.84  $\mu$ g (+)-ABA were extracted and the extraction was 15.6% efficient, as measured by the racemate dilution method, at the MeABA stage of purification. This represents 252.8  $\mu$ g (+)-ABA/kg fresh weight tomato fruit.

(e) Incubation of boiled tomato fruit (96.2g fresh weight) with ( $\pm$ )-[2- $^{14}$ C] 2-trans epoxide (1.3mCi/mmole) in darkness for 48 hours at 20°C.

Experiment commenced 28th April, 1969.

Boiled tomato fruit were prepared as for experiment (d). Ten tomatoes (total fresh weight 96.2g) were each injected with 10  $\mu$ l of an ethanol solution containing 23.3  $\mu$ mole/ml ( $\pm$ )-[2- $^{14}$ C] 2-trans epoxide (1.3mCi/mmole,  $6.41 \times 10^6$  d.p.m.) which had been freed of contamination with the alternate isomer.

After 48 hours incubation, in darkness at 20°C, the tomatoes were macerated in 1 litre methanol containing 156  $\mu$ g ( $\pm$ )-ABA and thoroughly extracted with 2 litres of a methanol:water (4:1, v/v) mixture. Radioactivity co-chromatographing with authentic ABA and its authentic derivatives is shown in Table 24. Material from the ether-soluble-acid fraction was found to co-chromatograph with marker epoxide and with marker 2-trans epoxide on the first chromatogram (Table 24). These materials were eluted separately



**Fig 16** Incubation of  $(\pm)$ -[2- $^{14}\text{C}$ ]epoxide (1.3 m.Ci/m.mole) with boiled tomato fruit. Radioactivity from the ether soluble acid fraction chromatographed with marker 1,2'-dihydroxy derivative of epoxide in a benzene-ethyl acetate-acetic acid (75:10:1, v/v) solvent system. On methylation, the labelled material co-chromatographed with authentic methyl ester of the 1,2'-dihydroxy derivative of epoxide in a hexane-ethyl acetate (1:1, v/v) solvent system, as shown by autoradiography of the t.l.c. plate after development.

Table 24. Incubation of ten boiled green tomato fruit (total fresh weight 96.2g) with  $(\pm)$ -[2- $^{14}\text{C}$ ]-2-trans epoxide (1.3mCi/mole,  $6.41 \times 10^6$  d.p.m.) at 20°C in darkness for 48 hours. Radioactivity chromatographing with ABA and its authentic derivatives. The efficiency of counting was 52.7% and the background 53.8 c.p.m.

Source of Material	Chromatographic solvents used	c.p.m. in subsample	d.p.m. in total sample	(+)-ABA by R.D. method, $\mu\text{g}$	Specific activity of ABA, d.p.m./ $\mu\text{g}$
Acid fraction, zone on t.l.c. plate adjacent to marker ABA	benzene-ethyl acetate-acetic acid (75:10:1, v/v)	69.0	22,913		
Sample from 1st t.l.c., zone on t.l.c. plate adjacent to marker ABA	propan-1-ol-butan-1-ol-aq. ammonia (sp.gr.0.88) -water (6:2:2:1, v/v)	70.0	10,626		
Methylated sample from 2nd t.l.c., zone on t.l.c. plate adjacent to marker MeABA	hexane-ethyl acetate (1:1, v/v)	10.0	206.5 (918.0 - R.D. value)	27.3 (R.D. value)	33.6
Sample from 3rd t.l.c. treated with aq. methanolic $\text{NaBH}_4$ solution, zone on t.l.c. plate adjacent to:- a) <u>cis</u> -1',4'-diol MeABA b) <u>trans</u> -1',4'-diol MeABA	hexane-ethyl acetate (1:1, v/v)	13.7 17.0	52.0 64.5		

and their u.v. spectra were identical with the u.v. spectra of authentic epoxide and 2-trans epoxide. An aliquot of each eluate was taken for [ $^{14}\text{C}$ ] assay (Table 25). On methylation they co-chromatographed with the authentic methyl esters when developed in a hexane-ethyl acetate (1:1, v/v) solvent system and autoradiography of the t.l.c. plates on which the methyl esters were separated showed that the radioactivity coincided with the methyl ester.

[ $^{14}\text{C}$ ]-Material from the ether-soluble-acid fraction was found to chromatograph between ABA and the epoxide on the first chromatogram (Table 24). This band was eluted and the profile of its u.v. spectrum was identical with that of the 1',2'-dihydroxy derivative of 2-trans epoxide, an aliquot of the eluate was counted (Table 25). On methylation, the material co-chromatographed with authentic methyl ester of the 1',2'-dihydroxy derivative when chromatographed in a hexane-ethyl acetate (1:1, v/v) solvent system. Autoradiography of this t.l.c. plate showed that the radioactivity coincided with the position of the methyl ester of the 1',2'-dihydroxy derivative.

2.33 $\mu\text{mole}$  ( $\pm$ )-[2- $^{14}\text{C}$ ] 2-trans epoxide (1.3mCi/mmole,  $6.41 \times 10^6$  d.p.m.) incubated with 96.2g (fresh weight) tomato fruit for 48 hours in darkness at 20°C gave 0.34nmole (918 d.p.m.) ABA which represents 0.014% incorporation. 6.15 $\mu\text{g}$  (+)-ABA were extracted and the extraction was 22.5% efficient, as measured by the racemate dilution method, at the MeABA stage of purification. This represents 283.3 $\mu\text{g}$  (+)-ABA/kg fresh weight of tomato fruit.



Table 25. Radioactivity chromatographing with, epoxide, 2-trans epoxide and the 1',2'-dihydroxy derivative of 2-trans epoxide after incubation of boiled tomato fruit with ( $\pm$ )-[2-<sup>14</sup>C]-2-trans epoxide (1.3mCi/mmole, 6.41 x 10<sup>6</sup> d.p.m.). The acid fraction was chromatographed on a Merck F254 precoated plate. The efficiency of counting was 52.7% and the background was 55.4 c.p.m.

Source of material	Chromatographic solvent used	c.p.m. in subsample	d.p.m. in total sample
Acid fraction, zone on t.l.c. plate adjacent to marker epoxide	benzene-ethyl acetate-acetic acid, (75:10:1, v/v)	1950	600,000
Acid fraction, zone on t.l.c. plate adjacent to marker 2-trans epoxide		3226	1,054,654
Acid fraction, zone on t.l.c. plate adjacent to marker 1',2'-dihydroxy derivative of 2-trans epoxide		8872	1,492,890

In the preceding experiments, (b) - (e), it was found that the  $(\pm)$ -[2-<sup>14</sup>C] 2-trans epoxide was converted to its 1',2'-dihydroxy derivative, (Table 1, XXXI), in boiled tomato fruit. The  $(\pm)$ -[2-<sup>14</sup>C]epoxide was converted to its 1',2'-dihydroxy derivative, (Table 1, XI), in boiled ██████ tomato fruit. As these 1',2'-dihydroxy products were also formed from the epoxides by treatment with dilute mineral acids, the large amounts found in boiled fruit were probably formed by non-enzymic reactions. The 1',2'-dihydroxy derivative is not inhibitory to coleoptile extension growth (Tamura & Nagao, 1969(b)) and it was unlikely, therefore, to be an intermediate in the series of reactions between the epoxide and ABA.

Results are presented here of two experiments involving the incubation of green tomato fruit with the  $(\pm)$ -[2-<sup>14</sup>C] 1',2'-dihydroxy derivative of epoxide (1.3mCi/mmole) and the  $(\pm)$ -[2-<sup>14</sup>C] 1',2'-dihydroxy derivative of 2-trans epoxide (1.3mCi/mmole).

(f) Incubation of  $(\pm)$ -[2-<sup>14</sup>C] 1',2'-dihydroxy derivative of epoxide (1.3mCi/mmole) with 5 tomato fruit (total weight 82.7g) for 48 hours in darkness at 20°C.

Experiment commenced 20th February, 1970).

Five green tomato fruit (total fresh weight 82.7g) were each injected with 20μl of an ethanol solution containing 677nmole/ml  $(\pm)$ -[2-<sup>14</sup>C] 1',2'-dihydroxy derivative of epoxide (1.3mCi/mmole,  $2.0 \times 10^5$  d.p.m.). After 48 hours incubation in darkness at 20°C the tomatoes were macerated in methanol containing 78μg  $(\pm)$ -ABA and thoroughly extracted with 2 litres of a methanol:water (4:1, v/v)

mixture. Radioactivity from the ether-soluble-acid fraction co-chromatographing with authentic ABA and its authentic derivatives is shown in Table 26.

The distribution of radioactivity, along the first chromatogram, developed in propan-1-ol-butan-1-ol-aq. ammonia (sp.gr. 0.88)-water (6:2:2:1, by vol.), from the ether-soluble acid fraction of tomatoes incubated with  $(\pm)$ -[2- $^{14}$ C] 1',2'-dihydroxy derivative of epoxide (1.3mCi/mmole) is shown in Figure 17. Material co-chromatographing with marker ABA was eluted from the plate and chromatographed in a toluene-ethyl acetate-acetic acid (75:10:1, by vol.) solvent system, the distribution of radioactivity along the chromatogram is shown in Figure 18. 67.7nmole  $(\pm)$ -[2- $^{14}$ C] 1',2'-dihydroxy derivative of epoxide (1.3mCi/mmole,  $2.0 \times 10^5$  d.p.m.) incubated with 82.7g of tomato fruit for 48 hours in darkness at 20°C gave 50.4pmole (148 d.p.m.) ABA which represents 0.074% incorporation. 8.3µg (+)-ABA were extracted and the extraction was 23.0% efficient as measured by the racemate dilution method, at the MeABA stage of purification. This represents 436.5µg (+)-ABA/kg fresh weight tomato fruit.

(g) Incubation of  $(\pm)$ -[2- $^{14}$ C] 1',2'-dihydroxy derivative of 2-trans epoxide with 5 green tomato fruit (total fresh weight 88.0g) for 48 hours in darkness at 20°C.

Experiment commenced 20th February, 1970.

Five green tomato fruit (total fresh weight 88.0g) were each injected with 20µl of an ethanol solution containing 759nmole/ml  $(\pm)$ -[2- $^{14}$ C] 1',2'-dihydroxy derivative of 2-trans epoxide 1.3mCi/mmole,  $2.24 \times 10^5$  d.p.m.). After 48 hours incubation in darkness at 20°C

Table 26. Incubation of 5 green tomato fruit (total fresh weight 82.7g) with  $(\pm)$ -[2- $^{14}\text{C}$ ] 1',2'-dihydroxy derivative of epoxide (1.3mCi/mmole,  $2.0 \times 10^5$  d.p.m.) at 20°C in darkness for 48 hours. Radioactivity chromatographing with ABA and its authentic derivatives. The efficiency of counting was 78.7% and the background was 20.2 c.p.m.

Source of material	Chromatographic solvents used	c.p.m. in subsample	d.p.m. in total sample	(+)-ABA by R.D. method, $\mu\text{g}$	Specific activity of ABA d.p.m./ $\mu\text{g}$
Acid fraction, zone on t.l.c. plate adjacent to marker ABA	propan-1-ol-butan-1-ol-aq. ammonia (sp. gr. 0.88)-water (6:2:2:1, by vol.)	NOT COUNTED			
Sample from 1st t.l.c. zone on t.l.c. plate adjacent to marker ABA	toluene-ethyl acetate-acetic acid (75:10:1, by vol.)	31.5	416.8		
Methylated sample from 2nd t.l.c. plate, zone on t.l.c. plate adjacent to marker MeABA	hexane-ethyl acetate, 1:1, v/v)	6.7	34.0 (148.0 - R.D. value)	36.1	10

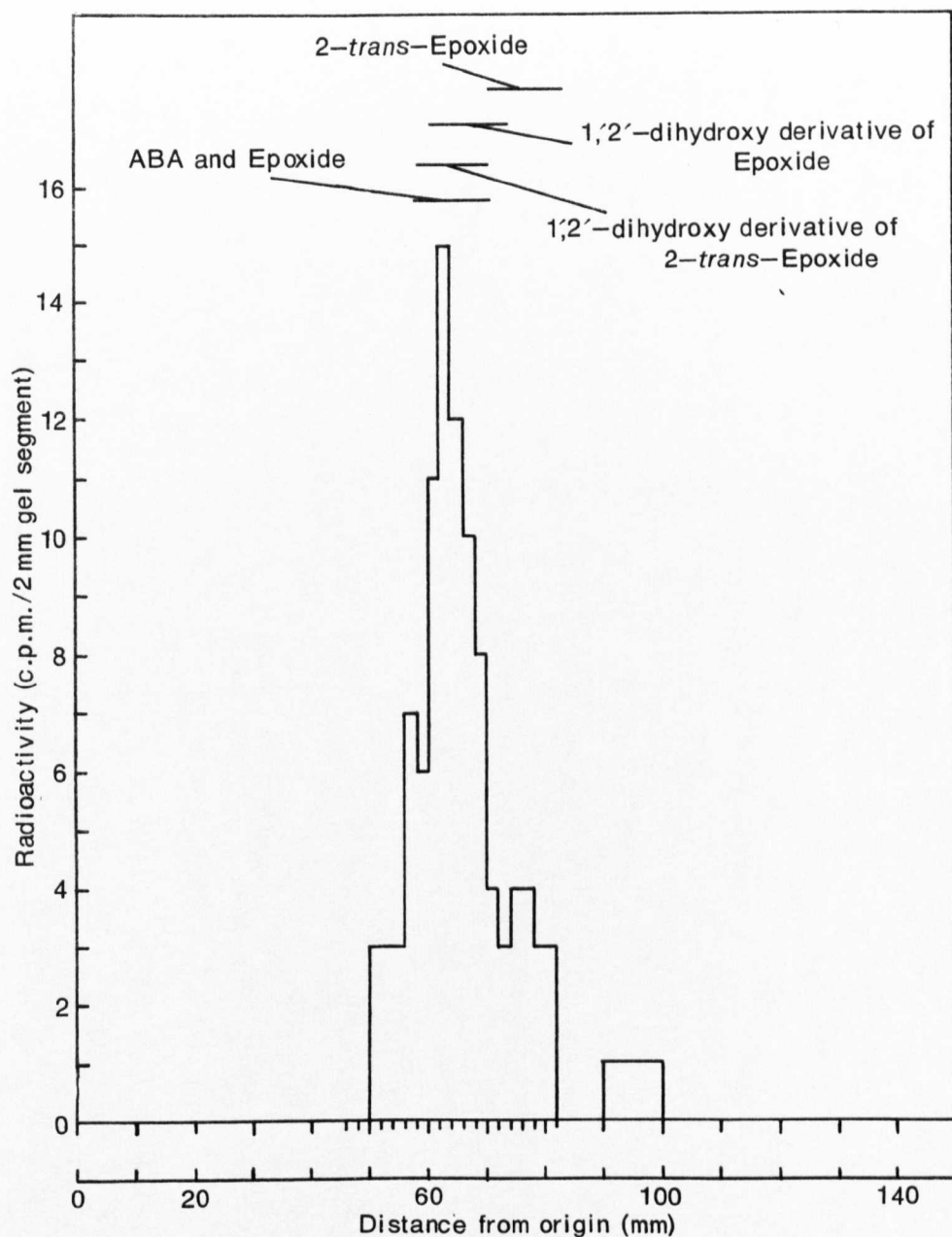
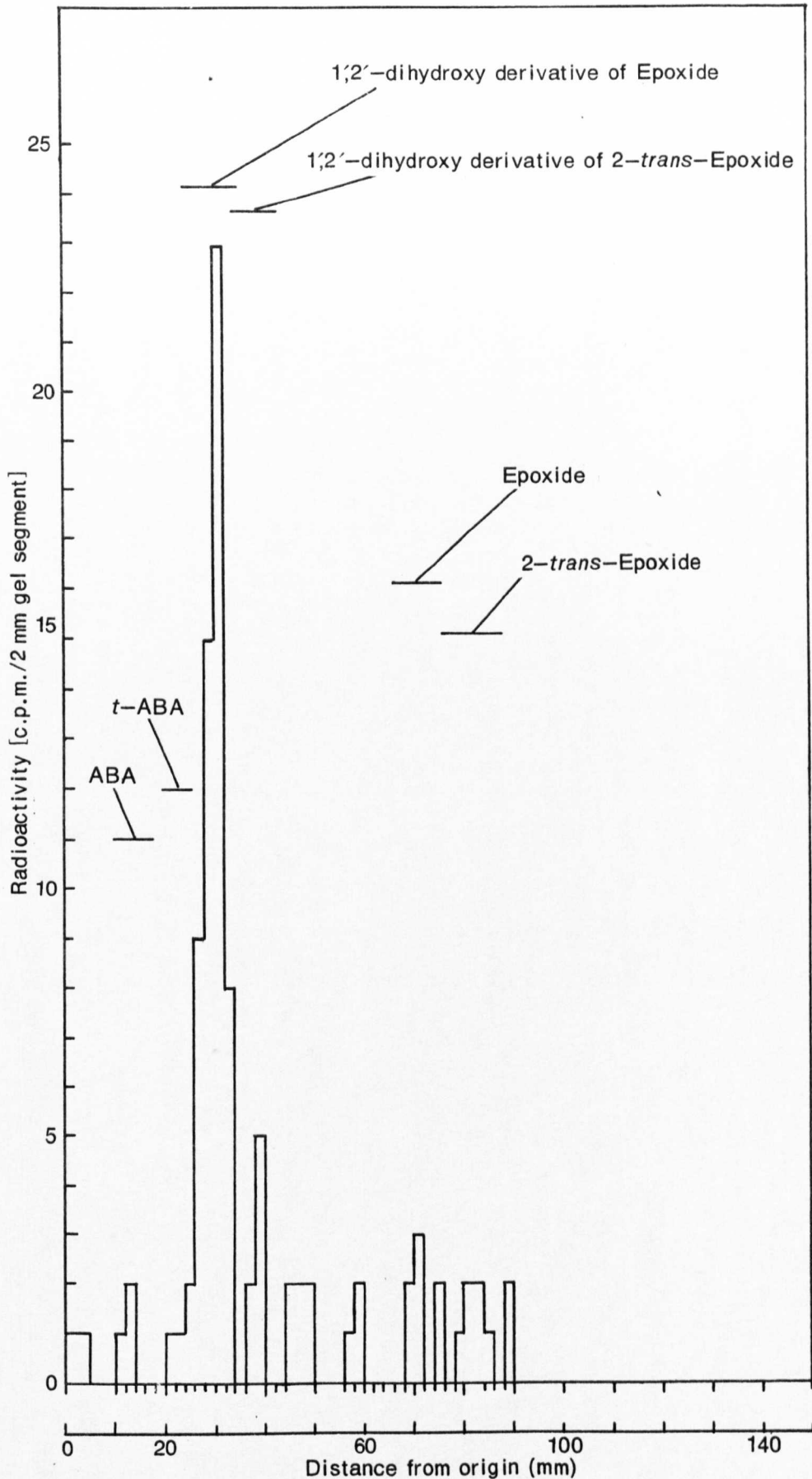


Fig 17. Histogram of the distribution of [ $^{14}\text{C}$ ] from (+)-[2- $^{14}\text{C}$ ] 1,2'-dihydroxy derivative of epoxy (1.3 mCi/m mole) in the ether-soluble acid fraction from tomato fruit. The extract was chromatographed in propan-1-ol-butan-1-ol-aq. ammonia (sp. gr. 0.88)-water (6:2:2:1, by vol) on a precoated silica-gel F<sub>254</sub> t.l.c. plate. The silica gel was put in the scintillation solution and counted.



**Fig 18.** Incubation of (+)-[2-<sup>14</sup>C] 1,2'-dihydroxy derivative of epoxide in tomato fruit. The zone on the t.l.c. plate in Fig 17, corresponding with ABA was eluted with methanol and chromatographed in toluene-ethyl acetate-acetic acid (75:10:1, by vol).

the tomatoes were macerated in 1 litre methanol containing 78 $\mu$ g ( $\pm$ )-ABA and thoroughly extracted with 2 litres of a methanol:water (4:1, v/v) mixture. Radioactivity co-chromatographing with authentic ABA and its authentic derivatives is given in Table 27.

No radioactivity was found to chromatograph with marker MeABA (Table 27), therefore the 1',2'-dihydroxy derivative of 2-trans epoxide was not incorporated into ABA. 8.4 $\mu$ g (+)-ABA were extracted and the extraction was 31.1% efficient, as measured at the MeABA stage of purification. This represents 308.0 $\mu$ g (+)-ABA/kg fresh weight tomato fruit.

(h) Incorporation of ( $\pm$ )-[2-<sup>3</sup>H]mevalonic acid and ( $\pm$ )-[2-<sup>14</sup>C] epoxide into abscisic acid by cut wheat shoots.

Experiment commenced on 23rd October, 1969.

(This experiment was performed in collaboration with Dr. B. V. Milborrow, Milstead Laboratory, Shell Research Limited, Sittingbourne, Kent.)

In experiment (i)(d) it was shown that wheat plants previously supplied with ( $\pm$ )-[2-<sup>3</sup>H]MVA incorporated more of the labelled precursor into ABA when wilted than did wheat shoots which were kept turgid. These results suggested a novel way of investigating whether the conversion of epoxide (X) into ABA was regulated in the same way as the pathway by which ABA is made in vivo. In this experiment the ( $\pm$ )-[2-<sup>3</sup>H]MVA and the ( $\pm$ )-[2-<sup>14</sup>C] epoxide were supplied to wheat shoots either alone or as a mixture containing [<sup>3</sup>H] and [<sup>14</sup>C] labelled material. The labelled material was dissolved in 0.01M potassium phosphate buffer, pH 7.3, the shoots were placed in the solution and allowed to take up the labelled

Table 27. Incubation of 5 green tomato fruit (total fresh weight<sub>5</sub> 88.0g) with (±)-[2-<sup>14</sup>C] 1',2'-dihydroxy derivative of 2-trans epoxide (1.3mCi/mmole, 2.24 x 10<sup>5</sup> d.p.m.) at 20°C in darkness for 48 hours. Radioactivity chromatographing with ABA and its authentic derivatives. The efficiency of counting was 78.7% and the background was 20.0 c.p.m.

Source of material	Chromatographic solvents used	c.p.m. in subsample	d.p.m. in total sample	(+)-ABA, by R.D. method, µg	Specific activity of ABA, d.p.m./µg
Acid fraction, zone on t.l.c. plate adjacent to marker ABA	propan-1-ol-butan-1-ol-aq. ammonia (sp.gr. 0.88) water (6:2:2:1, v/v)	NOT COUNTED	NOT COUNTED		
Sample from 1st t.l.c., zone on t.l.c. plate adjacent to marker ABA	toluene-ethyl acetate-acetic acid (75:10:1, v/v)				
Methylated sample from 2nd t.l.c. plate, zone on t.l.c. plate adjacent to marker MeABA	hexane-ethyl acetate, (1:1, v/v)	0	0	27.1	



precursor(s) for 2 hours at room temperature. The amount of each precursor taken up during this period is shown in Table 28. The plants were then removed from the solution, the submerged parts rinsed with water and blotted. In each of the 4 separate experiments, i.e. control (no labelled precursor supplied), wheat shoots supplied with  $(\pm)$ -[2-<sup>3</sup>H]MVA, wheat shoots supplied with a mixture of  $(\pm)$ -[2-<sup>3</sup>H]MVA and  $(\pm)$ -[2-<sup>14</sup>C]epoxide, and wheat shoots supplied with  $(\pm)$ -[2-<sup>14</sup>C]epoxide approximately half of the batch was placed with the cut ends in water and kept in a damp atmosphere at 20°C. The other half of the batch was spread out on filter paper and exposed to a draught of air for 30 minutes and then covered with transparent polythene. The plants were harvested after 6 hours. The wilted plants were found to have lost 29% of their original fresh weight. Small sub-samples of two batches of wheat shoots, one previously supplied with  $(\pm)$ -[2-<sup>3</sup>H]MVA the other with  $(\pm)$ -[2-<sup>14</sup>C]epoxide were not harvested after 6 hours and were kept in a wilted condition for a further 18 hours (a total of 24 hours wilt).

The identification of one of the radioactive acids, in the ether-soluble acid fractions, as ABA, was performed according to previously described methods. Labelled material from each of the ether-soluble acid fraction co-chromatographed with authentic ABA in two different solvent systems, one basic the other acidic, on methylation the labelled material co-chromatographed with authentic MeABA in a hexane-ethyl acetate (1:1, v/v) solvent system. In each case the labelled material chromatographing with MeABA was eluted from the t.l.c. gel with methanol. The profile

Table 28. Incorporation of  $(\pm)$ -[2- $^3\text{H}$ ]MVA and  $(\pm)$ -[2- $^{14}\text{C}$ ]epoxide into ABA by cut wheat shoots. The plants were treated as in experiment (i) (d) except that the wilted batches lost 29% of their original weight during 6 hours of treatment and ethanol was omitted. Small subsamples of two, marked\*, were kept for a further 18 hours before extraction (24 hr.).  $10.8\mu\text{M}$   $(\pm)$ -[2- $^3\text{H}$ ]MVA ( $93\text{mCi}/\text{mM}$ ) was supplied as the potassium salt, as was  $(\pm)$ -[2- $^{14}\text{C}$ ]epoxide ( $1.3\text{mCi}/\text{mM}$ ). Incorporation data are calculated from the amount of racemate taken up by the plants and assuming that 4 [ $^3\text{H}$ ] atoms from MVA are incorporated into one molecule of ABA. The relatively small amounts of the precursors incorporated in the 6 hr. part of this experiment are attributed to their slow rates of penetration into the cells.

Treatment	original fresh wt., g.	Weight absorbed, [ $^3\text{H}$ ] $\mu\text{g.}$ [ $^{14}\text{C}$ ]	( $\pm$ )-ABA extracted $\mu\text{g.}/\text{kg}$	(+)-ABA by R.D. method $\mu\text{g.}/\text{kg}$	d.p.m. in ABA after R.D. correction [ $^3\text{H}$ ] [ $^{14}\text{C}$ ] /sample	% incorporation MVA Epoxide	% of ABA formed from precursor MVA Epoxide
Wet control	56.6	-	1.1	19.4	-	-	-
Wilted control	43.8	-	29.2	670	-	-	-
Wet+MVA	33.1	7.72	0.37	15.1	280	0.000262	0.049
Wilted+ MVA*	57	6.84	29.6	520	6771	0.0072	0.0015
* 24 hr	1.4	0.168	-	-	138 (not R.D.)	0.054	-
Wet+MVA+ Epoxide	53.5	11.18	0.62	15.0	310	0.000192	0.033
Wilted+MVA +Epoxide	37.2	9.99	22.1	590	1302	0.0094	0.0038
Wet+ Epoxide	57.0	-	1.7	31.6	-	-	-
Wilted+ Epoxide*	57.0	-	23.7	415	-	-	-
*24 hr	2.0	-	-	-	(not R.D.)	36	-

of its u.v. absorption spectrum was identical with that of authentic MeABA and the profile of its o.r.d. spectrum identical with that of (+)-MeABA. In the first experiment with wilted wheat there was nine times as much label in ABA from the wilted leaves as in the turgid ones whereas there was about seventeen times as much (+)-ABA. This could arise by the conversion of a small amount of unlabelled precursor into ABA, by the failure of the [ $^3\text{H}$ ]MVA to penetrate to the site of synthesis of ABA before the wilt stimulated rapid synthesis of ABA or by the conversion of the [ $^3\text{H}$ ]MVA to other products before the commencement of the rapid synthesis of ABA. The subsamples of the wilted leaves were kept for a longer period to investigate these possibilities. The higher percentage incorporation with time suggests that the mevalonic acid had failed to reach the sites of ABA biosynthesis during the period of pretreatment.

Discussion of results. ( $\pm$ )-[2- $^{14}\text{C}$ ]epoxide was converted to ABA by tomato fruit in 1.8% yield (or 3.6% of one enantiomer, if only one is utilized) and 15% of the ABA was derived from the precursor. Incubation of ( $\pm$ )-[2- $^{14}\text{C}$ ] 2-trans epoxide with tomato fruit resulted in conversion of the precursor to ABA in 0.042% yield and 0.5% of the ABA was derived from the precursor. The amount of 2-trans epoxide incorporated into ABA was so small, in comparison with the epoxide, that it may be attributed to photolytic isomerization during isolation or residual contamination of the starting material with ( $\pm$ )-[2- $^{14}\text{C}$ ]epoxide.

A solution of ( $\pm$ )-2-trans epoxide in dry acetone which had been exposed to sunlight for 21 days in a glass bottle was converted to a mixture containing 39.0% epoxide and 61.0% 2-trans epoxide, there were no other products. Thus light can photoisomerize the 2-trans double bond and although care was taken to exclude light as much as possible during the course of this experiment, the faint illumination which had to be used at times, eg. when removing the gel from the t.l.c. plate, could have been responsible for some photoisomerization.

The ( $\pm$ )-[2-<sup>14</sup>C]epoxide and ( $\pm$ )-[2-<sup>14</sup>C]-2-trans epoxide apparently incorporated into ABA after incubation with boiled tomato fruit, 0.038% and 0.014% respectively, were so small that they may be attributed to contamination of the extracted ABA with radioactive impurity. During the purification of the ABA, extracted from the boiled tomato fruit, the loss of radioactivity as the various derivatives were formed and purified was much more pronounced than during the purification of the ABA extracted from the living tomato fruits.

( $\pm$ )-[2-<sup>14</sup>C]epoxide and 2-trans epoxide were converted into their respective 1',2'-dihydroxy derivatives following incubation with boiled tomato fruit. These products were also formed by treatment of the epoxides with dilute sulphuric acid and the large amounts found in the boiled fruit were probably formed by non-enzymic acid catalysed hydrolysis.

( $\pm$ )-[2-<sup>14</sup>C] 1',2'-dihydroxy derivative of epoxide and 2-trans epoxide were not converted to ABA when incubated with tomato fruit.

The inhibitory activity of epoxide reported by Tamura and Nagao (1969(b)) may be explained by its conversion to ABA within the plant. The lack of inhibitory activity of 2-trans epoxide and the 1',2'-dihydroxy derivatives of epoxide and 2-trans epoxide, reported by the same workers, may be attributed to the fact that they are not converted to ABA.

It was observed that wilted wheat shoots, previously supplied with  $(\pm)$ -[2-<sup>3</sup>H]MVA for 2 hours, converted nine times as much MVA into ABA as did unwilted plants. Parallel determinations of the amount of (+)-ABA in the extracts showed that the concentration rose from less than 4 $\mu$ g in the unwilted wheat to 74 $\mu$ g/kg (original fresh weight) in the wilted wheat. Wilted wheat shoots previously supplied with  $(\pm)$ -[2-<sup>14</sup>C]epoxide were found to convert a greater proportion of epoxide into ABA than unwilted plants. This suggests that epoxide or a close derivative of it, is a natural intermediate because the amount incorporated is regulated in the same way as the endogenous biosynthesis of ABA. If the conversion were brought about by an adventitious series of reactions this parallelism would not be expected to occur. The response to an environmental factor of the endogenous synthesis of ABA (in this case wilting) and the incorporation of the epoxide indicates that a site of regulation of biosynthesis exists between epoxide and ABA.

- (j) Incorporation of  $(\pm)$ -[2- $^{14}\text{C}$ ; 1',2'- $^{18}\text{O}$ ]epoxide (24.4  $\mu\text{Ci}/\text{mmole}$   $^{14}\text{C}$ ; 33.2 atoms %  $^{18}\text{O}$ ) into abscisic acid by wheat leaves which were subjected to 4 hours wilting, in light at room temperature.

Experiment commenced 19th December, 1969.

The incorporation of [ $^{14}\text{C}$ ] labelled epoxide into ABA showed that the carbon skeleton of epoxide became the carbon skeleton of ABA. The fate of the oxygen of the epoxy-group was investigated using [1',2'- $^{18}\text{O}$ ] labelled epoxide (Methods, section (x)(a); mass spectral data - Appendix I, Fig. 22-25). Wilting wheat shoots were used for this experiment because previous experiments had shown that a large proportion of the ABA formed during wilting by plants which had been supplied with epoxide was derived from this precursor. Any [ $^{18}\text{O}$ ] labelled ABA formed from the [1',2'- $^{18}\text{O}$ ] epoxide under these conditions would not, therefore, be diluted with large amounts of endogenous [ $\text{O}^{16}$ ]ABA (Wright and Hiron, 1969). A mixture of  $(\pm)$ -[2- $^{14}\text{C}$ ]epoxide (33.2 atoms %  $^{18}\text{O}$ ) was used so that a comparison could be made between the amount of [ $^{18}\text{O}$ ] and [ $^{14}\text{C}$ ] derived from the epoxide which was present in the ABA. The amount of optically active ABA was determined spectropolarimetrically.

1,647  $\mu\text{g}$  [1',2'- $^{18}\text{O}$ ]epoxide were dissolved in 2.0ml ethanol containing 33.4  $\mu\text{g}$  [2- $^{14}\text{C}$ ]epoxide (1.3mCi/mmmole). After subsamples had been removed 1574  $\mu\text{g}$  of the  $(\pm)$ -[2- $^{14}\text{C}$ ; 1',2'- $^{18}\text{O}$ ] epoxide remained. This was made up to 2.5ml ethanol and then to 50ml with 0.01M potassium phosphate buffer, pH 7.3. The cut ends of 103g (fresh weight) of wheat shoots were immersed in the solution for 18 hours at 7°C. During this time they took up

Table 29.  $R_f$ s of a [ $^{14}\text{C}$ ] material (ABA) isolated from the ether soluble acid fraction of wheat shoots, which co-chromatographed with authentic ABA and authentic MeABA in several solvent systems. The  $R_{fs}$  of the epoxide are also given.

Solvent System	Sample	$R_f$
toluene-ethyl acetate-acetic acid, (80:10:0.5, by vol.)	ABA epoxide	0.06 0.40
propan-1-ol-butan-1-ol-aq. ammonia (sp.gr. 0.88)-water, (5:5:1:1, by vol.)	ABA epoxide	0.49 0.52
hexane-ethyl acetate (1:1, v/v)	MeABA Me epoxide	0.50 0.79
toluene-ethyl acetate-acetic acid, (80:10:0.5, by vol.)	MeABA Me epoxide	0.16 0.40

1127 $\mu$ g of the doubly labelled epoxide. The shoots were removed from the solution and wilted for 20 minutes during which time they lost 50% of their fresh weight. The shoots were kept at this stage of wilt for 3 hours and then immersed in 1 litre methanol (containing 10mg B.H.T.) and cut up into 1cm lengths. The shoots were thoroughly extracted with a further 2 litres of a methanol:water (4:1, v/v) mixture, containing 10mg B.H.T./litre.

One of the acids in the ether-soluble-acid fraction was identified as ABA by co-chromatography with authentic ABA and its authentic derivatives (Table 29) and by measurement of the o.r.d. spectrum and the u.v. spectrum of material which chromatographed at the same  $R_f$  as authentic MeABA.

The radioactivity of the material co-chromatographing with authentic MeABA in a toluene-ethyl acetate acetic acid (80:10:0.5 by vol.) solvent system, after methylation of the extracted labelled ABA, is given in Table 30.

Table 30. Conversion of ( $\pm$ )-[2- $^{14}$ C; 1',2'- $^{18}$ O]epoxide to ABA by 103g wheat shoots supplied with 1.13mg (24.4 $\mu$ Ci/mmmole [ $^{14}$ C], 33.2 atoms per cent [ $^{18}$ O]) epoxide. Radioactivity was detected at the same  $R_f$  as authentic MeABA after treatment of the extracted material with ethereal diazo-methane. The efficiency of counting was 78.1% and the background was 23.3 c.p.m.

Source of material	Chromatographic solvent used	c.p.m. in subsample	d.p.m. in total sample	Wt. of MeABA by $^{14}$ C, $\mu$ g
Material co-chromatographing with authentic MeABA	toluene-ethyl acetate-acetic acid, (80:10:0.5, by vol)	979.6	6421	30.48



Methylated ABA obtained from the wheat was assayed for [ $^{18}\text{O}$ ] using a combined gas chromatograph-mass spectrometer.

Unpublished results obtained at Milstead Laboratory by Dr. G. Ryback, Mr. R. Mallaby and Mr. V. P. Williams, show that the mass spectra of [ $1'-^{16}\text{O}$ ]MeABA possesses characteristic ions at  $\frac{m+}{e}$  190, 162, 134 and 125 which are very prominent. The ion at  $\frac{m+}{e}$  190 has been shown to retain the tertiary hydroxyl group. However it was suggested that the formation of the ion at  $\frac{m+}{e}$  190 could result from two separate fragmentation paths. In one, the tertiary hydroxyl oxygen is lost. The ion at  $\frac{m+}{e}$  125 ( $\frac{m+}{e}$  111 in the free acid) has been identified as the dienoic acid side chain (also, Gaskin and MacMillan, 1968).

If the [ $1',2'-^{18}\text{O}$ ]-epoxy oxygen of the epoxide is retained as the tertiary hydroxyl group of MeABA, an increase in mass number would be expected both in the parent ion, to  $\frac{m+}{e}$  280 (Parent ion  $\frac{M+}{e}$  278 + 2) and in the ion at  $\frac{m+}{e}$  190, to  $\frac{m+}{e}$  192. On the other hand no increase in the ion at  $\frac{m+}{e}$  125 would be expected if the [ $^{18}\text{O}$ ] were retained as the tertiary hydroxyl group of MeABA.

Measurements of the ratio of the peak heights of the parent ion (P)  $\frac{M+}{e}$  278 and the ion at  $\frac{M+}{e}$  280 (Parent + 2, P + 2) of the biosynthesised methylated ABA indicated a 32.2% abundance of [ $^{18}\text{O}$ ] in the molecule, Table 31. Measurement of the ratio of ions at  $\frac{m+}{e}$  190 and  $\frac{m+}{e}$  192 indicated a 14.0% abundance of [ $^{18}\text{O}$ ]. The ratio of ions at  $\frac{m+}{e}$  125 and  $\frac{m+}{e}$  127 was the same as the ratio observed in [ $1'-^{16}\text{O}$ ]-MeABA indicating that no [ $^{18}\text{O}$ ] had been incorporated into the dienoic acid side chain. Measurement of the ratio of ions at  $\frac{m+}{e}$  134 and  $\frac{m+}{e}$  136 and the ratio of ions at

Table 31. Measurement of the proportion of [ $^{18}\text{O}$ ] in the biosynthesised, methylated, ABA from the ratio of the parent ions  $\frac{M+}{e}$  278 and  $\frac{M+}{e}$  280. The  $\frac{M+}{e}$  parent region of authentic [ $1'-^{16}\text{O}$ ]MeABA is also given.

	$\frac{M+}{e}$	[ $1'-^{16}\text{O}$ ]MeABA Relative intensity	Biosynthesised [ $^{18}\text{O}$ ] MeABA, relative intensity
Parent (P)	278	100	100
P + 1	279	25.3	7.9
P + 2	280	3.6	47.4
P + 3	281	0.0	1.3

The proportion of [ $^{18}\text{O}$ ] found in the biosynthesised methylated ABA, 32.3 atoms per cent, was 97% of the proportion of [ $^{18}\text{O}$ ] in the ( $\pm$ )-[ $1'-^{18}\text{O}$ ]-epoxide (33.2 atoms per cent [ $^{18}\text{O}$ ]; see Methods, section (x)a, and Figs. 22 to 25 in Appendix 1) supplied to the wheat shoots.

$\frac{m+}{e}$  162 and  $\frac{m+}{e}$  164 indicated 20.0% and 17.5% respective abundance of [ $^{18}\text{O}$ ] in the molecule. The 14.0% abundance of [ $^{18}\text{O}$ ] in the molecule, measured from the ratio of ions at  $\frac{m+}{e}$  190 and  $\frac{m+}{e}$  192 indicates that the [ $1',2'-^{18}\text{O}$ ]-epoxy oxygen is retained in the tertiary hydroxyl group of ABA and is consistent with the suggestion (V. P. Williams, unpublished work) that the ion at  $\frac{m+}{e}$  190 is formed as a result of two separate fragmentation paths; one of which involves the retention of the tertiary hydroxyl group and suggests that the two fragmentation paths occur at the same rate.

The o.r.d. spectrum of an ethanol solution of the methylated ABA extracted from the wheat shoots indicated that they contained 20.0 $\mu\text{g}$  (+)-MeABA. Quantitative g.l.c. of the same solution of MeABA gave a value of 22.1 $\mu\text{g}$  MeABA; the g.l.c. trace is shown in Figure 19. Peak I, was unidentified material; peak II, had the same retention time as MeABA.

Calculations based on the [ $^{14}\text{C}$ ] radioactivity in the material co-chromatographing with authentic MeABA (Table 30) indicated that the wheat shoots contained 30.5 $\mu\text{g}$  methylated ABA.

The discrepancy between the amount of methylated ABA extracted from the wheat, as calculated by o.r.d. and g.l.c. measurements and as calculated by measurement of [ $^{14}\text{C}$ ] in the same material was investigated.

The biosynthesised, methylated, ABA was treated with  $\text{NaBH}_4$  solution (see Methods, section (x)(c)) and the products separated by t.l.c. in a hexane-ethyl acetate (1:1, v/v) solvent system. Distribution of [ $^{14}\text{C}$ ]-radioactivity along the t.l.c. plate was measured using a radiochromatogram scanner (see Methods,

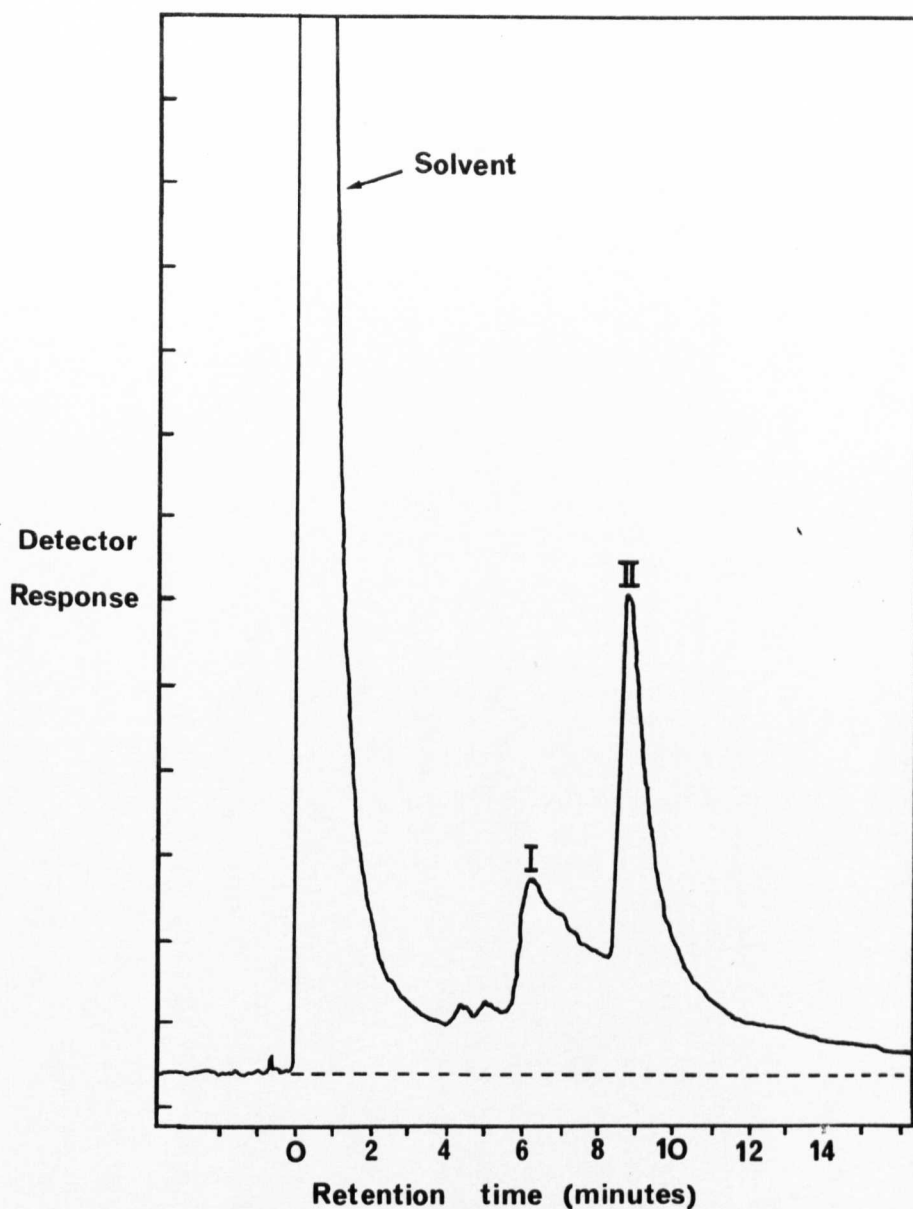


Fig 19. G.L.C. of esters produced on methylation of (+)-ABA extracted from wilted wheat shoots previously supplied with 1.13 mg (+)-(24.4  $\mu$ Ci/m mole [2- $^{14}$ C]; 33.2 atom per cent [1,2- $^{18}$ O]) epoxide. Oven temperature 225°C Isothermal.

I unidentified material

II material having the retention time of authentic MeABA

Conditions for G.L.C. of methyl esters

Gas chromatograph	: Varian aerograph series 1520 coupled to a Honeywell recorder
Column	: 5' $\times$ 1/8" i.d. stainless steel coil
Packing	: 5% S.E.30 coated on Chromosorb W (60-80 mesh)
Carrier gas	: Helium, 15 ml min $^{-1}$
Flame ionization detector	: H $_2$ , 25 ml min $^{-1}$ , air, 300 ml min $^{-1}$
Injection temperature	: 230°C
Detection temperature	: 230°C
Chart speed	: 4 mins/inch
Sensitivity	: Range 32 attenuation $\times$ 0.1

A symmetrical peak whose height was exactly equal to full scale deflection was produced under these conditions by 11.0  $\mu$ g of MeABA

section (viii)). [ $^{14}\text{C}$ ]-radioactive materials, in equal amounts, were found to have co-chromatographed with the cis-1',4' and trans-1',4'-diols of MeABA and slightly more radioactive material was found to have co-chromatographed with MeABA; material co-chromatographing with each of the authentic compounds was eluted with ethanol and an aliquot counted (Table 32). 4.2 $\mu\text{g}$  ( $\pm$ )-MeABA were added to the eluted material which had co-chromatographed with MeABA on the previous t.l.c. plate (Table 32), and the mixture treated with  $\text{NaBH}_4$ , as before. The products were separated by t.l.c. in a hexane-ethyl acetate (1:1, v/v) solvent system. Sample material co-chromatographed with the authentic diol esters and with MeABA. Distribution of [ $^{14}\text{C}$ ]-radioactive material along the plate was measured using a radiochromatogram scanner. All the radioactivity had co-chromatographed with MeABA, although the unlabelled MeABA added to the sample had been converted into the 1',4'-diols. The silica gel was divided into segments, transferred to vials containing 10ml of scintillation solution and assayed for [ $^{14}\text{C}$ ]-radioactivity. A histogram of the [ $^{14}\text{C}$ ] activity along the plate was drawn - Figure 20. The data confirmed that [ $^{14}\text{C}$ ]-radioactive material had co-chromatographed with MeABA but not with the 1',4'-diols of MeABA, from which it was concluded that the MeABA was unlabelled but chromatographed with the same  $R_f$  as a [ $^{14}\text{C}$ ]labelled impurity. In Table 32, the radioactivity in the 1',4'-diols represented the radioactivity in the methylated, ABA extracted from the wheat; the radioactive material chromatographing with authentic MeABA was an impurity which accounted for 37% of the radioactivity found in the material produced on methylation of the ABA (Table 30) extracted from the wheat shoots. Therefore the amount of [ $^{14}\text{C}$ ]-labelled,

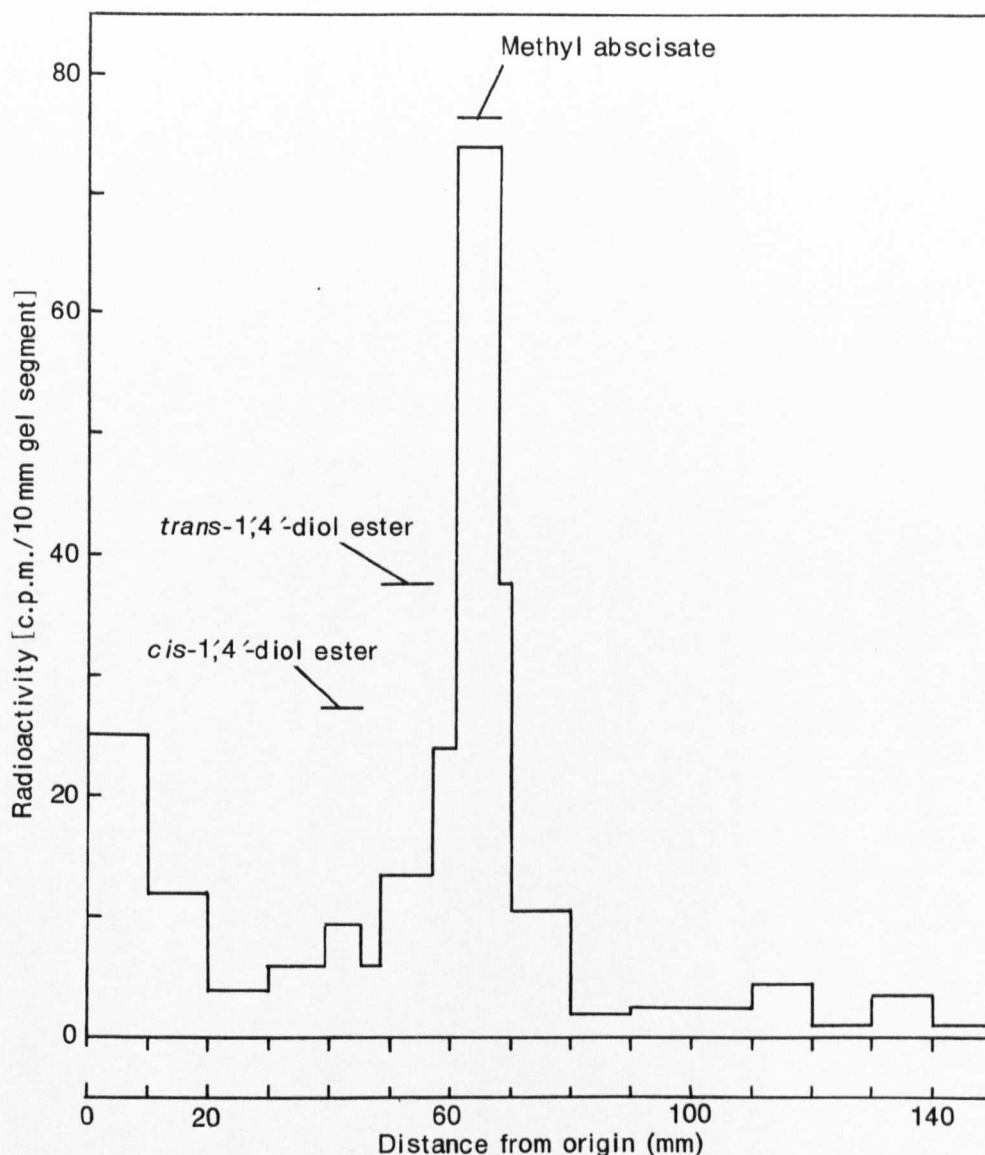


Fig 20. ABA was extracted from wilted wheat shoots previously supplied with 1.13 mg ( $\pm$ )-(24.4  $\mu$ Ci/m mole [ $2\text{-}^{14}\text{C}$ ], 33.2 atoms %-[ $1,2\text{-}^{18}\text{O}$ ]) epoxide and methylated. The zone on the t.l.c. plate chromatographing with marker MeABA, in hexane-ethyl acetate (1:1, by vol), was eluted with methanol and reduced with sodium borohydride. Radioactive material chromatographed with marker *cis*-1,4'- and *trans*-1,4'-diols of MeABA and with marker MeABA in hexane-ethyl acetate (1:1, by vol). The radioactive zone on the t.l.c. plate adjacent to marker MeABA was eluted with methanol and 4.2  $\mu$ g "cold" ( $\pm$ ) MeABA added. On treatment of this mixture with sodium borohydride the radioactive material was unaffected in that it again chromatographed with marker MeABA; u.v. light revealed that the unlabelled sample material chromatographed with marker *cis*-1,4'- and *trans*-1,4'-diols of MeABA, in hexane-ethyl acetate (1:1, by vol). The silica-gel was put into scintillation solution and counted.

Table 32. Conversion of (±)-([2-<sup>14</sup>C]; [1',2'-<sup>18</sup>O])-epoxide to ABA by wheat shoots supplied with 1.13mg (24.4μCi/mmole [<sup>14</sup>C]; 33.2 atom per cent [<sup>18</sup>O]) epoxide. Radioactivity co-chromatographing with authentic cis-1',4' and trans-1',4' diols of MeABA and with MeABA after treatment of the extracted, methylated, ABA with NaBH<sub>4</sub> solution.

Source of material	Chromatographic solvent used	c.p.m. in subsample	d.p.m. in total sample
Zone on t.l.c. plate adjacent to marker <u>cis</u> 1',4'-diol MeABA	hexane-ethyl acetate (1:1, v/v)	144.1	369.0
Zone on t.l.c. plate adjacent to marker <u>trans</u> -1',4'-diol MeABA		107.1	274.3
Zone on t.l.c. plate adjacent to marker MeABA		14.8	379.0

methyated, ABA (after correction for the impurity) derived from the epoxide compared with the total optically active, methyated, ABA determined by o.r.d. analysis is in close agreement with the results obtained by mass spectrometry (Table 33).

Table 33. Formation of the tertiary hydroxyl group of ABA from a 1',2' epoxy oxygen. 1.13mg ( $\pm$ )-[2- $^{14}\text{C}$ ; 1',2'- $^{18}\text{O}$ ]epoxide (24.4 $\mu\text{Ci/mmole}$  [ $^{14}\text{C}$ ]; 33.2 atoms % [ $^{18}\text{O}$ ]) was taken up by 103g wheat shoots in 35ml 0.01M potassium phosphate buffer, pH 7.3 during 18 hours at 7°C. The plants were wilted so that they lost 48% of their weight during the first 20 minutes of a 4 hour treatment at 20°C.

(+)-ABA by o.r.d., $\mu\text{g}$	Mean atoms % [ $^{18}\text{O}$ ] in MeABA $\frac{\text{M}+}{\text{e}}, \frac{280}{278 + 280}$	S.E.	% ABA formed from epoxide by [ $^{18}\text{O}$ ]	Wt. of ABA formed from [ $^{14}\text{C}$ ] epoxide, $\mu\text{g}$	% ABA formed from epoxide by [ $^{14}\text{C}$ ] and o.r.d.,
20.0	32.2	2	97	19.2	96

Discussion of results. The ABA extracted from wilted wheat shoots previously supplied with a solution of ( $\pm$ )-[2- $^{14}\text{C}$ ; 1',2'- $^{18}\text{O}$ ] epoxide was shown to contain [ $^{18}\text{O}$ ]. The proportion of [ $^{18}\text{O}$ ] in the molecule was determined from the ratio of the parent ions at  $\frac{\text{M}+}{\text{e}}$  278 and  $\frac{\text{M}+}{\text{e}}$  280 of the methyl ester. Comparison of the mass spectrum of this material with that of authentic [ $^{16}\text{O}$ ] MeABA shows that the 1',2'-epoxy group becomes the tertiary hydroxyl group of ABA and the conversion is quantitative (Table 33). Furthermore, the weight of ABA calculated to be derived from the [ $^{14}\text{C}$ ]epoxide



(of known specific activity) together with the weight of ABA determined by o.r.d., and in agreement with the proportion of [ $^{18}\text{O}$ ] in the ABA, excludes the presence of any but trace amounts of racemic ABA.

The MeABA obtained in the present experiment was found to contain another [ $^{14}\text{C}$ ]labelled compound, which was unaffected by treatment with  $\text{NaBH}_4$ , and accounted for 37% of the [ $^{14}\text{C}$ ] in the methylated ABA extracted from the wheat.

In subsequent experiments wilted wheat plants, previously supplied with ( $\pm$ )-[2- $^{14}\text{C}$ ]epoxide, have been found to produce only one other radioactive material besides ABA (B. V. Milborrow, personal communication). This material chromatographs with MeABA after methylation of the extract and is unaffected by treatment with  $\text{NaBH}_4$ . It forms an acetyl derivative, when treated with acetic anhydride in pyridine, because after acetylation a derivative chromatographs at  $R_f$  0.77 whereas MeABA chromatographs at  $R_f$  0.58 on silica gel in the same hexane-ethyl acetate (1:1, v/v) solvent system.

Conversion of the epoxide to ABA requires the net addition of a keto-group on C-4' and this could occur by the insertion of a hydroxyl group at this position, followed by oxidation. The acetyltable material isolated is the only derivative of the epoxide, other than the biologically inactive 1',2'-dihydroxy compound to have been detected. Its unreactivity to  $\text{NaBH}_4$ , as assessed by its having the same  $R_f$  before and after treatment, excludes the presence of a keto-group so this compound may be the 4'-hydroxy derivative of the epoxide and a precursor of ABA.

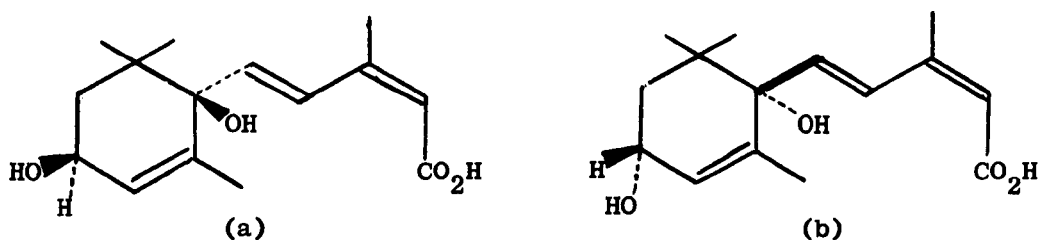
- (iii) Incorporation of ( $\pm$ )-[2- $^{14}$ C]-cis-1',4'-diol and ( $\pm$ )-[2- $^{14}$ C]-trans-1',4'-diol of ABA into ABA by wheat shoots subjected to wet and wilt treatments.

Experiments commenced 12th May, 1970.

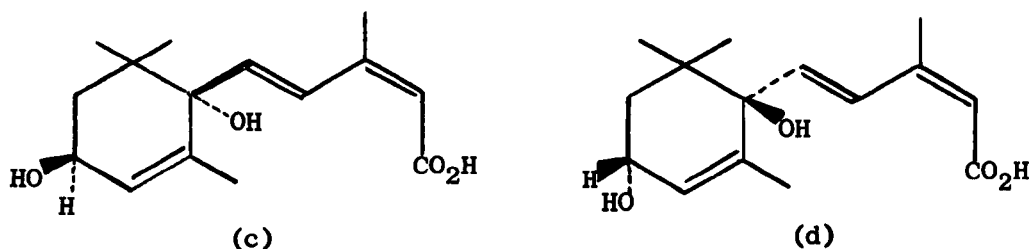
The demonstration of the conversion of the epoxide into (+)-ABA, which entails the introduction of a 4'-keto group which, by analogy with similar oxidations (Murphy and West, 1969) probably takes place via a hydroxylation, suggested that plants were able to oxidise a hydroxyl group on the C-4' of the carbon skeleton of ABA to a 4' ketone. When synthetic racemic abscisic acid became available the 1',4'-diols of ABA were prepared and found to inhibit the growth of a number of plants (personal communication, Mr. R. Leach and Dr. M. Anderson) although they were less potent than ABA. The treatment of the ether-soluble acid fraction extracted from sycamore leaves (whose growth inhibitory activity has been shown to be due to the presence of (+)-ABA; Cornforth, Milborrow, Ryback and Wareing, 1965) with aqueous methanolic  $\text{NaBH}_4$  solution was reported by Robinson (1962), and Robinson & Wareing (1964). The sycamore extract was no longer inhibitory after treatment with  $\text{NaBH}_4$ . The loss of activity recorded by Robinson (1962) was attributed by him to the reduction of a keto group in the inhibitory molecule. Treatment of synthetic ABA with aqueous methanolic  $\text{NaBH}_4$  solution in the cold produces approximately equal amounts of the cis-1',4'- and trans-1',4'-diols of ABA. However when synthetic ABA is treated under the more severe conditions employed by Robinson, another biologically inactive product is formed, which has been tentatively identified as a 1',4'-diol in which the 2'-double bond is saturated (Dr. G. Ryback - unpublished results).

The reduction of ( $\pm$ )-ABA by an aqueous methanolic  $\text{NaBH}_4$  solution, when performed at  $0^\circ\text{C}$  for 60 minutes gives two major products, the 1',4'-diols and several minor, unidentified products. The yield of the 1',4'-diols combined is 60 to 70% of the starting material. An increase of either the reaction time or the temperature at which the reaction is performed decreases the yield of the 1',4'-diols and increases the amounts of the other products.

As with the epoxide, it was of interest to discover whether the growth inhibitory action of the diols could be attributed to abscisic acid formed from them by oxidation in vivo or whether they can cause inhibition of growth in the absence of any conversion to ABA. The cis-1',4'-diol formed from ( $\pm$ )-ABA exists in two enantiomeric forms, here shown as (a) and (b).



The two enantiomers of the trans-1',4'-diol of ( $\pm$ )-ABA are shown as (c) and (d).



If the diols are oxidised to ABA then it is possible that there is a selection of one stereoisomer. The enzyme which was postulated to perform the oxidation of the 4'-hydroxyl group to a 4'-ketone during the biosynthesis of ABA would be expected to be specific for a particular configuration of a hydroxyl group at C-4' and could also be affected by the configuration of the tertiary hydroxyl on C-1' of the carbon skeleton of the diol. Only the diol having the correct configuration at C-4', or C-4' and C-1' could be expected to undergo oxidation to ABA by the action of the enzyme which catalyses this step in normal biosynthesis. If the conversion of only one enantiomer of only one diol to ABA occurred then it could be concluded that the 4'-hydroxyl-oxidising enzyme is completely stereospecific, both for the hydroxyl at C-1' and at C-4'. The equal growth-inhibitory activity of the 1',4'-diols indicated that it was not. However, if the enzyme were specific in its stereochemical requirements at C-4' only, then the optical activity of the residual diol, and of the ABA formed, would indicate the relative stereochemistry of the C-4' and C-1' hydroxyl groups required by the enzyme. One enantiomer of the 1',4'-cis- and the opposite enantiomer of the 1',4'-trans-diol would be converted to ABA. The ABA formed from the two would be of opposite stereochemistry. Further, the residual diol would be expected to show optical activity with a preponderance of the opposite enantiomer from that which had been oxidised to ABA.

Chemical oxidation, with manganese dioxide in dry chloroform, would convert the residual diol to ABA, whose o.r.d. spectrum could be more readily detected in the presence of racemic

material than could that of the diol, because ABA has a specific rotation approximately six times greater than the diols and their u.v. extinction coefficients are similar (Table 2).

( $\pm$ )-cis-1',4'-diol and ( $\pm$ )-trans-1',4'-diol were supplied to wheat plants which were either wilted or kept turgid. This was done to discover whether or not the oxidation of the diols to ABA were affected by the change in turgor which so dramatically alters the rate of biosynthesis of ABA and had been found to effect the incorporation of the epoxide. If more diol were converted to ABA in wilted wheat plants than in turgid ones then this would indicate that the oxidation of the diol to ABA is regulated by the same mechanism that controls ABA biosynthesis and the conversion of the 4'-hydroxyl to a 4'-ketone is one of the steps which is regulated. If the diols were converted to ABA equally in wet and in wilt treatments, then it may be concluded either that the diol is a natural precursor of ABA and there is no control of its conversion to ABA, or that it is not a natural precursor of ABA and its conversion to ABA is effected by an adventitious dehydrogenase enzyme system.

(a) Incorporation of ( $\pm$ )-[2-<sup>14</sup>C]-cis-1',4'-diol of ABA (21.6  $\mu$ Ci/mmole) into ABA by cut wheat shoots.

200g of wheat shoots were placed in a solution containing 586.5  $\mu$ g ( $\pm$ )-[2-<sup>14</sup>C]cis-1',4'-diol of ABA (21.6  $\mu$ Ci/mmole) dissolved in 100ml 0.01M potassium phosphate buffer pH 7.3, containing 1% ethanol. The shoots were left for 16 hours at 4°C and a further 3 hours at 20°C during which time they took up 298.7  $\mu$ g of the ( $\pm$ )-[2-<sup>14</sup>C] diol. The shoots were removed from the solution and

divided into two equal batches. One batch was wilted so that it lost 30% of its fresh weight (referred to as wilt treatment). The other batch was placed with the cut ends in water for 6 hours (referred to as the water treatment). At the end of 6 hours the wheat shoots were immersed in 1 litre methanol (containing 10mg B.H.T.) and cut into 1cm lengths and left for 48 hours. Both batches were thoroughly extracted with a further 2 litres of a methanol:water (4:1, v/v) mixture, containing 10mg B.H.T. per litre.

The ether-soluble acid fraction from the wilt and wet treatments were chromatographed separately. In each case one of the radioactive acids in the crude ether-soluble acid fraction was identified as ABA by its co-chromatography with marker ABA and, after methylation, with marker MeABA on Merck F<sub>254</sub> precoated plates. The  $R_f$  values of authentic ABA, cis-1',4'-diol of ABA, trans-1',4'-diol of ABA, MeABA and the methyl esters of the two diols of ABA in the different solvent systems used to purify the extracts are given in Table 34.

Material which co-chromatographed adjacent to MeABA (Table 34) was eluted with ethanol and aliquots were taken for [<sup>14</sup>C] assay. The amounts of MeABA extracted were calculated from the u.v. absorption spectra, the o.r.d. spectra and the [<sup>14</sup>C] data (Table 35). The profiles of the o.r.d. curves were characteristic of (-)-ABA (Figure 21).

Recovery of unchanged [2-<sup>14</sup>C]cis-1',4'-diol of ABA (21.6μCi/mmmole) from the water and wilt treatments

The material from the ether-soluble acid fraction which co-chromatographed with cis-1',4'- and trans-1',4'-diol markers in

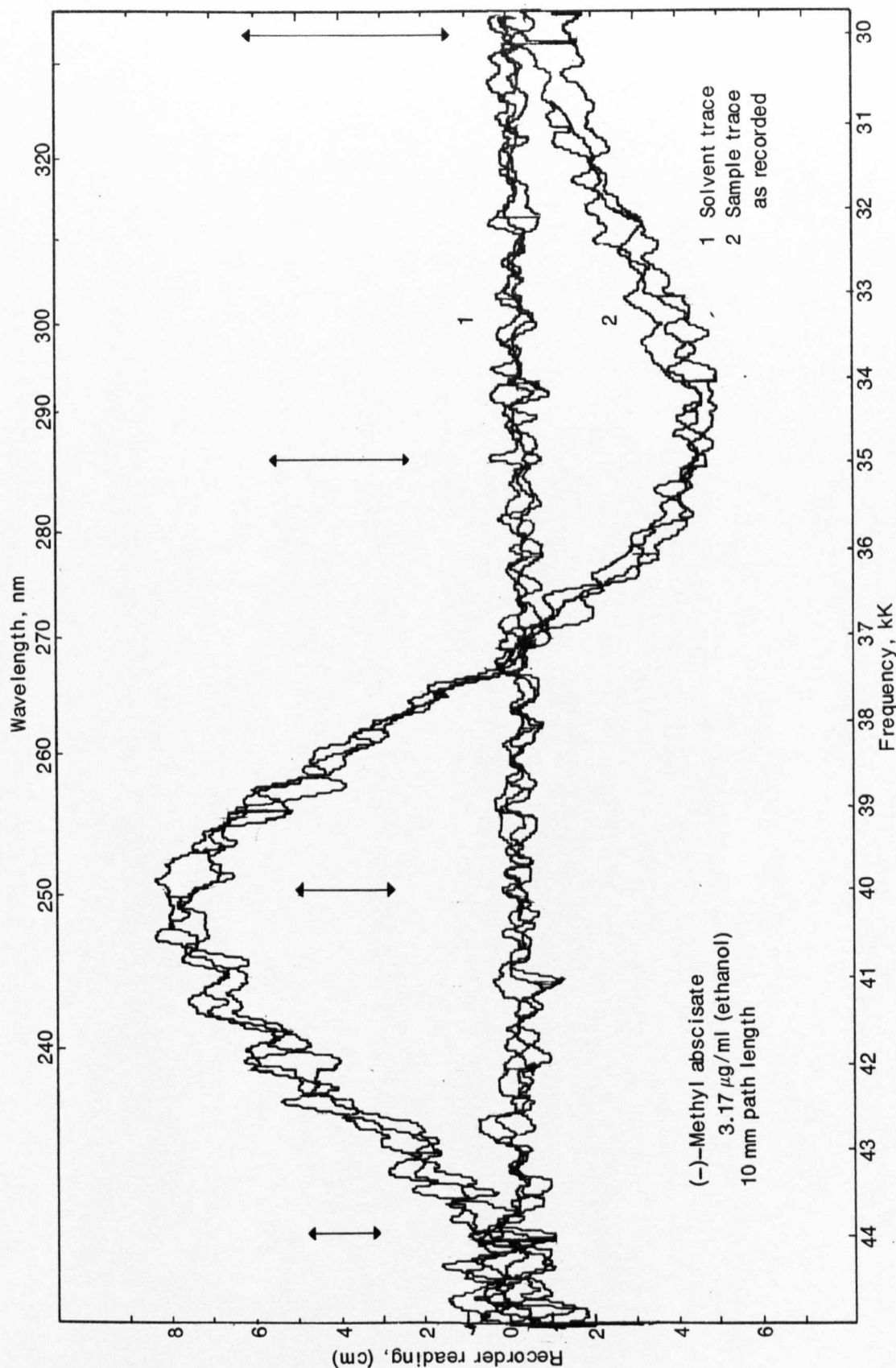


Fig 21 The optical rotatory dispersion curve of methylated ABA recovered from wheat shoots supplied with (+)-[2- $^{14}\text{C}$ ]-*cis* 1',4'-diol of ABA (21.6  $\mu\text{Ci/m mole}$ ) and left in water for 6 hours, (see text for details), as obtained from the "Polarmatic 62". The sensitivity of the instrument varies with frequency as indicated by the length of the arrows which correspond to a rotation of 5 millidegrees at the frequencies shown. At 35 w.n.  $5 \text{ m}^\circ = 2.1 \mu\text{g (-)-methyl abscisate/ml}$ .

Table 34.  $R_{fs}$  of ABA and its derivatives on Merck F<sub>254</sub> precoated silica gel t.l.c. plates.

Authentic compound	$R_f$ values in four solvent systems			
	toluene-ethyl acetate- acetic acid, (75:30:3, v/v)	toluene-ethyl acetate- acetic acid, (75:40:4, v/v)	propan-1-ol-butan-1-ol- -aq. ammonia (sp.gr. 0.88) -water (6:2:1:2)	hexane-ethyl acetate (1:1, v/v)
ABA				
cis-1',4'-diol of ABA	0.34	0.57	0.67	0.06
trans-1',4'-diol of ABA	0.13	0.31	0.60	
	0.25	0.50	0.63	
MeABA				
cis-1',4'-diol of MeABA				0.39
trans-1',4'-diol of MeABA				0.20
				0.32



Table 35. Incorporation of  $(\pm)$ -[2- $^{14}\text{C}$ ]-cis-1',4'-diol of ABA (21.6 $\mu\text{Ci/mmole}$ ) into ABA by 200g of cut wheat shoots. The wheat shoots absorbed 298.7 $\mu\text{g cis-1',4'-diol}$  (54,244 d.p.m.) in 60ml 0.01M potassium phosphate buffer pH 7.3 1% ethanol (v/v) during 19 hours. The batch was then divided and half wilted so that it lost 30% of its fresh weight; the other half was placed with the cut ends in water for 6 hours. Then both samples were extracted with methanol. Radioactivity co-chromatographing with marker MeABA after methylation of the extracted ABA. The efficiency of counting was 43.9% and the background was 18.4 c.p.m.

Treatment	c.p.m. in subsample	d.p.m. in total sample	MeABA by [ $^{14}\text{C}$ ], $\mu\text{g}$	MeABA excess (-) from o.r.d. curve $\mu\text{g}$	MeABA by U.V., $\mu\text{g}$	% $(\pm)$ - <u>cis-1',4'-diol</u> incorporated into ABA by plant	% ABA formed from <u>cis-1',4'-diol</u> by [ $^{14}\text{C}$ ]/o.r.d.
Water	23.9	1088.8	6.00	5.70	<11.1*	4.2	95.0
Wilt	20.1	915.6	5.10	4.90	<11.0*	3.5	96.0

\* The U.V. spectra were contaminated with unidentified material which had a high absorption between 230-220nm and distorted the normal profile exhibited by MeABA therefore these values are maxima.

the first solvent system (Table 34) were eluted separately and methylated. The products were chromatographed in a hexane-ethyl acetate (1:1, v/v) solvent system. No material from the trans-1',4'-diol sample was found to co-chromatograph with either of the authentic diol methyl esters. Material from the cis-1',4'-diol sample co-chromatographed with marker cis-1',4'-diol of MeABA. This material was purified by chromatography in a toluene-ethyl acetate-acetic acid (75:40:4, v/v) solvent system and then oxidized (using finely divided  $\text{MnO}_2$  in dry chloroform), the product co-chromatographed with marker MeABA in a hexane-ethyl acetate (1:1, v/v) solvent system. It was eluted from the silica-gel with ethanol and measurements of its o.r.d. curve and  $^{14}\text{C}$  content were made (Table 36).

The unchanged cis-1',4'-diol recovered from both the water and wilt treated wheat shoots was oxidised to MeABA with  $\text{MnO}_2$  and its o.r.d. curve was identical with that of (-)-ABA. Accurate quantitative measurements of this MeABA formed by oxidation of the 1',4'-diol, could not be obtained by ultraviolet absorption photometry because the amount of material was too small. The specific rotation of the optically active 1',4'-diols is approximately 1/6th that of ABA while their molar extinction coefficients are similar. The optical rotatory dispersion spectrum of  $1\mu\text{g}$  (+)-ABA can be detected in solutions whose optical density does not exceed 1.5 O.D. units from 240 to 300nm. This optical density is produced by an  $8\mu\text{g}/\text{ml}$  solution of ABA (10.00mm path length) therefore optically active ABA can only be detected when one part is present with 8 parts, or less, of racemic material. With the 1',4'-diols

Table 36. Unchanged [2-<sup>14</sup>C]cis-1',4'-diol of ABA (21.6μCi/mmole), recovered from cut wheat shoots subjected to water and wilt treatments. The unchanged cis-1',4'-diol of ABA was isolated by t.l.c., methylated, and oxidized to MeABA. Measurement was then made of its o.r.d. spectrum and [<sup>14</sup>C] content. The efficiency of counting was 43.9% and the background 18.4 c.p.m.

Treatment	c.p.m. in subsample	d.p.m. in total sample	MeABA by [ <sup>14</sup> C], μg	MeABA, excess (-) from o.r.d. curve, μg
Water	28.2	128.4	0.71	0.73
Wilt	73.3	334.0	1.84	0.51

this ratio is 1:1.5. Hence if a small excess of an optically active 1',4'-diol is present in a mixture with a large proportion of racemic material it may be impossible to detect. However, after the mixture has been oxidised to ABA the optical activity may be measureable. For this reason the unmetabolized 1',4'-diols recovered from the wheat were oxidized to ABA.

(b) Incorporation of  $(\pm)$ -[2- $^{14}$ C]-trans-1',4'-diol of ABA  
(21.6  $\mu$ Ci/mmmole) into ABA by cut wheat shoots

200g of cut wheat shoots were placed in a solution containing 630  $\mu$ g  $(\pm)$ -[2- $^{14}$ C]-trans-1',4'-diol of ABA (21.6  $\mu$ Ci/mmmole) dissolved in 100ml 0.01M potassium phosphate buffer, pH 7.3, containing 1% ethanol (v/v). The shoots were left for 16 hours at 4°C and a further 3 hours at 20°C during which time they took up 409.6  $\mu$ g of the  $(\pm)$ -[2- $^{14}$ C]diol. The subsequent treatment of the wheat shoots and the extraction of ABA were the same as described in the previous experiment ((iii) (a)). The results obtained are presented in Table 37. The profiles of the o.r.d. curves were characteristic of (-)-ABA.

(c) Recovery of unchanged [2- $^{14}$ C]-trans-1',4'-diol of ABA  
(21.6  $\mu$ Ci/mmmole) from the water and wilt treatments

Unchanged trans-1',4'-diol was recovered from the two treatments by the methods described in the previous experiment, ((iii) (a)). No cis-1',4'-diol was detected in ether-soluble acids extracted.

MeABA, formed from unmetabolized trans-1',4'-diol, by MnO<sub>2</sub> oxidation, from both the water and wilt treatments did not show optical activity. The u.v. absorption spectra and [ $^{14}$ C] content of these samples of MeABA were measured (Table 38).

Table 37. Incorporation of  $(\pm)$ -[2- $^{14}\text{C}$ ]trans-1',4'-diol of ABA (21.6 $\mu\text{Ci}/\text{mmole}$ ) into ABA by 200g cut wheat shoots. The wheat shoots absorbed 409.6 $\mu\text{g}$  trans 1',4'-diol (74,383 d.p.m.) in 66ml 0.01M potassium phosphate buffer, pH 7.3, containing 1% ethanol (v/v) during 19 hours. The batch was then divided and half wilted so that it lost 30% of its fresh weight; the other half was placed with the cut ends in water for 6 hours when both samples were extracted with methanol. Radioactivity co-chromatographing with marker MeABA after methylation of the extracted ABA. The efficiency of counting was 43.9% and the background 18.4 c.p.m.

Treatment	c.p.m. in subsample	d.p.m. in total sample	MeABA by [ $^{14}\text{C}$ ], $\mu\text{g}$	MeABA, excess (-) from o.r.d. curve, g	MeABA by U.V., $\mu\text{g}$	% trans-1',4'-diol incorporated into ABA by plant	% ABA formed from trans-1',4'-diol by [ $^{14}\text{C}$ ]/o.r.d.
Water	20.8	947.6	5.2	4.60	29.75*	2.5	88.5
Wilt	15.8	719.8	4.0	3.90	11.0*	1.9	97.5

\* The values of samples determined by their U.V. absorption are maximum because they were contaminated with unidentified material.

Table 38. Unchanged  $[2-^{14}\text{C}]\text{trans-1',4'-diol}$  of ABA ( $21.6\mu\text{Ci}/\text{mmole}$ ) recovered from the cut wheat shoots subjected to water and wilt treatments. The unchanged trans-1',4'-diol of ABA was isolated by t.l.c., methylated, and oxidized to MeABA. Its u.v. absorption and  $[^{14}\text{C}]$  content were then determined. The efficiency of counting was 43.9% and the background 18.4 c.p.m.

Treatment	c.p.m. in subsample	d.p.m. in total sample	MeABA by $[^{14}\text{C}]$ , $\mu\text{g}$	MeABA by u.v., $\mu\text{g}$
Wet	26.5	122.2	0.67	2.6
Wilt	494.9	2,286.2	12.59	14.6

Discussion of results. The cis-1',4'-diol and trans-1',4'-diol of ABA were both metabolized to ABA by wheat shoots which were either wilted or kept turgid. There was no apparent difference between the wilt and turgid treatments on the formation of ABA from the 1',4'-diols and it was concluded that there is no biological control i.e. feed-back mechanism on the conversion of the 1',4'-diols to ABA in the plant, and that the diol is not a natural precursor of ABA in plants.

Wheat shoots which had previously been supplied with either  $(\pm)\text{-}[2-^{14}\text{C}]\text{cis-1',4'-diol}$  or  $(\pm)\text{-}[2-^{14}\text{C}]\text{trans-1',4'-diol}$  contained approximately equal amounts of  $[^{14}\text{C}]\text{ABA}$  and in which there was an excess of the (-)-enantiomer when the plants had

been subjected either to the water or the wilt treatments. 5.7 $\mu$ g of free (-)-ABA were detected in the ether-soluble acid fraction extracted from wheat shoots previously supplied with ( $\pm$ )-[2- $^{14}$ C]cis-1',4'-diol which had received the water treatment; however, the amount of [ $^{14}$ C]ABA in the final isolate indicated that a total of 6.0 $\mu$ g [ $^{14}$ C]ABA were present. Therefore 0.3 $\mu$ g [ $^{14}$ C]ABA were unaccounted for as (-)-[ $^{14}$ C]ABA and must have been present either as (-)-ABA or a mixture of (-)-[ $^{14}$ C]ABA and (+)-[ $^{14}$ C]ABA. A similar result was obtained with the wheat shoots which were wilted. ABA which was purified from the ether-soluble acid fraction extracted from the wheat contained 4.9 $\mu$ g excess of free (-)-[ $^{14}$ C]ABA whereas the amount of [ $^{14}$ C] in the ABA indicated that a total of 5.1 $\mu$ g ABA had been extracted. Therefore there were 0.2 $\mu$ g [ $^{14}$ C]ABA present as either (-)-[ $^{14}$ C]ABA or a mixture of (-)-[ $^{14}$ C]ABA and some (+)-[ $^{14}$ C]ABA.

The evidence which suggests that (-)-ABA does not occur naturally has already been discussed (Milborrow, 1970(a)), the (-)-ABA observed in this experiment, therefore, is considered to have been derived from the (-)-[2- $^{14}$ C]1',4'-diol.

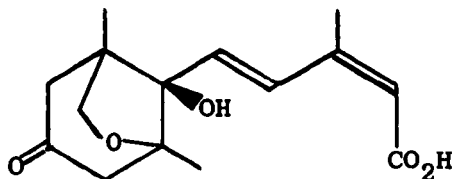
The cis- and trans-1',4'-diols of (-)-ABA have 4'-hydroxyl groups of opposite stereochemistry and (-)-ABA is formed from both, therefore the enzyme or enzymes oxidising the 4'-hydroxyl group to ketone can accommodate both 4'-hydroxyl epimers.

The unchanged [2- $^{14}$ C]cis-1',4'-diol recovered in the ether-soluble acid fraction extracted from the wheat, contained an excess of the (-) enantiomer which accounted for all the [ $^{14}$ C] activity in the 1',4'-diol recovered from wheat given the water

treatment and approximately  $\frac{1}{4}$  of the [ $^{14}\text{C}$ ] activity in the 1',4'-diol recovered from the wheat given the wilt treatment.

These results suggest that the (+) enantiomer of ( $\pm$ )-[2- $^{14}\text{C}$ ]cis-1',4'-diol is more rapidly metabolized than the (-). No labelled material in a parallel experiment was detected in the ether-soluble neutral fraction nor in the aqueous residue after the ether-soluble acid fraction had been removed. Racemic [2- $^{14}\text{C}$ ]ABA has been shown to undergo stereochemically selective metabolism when supplied to tomato plants, the (+) enantiomer is hydroxylated and during isolation it usually rearranges to give phaseic acid (XXXIII) (Milborrow, 1969(a); 1970(a)) whereas both (+)- and (-)-ABA forms of ABA are converted to glucose ester.

(XXXIII)



Phaseic acid

No [ $^{14}\text{C}$ ] labelled glucose ester of either the 1',4'-diol or ABA was detected, therefore the "missing" (+)-ABA was sought as phaseic acid. [ $^{14}\text{C}$ ] labelled material was detected at the position on the silica gel TLC plate at which phaseic acid occurs but the compound had been destroyed by remaining in contact with the silica-gel for some days and could not be eluted from the silica-gel with methanol. These observations suggest that both (+) and (-) enantiomers of both diols are oxidised to ABA and that the (+)-ABA is then further metabolized and can be isolated as phaseic acid. There was little difference between the amounts



of (+)-ABA in the wilted as compared with the unwilted wheat; this suggests that either ABA produced from the  $(\pm)$ -[2- $^{14}\text{C}$ ]cis-1',4'-diol, or the diol itself inhibited the rapid biosynthesis of (+)-ABA which would normally have occurred in response to the wilting stimulus (Wright and Hiron, 1969). At present it is impossible to exclude the possibility that the diols themselves are able to inhibit the biosynthesis of ABA.

Attempts to isolate [ $^{14}\text{C}$ ] labelled diols from extracts of avocado supplied with [2- $^{14}\text{C}$ ]MVA have been entirely unsuccessful which indicates, although not conclusively, that the diols are not normal precursors of ABA biosynthesis and are less likely, therefore, to operate a feedback mechanism. The similar maximum concentrations of (+)-ABA isolated from wheat in several experiments would also indicate that it is the ABA itself which is effective.

The total amounts of ABA isolated in this experiment are similar to those obtained in other experiments in which increased amounts of (+)-ABA were isolated from wilted wheat plants. The ABA derived from the diols shows a preponderance of the (-) enantiomer and the smaller amounts of (+)-ABA which were present, and present in approximately equal amounts in both water and wilt treatments, indicate that the natural biosynthetic pathway can be inhibited by (-)-ABA when it is present in the tissues. The stage or stages in the biosynthetic sequence at which this feedback mechanism operates may be indicated by a change in the relative amounts of one or several precursor and should thereby provide a means of identifying intermediates in ABA biosynthesis.

The failure to detect any labelled products formed from the  $(\pm)$ -[2- $^{14}\text{C}$ ]1',4'-diols other than ABA or phaseic acid and the preponderance of (-)-enantiomer in both the residual diol and ABA suggests that the (+)-cis-1',4'-diol is oxidised to (+)-ABA more rapidly than the (-)-[2- $^{14}\text{C}$ ]cis-1',4'-diol is oxidised to (-)-ABA. This is in agreement with the findings that (+)-ABA is the naturally occurring enantiomer and (-)-ABA, although biologically as active as (+)-ABA, is not a natural plant product (Milborrow, 1970(a); 1969(a) and 1968). The fact that the abscisic acid isolated contained a preponderance of the (-) enantiomer agrees with previous observations that (+)-ABA is more rapidly destroyed, in plants, than (-) ABA when both are present.

Wheat shoots subjected to water and wilt treatments, which were previously supplied with  $(\pm)$ -[2- $^{14}\text{C}$ ]trans-1',4'-diol of ABA contained an excess of free (-)-ABA, but the unchanged [2- $^{14}\text{C}$ ]-trans-1',4'-diol recovered from the wheat had no optical activity and was therefore presumed to be a racemic mixture.

No glucose ester of either (+) or (-) ABA was detected, the presence of glucose ester of trans-1',4'-diol was not investigated but its physical properties would be expected to be similar to those of ABA glucose ester and no radioactivity was found in an aqueous extract which should have contained the material, if present. It appears that the (+) and (-) enantiomers of trans-1',4'-diol were converted to (+)-ABA and (-)-ABA respectively at the same rate and the (+)-ABA is then preferentially destroyed.

From these results it appears that at least some of the biological activity of the cis-1',4'-diol and the trans-1',4'-diol of ABA can be attributed to the ABA formed from them. Whether or not the diols are active per se or by exerting a sparing action on ABA (Milborrow, 1969(b)) cannot be decided at present.

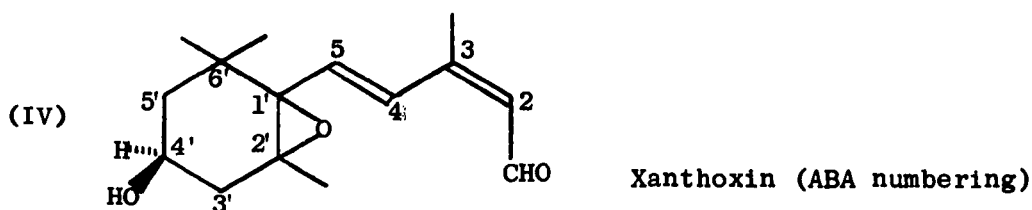
The observation that (+)-[2-<sup>14</sup>C]cis-1',4'-diol was converted to (+)-ABA (and then further metabolised) so rapidly that the unchanged [2-<sup>14</sup>C]cis-1',4'-diol recovered at the end of the experiments consisted almost entirely of the (-) enantiomer suggested that the conversion of cis-1',4'-diol to ABA is preferentially stereospecific but not completely so because an excess of free (-)-ABA was found in the acid fraction of wheat supplied with racemic cis-1',4'-diol.

On the other hand the oxidation of the trans-1',4'-diol to ABA does not appear to be stereospecific because the residual material recovered at the end of the experiment lacked optical activity. It is concluded from this that (+) and (-) forms of the trans-1',4'-diol are converted to (+)- and (-)-ABA at equal rates.

Both the (+) and (-) enantiomers of both cis-1',4'- and trans-1',4'-diols are oxidised to ABA therefore either the enzyme is not stereospecific and can oxidise all epimers at C-4' or more than one form of the enzyme catalyzes the reaction.

Although the residual trans-1',4'-diol recovered at the end of the experiment was racemic, or contained so little excess of one enantiomer that optical activity could not be detected, the residual cis-1',4'-diol was optically active (Table 36) and

contained a considerable excess of the (-) enantiomer (ORD and [ $^{14}\text{C}$ ] measurements indicate that the diol was almost entirely (-)). The more rapid oxidation of the (+)-cis-1',4'-diol (a) than the (-)-cis-1',4'-diol (b) to ABA may indicate that the relative configuration of hydroxyl groups in (a) represents the relative configuration of the oxygen atoms of a natural precursor of ABA. It may also be significant that a strongly growth-inhibitory compound xanthoxin (IV), first isolated as a degradation product



of violaxanthin and recently isolated from bean plants (Phaseolus vulgaris) (Taylor and Burden, 1970) also has a hydroxyl group at C-4' (ABA numbering) with the same stereochemistry as the 4'-hydroxyl group of the (+)-cis-1',4'-diol.

Although the absolute stereochemistry of the 1',2'-epoxy group of xanthoxin (IV), like that of the 1',2'-epoxy group of the parent violaxanthin, remains uncertain, Taylor (personal communication) has converted the 2-trans isomer of xanthoxin to (+)-ABA. The 1',2'-epoxy group, therefore, has the same stereochemistry at C-1' as the C-1' hydroxyl group of (+)-ABA.

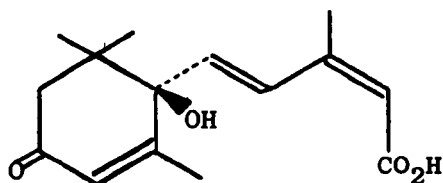
The definitive designation of the stereochemistry of these compounds awaits the determination of the absolute configuration of (+)-ABA by X-ray analysis, which is now in hand (B. V. Milborrow, personal communication).

## CHAPTER V

### DISCUSSION

The absolute configuration of (+)-abscisic acid was reported in 1967 by Cornforth, Draber, Milborrow and Ryback (1967) and was determined by the application of Mills' rule to the optical rotatory dispersion spectra of the cis-1',4'- and trans-1',4'-diols of both (+)- and (-)-abscisic acid methyl esters. The stereochemistry of natural (+)-abscisic acid was deduced to be as shown in (Ia).

(Ia) natural (+)-abscisic acid



However this configuration was deduced by an indirect method and it has not been confirmed by a direct method and to that extent some doubt remains.

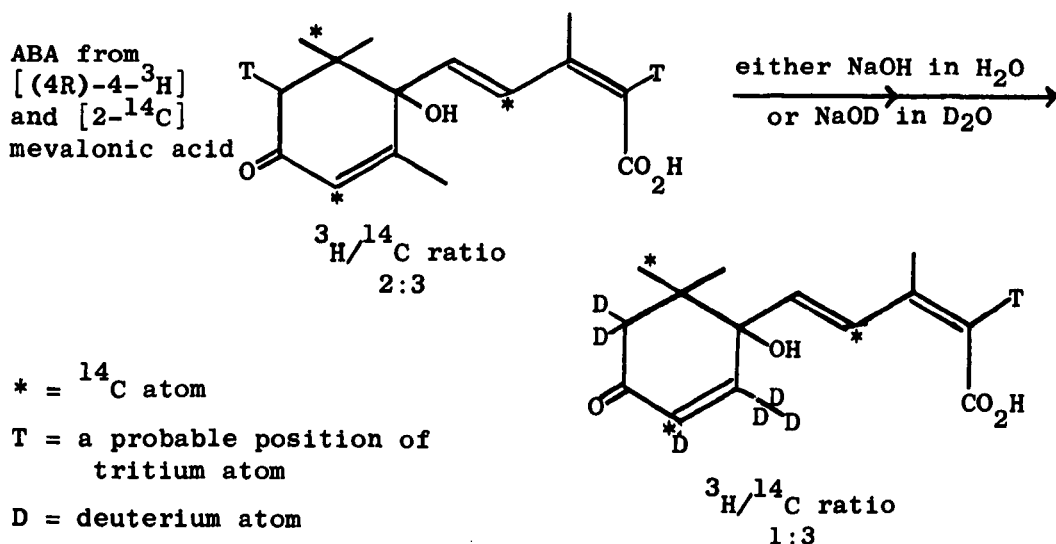
Application of the Cahn-Ingold-Prelog conformation rules, as enunciated in their original papers (Cahn and Ingold, 1951; Cahn, Ingold and Prelog, 1956) gave the notation S to the above formulation of natural (+)-abscisic acid. However, in 1966 Cahn, Ingold and Prelog proposed a slight modification to their rules (1966, p.391 et seq.). One effect of these changes was to reverse the notation of (+)-abscisic acid. Thus, assuming that

Mills' rule is obeyed, and therefore that the stereochemistry depicted in the above structure is correct, then natural (+)-abscisic acid is S according to the Cahn, Ingold and Prelog rules of 1956; R according to the Cahn, Ingold and Prelog rules of 1966. Because the stereochemistry of abscisic acid has not yet been confirmed by a direct measurement no attempt will be made to bring the terminology into line with the more recent set of rules, until the absolute stereochemistry of abscisic acid has been unambiguously determined by X-ray analysis of a crystal of a derivative containing a heavy atom.

For the purposes of this thesis therefore, natural (+)-abscisic acid will be referred to as S (according to the Cahn, Ingold and Prelog rules of 1956) and to have the stereochemistry depicted in Figure (Ia).

The first requirement for an investigation into the biosynthesis of a compound is to have a system which will make it. When the present work began no system, in which the biosynthesis of ABA had been shown, was available. However, there were numerous reports indicating that the concentration of abscisic acid increased in leaves of perennials in autumn and in fruit during ripening. However, abscisic acid had been isolated from roots, stems, fruits, seeds, leaves and tubers of higher plants, so it was possible that synthesis occurred in all of these tissues. Tomato fruit were chosen for the first experiments because they can easily be grown and because they had been shown to incorporate [ $^{14}\text{C}$ ] from [ $2\text{-}^{14}\text{C}$ ]mevalonic acid into carotenoids.

The structure of abscisic acid suggests that it is assembled from three mevalonic acid residues and experiments in Chapter IV, section (i) show that [ $^{14}\text{C}$ ] label from [ $2\text{-}^{14}\text{C}$ ] labelled mevalonic acid, incubated with green tomato fruit and with ripe avocado pear fruit, is incorporated into ABA. Subsequent experiments using mixtures of [ $2\text{-}^{14}\text{C}$ ]mevalonic acid with [(4R)-4- $^3\text{H}$ ] and [(4S)-4- $^3\text{H}$ ]mevalonic acid (Robinson and Ryback, 1969) incubated with avocado pear fruit showed that the ABA isolated from the [(4R)-4- $^3\text{H}$ ]MVA experiment had a  $^3\text{H}/^{14}\text{C}$  ratio close to 2:3 (relative to a 1:1 ratio in the substrate) and that biosynthesis from [(4S)-4- $^3\text{H}$ ]MVA gave negligible incorporation of tritium. When the ABA derived from [ $2\text{-}^{14}\text{C}$ , (4R)-4- $^3\text{H}$ ]mevalonic acid was treated with NaOD in  $\text{D}_2\text{O}$  the  $^3\text{H}/^{14}\text{C}$  ratio fell to 1:3. (Treatment of ABA with either NaOH in  $\text{H}_2\text{O}$  or NaOH in  $\text{D}_2\text{O}$  facilitates the exchange of six carbon-atom bound hydrogen atoms of the ring moiety but none of the side chain.) This indicated that the initially observed  $^3\text{H}/^{14}\text{C}$  ratio 2:3 was correct for the predicted incorporation of  $^{14}\text{C}$  and  $^3\text{H}$  from MVA (see Scheme 1 below):



Scheme 1

This was not a rigorous proof of the position of the labelled atoms but the results were consistent with the retention of  $^3\text{H}$  at C-2 and C-5' when [(4R)-4- $^3\text{H}$ ]mevalonic acid was substrate and the corresponding expulsion of tritium from [(4S)-4- $^3\text{H}$ ]mevalonic acid. (The third carbon atom carrying tritium derived from C-4 of mevalonic acid is C-1' of abscisic acid and from this both of the hydrogen atoms are lost during biosynthesis. From the pattern of retention and elimination of the other tritium label, presumed to be retained at C-2 of ABA, it was concluded that ABA is biosynthesised from a precursor having a trans double bond corresponding in position to the 2-cis double bond of abscisic acid and that at some stage of the biosynthesis this is isomerized to the 2-cis double bond of abscisic acid.

Recent work (Dr. Robinson, personal communication) has shown that [ $^{14}\text{C}$ ]labelled trans-trans and cis-trans isomers of both farnesol and farnesal are converted to labelled ABA in avocado tissue. The indication from these observations is that the plants can isomerize the 2-double bond of ABA at a stage of biosynthesis close to farnesol. The isomerization takes place before the terminal oxygen function (C-1) is oxidised to a carboxylic acid because it has been found that plants are unable to isomerize 2-trans-ABA to ABA (Milborrow, 1970).

Abscisic acid is particularly resistant to oxidative degradation and no method could be found which converted ABA into identifiable subunits. The demonstration of the incorporation of mevalonic acid into abscisic acid had to rely, therefore, on the identity of behaviour during chromatography of abscisic acid and



the labelled material derived from labelled mevalonic acid. Thus not only did [ $^{14}\text{C}$ ]labelled material from [ $2\text{-}^{14}\text{C}$ ]mevalonic acid chromatograph at the same  $R_f$  as abscisic acid in 2 quite different solvent systems, one basic the other acidic, but its derivatives could not be separated from derivatives of abscisic acid. Thus [ $^{14}\text{C}$ ] labelled material ran at the same  $R_f$  as methyl abscisate after methylation with an ethereal solution of diazomethane, it was divided into two compounds by treatment with aqueous methanolic sodium borohydride solution in the same proportions as methyl abscisate is reduced by this treatment to its two 1',4'-diols. The 1',4'-diols of methyl abscisate, together with the material containing radioactivity from [ $2\text{-}^{14}\text{C}$ ]mevalonic acid, were oxidized back to methyl abscisate by finely divided manganese dioxide in dry chloroform. The parallelism of the behaviour of abscisic acid and the labelled material subjected to these treatments leaves little doubt but that the [ $2\text{-}^{14}\text{C}$ ]mevalonic acid has been incorporated into abscisic acid by tomato fruit and by avocado pear.

The metabolism of mevalonic acid by microorganisms and by yeast enzyme systems (Siddiqi and Rodwell, 1962) to acetoacetate proceeds in a sequence resembling closely the reverse of that proposed for the biosynthesis of mevalonic acid in yeast and mammalian tissue. [ $2\text{-}^{14}\text{C}$ ] labelled mevalonic acid was shown to be degraded by microorganisms into [ $^{14}\text{C}$ ] labelled acetoacetate and unlabelled acetyl CoA. [ $2\text{-}^{14}\text{C}$ ] labelled acetic acid was not incorporated into abscisic acid by avocado pear (Dr. Robinson - Personal communication) therefore subsequent metabolism of labelled acetoacetate, derived from [ $2\text{-}^{14}\text{C}$ ]mevalonic acid, would not be expected

to contribute to the formation of labelled abscisic acid. The degradation of mevalonic acid in plant tissue has yet to be demonstrated.

[2-<sup>14</sup>C] labelled mevalonic acid has been incorporated into abscisic acid by slices of banana and strawberry fruit (Noddle and Robinson, 1969).

Although abscisic acid concentrations have been reported to increase during the ripening of various fruits, such as apple, strawberry and tomato; abscisic acid has not been claimed as being implicated in fruit development and ripening nor have applications of abscisic acid to fruit produced observable effects. Applications of 1.0 µg of abscisic acid to the emasculated buds of Rosa sheradii produced some fruit set and parthenocarpic development (Jackson and Blundell, 1966) while abscisic acid has also been shown to induce fruit fall in olive (Barnsley, Gabbott and Milborrow, 1968).

Most of the observations of abscisic acid levels and its attribution to various physiological changes have been concerned with leaves. This investigation also showed that abscisic acid was formed from [2-<sup>4</sup>H]mevalonic acid in wheat leaves. Wright and Hiron (1969) reported that the abscisic acid concentration in wheat leaves increased 40-fold during the first 4 hours of wilting. The majority of the increase was due to the biosynthesis of abscisic acid rather than by its release from a conjugate or precursor. This was shown by the increased incorporation of (±)-[2-<sup>3</sup>H]mevalonic acid into abscisic acid by wilted wheat plants in comparison with turgid plants which had absorbed equal amounts of (±)-[2-<sup>3</sup>H]mevalonic acid. Wilted wheat shoots previously supplied with (±)-[<sup>3</sup>H]mevalonic

acid solution for 2 hours converted 9 times as much mevalonic acid into abscisic acid as did wilted plants. Parallel determinations of the amount of (+)-abscisic acid in the extracts by means of its o.r.d. spectra showed that the concentration rose from less than 7 $\mu$ g to 120 $\mu$ g/kg original fresh weight. The discrepancy between the ratios of incorporation of labelled mevalonic acid in turgid and wilted plants in comparison with the amounts of (+)-abscisic acid determined by o.r.d., is considered to result from slow penetration of the labelled mevalonate. In a second experiment, approximately twice the amount of mevalonic acid was incorporated into abscisic acid in wilted plants although the concentration of (+)-abscisic acid, determined by o.r.d., showed an approximate 25-fold difference. A sub-sample of these wilted plants was maintained in a wilted condition for a further 18 hours and incorporated 8 times as much label from mevalonate into abscisic acid as during the first 4 hours.

The observation that applications of synthetic ( $\pm$ )-abscisic acid to the excised leaves of wheat and of barley caused stomatal closure and inhibition of transpiration were followed by reports (Mittelheuser and Van Steveninck, 1969) that the concentration of (+)-abscisic acid increased in wheat leaves which were induced to wilt; leaves which were kept turgid in a damp atmosphere showed no increase in (+)-abscisic acid concentration. This leads to the speculation that (+)-abscisic acid has a role in preventing undue water loss through its induction of stomatal closure and hence the restriction of transpiration of

leaves. At present little information is available regarding the effects of the other plant hormones, the auxins, the gibberellins and the cytokinins on stomatal movement and transpiration rate in leaves. However applications of kinetin to excised wheat leaves has been shown to stimulate transpiration by increasing the stomatal aperture of the treated leaves and decreasing their stomatal resistance (Pallas and Box, 1970). The possible interaction between kinetin and abscisic acid on the rate of transpiration and the effect on stomatal closure was investigated by Mittelheuser and Van Steveninck (1969). They found that  $3.8 \times 10^{-6}$  M racemic abscisic acid supplied to 14 day old, excised, wheat leaves caused a 37% reduction in transpiration rate and resulted in complete closure of the stomata. The inhibitory effect of  $1.9 \times 10^{-6}$  M racemic abscisic acid reduced, but did not completely suppress, the stimulation of transpiration caused by  $2.5 \times 10^{-5}$  M kinetin; other concentrations were not tested. Mittelheuser and Van Steveninck (1969) concluded that "although exogenously supplied plant growth substances can effect stomatal apertures and transpiration rates, there is no evidence yet available indicating that concentrations of endogenous hormones may influence these processes". The work of Wright and Hiron (1969) and the work described in this thesis suggests that endogenous synthesis of abscisic acid occurs in wheat leaves and its action may be similar to that of the hormone when applied exogenously. The movements of stomatal guard cells have been claimed to operate by changes in their suction pressure; when the guard cells have a high suction pressure they open and when it is low they close. These changes

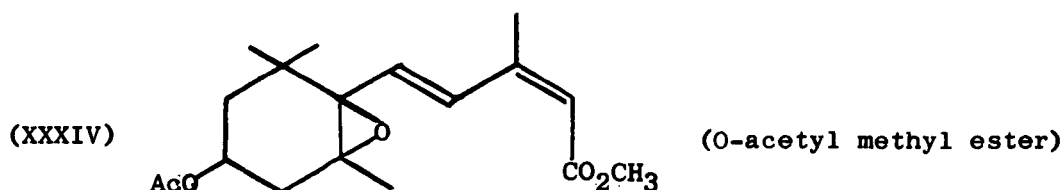
have been attributed to the interconversion of starch and glucose (Heath, 1952). The stomatal closure observed with abscisic acid could therefore be interpreted in terms of the effect of abscisic acid in stopping the synthesis of  $\alpha$ -amylase (Chrispeels and Varner, 1967) in which gibberellic acid and abscisic acid have opposing actions. Inhibition of amylase synthesis by high concentrations of ABA would be expected to inhibit amylase synthesis and thereby prevent the hydrolysis of starch. This might keep the osmotic pressure of the guard cells low and the stomata closed. Measurement of the concentration of auxin, gibberellin and cytokinins, in turgid and in wilted wheat leaves, is necessary for a full understanding of the mechanism of stomatal response to increased transpiration rates and the possible interaction of hormones in its control.

Having found tissues which biosynthesised abscisic acid, tomato fruit and wheat leaves were subsequently used to test whether some analogues of abscisic acid, which had been made by Dr. M. Anderson at Shell Research Limited, Woodstock Agricultural Research Centre, and which produced similar growth inhibitory activity to ABA, were active per se or due to their conversion to abscisic acid. Of considerable interest was the epoxide (X) which had shown considerable biological activity in inhibiting germination of seeds and inhibiting the elongation of rice coleoptiles. It was possible to consider, on the basis of structural similarities between epoxide and abscisic acid, and the similarity of substitution of the two molecules, that epoxide was active through its conversion to abscisic acid. This could be achieved either, or in part, by an adventitious series of enzyme-catalysed reactions or by the

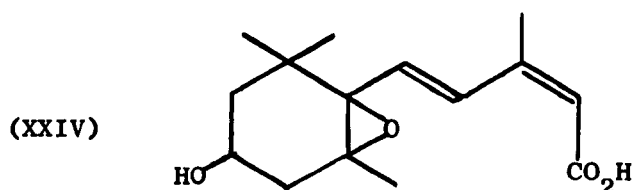
enzymes of the normal biosynthetic pathway. Alternatively the epoxide could be active per se. [2-<sup>14</sup>C] labelled epoxide supplied to either ripening tomato fruit kept in light or to unripe tomato fruit kept in darkness, was converted to labelled material which was identified as abscisic acid. Identification of the labelled material depended on its similarity in behaviour to authentic abscisic acid and derivatives of authentic abscisic acid, and from a comparison of its spectral properties with those of authentic (+)-abscisic acid. All data matched exactly.

Thus the C<sub>15</sub> carbon skeleton of epoxide is converted to abscisic acid and the biological activity of epoxide can be attributed to its conversion to abscisic acid, although activity of the epoxide per se cannot yet be excluded. The biosynthesis of abscisic acid by wheat shoots in response to the stimulus of wilting provided a means of determining whether epoxide, or a derivative, was a natural precursor of abscisic acid, or whether epoxide was converted to abscisic acid by an adventitious series of reactions. Wilted wheat shoots were found to incorporate more [<sup>14</sup>C]labelled epoxide into abscisic acid than turgid plants supplied with [<sup>14</sup>C]epoxide and therefore the conversion of epoxide to abscisic acid is probably regulated by at least one of the mechanisms which regulate the biosynthesis of abscisic acid. Epoxide extracted from avocado tissue which had been incubated with [2-<sup>14</sup>C]mevalonic acid and "cold epoxide" and which had synthesised [<sup>14</sup>C] labelled abscisic acid, was unlabelled (D. R. Robinson, personal communication). Epoxide, therefore, is probably not a natural intermediate although it could exist in a bound form as the acetyl CoA derivative or as the glucose ester and so elude the "cold trap".

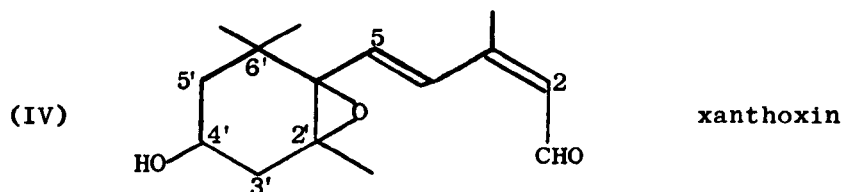
The conversion of epoxide to abscisic acid requires the net addition of a C-4'-keto group, which probably, by analogy with known reactions, comes from the addition of a hydroxyl group to C-4'. Incubation of [2-<sup>14</sup>C] labelled epoxide with plant tissue resulted in the production of only one other labelled product, besides the 2-trans isomer and the 1',2'-dihydroxy derivatives of the two isomers. This labelled material exhibited the same chromatographic properties as abscisic acid, and after methylation, as methyl abscisate. However it was not reduced by treatment with aqueous methanolic sodium borohydride solution which converted methyl abscisate into the cis-1',4'- and trans-1',4'-diols of methyl abscisate. The methylated unidentified [<sup>14</sup>C] labelled material, treated with acetic anhydride in pyridine, produced a derivative having a higher R<sub>f</sub> than methyl abscisate. From its observed properties it has tentatively been suggested that the structure of the esterified material is as shown below (XXXIV),



(Work is in progress to further characterize and identify this material; B. V. Milborrow - unpublished results). This [<sup>14</sup>C] labelled material derived from [2-<sup>14</sup>C]epoxide for which the proposed structure is (XXIV)



is similar to the material extracted from the seedlings of Phaseolus vulgaris and Triticum vulgare and which is produced on photooxidation of natural violaxanthin in vitro. This material has been designated xanthoxin (IV) (Taylor and Burden, 1970).



(The trans-2-trans-4-isomer has also been isolated from seedlings.)

If xanthoxin is a naturally-occurring intermediate in abscisic acid biosynthesis then the next step could be the oxidation of the C-1 aldehyde group to a carboxylic acid group. If this were so and if the epoxide were hydroxylated at C-4' then this product derived from the precursor could be a natural precursor. Alternatively the intermediates between epoxide and abscisic acid, although differing in the chemistry of their side chain, might still undergo the same series of oxidations and rearrangements of the ring and these reactions could be the ones in wheat leaves that are affected by the change from wet to dry conditions.

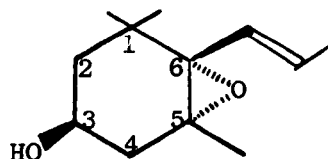
The biological activity of the xanthoxin (and the rise in the concentration of abscisic acid in seeds treated with it - Taylor, 1967) suggest that it is converted to abscisic acid. In the absence of the demonstration of the conversion of labelled xanthoxin into ABA, the possibility remains that applications of xanthoxin stimulate the biosynthesis of abscisic acid or prevent its normal degradation.



If xanthoxin is converted to abscisic acid by the rearrangement of the epoxy-group to an  $\alpha$ - $\beta$  unsaturated hydroxyl-group, the oxygen of the epoxy-group would become the oxygen of the tertiary hydroxyl group; furthermore this reaction would retain the configuration of the epoxy-bond if the C-1' oxygen bond were not broken. It was of interest to see whether the epoxy oxygen of epoxide were retained as the tertiary hydroxyl oxygen of ABA. The fate of the epoxy oxygen was investigated with wilting wheat because any [ $^{18}\text{O}$ ] containing abscisic acid formed would not be diluted by a large pool of endogenous [ $^{16}\text{O}$ ] material. A mixture of ( $\pm$ )-[1',2'- $^{18}\text{O}$ ] and ( $\pm$ )-[2- $^{14}\text{C}$ ] labelled epoxide was fed to wheat shoots which were subsequently wilted. The proportion of [ $^{18}\text{O}$ ] to [ $^{16}\text{O}$ ] in the methylated abscisic acid extracted showed that 97% of the abscisic acid had been derived from the [ $^{18}\text{O}$ ] precursor. The amount [ $^{14}\text{C}$ ] labelled abscisic acid derived from the epoxide compared with the total optically active abscisic acid determined by o.r.d. was in close agreement with the result obtained by mass-spectrometry. The oxygen of the 1',2' epoxy group therefore becomes the tertiary hydroxyl group of abscisic acid and as the same [ $^{18}\text{O}$ ] to [ $^{14}\text{C}$ ] ratio is present in the ABA as in the epoxide there was no loss of [ $^{18}\text{O}$ ] during the conversion. Further, the weight of abscisic acid calculated to be derived from the epoxide of known specific radioactivity, together with the weight of abscisic acid determined by o.r.d. and in agreement with the proportion of [ $^{18}\text{O}$ ] present excludes the presence of all but trace amounts of racemic abscisic acid. For the [ $^{18}\text{O}$ ] to be retained the C-1'-oxygen

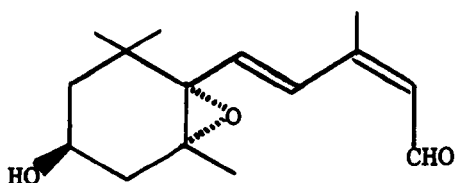
bond must remain intact and epimerization at C-1' is not possible. It follows that only one enantiomer of the epoxide is converted into abscisic acid; that one in which the epoxide is on the same side of the six membered ring as the hydroxyl group in abscisic acid. However it is not known at which stage in the conversion of epoxide to abscisic acid the stereospecific selection occurs which allows only that enantiomer, which gives rise to (+)-abscisic acid, to be further metabolized. It is probable that the mechanism which selects one enantiomer of the epoxide for further metabolism is associated with the insertion of the C-4' oxygen function. If the stereoselective mechanism operated after this stage then an equivalent amount of [2-<sup>14</sup>C] labelled intermediate of the unmetabolizable configuration would accumulate in the plant. Apart from abscisic acid and the trace amounts of labelled material which co-chromatographed with abscisic acid, but was not affected by borohydride, no other radioactive compounds derived from the epoxide were found in extracts of living tissues.

The selection of one enantiomer to form (+)-abscisic acid with retention of configuration suggests that in xanthoxin also (which has been proposed as a precursor of abscisic acid) the configuration of the 1',2'-epoxy group would be expected to be the same as the tertiary hydroxyl group of abscisic acid. However the absolute configuration of the 1',2'-epoxy group of violaxanthin (from which xanthoxin is formed photolytically) has the S configuration (Bartlett et al., 1970), in which the epoxide group is trans to the C-4' hydroxyl group as shown in Fig. (1).



- (i) Configuration of ring moiety in violaxanthin (Bartlett et al., 1970) (3S) (5R) (6S)

Therefore the conversion of xanthoxin, which must be presumed to have the structure (ii)



- (ii) proposed configuration of xanthoxin.

to natural abscisic acid suggests that either the assignment of the absolute configuration of abscisic acid or violaxanthin is wrong.

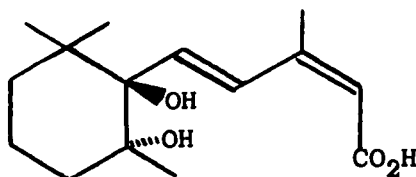
In studying the activity of analogues of abscisic acid (Tamura and Nagao, 1969(a) and 1969(b)) were primarily concerned with structure/activity relationships and with the inhibitory activity of the analogues compared with the inhibitory activity of abscisic acid. The inhibitory activity of the analogues which they studied was attributed to the compound as applied to the test plants; no consideration was given to the possibilities that they were active by virtue of their conversion to abscisic acid, that they exhibited a sparing effect on abscisic acid, that they blocked the degradation of abscisic acid or that they stimulated the synthesis of more abscisic acid. In subsequent work Tamura and Nagao (1969(c)) synthesised analogues having only the

3-methyl penta-cis-2-trans-4 dienoic acid side chain as a consequence of their earlier work, in which inhibitory activity was restricted to those analogues having this side chain; analogues with the 3-methyl penta-trans-2-trans-4-dienoic acid side chain were inactive, and no consideration was given to the possibility that an all-trans alcohol or aldehyde derivative might be active. If, as seems likely, the biosynthesis of abscisic acid involves the isomerization of a trans double bond to a cis double bond, then active analogues of abscisic acid may well be found with an all-trans side chain possessing either an alcohol or an aldehyde group at C-1. Sondheimer and Walton (1970) studied the structure-activity correlations with compounds related to abscisic acid in which the test compounds "were used to provide information on the role of the stereochemistry, functional groups and carbon skeleton in determining the activity of ABA as a growth inhibitor". It was the conclusion of these authors that present evidence suggested that the activity of analogues of abscisic acid could not be interpreted on the basis of their conversion to abscisic acid within the plant, but rather (X) on their conversion to epoxide/which they suggested was active per se.

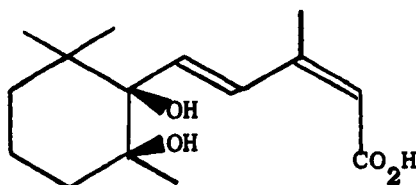
It was found that a small amount of [ $^{14}\text{C}$ ] labelled material was incorporated into abscisic acid from  $(\pm)$ -[2- $^{14}\text{C}$ ]2-trans epoxide incubated with tomato fruit in darkness. The small amount of [ $^{14}\text{C}$ ] found in the ABA is attributed to either photoisomerization of traces of 2-trans epoxide to epoxide during the manipulations before the material was applied to the fruit or to imperfect removal of  $(\pm)$ -[2- $^{14}\text{C}$ ]epoxide contaminant from  $(\pm)$ -[2- $^{14}\text{C}$ ]2-trans

epoxide. Negligible amounts of [ $^{14}\text{C}$ ] activity were detected in the abscisic acid recovered from tomato fruit which had been incubated in darkness with either the 1',2'-dihydroxy derivative of epoxide or of 2-trans epoxide; this was in accord with the published biological activity of these compounds.

(XIa) 1',2'-dihydroxy derivative with trans configuration of hydroxyl groups at C-1' and C-2' (one enantiomer of a racemic mixture shown).



(XIb) 1',2'-dihydroxy derivative with cis configuration of hydroxyl groups at C-1' and C-2' (one enantiomer shown).

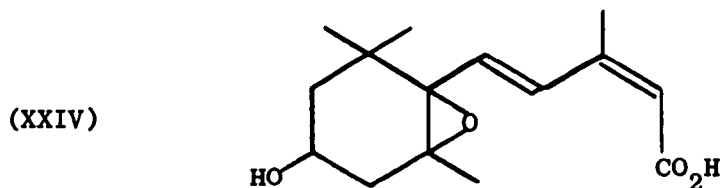


Hydrolysis of an epoxide by mineral acids produces a 1',2'-dihydroxy derivative with the hydroxyl groups in the trans configuration (XIa) and the possibility that the biological conversion of epoxide into ABA occurs via an intermediate (XIb) in which the hydroxyl groups at C-1' and C-2' are in a cis configuration cannot be dismissed. If this occurs then the 1',2'-trans compound might be expected to be inactive. The chemical hydrolysis of epoxides produces trans dihydroxy derivatives, furthermore no report of a naturally-occurring example of a cis 1,2-dihydroxy derivative formed from an epoxide has been found. It is considered, therefore, that the participation of a 1',2'-cis intermediate is unlikely.

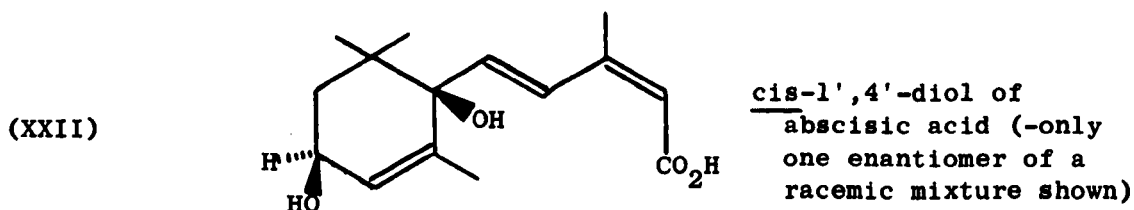
In the early work on the inhibitor- $\beta$  fraction extracted from sycamore leaves some idea of the functional groups of the active constituent was obtained by reducing the extract with sodium borohydride solution (Robinson and Wareing, 1964). This treatment caused a loss of growth inhibitory activity which was interpreted as showing the presence of a ketone group in the active molecule. When the active component of inhibitor  $\beta$  from sycamore leaves was identified later as abscisic acid, the sodium borohydride reduction products were identified as the cis-1',4'- (XXII) and trans-1',4' (XXIII)-diols of abscisic acid. Using synthetic racemic abscisic acid the cis-1',4'- and trans-1',4'-diols of abscisic acid were shown to have marked inhibitory activity (Dr. M. Anderson and Mr. R. Leach, in preparation) which was slightly less than that of abscisic acid. It has been shown that the conditions employed in the reduction of inhibitor- $\beta$ , by Robinson and Wareing, produce other unidentified products besides the 1',4'-diols, whose inhibitory activity has not been assayed but which may account for the loss of inhibitory activity detected by Robinson and Wareing. It was of interest to find out if the growth inhibitory activity of the 1',4'-diols of abscisic acid could, like that of the epoxide, be attributed to their conversion to abscisic acid. No [ $^{14}\text{C}$ ] labelled material from [ $^{14}\text{C}$ ] labelled epoxide, incubated with tomato fruit or wheat shoots, had been found on any chromatogram at the  $R_f$  of either of the 1',4'-diols so neither was expected to be on the pathway from epoxide to abscisic acid.

An attempt was made to discover whether the oxidation of the 1',4'-diols of abscisic acid to abscisic acid, if it occurred, was affected by the change from a turgid to a wilt condition in wheat plants. No significant effect was observed and so this reaction cannot be one of those controlled in the biosynthetic sequence which is affected by the change in environmental conditions.

An indication of the possible stereospecific conversion of the 1',4'-diols of abscisic acid to abscisic acid was also possible in the extent to which the cis-1',4'-diol and trans-1',4'-diol of abscisic acid were converted to abscisic acid. This was of interest since a molecule such as (XXIV)



may be a biosynthetic precursor and the conversion of the 1',4'-diols of abscisic acid might indicate the preferred configuration for the hydroxyl group at C-4'. Although not conclusive, the results suggested that the preferred configuration for the hydroxyl group at C-4' was S, as shown below (XXII), because most of the residual cis-1',4'-diol was oxidised chemically to (-)-ABA while the ABA formed from the trans-1',4'-diol was racemic.



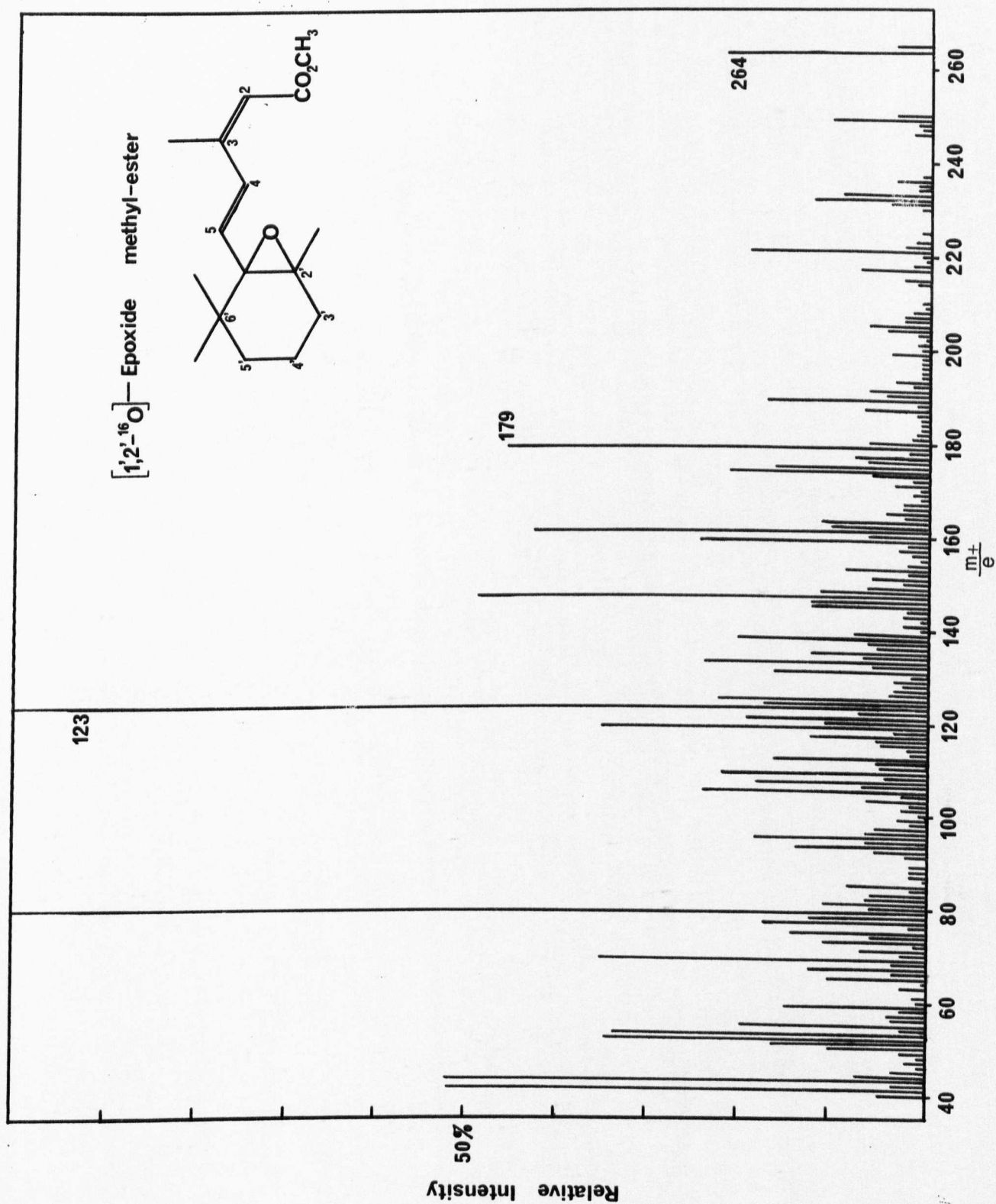
This configuration at C-4' is the same as that presumed for C-4' of xanthoxin. Although the absolute configuration of xanthoxin has not been reported, it is formed from natural violaxanthin and the configuration of the hydroxy group of this compound has been related to that of fucoxanthin which has been determined by X-ray analysis.

However the possibility that more than one enzyme was responsible for the conversion of the 1',4'-diols of abscisic acid to abscisic acid was suggested by the conversion of both cis-1',4'- and trans-1',4'-diol to abscisic acid. A "background" of non-specific conversion superimposed on the stereospecific conversion may account for these results. Both cis-1',4'- and trans-1',4'-diol of abscisic acid were converted to (-)-abscisic acid, a comparison of the specific activity and optical activity of this abscisic acid indicated that very little (+)-abscisic acid is biosynthesized from endogenous intermediates during wilting when there is a large amount of (-)-abscisic acid present, which had been formed by oxidation of the 1',4'-diols. This suggests that the extremely rapid biosynthesis of (+)-abscisic acid, which occurs in wilting wheat, was prevented either by high concentration of abscisic acid formed from the diols, the diols themselves, or by the two molecules together. The possibility that the diols are able to inhibit the rapid synthesis of ABA induced by wilting cannot be discounted by the results of the experiments reported here. The data provide evidence for the operation of a "feedback mechanism" in the biosynthesis of abscisic acid and give further indication that the biosynthesis of this hormone is subject to complex physiological control.



APPENDIX 1

DIAGRAMS OF MASS SPECTRA



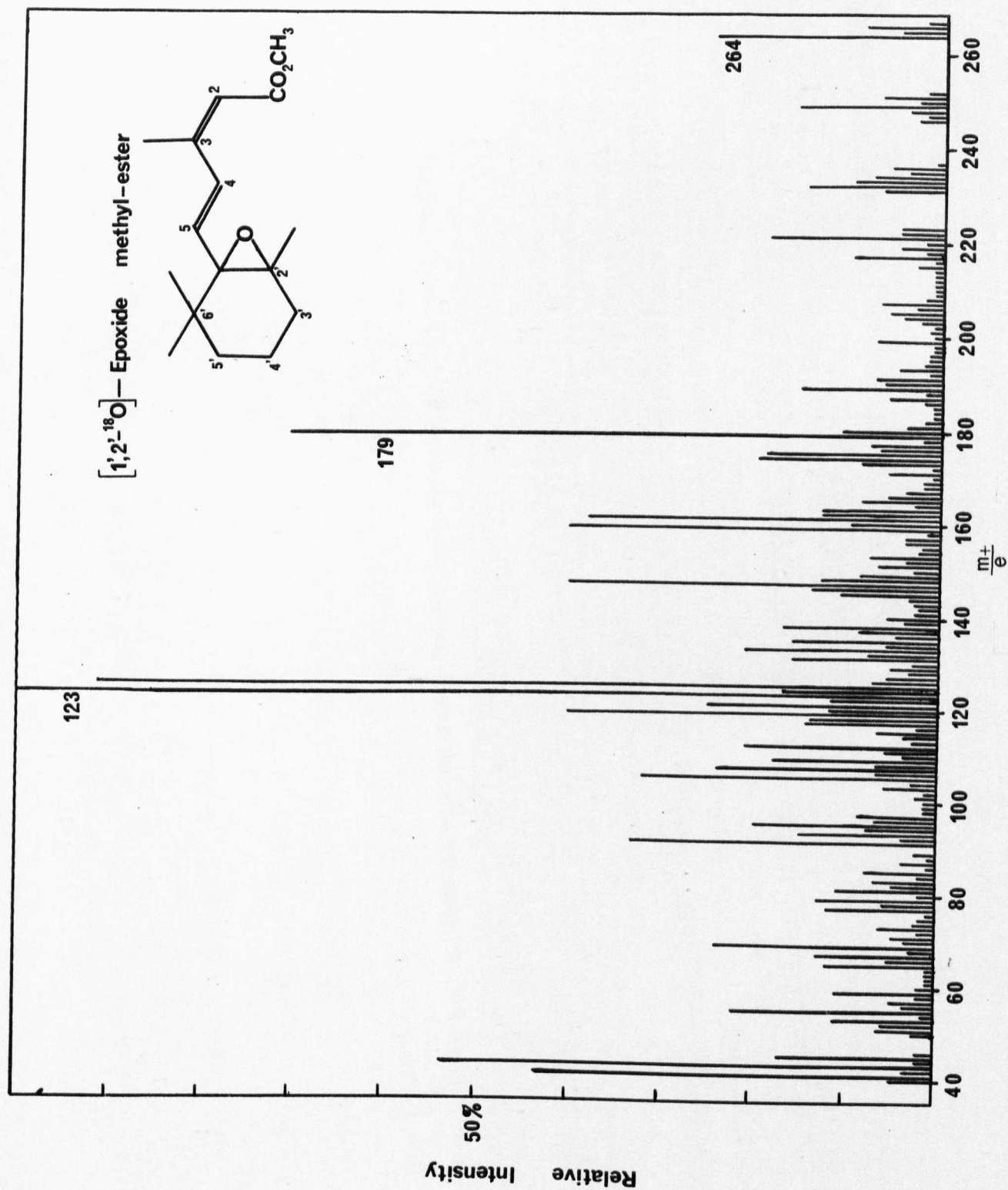


Fig 23

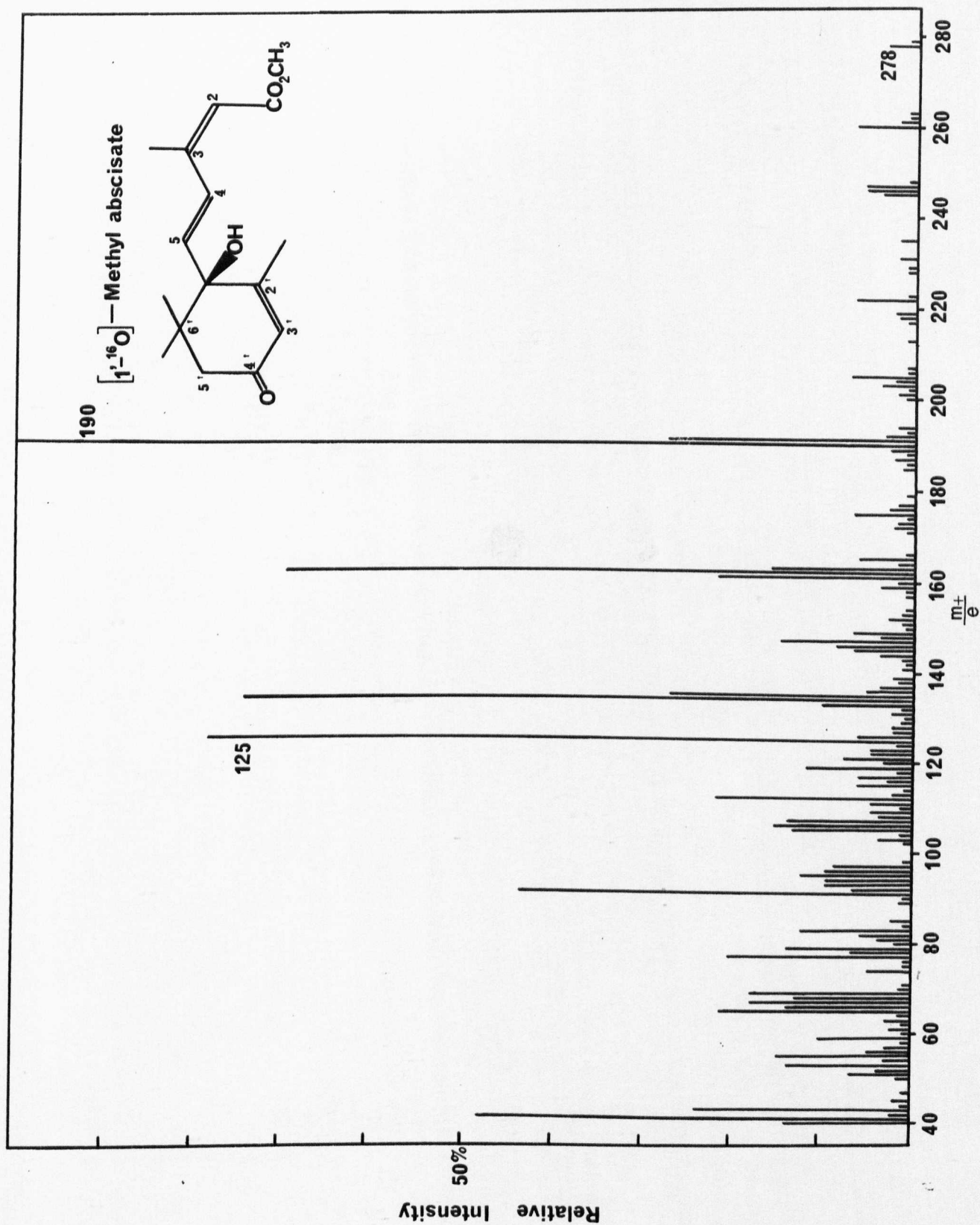


Fig 24

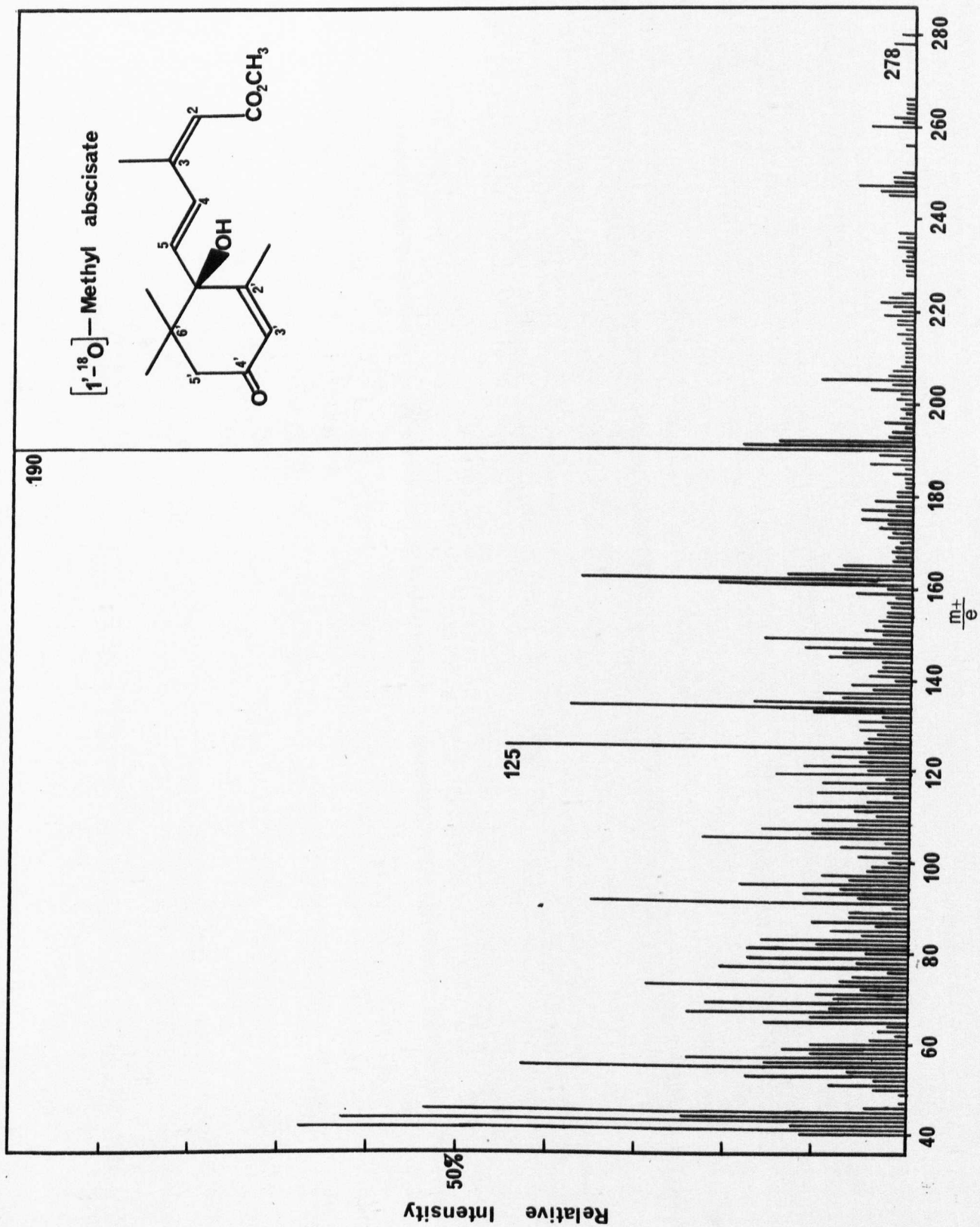


Fig 25

APPENDIX 2

Publications which include information  
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# Biosynthesis of Absciscic acid: Incorporation of Radioactivity from [2-<sup>14</sup>C]Mevalonic Acid by Intact Fruit

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As the first stage of a programme to elucidate the pathway of biosynthesis of absciscic acid, we have looked for a system capable of synthesizing this sesquiterpenoid hormone from <sup>14</sup>C-labelled mevalonic acid, presumed to be a natural precursor. The ripening fruits of four species, avocado (*Persea gratissima*), tomato (*Lycopersicon esculentum*), banana (*Musa sapientum*) and strawberry (*Fragaria* cultivar Talisman), were examined for this property chiefly because of the comparatively large amounts of absciscic acid found in fruits (see, e.g., Milborrow, 1967) and the indication that the concentrations can increase during maturation (Rudnicki, Pieniazek & Pieniazek, 1968). Tomato fruits can also give good synthesis of carotenoids from mevalonic acid (see, e.g., Purcell, Thompson & Bonner, 1959) and one possible pathway to the structure of absciscic acid could have a carotenoid as an intermediate. With established techniques (Milborrow, 1967) we have preliminarily determined (+)-absciscic acid in tomato fruits and banana fruit pulp in quantities of 0.036–0.096 and 0.535 mg./kg. fresh wt. respectively. This is the first report of the occurrence of the hormone in these plants. The presence of absciscic acid had previously been shown in avocado fruit (Milborrow, 1967) and in strawberry fruit (Rudnicki *et al.* 1968). Radioactivity from <sup>14</sup>C-labelled mevalonic acid was incorporated into absciscic acid by all these fruits, and we present here details of the procedures employed with avocado and tomato.

Avocado fruits, imported from Swaziland, were split lengthwise and the large single seeds removed. A solution of the potassium salt of (RS)-[2-<sup>14</sup>C]-mevalonic acid (1–2 ml. containing 1.5–4.0 μmoles, 4.8–6.4 μC/μmole) in 0.05 M-potassium phosphate buffer, pH 7.3, containing 2% (v/v) of ethanol, was placed in the cavity of one fruit half (fresh wt. approx. 120 g.). The cut surface was then covered, either with the other fruit half or with a glass plate, and the preparation was kept at room temperature, in normal laboratory light, for 1–5 days. As liquid was absorbed (approx. 0.5 ml. in 12 hr.) additional phosphate buffer, or water, was provided to prevent undue desiccation of the exposed surface. A second

method of mevalonate application employed with avocado fruit was to wet 5 mm.-thick slices with substrate solution held in Petri dishes. This procedure, which was also used with banana and strawberry fruits, did not improve yields and was later abandoned.

Ten green tomato fruits (about 23 g. each) were harvested from plants growing in a glass-house, and each was injected with 10 μl. of a solution of the potassium salt of (RS)-[2-<sup>14</sup>C]mevalonic acid (2.09–10.4 μmoles, 4.82 μC/μmole) in 0.05 M-potassium phosphate buffer, pH 7.3, containing 10% (v/v) of ethanol, with a Hamilton syringe. The tomato fruits were incubated for 3 days in darkness. Similar results were obtained with fruit developing red coloration under normal laboratory light.

At the conclusion of incubation the avocado or tomato fruits were macerated and thoroughly extracted with methanol. The ether-soluble acid fraction was then isolated as usual (Milborrow, 1967) and chromatographed on silica gel F<sub>254</sub> t.l.c. plates. Identification of one of the radioactive acids as absciscic acid was made principally by t.l.c. comparisons with authentic reference compounds. Precise co-chromatography of the acid with authentic absciscic acid was achieved in acetic acid-based (Fig. 1a) and ammonia-based (Fig. 1b) solvent systems. After methylation with ethereal diazomethane the material migrated with authentic methyl abscisate (Fig. 1c); this was followed by treatment with excess of NaBH<sub>4</sub> in aqueous methanol, which generated two radioactive entities migrating with the epimeric diol esters that are formed on the like treatment of methyl abscisate (Cornforth, Draber, Milborrow & Ryback, 1967) (Fig. 1d).

The extracts of these tissues contained sufficient absciscic acid for determinations to be made from u.v.-absorption spectra and optical rotatory dispersion curves. Thus the observed coincidence of radioactivity in different chromatographic systems with material exhibiting the highly characteristic spectral properties of absciscic acid, the methyl ester and the epimeric diol esters (Cornforth, Milborrow & Ryback, 1966; Cornforth *et al.* 1967; and comparison with authentic samples), plus the demonstration of reoxidation of each diol

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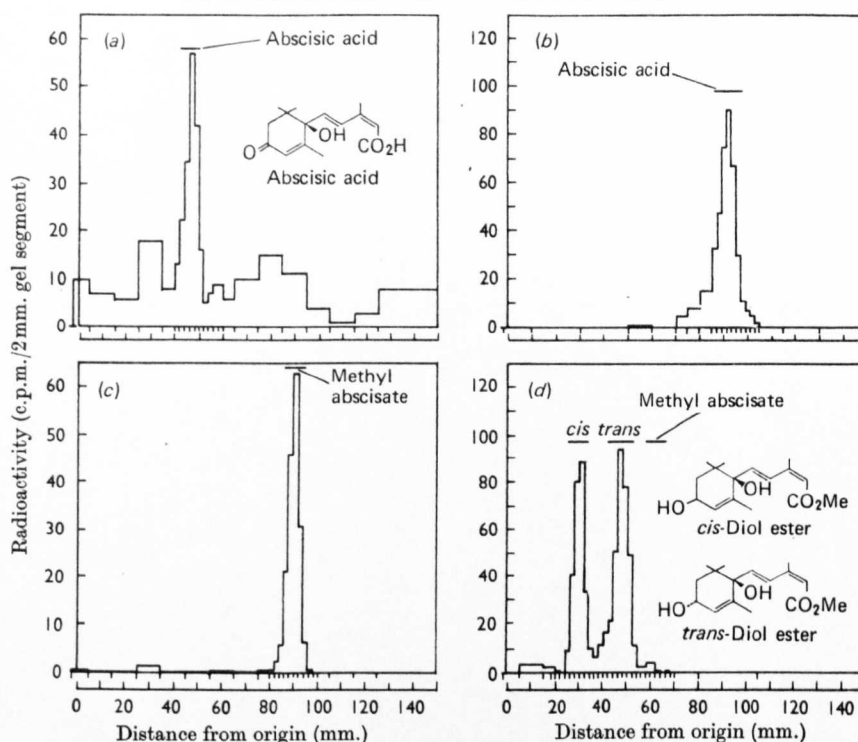


Fig. 1. Co-chromatography of radioactive (+)-abscisic acid formed in avocado fruit, and its derivatives, with authentic racemic compounds. Microgram quantities of appropriate reference compounds were mixed with radioactive material and chromatographed on silica gel t.l.c. plates. Short-wavelength u.v. light revealed the location of the reference compounds (represented by horizontal bars in the histograms) and the radioactivity in gel segments was assayed by liquid-scintillation spectrometry: (a) part of the crude acid extract of the fruit chromatographed in toluene-ethyl acetate-acetic acid (15:3:1, by vol.); (b) recovered abscisic acid zone from (a) rechromatographed in propan-1-ol-butan-1-ol-aq. ammonia (sp.gr. 0.88)-water (6:2:2:1, by vol.); (c) abscisic acid zone recovered from (b) converted into methyl ester and chromatographed in ethyl acetate-*n*-hexane (1:1, v/v); (d) products from the treatment of the methyl ester recovered from (c) with  $\text{NaBH}_4$  chromatographed in ethyl acetate-*n*-hexane (1:1, v/v).

ester with  $\text{MnO}_2$  to labelled material exhibiting the chromatographic and spectral properties of methyl abscisate, may all be considered as additional evidence for the identity of the biosynthetic product. Moreover, the specific radioactivity from spectropolarimetric measurements remained the same throughout these transformations.

In average experiments  $3.04 \mu\text{moles}$  of (*RS*)-mevalonate ( $32.50 \times 10^6 \text{d.p.m.}$ ) applied to one avocado fruit half for 4 days gave  $1.9 \text{nmole}$  ( $51500 \text{d.p.m.}$ ) of abscisic acid, and  $2.01 \mu\text{moles}$  ( $21.60 \times 10^6 \text{d.p.m.}$ ) of (*RS*)-mevalonate applied to 230g. of tomato fruit for 3 days gave  $0.2 \text{nmole}$  ( $6150 \text{d.p.m.}$ ) of abscisic acid (these values are calculated from measurements of radioactivity assuming that 1 mole of abscisic acid is formed from 3 moles of mevalonic acid). The yields of bio-synthesized abscisic acid represent 0.5% (avocado) and 0.6% (tomato) of the total abscisic acid extracted (determined quantitatively by spectro-

polarimetry). The proportion of [ $^{14}\text{C}$ ]mevalonic acid recovered as abscisic acid varied for reasons yet unknown, even in experiments with the same variety of fruit. The rapid metabolism of abscisic acid that occurs under certain conditions (B. V. Milborrow, unpublished work) may be a contributory factor.

We thank Dr G. Ryback for many helpful discussions. R.C.N. acknowledges the award of a Post-Graduate Studentship from 'Shell' International Petroleum Co. Ltd.

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## Conversion of 5-(1,2-Epoxy-2,6,6-trimethylcyclohexyl)-3-methylpenta-*cis*-2-*trans*-4-dienoic Acid into Absciscic Acid in Plants

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(±)-5-(1,2-Epoxy-2,6,6-trimethylcyclohexyl)-3-methyl[2-<sup>14</sup>C]penta-*cis*-2-*trans*-4-dienoic acid is converted into absciscic acid by tomato fruit in 1.8% yield (or 3.6% of one enantiomer if only one is utilized) and 15% of the absciscic acid is derived from the precursor. The 2-*trans*-isomer is not converted. The amounts of [2-<sup>3</sup>H]mevalonate incorporated into absciscic acid have shown that the 40-times higher concentration of (+)-absciscic acid in wilted wheat leaves in comparison with unwilted ones reported by Wright & Hiron (1969) arises by synthesis. The conversion of (±)-5-(1,2-epoxy-2,6,6-trimethylcyclohexyl)-3-methyl-[2-<sup>14</sup>C]penta-*cis*-2-*trans*-4-dienoic acid into absciscic acid by wheat leaves is also affected in the same way by wilting and it is concluded from this that the epoxide or a closely related compound derived from it is on the biosynthetic pathway leading to absciscic acid. The oxygen of the epoxy group was shown, by <sup>18</sup>O-labelling, to become the oxygen of the tertiary hydroxyl group of absciscic acid.

The growth-inhibitory activity of some analogues of absciscic acid (I) has been reported (Anderson, 1969; Tamura & Nagao, 1969*a,b*) but there has been no indication whether these compounds are active *per se* or because they give rise to absciscic acid. The strong inhibitory activity of (±)-5-(1,2-epoxy-2,6,6-trimethylcyclohexyl)-3-methylpenta-*cis*-2-*trans*-4-dienoic acid (II) 'epoxide' on plant growth could arise from the compound's molecular structure mimicking that of absciscic acid. Alternatively it could be converted into absciscic acid. (±)-5-(1,2-Epoxy-2,6,6-trimethylcyclohexyl)-3-methyl[2-<sup>14</sup>C]penta-*cis*-2-*trans*-4-dienoic acid has been synthesized by Dr G. Ryback and Mr R. Mallaby in this laboratory and we have found that it is rapidly metabolized to absciscic acid by tomato fruit. The rapid rate of this conversion suggested that the epoxide itself or a closely related product formed from it could be an intermediate in the biosynthesis of absciscic acid.

Wright & Hiron (1969) have reported that the absciscic acid concentration in wheat leaves increases 40-fold during the first 4 h of wilting and we find that this increase is caused by synthesis of absciscic acid rather than by its release from a conjugate or an immediate precursor. This was shown by the increased incorporation of [2-<sup>3</sup>H]mevalonic acid into absciscic acid by wilted wheat plants in com-

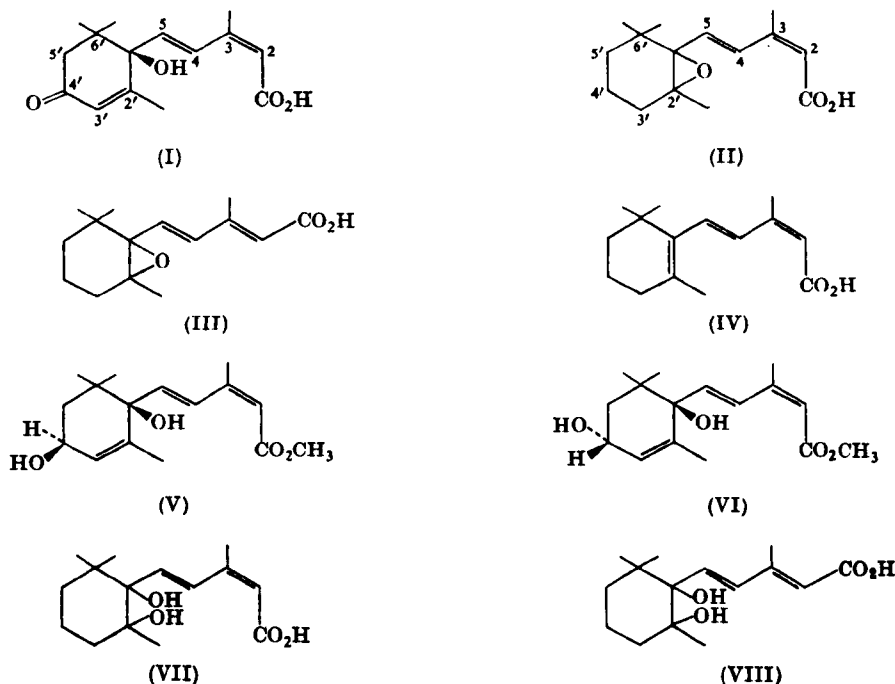
parison with turgid plants that had absorbed equal amounts of [2-<sup>3</sup>H]mevalonic acid. When racemic 2-<sup>14</sup>C-labelled epoxide (II) was supplied, larger amounts of labelled material were again incorporated into absciscic acid by wilted plants.

### MATERIALS

Racemic 5-(1,2-epoxy-2,6,6-trimethylcyclohexyl)-3-methylpenta-*cis*-2-*trans*-4-dienoic acid (II) and 3-methyl-5-(2,6,6-trimethylcyclohex-1-enyl)-penta-*cis*-2-*trans*-4-dienoic acid (IV) were synthesized by Dr M. Anderson at Woodstock Agricultural Research Centre, Sittingbourne, Kent, U.K. (Anderson, 1969); the racemic epoxide (II) labelled with <sup>14</sup>C at C-2 was synthesized by Dr G. Ryback and Mr R. Mallaby in this laboratory and we thank them for providing the material.

Racemic 5-(1,2-[<sup>18</sup>O]epoxy-2,6,6-trimethylcyclohexyl)-3-methylpenta-*cis*-2-*trans*-4-dienoic acid was synthesized from <sup>18</sup>O-enriched oxygen and the methyl ester of compound (IV) as described by Tamura & Nagao (1969*b*). No reaction occurred in darkness but the <sup>18</sup>O-labelled epoxide was formed in 25% yield when the material was illuminated by diffuse sunlight for 5 days. The product was identified by co-chromatography with authentic material and with an authentic sample of the free acid after hydrolysis in 10% (w/v) KOH in aq. (1:1, v/v) ethanol for 24 h at 20°C in the dark. Racemic absciscic acid was as used previously (Cornforth, Milborrow & Ryback, 1965). (±)-[2-<sup>3</sup>H]Mevalonic acid (93 mCi/mmol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. A.R.-grade chemicals were obtained from B.D.H. Chemicals Ltd., Poole, Dorset, U.K., Hopkin and

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Williams, Chadwell Heath, Essex, U.K., or CIBA (A.R.L.) Ltd., Duxford, Cambs., U.K.

Plants were grown in a glasshouse at approximately 23°C and with mercury vapour lamps providing supplementary illumination when necessary. Green tomato fruit (*Lycopersicon esculentum*, cultivar. F<sub>1</sub> Supercross) were used for some experiments; for other experiments wheat seedlings (*Triticum vulgare*, cultivar. Capelle) were harvested 14 days after sowing and when the shoots, cut at ground level, weighed an average of 0.37g and measured 12cm in height.

**Application of labelled compounds.** An ethanolic solution of the epoxide (II) (1mg in 1ml) was supplied to green tomatoes (126g) by multiple injections; the fruits were then kept in darkness for 63h at 24°C. Wheat shoots were placed in solutions of the [2-<sup>14</sup>C]epoxide or [2-<sup>3</sup>H]-mevalonate in 0.01M-potassium phosphate buffer, pH7.3, (50ml) and ethanol (1ml). After 2h at room temperature in the first two experiments, or 18h at 7°C in the third, the plants were removed, the submerged parts rinsed in water, and blotted. Half of the batch from each solution was placed with the cut ends in water and kept in a damp atmosphere at 20°C whereas the other half was spread out on filter paper and exposed to a draught of air for 30min, by which time the plants had lost 25–30% of their weight. They were then covered with a sheet of transparent polythene for 3.5h; both sets were illuminated until harvested.

The experiments with 2-*cis*- (II) and 2-*trans*-isomers (III) of the epoxide were carried out in darkness except for a few essential manipulations; these were done at less than 300lx. Boiled tomatoes with intact cuticles were prepared

in a steam bath for 5h, allowed to cool and treated in the same way as the living fruit.

**Extractions.** Ten tomatoes (125g total) were macerated in methanol (250ml) containing (±)-abscisic acid (156μg) and the slurry was filtered through cellulose powder and re-extracted with methanol (3×250ml). Saturated NaHCO<sub>3</sub> solution (100ml) was added to the combined extracts and the methanol was evaporated.

Wheat leaves (60g) were chopped and plunged into ice-cold methanol (2l) containing 0.05% of 2,6-di-*tert*-butyl-4-methylphenol, 2ml of saturated aq. NaHCO<sub>3</sub> and unlabelled, racemic abscisic acid (20μg/60g of turgid leaves; 50μg/60g of wilted leaves). The solution was stirred at intervals for 5 days and then the leaves were extracted a second time with 1 litre of methanol.

The methanol from both extractions was removed at 30°C, 250ml of water was added, the pH was adjusted to 8.0 and neutral materials were removed with ether. The pH was then adjusted to 3.0 with dil. H<sub>2</sub>SO<sub>4</sub> and the ether-soluble acid fraction (approx. 52μg/g of fruit) was extracted, dried and chromatographed. Any abscisic acid glucose ester (Koshimizu, Inui, Fukui & Mitsui, 1968; Milborrow, 1970) remaining in the aqueous phase was hydrolysed by heating at pH11 for 30min after a further sample of (±)-abscisic acid and 2,6-di-*tert*-butyl-4-methylphenol had been added. Acids released by this treatment were extracted with ether at pH3.0.

**Chromatographic separation and identification of abscisic acid.** The acid fractions were chromatographed on Merck precoated, silica-gel F<sub>254</sub> t.l.c. plates in benzene-ethyl acetate-acetic acid (15:3:1, by vol.) (Fig. 1) or in toluene-ethyl acetate-acetic acid (25:15:2, by vol.). The zones

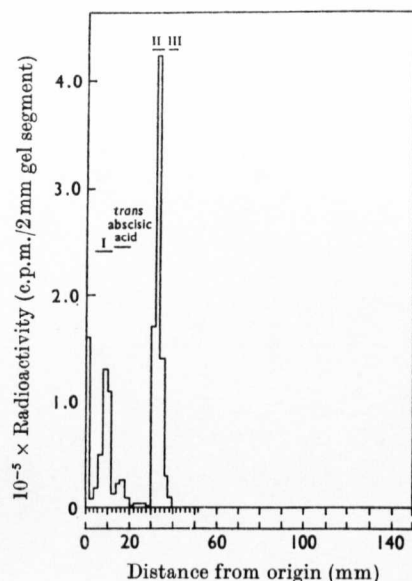


Fig. 1. Histogram of the distribution of  $^{14}\text{C}$  from  $(\pm)$ -[2- $^{14}\text{C}$ ]epoxide (II) (1.3 mCi/mmol) in the ether-soluble acid fraction from tomato fruit. The extract was chromatographed in benzene-ethyl acetate-acetic acid (15:3:1, by vol.) on a precoated silica-gel F<sub>254</sub> t.l.c. plate. The silica gel was put in the scintillation solution, which gave 50% counting efficiency.

adjacent to authentic markers were eluted with methanol, dried, and methylated with ethereal diazomethane. The methyl esters were chromatographed in hexane-ethyl acetate (1:1, v/v) (Fig. 2). Methyl abscisate was also converted into its two 1',4'-diol esters (V, VI) in ice-cold water-methanol (1:2, v/v) when a crystal of  $\text{NaBH}_4$  was added. They were separated by chromatography in the hexane-ethyl acetate solvent: *cis*-1',4'-diol (V),  $R_F$  0.38; *trans*-1',4'-diol (VI),  $R_F$  0.54 (Fig. 3).

Finally each 1',4'-diol methyl ester was eluted with methanol, dried and dissolved in dry chloroform containing finely divided  $\text{MnO}_2$ . After being stirred at room temperature, in darkness, for 3 days both diols were oxidized to methyl abscisate, which was identified by chromatography and optical-rotatory-dispersion analysis. The optical-rotatory-dispersion spectra of all compounds were measured and compared with those of authentic materials. The circular-dichroism spectra (Milborrow, 1967) and mass spectra of some samples were also measured.

The [ $^{14}\text{C}$ ]epoxide methyl ester was converted into 5-(1,2-dihydroxy-2,6,6-trimethylcyclohexyl)-3-methylpenta-*cis*-2-*trans*-4-dienoic acid (VII) methyl ester when treated with 2M- $\text{H}_2\text{SO}_4$  in aq. (1:4, v/v) methanol (Tamura & Nagao, 1969a). It was hydrolysed in ethanolic KOH and the free acids were purified by chromatography (Table 1).

Radioactively labelled 2-*cis*- and 2-*trans*-epoxides were freed of contamination with the unwanted radioactive

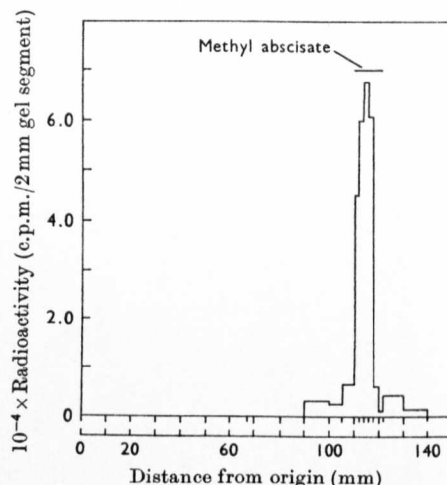


Fig. 2. Incorporation of  $^{14}\text{C}$  from  $(\pm)$ -[2- $^{14}\text{C}$ ]epoxide into abscisic acid. The zone of the t.l.c. plate in Fig. 1 corresponding to abscisic acid was eluted with methanol and the acids were methylated and chromatographed in hexane-ethyl acetate (1:1, v/v).

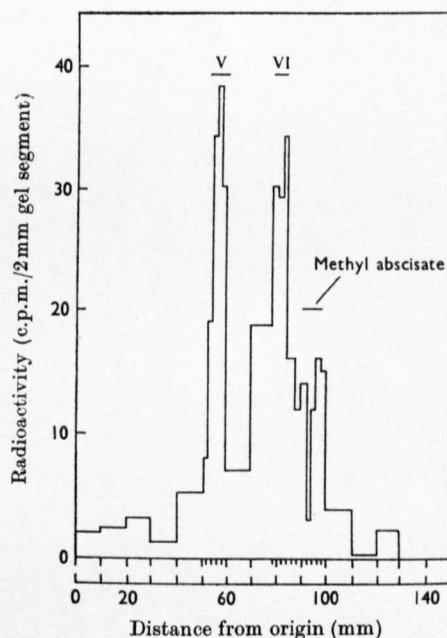


Fig. 3. Incorporation of  $^{14}\text{C}$  from  $(\pm)$ -[2- $^{14}\text{C}$ ]epoxide into abscisic acid. The zone of the t.l.c. plate in Fig. 2 corresponding to methyl abscisate was eluted and reduced with  $\text{NaBH}_4$ . The  $^{14}\text{C}$  incorporated into abscisic acid from the epoxide co-chromatographed with authentic 1',4'-*cis*- (V) and 1',4'-*trans*-diol (VI) markers in benzene-ethylacetate-acetic acid (15:3:1, by vol.).

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Table 1.  $R_F$  values of methyl esters of abscisic acid and related compounds

Compounds were subjected to t.l.c. in hexane-ethyl acetate (1:1, v/v) on Merck precoated  $F_{254}$  plates.

Methyl ester	$R_F$
Abscisic acid (I)	0.41
2- <i>trans</i> -Abscisic acid	0.42
Epoxide (II)	0.65
2- <i>trans</i> - Epoxide (III)	0.66
1',2'-Dihydroxy derivative (VII)	0.54
2- <i>trans</i> -1',2'-Dihydroxy derivative (VIII)	0.57
2- <i>cis</i> -1',4'-Diol of abscisic acid (V)	0.19
2- <i>trans</i> -1',4'-Diol of abscisic acid (VI)	0.31

isomer by an addition of 10% of the unlabelled impurity; this was followed by a chromatographic separation.

The 2-*trans*-isomer of the epoxide (III) was converted into its 1,2-dihydroxy-2,6,6-trimethyl-1-cyclohexyl derivative (VIII) in boiled and in living tomato fruit. The [ $^{14}\text{C}$ ]epoxide was converted into its 1,2-dihydroxy-derivative in boiled but not in living tomato fruit. These products and their methyl esters were identified by co-chromatography with authentic materials and by their u.v. spectra.

**Measurement of abscisic acid.** The quantity of (+)-abscisic acid in a purified extract was measured by its rotation of polarized light at 289nm (Cornforth, Milborrow & Ryback, 1966). The spectropolarimeter used, a modified Bellingham-Stanley (Bendix) Polarmatic 62 coupled to a Bryans  $x$ - $y$  plotter (model 20180 S), could produce a deflexion of 2cm for 1 $\mu\text{g}$  of (+)-abscisic acid/ml per 1cm path length in acidic methanol. The (+)-abscisic acid methyl ester was dissolved in neutral methanol because its optical rotatory dispersion spectrum is unaffected by pH and is identical with that of the free acid in acidic methanol (2.5mm- $\text{H}_2\text{SO}_4$ ). Amounts as small as 0.3 $\mu\text{g}$  of (+)-abscisic acid could be determined.

The quantities of extractable (+)-abscisic acid present in the plant were determined by the 'Racemate Dilution' method (Milborrow, 1967). This technique depends on the optical activity of the natural abscisic acid and the absence of optical activity in the synthetic compound. A ratio is set up between an unknown quantity of (+)-abscisic acid in the plant tissue and a known amount of ( $\pm$ )-abscisic acid in the solution in which it is macerated. The total abscisic acid in a highly purified extract is measured from its u.v. absorption ( $\epsilon_{260.5}$  21400) in a Unicam SP. 800 or a Cary 14 spectrophotometer and the proportion of (+)-abscisic acid in the solution is measured by its optical rotatory dispersion. Provided no differential loss of one enantiomer occurs, e.g. by enzyme action or crystallization, then these data give the ratio, and hence the amount of free (+)-abscisic acid originally present in the tissue can be calculated. This value is unaffected by losses during purification.

**Assay of radioactivity.** Samples containing  $^{14}\text{C}$  were dissolved in a scintillation solution of 2,5-bis-(5-*tert*-butylbenzoxazol-2-yl)thiophen (4g/l) in redistilled methanol-toluene (1:1, v/v) and measured in a Packard Tri-Carb scintillation spectrometer (series 314E) that gave

50% efficiency of counting. Simultaneous measurements of  $^3\text{H}$  and  $^{14}\text{C}$  were carried out in a solution containing 2,5-bis-(5-*tert*-butylbenzoxazol-2-yl)thiophen (6g/l) and naphthalene (80g/l) in 1 litre of methoxyethanol-toluene (2:3, v/v) in a Packard Tri-Carb model 3375 that gave 56.6% counting efficiency for  $^{14}\text{C}$  and 0.09% for  $^3\text{H}$  in one channel and 18%  $^{14}\text{C}$ , 24%  $^3\text{H}$  in the other. Radioactive spots on precoated silica-gel t.l.c. plates were located either with a Packard radiochromatogram scanner (model 7200), rate-meter (model 385) and Honeywell Electronik 18 recorder or by covering the plates with Kodak Kodirex X-ray film and preparing the radioautograms with D II developer and Amfix fixative (May and Baker Ltd).

**Assay of  $^{18}\text{O}$ .** The  $^{18}\text{O}/^{16}\text{O}$  ratio in the epoxide, and in abscisic acid formed from it, was determined by mass spectrometry of their methyl esters. This was carried out with a Varian G.L.C. (series 1200) coupled to an A.E.I. MS9 via a Llewellyn-Type separator (Hawes, Mallaby & Williams, 1969). The mean intensity of the parent ion of the  $^{18}\text{O}$ -labelled epoxide at  $m/e$  266 was 66% of the height of the parent ion at  $m/e$  264 (40 atoms % enrichment with  $^{18}\text{O}$ ).  $^{18}\text{O}$  was absent from the side chains of (I) and (II) because there was no satellite peak two mass units greater than the  $m/e$  125 peak which is attributed to the methyl dienolate side-chain-fragment ion in both compounds. The mass spectrum of the epoxide methyl ester contained peaks characteristic of authentic material at  $m/e$  232, 189, 179 (25%), 174, 147 (23%), 138, 133, 125 (10%), 123 (100%), 95, 93, 79 (38%), 69 (17%) and 52 (20%).

## RESULTS AND DISCUSSION

Green tomato fruit have already been shown to synthesize abscisic acid from mevalonic acid (Noddle & Robinson, 1969) and were, therefore, used for the first experiments to find whether 5-(1,2-epoxy-2,6,6-trimethylcyclohexyl)-3-methyl-[2- $^{14}\text{C}$ ]penta-*cis*-2-*trans*-4-dienoic acid (II) can be converted into abscisic acid (I).

The criteria we use to demonstrate incorporation of a labelled compound into abscisic acid require that the radioactivity should not only co-chromatograph with abscisic acid but also with methyl abscisate after methylation, should be divided between the methyl esters of the 1',4'-*cis*- (V) and 1',4'-*trans*- (VI) diols formed from methyl abscisate by treatment with methanolic borohydride, and should reappear in methyl abscisate after oxidation of both 1',4'-diols (Figs. 1, 2 and 3; Table 2). Optical-rotatory-dispersion spectra of the zones of the chromatograms opposite authentic markers were also obtained and when they contained natural (+)-abscisic acid, or the products derived from it, they exhibited the characteristic optical-rotatory-dispersion and u.v. spectra (Cornforth, Draber, Milborrow & Ryback, 1967).

The 2-*trans*-isomer, 5-(1,2-epoxy-2,6,6-trimethylcyclohexyl)-3-methylpenta-*cis*-2-*trans*-4-dienoic acid (III), was not converted into abscisic acid; there was a progressive loss of radioactivity as the various derivatives were formed and purified. The

Table 2. Incorporation of  $(\pm)$ -[2- $^{14}\text{C}$ ]epoxide (II) and its 2-trans-isomer (III) into abscisic acid

The compounds (1.3 mCi/mmol) were injected in ethanolic solution into green or boiled green tomato fruits (125 g)

	Dose injected (d.p.m.)	Radioactivity in abscisic acid (methyl ester) after racemate dilution correction (d.p.m.)	Incorporation (%)	(+)-Abscisic acid isolated ( $\mu\text{g}/125\text{ g}$ , assayed by racemate dilution method)	Radioactivity in 1',2'- dihydroxy derivative (d.p.m.)
Epoxide	11 687 000	206 300	1.8	129	—
2-trans-Epoxide	12 191 000	5124	0.042	97	—
Epoxide into boiled tomatoes	5 747 000	2196	0.038	24.6	200 000
2-trans-Epoxide into boiled tomatoes	6 411 000	920	0.014	27.3	1 392 000

2-*cis*-epoxide and 2-*trans*-epoxide were also injected into boiled tomatoes and both compounds were converted into the 1,2-dihydroxy-2,6,6-trimethylcyclohexyl derivatives (VII, VIII) (Table 2).

As these products were also formed from the epoxides by treatment with dilute acids, the large amounts found in boiled fruit were probably formed by non-enzymic reactions. The 1',2'-dihydroxy derivative (VII) is not inhibitory to coleoptile extension growth (Tamura & Nagao, 1969c), and is unlikely, therefore, to be an intermediate in the series of reactions between the epoxide and abscisic acid.  $(\pm)$ -5-(1,2-Dihydroxy-2,6,6-trimethylcyclohexyl)-3-methyl[2- $^{14}\text{C}$ ]penta-*cis*-2-*trans*-4-dienoic acid was not converted into abscisic acid by tomatoes (Table 3).

All isolates of naturally-occurring abscisic acid have given almost identical, positive, specific rotations so it appears that only (+)-abscisic acid is synthesized by plants. Racemic 5-(1,3-epoxy-2,6,6-trimethylcyclohexyl)-3-methylpenta-*cis*-2-*trans*-4-dienoic acid (II) was injected into the tomatoes but it is most unlikely that both enantiomers of the epoxide are converted into abscisic acid. Measurements of radioactivity found in abscisic acid indicate that 1.8% of the total epoxide (or 3.6% if one enantiomer only is metabolized to abscisic acid) is recovered as abscisic acid after 48 h. This amount represents 15% of the abscisic acid in the plant. The unchanged epoxide recovered from the tomatoes does indeed show slight optical activity and this could result from the conversion of one enantiomer only into abscisic acid, leaving an excess of the other; the rotatory power of the pure epoxide enantiomers would in any case be expected to be far less than that of abscisic acid. On the other hand the optical activity of the epoxide remaining in the plant could also arise from a stereospecific metabolism of either enantiomer to some other product. The amount of  $^{14}\text{C}$ -labelled 2-*trans*-epoxide (III) incorporated into abscisic acid by tomato fruit was so small in comparison with the amount of the 2-*cis*-isomer (0.0016% compared to 0.44%) that it may be attributed to photolytic isomerization during isolation or residual contamination of the starting material and it is concluded that the 2-*trans*-isomer (III) cannot act as a precursor of abscisic acid (Table 2). The 2-*cis* double bond of abscisic acid is probably first formed in the *trans* configuration (Robinson & Ryback, 1969) and no enzymic isomerization of 2-*trans*-abscisic acid has been detected (Milborrow, 1970). The non-incorporation of the 2-*trans*-epoxide puts the stage at which this bond is isomerized to its final *cis* configuration to an earlier phase of the biosynthesis.

Wright & Hiron (1969) have found that wheat seedlings normally contain about 20  $\mu\text{g}$  of extractable (+)-abscisic acid/kg fresh weight but when

Table 3. Incorporation of ( $\pm$ )-(1,2-dihydroxy-2,6,6-trimethylcyclohexyl)-3-methyl-[2- $^{14}$ C]penta-cis-2-trans-4-dienoic acid (1.3mCi/mmol) and its 2-trans-isomer (1.3mCi/mmol) into abscisic acid by green tomato fruit (83 and 99g) during 48h at 20°C in the dark

	Radioactivity (d.p.m.)		(+)-Abscisic acid isolated, assayed by racemate dilution method ( $\mu$ g)
	Injected	In abscisic acid methyl ester	
2-cis-1',2'-Dihydroxy derivative (VII)	199700	34	36
2-trans-1',2'-Dihydroxy derivative (VIII)	223800	0	27

caused to wilt for 4h the concentration increases to 500 $\mu$ g/kg. This rise could be caused by the release of bound abscisic acid or by its formation from a large pool of a precursor but we have found that wilted wheat shoots, previously supplied with ( $\pm$ )-[ $^3$ H]mevalonic acid solution for 2h, convert nine times as much mevalonate into abscisic acid as do unwilted plants. Parallel determinations of the amount of (+)-abscisic acid in the extracts by means of its optical rotatory dispersion showed that the concentration rose from less than 7 $\mu$ g to 120 $\mu$ g/kg original fresh wt. (Table 4). The discrepancy between the ratios of incorporation of labelled mevalonate in turgid and wilted plants in comparison with the amounts of (+)-abscisic acid determined by optical rotatory dispersion is considered to result from slow penetration of the labelled mevalonate because in a second experiment approximately twice the amount of mevalonate was incorporated into abscisic acid in wilted seedlings although the concentrations of abscisic acid determined by optical rotatory dispersion showed an approximately 25-fold difference. A subsample of these wilted plants was maintained in a wilted condition for a further 18h and incorporated 8 times as much label from mevalonate into abscisic acid as during the first 4h (Table 5).

These results suggested a novel way of investigating whether the conversion of the epoxide (II) into abscisic acid was regulated in the same way as the pathway by which abscisic acid is made *in vivo*. In a second wheat experiment with [ $^3$ H]mevalonate the  $^{14}$ C-labelled epoxide was included, alone and mixed with [ $^3$ H]mevalonate. The samples of plants that had absorbed these solutions were divided and exposed to wet and dry conditions and analysed as before. The greater incorporation of [2- $^{14}$ C]epoxide into abscisic acid by wilting leaves suggests that the epoxide, or a close derivative of it, is a natural intermediate because the amount incorporated is regulated in the same way as the endogenous biosynthesis of abscisic acid. If the conversion were brought about by an adventitious series of reactions this parallelism would not be expected to occur. The response to an environmental factor of the endo-

genous synthesis of abscisic acid and of the incorporation of the epoxide indicates that a site of regulation of biosynthesis exists between the epoxide and abscisic acid.

The incorporation of  $^{14}$ C-labelled epoxide into abscisic acid shows that the carbon skeleton of the epoxide becomes the carbon skeleton of abscisic acid. The fate of the epoxy oxygen was investigated with wilting wheat because any  $^{18}$ O-containing abscisic acid formed during the experiment would not be diluted by a large pool of endogenous  $^{16}$ O material. The plants took up a solution of ( $\pm$ )-[2- $^{14}$ C,1',2'- $^{18}$ O]epoxide for 18h at 7°C before they were wilted at 20°C. The abscisic acid formed during the 4h of wilting was isolated and the proportion of  $^{18}$ O determined from the ratio of the parent ions, at  $m/e^+$  278 and  $m/e^+$  280, of the methyl ester. There is no indication of an  $m/e$  127 peak in the mass spectrum of either the epoxide methyl ester or the abscisic acid methyl ester, therefore there was no  $^{18}$ O in the dienoid acid side chain of either compound. Unpublished results in our laboratory show that the oxygen of the tertiary hydroxyl group is retained in the fragment ion of  $m/e$  190 (base peak) in abscisic acid and its methyl ester and, as expected, the mass spectrum of  $^{18}$ O-labelled abscisic acid showed a new peak at  $m/e$  192. The proportion of  $^{18}$ O to  $^{16}$ O in the methyl abscisic acid showed that 97% had been derived from the  $^{18}$ O-labelled precursor. The amount of  $^{14}$ C-labelled abscisic acid derived from the epoxide compared with the total optically active abscisic acid determined by optical-rotatory-dispersion analysis is in close agreement with the result obtained by mass spectrometry (Table 6). The oxygen of the 1,2-epoxy group, therefore, becomes the tertiary hydroxyl of abscisic acid and the conversion is quantitative. Further, the weight of abscisic acid calculated to be derived from the epoxide of known specific radioactivity, together with the weight of abscisic acid determined by optical rotatory dispersion, and in agreement with the proportion of  $^{18}$ O present, excludes the presence of any but trace amounts of racemic abscisic acid. For the  $^{18}$ O to be retained the C-1'-oxygen bond

Table 4. Incorporation of  $(\pm)$ -[2- $^3$ H]mevalonate (93mCi/mmol) into  $(+)$ -abscisic acid by cut wheat leaves

The wheat plants absorbed 19  $\mu$ g of mevalonate in 20 ml of 0.01 M-potassium phosphate buffer, pH 7.3, 3% (v/v) in ethanol during 2 h. The batch was then divided and one half was allowed to wilt so that it lost 20% of its weight; the other half was placed with the cut ends in water for 5 h.

Treatment	Original wt. (g)	(+)-Abscisic acid ( $\mu$ g/kg)	(+)-Abscisic acid (assayed by racemate dilution method) ( $\mu$ g/kg)	Radioactivity in abscisic acid (assayed by racemate dilution method) (d.p.m.)	s.e. (%)	% ( $\pm$ )-mevalonate incorporated	% abscisic acid formed from mevalonate
Water	75	<4	<6.6	286	1	0.0013	—
Wilt	70	80	120	2600	1	0.012	0.012

Table 5. Incorporation of  $(\pm)$ -[2- $^3$ H]mevalonate and  $(\pm)$ -[2- $^{14}$ C]epoxide into abscisic acid by cut wheat shoots

The plants were treated as before except that the wilted batches lost 28% of their original weight during 6 h of treatment and ethanol was omitted. Small subamples of two (\*) were kept for a further 18 h before extraction (24 h).  $(\pm)$ -[2- $^3$ H]Mevalonate (10.8  $\mu$ mol) (93 mCi/mmol) and  $(\pm)$ -[2- $^{14}$ C]epoxide (1.3 mCi/mmol) were supplied as the potassium salt. Incorporation results are calculated from the amount of racemate taken up by the plants and assuming that four  $^3$ H atoms from mevalonate are incorporated into one molecule of abscisic acid. The relatively small amounts of the precursors incorporated in 6 h are attributed to their slow rates of penetration into the cells. R.D., racemate dilution.

Treatment	Original fresh wt. (g)	Weight absorbed ( $\mu$ g)		(+)-A abscisic acid extracted ( $\mu$ g/kg)	(+) -Abscisic acid assayed by R.D. method ( $\mu$ g/kg)	Radioactivity in abscisic acid after R.D. correction (d.p.m./sample)		% incorporation		% of abscisic acid formed from precursor	
		$^3\text{H}$	$^{14}\text{C}$			$^3\text{H}$	$^{14}\text{C}$	Mevalonate	Epoxide	Mevalonate	Epoxide
Wet control	58.6	—	—	1.1	19.4	—	—	—	—	—	—
Wilted control	43.8	—	—	29.2	670	—	—	—	—	—	—
Wet + mevalonate	33.1	7.72	—	0.37	15.1	280	—	0.000262	—	0.049	—
Wilted + mevalonate*	57.0	6.84	—	29.6	520	6771	—	0.0072	—	0.0015	—
* 24 h	1.4	0.168	—	—	—	138	(not R.D.)	0.054	—	—	—
Wet + mevalonate + epoxide	53.5	11.18	9.5	0.62	15.0	310	1339	0.000192	1.28	0.033	15
Wilted + mevalonate + epoxide	37.2	9.99	10.6	22.1	590	1302	2068	0.0094	1.77	0.0038	0.90
Wet + epoxide	57.0	—	8.6	1.7	31.6	—	1054	—	1.12	—	5.6
Wilted + epoxide*	57.0	—	8.0	23.7	415	—	3018	—	3.42	—	1.2
* 24 h	2.0	—	0.028	—	—	(not R.D.)	111	—	36	—	—

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