Phytochemistry, 1973, Vol. 12, pp. 1597 to 1608. Pergamon Press. Printed in England.

FORMATION OF (−)-1′,2′-*EPI*-2-*CIS*-XANTHOXIN ACID FROM A PRECURSOR OF ABSCISIC ACID

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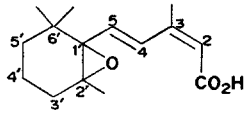
(*Received* 21 *December* 1972. *Accepted* 1 *February* 1973)

Key Word Index—*Lycopersicon esculeutum*; solanaceae; tomato; *Persea gratissima*; Lauraceae; avocado; biosynthesis of abscisic acid; violaxanthin; xanthoxin acid; stereochemistry.

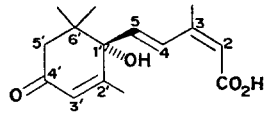
Abstract—Tomato shoots and avocado mesocarp supplied with (±)-[2-^{14}C]-5-(1,2-epoxy-2,6,6-trimethylcyclohexyl)-3-methylpenta-*cis*-2-*trans*-4-dienoic acid metabolize it into (+)-abscisic acid and a more polar material that was isolated and identified as (−)-*epi*-1′(*R*),2′(*R*)-4′(*S*)-2-*cis*-xanthoxin acid. The (+)-1′(*S*),2′(*S*)-4′(*S*)-2-*cis*-xanthoxin acid recently synthesized from natural violaxanthin, has the 1′,2′-epoxy group on the opposite side of the ring to that of the 4′(*S*)-hydroxyl group and the compound is rapidly converted into (+)-abscisic acid. The 1′,2′-epoxy group of (−)-1′,2′-*epi*-2-*cis*-xanthoxin acid is on the same side of the ring as the 4′(*S*) hydroxyl group: the compound is not metabolized into abscisic acid. The configuration of the 1′,2′-epoxy group probably controls whether or not the 4′(*S*) hydroxyl group can be oxidized. (+)-2-*cis*-Xanthoxin acid is probably not a naturally occurring intermediate because a 'cold trap', added to avocado fruit forming [^{14}C]-labelled abscisic acid from [2-^{14}C]mevalonate, failed to retain [^{14}C] label.

INTRODUCTION

COMPOUND I[1–3] is converted into abscisic acid (II) by plants. It appears not to be a natural intermediate in the synthesis of abscisic acid because a conventional 'cold trap' of I, added to a sample of avocado mesocarp which incorporated [2-^{14}C]mevalonic acid (MVA) into abscisic acid (ABA) during the experiment, and a 'cold scavenger' of I, added to a similar extract after the tissue had been killed, both failed to retain any labelled compound I. The oxygen of the 1′,2′-epoxy group of I (ABA numbering) was shown, by [^{18}O] labelling, to become the oxygen of the tertiary hydroxyl group of abscisic acid.[4] Furthermore, the abscisic acid was, within the limits of the analytical method, pure (+)-enantiomer although compound I supplied was racemic.



(I)



(II) (+)-Abscisic acid
(revised configuration)

Thus it appears that even if it were not itself a natural precursor, one enantiomer of racemic I underwent stereo-selective transformations which converted it into (+)-ABA. It is possible that some of these reactions could be catalysed by the enzymes which operate on

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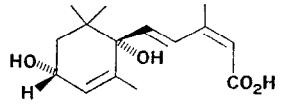
[1] ANDERSON, M. (1969) *Br. Pat.* No. 1164564.
[2] TAMURA, S. and NAGAO, M. (1969) *Planta* **85,** 209.
[3] TAMURA, S. and NAGAO, M. (1969) *Agr. Biol. Chem.* **33,** 1357.
[4] MILBORROW, B. V. and NODDLE, R. C. (1970) *Biochem. J.* **119,** 727.

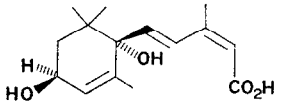
the natural precursors of ABA. Alternatively, as pointed out earlier,[1–3] I could be converted into a compound that is a normal biosynthetic intermediate. It is also possible, although unlikely, that there may not be a unique pathway for the final steps of ABA biosynthesis; the carbon skeleton could undergo the necessary transformations in a number of sequences. Consequently it was of interest to discover the nature of the metabolites of I and the factors influencing their conversion into ABA. We now report the isolation of one such compound and describe some stereochemical features which affect its metabolism.

RESULTS AND DISCUSSION

During experiments on the incorporation (±)-[2-^{14}C] compound I into ABA, the labelled abscisic acid was isolated by chromatography in butanol–propanol–ammonia–water (2:6:1:2) followed by toluene–ethyl acetate–acetic acid (50:15:1). It was then methylated and chromatographed in hexane–ethyl acetate (3:1) where only one UV absorbing zone, corresponding with marker ABA, was found to be radioactive. This zone contained (+)-abscisic acid as determined by its ORD and UV spectra, and, after it had been eluted and reduced with sodium borohydride, the bulk of the radioactivity chromatographed with the 1′,4′-*cis*-(III) and 1′,4′-*trans*-diols (IV) of ABA (Table 1). However, residual [^{14}C] labelled material again chromatographed with methyl ABA but was unaffected by further treatment with borohydride. Unlabelled racemic methyl abscisate was then added to this residue and when the mixture was treated with borohydride for a second time the resultant diols were unlabelled and the residual radioactive compound was unaffected. Treatment of this labelled residue with acetic anhydride in pyridine produced a less polar product—presumably an *O*-acetyl derivative (the tertiary hydroxyl group of ABA and analogues are not *O*-acetylated in the conditions used)—which was readily separable by TLC in hexane–ethyl acetate (3:1) and was obtained as a colourless gum. Apart from abscisic acid, and a biologically inactive acid hydrolysis product (V) of I, this material was the only other labelled product that had, at that time, been found to be formed from I in living tissues. The labelled metabolite was considered to be the 4′-hydroxy derivative of I and a possible intermediate between I and ABA.



(III) 1′,4′-*cis*-diol of (+)-ABA



(IV) 1′,4-*trans*-diol of (+)-ABA

Identification of the Metabolite

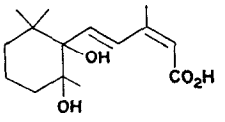
We were able to compare the physical properties of the metabolite, in the form of its *O*-acetyl methyl ester, with authentic *O*-acetyl-2-*cis*-xanthoxin acid methyl ester (VI) generously provided by Drs. Burden and Taylor of Wye College who had synthesized it from naturally occurring violaxanthin. Authentic (+)-*O*-acetylxanthoxin acid methyl ester and the material derived from I each gave one product on treatment with N H_2SO_4 and the hydrolysis products, believed to be 1′,2′,4′-trihydroxy derivatives, could not be separated by TLC. After chromatography in hexane–ethyl acetate (3:1) the *O*-acetyl methyl ester of the material derived from violaxanthin and the *O*-acetyl methyl ester of the metabolite formed biologically from I had the same R_f. However, after multiple development of a silica gel TLC plate that had been lightly loaded with linear zones of both materials, overlapping in

TABLE 1. FORMATION OF (+)-[2-^{14}C] 1′,4′-DIOLS OF ABSCISIC ACID METHYL ESTER FROM A MIXTURE OF [2-^{14}C]METHYL ABA AND [2-^{14}C]2-*cis-epi*-XANTHOXIN ACID METHYL ESTER SEPARATED FROM TOMATO SHOOTS SUPPLIED WITH I

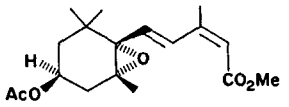
Compound I supplied (0·5 mg; 0·020 μCi/μM) to tomato shoots (107 g)		dpm
Exp. 1 (0·02 sample)	1′,4′-*cis*-diol methyl ester zone	588
	1′,4′-*trans*-diol methyl ester zone	609
	ABA and 2-*cis*-1′,2′-*epi*-xanthoxin acids methyl ester zone	629
Compound I supplied (1·2 mg; 0·035 μCi/μM) to tomato shoots (340 g)		dpm
Exp. 2 (0·1 sample)	1′,4′-*cis*-diol methyl ester zone	2645
	1′,4′-*trans*-diol methyl ester zone	2763
	ABA and 2-*cis*-1′,2′-*epi*-xanthoxin acids methyl ester zone	1062

Subsamples of the abscisic acid zones, after chromatography, were methylated and reduced to the 1′,4′-diols. The residual material containing [2-^{14}C]2-*cis-epi*-xanthoxin acid methyl ester was mixed with unlabelled methyl ABA and reduced again with $NaBH_4$. The diols found were unlabelled showing that the unreduced material from the first treatment contained no ABA

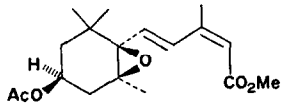
one part, the violaxanthin-derived compound ran at a slightly higher R_f. The IR spectra, obtained on films of the materials deposited by evaporation of a chloroform solution on NaCl discs, also showed very slight differences. The NMR and MS also were slightly different, but all spectral measurements of the two samples were so similar that there is no doubt that that the structures are very closely related.



(V)



(VI)



(VII) (−)-4′(*S*)-O-acetyl-2-*cis*-1′(*R*),2′(*R*)-*epi*-xanthoxin acid methyl ester

The Structure of the Metabolite

The MS of the metabolite shows a peak at *m/e* 322, which is the position of the parent ion of *O*-acetyl-2-*cis*-xanthoxin acid methyl ester. A prominent peak at *m/e* 125 in the spectra of both the metabolite and the standard is attributed to a fragment comprising the dienoic acid side-chain and ester methyl because the same fragment ion has been observed in mass spectra of the methyl esters of abscisic and phaseic acids[6] and I[7] and is replaced by a peak at *m/e* 111 in those of the free acids. Furthermore, the monodeuteriomethyl esters of abscisic acid and I formed by treatment of the free acids (which had exchanged their carboxyl protons in D_2O) with diazomethane in ether–CH_3OD (4:1), followed by washing with H_2O, showed parent ions at *m/e* 279 and 265 respectively and the *m/e* 125 fragment ion in both spectra was replaced by one at *m/e* 126. This confirms the identification of the *m/e* 125 peak as the side chain and the ester methyl. The peaks at *m/e* 43 are attributed to a fragment ion formed from an *O*-acetyl group. It follows that any difference between the two samples must arise from the ring and its other substituents.

[5] CORNFORTH, J. W., MILBORROW, B. V. and RYBACK, G. (1965) *Nature* **206**, 715.
[6] MACMILLAN, J. and PRYCE, R. J. (1968) *Chem. Commun.* 124.
[7] NODDLE, R. C. (1971) Ph.D.Thesis, University of Warwick.

Signals in the NMR spectra ($CDCl_3$) attributable to the protons on the dienoic acid side chains of *O*-acetyl-2-*cis*-xanthoxin acid methyl ester and the *O*-acetyl derivative of the methyl ester of the metabolite are superimposable (very similar signals were observed in the NMR spectra of abscisic and phaseic acids). The signals of the C-2′ and the geminal C-6′ methyl groups and the *O*-acetyl methyl differ slightly from those of the metabolite but the most marked difference lies in the position of the complex signal attributed to the methine proton of C-4′ of the two compounds (Table 2). It is probable, therefore, that this alteration arises from a difference in the structure at this position.

TABLE 2. CHEMICAL SHIFTS IN $CDCl_3$ OF PROTONS ATTRIBUTED TO THE RING AND ITS SUBSTITUENT GROUPS IN (+)-*O*-ACETYL-2-*cis*-XANTHOXIN ACID METHYL ESTER VI AND THE *O*-ACETYL, METHYL ESTER OF THE METABOLITE FORMED FROM COMPOUND I AND IDENTIFIED AS IX

Compound	C-2′ methyl	C-3′ and C-5′ methylene protons	C-6′ geminal methyls	C-4′ O-acetyl methyl	C-4′ methine proton
VI	*s* 1·21δ	Multiplets between 1·2δ and 2·5δ	*s* 1·01 & 1·15δ	*s* 2·018δ	*m* 4·94δ
VII	*s* 1·18δ	Multiplets between 1·2δ and 2·5δ	*s* 1·00 & 1·06δ	*s* 2·11δ	*m* 5·15δ

Neither the metabolite nor its *O*-acetyl methyl ester crystallized so some of the minor differences between their physical properties and the properties of the equivalent compounds derived from violaxanthin may be attributable to traces of impurities; nevertheless the IR spectra, in particular, are so similar as to support strongly the suggestion that the metabolite and the authentic (+)-2-*cis*-xanthoxin acid are stereoisomers.

ORD Measurements

The configuration of the 4′-hydroxyl group of (+)-2-*cis*-xanthoxin acid (ABA numbering) has been related by chemical synthesis to that of a derivative of fucoxanthin. It is (*S*) according to the Cahn, Ingold and Prelog rules,[8] as shown in VIII. (+)-2-*cis*-Xanthoxin acid has been converted chemically[9,10] into (+)-ABA by a method which retains the configuration of the 1′,2′-epoxide oxygen when it becomes the 1′-tertiary hydroxyl group of (+)-ABA and during the course of the experiments reported here the absolute configuration of the 1′-hydroxyl group of (+)-ABA was revised when it was unequivocally shown to be as depicted in II.[11] Similar conclusions were reached by other authors using other methods.[12,13] These results, therefore, define the absolute configuration of (+)-2-*cis*-xanthoxin acid to be as shown in VIII.

[8] CAHN, R. S., INGOLD, C. K. and PRELOG, V. (1966) *Angew. Chem. Internat. Edit.* **5**, 385.
[9] BURDEN, R. S. and TAYLOR, H. F. (1970) *Tetrahedron Letters* 4071.
[10] TAYLOR, H. F. and BURDEN, R. S. (1972) *Proc. Roy. Soc. (London)* **180B**, 317.
[11] RYBACK, G. (1972) *Chem. Commun.* 1190.
[12] ORITANI, T. and YAMASHITA, K. (1972) *Tetrahedron Letters* 2521.
[13] MILBORROW, B. V., BURDEN, R. S. and TAYLOR, H. F. unpublished.

The major component of the Cotton effect centred at 267 nm in the ORD spectrum of (+)-2-*cis*-xanthoxin acid is attributable, like that of ABA, to the dienoic acid side chain chromophore because the amplitude is greater, and the first extremum occurs at a shorter wavelength in acidic solutions than in alkaline ones. The wavelength of the UV maximum of the dienoic acid chromophore of ABA, (+)-2-*cis*-xanthoxin acid and sorbic acid show a similar bathochromic shift on acidification.

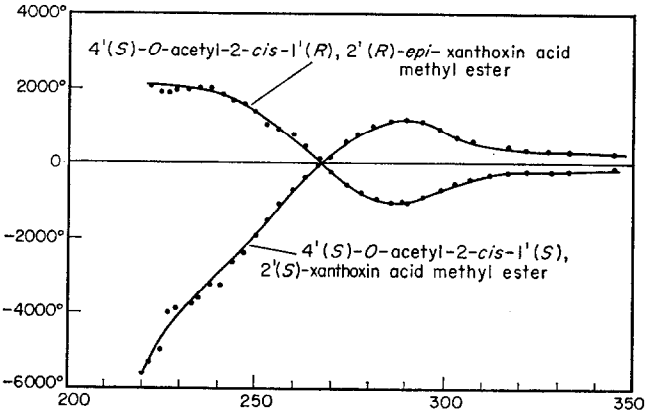
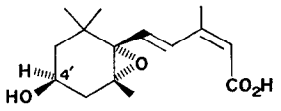
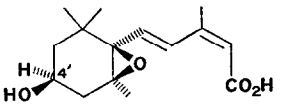


FIG. 1. ORD SPECTRA OF (+)-4'(*S*)-*O*-ACETYL-2-*cis*-1'(*S*),2'(*S*)-XANTHOXIN ACID METHYL ESTER (*c* 0·000011, EtOH) SYNTHESIZED BY BURDEN AND TAYLOR AND (−)-4'(*S*)-*O*-ACETYL-2-*cis*-1'(*R*),2'(*R*)-*epi*-XANTHOXIN ACID METHYL ESTER (*c* 0·000016, EtOH) ISOLATED FROM AVOCADO FRUIT WHICH HAD BEEN FED WITH RACEMIC COMPOUND I.

The ORD spectra of (+)-*O*-acetyl-2-*cis*-xanthoxin acid methyl ester VI and (−)-*O*-acetyl-2-*cis*-,1',2'-*epi*-xanthoxin acid methyl ester VII are almost perfect mirror images at wavelengths longer than 250 nm. Below 250 nm, where the influence of the 4'-*O*-acetyl groups should be detected, the specific (−) rotation of VI synthesized from VIII is greater than the (+) rotation of VII synthesized from IX isolated from plants fed with racemic compound I (Fig. 1). In view of the difference in other physical properties apart from ORD spectra, the two substances VIII and IX are *not* enantiomers. The signs of the Cotton effects being opposite, it can be concluded that they differ in absolute configuration at C-1' (and therefore at C-2l'), and hence they must have the *same* configurations at C-4', provided that the carbon skeletons and the position of the substituents are the same. The two epimeric compounds VI and VII would be expected to show such slight differences in physical properties as were observed.



(VIII) (+)-2-*cis*-xanthoxin acid

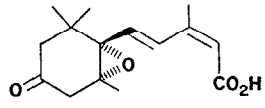


(IX) (−) 2-*cis*-1',2'-*epi*-xanthoxin acid

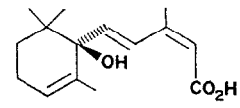
Formation of 'Xanthoxin Acids'

A possible route for the conversion of the (±)-[2-^{14}C] I to (+)-ABA, and one which does not involve xanthoxin acid as an intermediate, could be for the 1',2'-epoxide group of I to undergo isomerization as a first step. The 1'-hydroxy-α-ionylideneacetic acid produced

could then be hydroxylated at C-4′ and oxidized to give ABA. This route involves the formation of a 1′,4′-diol and as both 1′,4′-*cis*- and 1′,4′-*trans*-diols are rapidly oxidized to ABA in aqueous solutions there would be no stereospecific formation of (+)-ABA unless (i) the hydroxylation or (ii) the isomerization reactions were confined to the enantiomer of I which corresponded stereochemically at C-1′ with (+)-ABA. If the former alternative (i) occurred then labelled (*R*)-5-(1-hydroxy-2,6,6-trimethylcyclohex-2-enyl)-3-methylpenta-*cis*-2-*trans*-4-dienoic acid (XI) would be expected to accumulate (no indication of the presence of this compound was found on autoradiograms) while if the latter alternative occurred then the 1′(*R*),2′(*R*)-enantiomer of I would be expected to accumulate. The optical activity of residual I, reisolated from tomato plants, could hardly be detected and, as almost all of I absorbed is metabolized, these sequences of reactions (i and ii) probably do not occur.

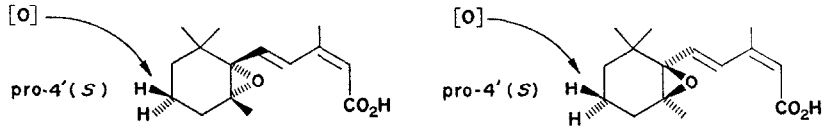


(X) 5-(1(*R*),2(*R*)-epoxy-4-oxo-2,6,6-trimethyl)-3-methylpenta-*cis*-2-*trans*-4-dienoic acid



(XI) (*R*)-hydroxy-α-ionylidene acetic acid

It appears more likely that the conversion of I into ABA proceeds by way of a 2-*cis*-xanthoxin acid. The hydroxylation required for the formation of (+)-2-*cis*-xanthoxin acid from 1′(*S*),2′(*S*)I would, by replacement of the exactly corresponding (pro-4′*S*) hydrogen in the enantiomer 1′(*R*),2′(*R*)I, give rise to two compounds (Scheme 1).



(VIII) (IX)

SCHEME 1. REPLACEMENT OF THE PRO-4′(*S*) HYDROGEN IN BOTH ENANTIOMERS OF I BY A 4′(*S*) HYDROXYL GROUP TO FORM VIII AND IX.

One is known (+)-2-*cis*-xanthoxin acid VIII. The other would be the 1′,2′-epimer of 2-*cis*-xanthoxin acid IX, and we suggest that this is the structure of our metabolite which for brevity* we shall here call 2-*cis*-1′,2′-*epi*-xanthoxin acid.

When the residual I recovered from tomato plant tissue was purified it was found to have barely detectable optical activity (a weak negative Cotton effect similar to that of xanthoxin acid but centred at *ca.* 268 nm). This confirms that both enantiomers of I are metabolized, the configuration of I that gives (+)-ABA being slightly preferred.

Formation of ABA

The conversion of xanthoxin acid to ABA requires (schematically only) the oxidation of the 4′-hydroxyl group to a ketone and the isomerization of the 1′,2′-epoxy group to a 1′-hydroxyl group and a 2′ double bond. Milborrow[14] and Noddle[7] reported that both the (+) and (−) components of racemic 1′,4′-*cis*- and 1′,4′-*trans*-diols were oxidized to ABA in

* The systematic name is: (−)-5-(1(*R*),2(*R*)-epoxy-4(*S*)-hydroxy-2,6,6-trimethylcyclohexyl-)3-methylpenta-*cis*-2-*trans*-4-dienoic acid.

[14] MILBORROW, B. V. (1972) in *Plant Growth Substances* 1970 (CARR, D. J., ed.), p. 281, Springer, Berlin.

wheat leaves and the residual, unoxidized diols were racemic so it appeared that both a 4′(*R*)- and 4′(*S*)-hydroxyl group could be oxidized. However, recent work has shown[15] that the 1′,4′-diols are oxidized to ABA spontaneously in aqueous solution in air, so the apparent lack of stereospecificity can be attributed to non-enzymatic oxidation. We find that 2-*cis*-xanthoxin acid is not oxidized under these conditions.

TABLE 3. [2-^{14}C]2-*cis*-1′,2′-*epi*-XANTHOXIN ACID (0·045 μCi/μM) WAS NOT CONVERTED IN SIGNIFICANT AMOUNTS INTO ABA BY TOMATO SHOOTS OR AVOCADO FRUIT

Compound	dpm supplied	dpm in methyl ABA analysed after 60 h†	Products from methyl ABA zone of avocado*	dpm
2-*cis*-1′,2′-*epi*-Xanthoxin acid (50 μg) supplied to 2 tomato shoots	24 450	10	1′,4′-*cis*-Diol methyl ester	65
			1′,4′-*trans*-Diol methyl ester	62
			Residual 2-*cis*-1′,2′-*epi*-Xanthoxin acid methyl ester acid hydrolysis product	2045
2-*cis*-1′,2′-*epi*-Xanthoxin acid (50 μg) supplied to half an avocado fruit	24 450	2230		

* The 'methyl ABA zone' of avocado was reduced with $NaBH_4$ to give the 1′,4′-diol methyl esters.
† The few dpm present in ABA may be attributable to traces of [2-^{14}C]2-*cis*-xanthoxin acid present in the solution because the *epi*-xanthoxin acid was prepared biologically from racemic compound I.

The accumulation of the (−)-2-*cis*-1′,2′-*epi*-xanthoxin acid in plants which had taken up a solution of I suggested that there was a stereospecific block to its further metabolism and this was confirmed in both tomato shoots and avocado fruit when (−)-[2-^{14}C]-2-*cis*-1′,2′-*epi*-xanthoxin acid, which had been isolated from avocado fruit fed (±)-[2-^{14}C] compound I, was refed to these tissues (Table 3). No significant amounts of label were found in the ABA although in a parallel experiment (+)-[2-^{14}C]-2-*cis*-xanthoxin acid was rapidly converted into ABA (Table 4).

TABLE 4. (+)-[2-^{14}C]-2-*cis*-XANTHOXIN ACID (0·38 μCi/μM) WAS CONVERTED INTO ABA IN TOMATO SHOOTS (24h); THE DATA FOR AVOCADO FRUIT WILL BE PUBLISHED ELSEWHERE[10,13]

Fraction	dpm	Fraction	dpm
(+)-2-*cis*-Xanthoxin acid supplied to 2 tomato shoots	150 000	Label in ABA (calculated from 1/10th subsample)	4320
Label in ether-soluble acid fraction other than ABA	12 700	Label in 1′,4′-diol methyl esters *cis*	894
Label in ether-soluble neutral + basic fraction	7645	*trans*	872
Label in aqueous residue	90 800	2-*cis*-Xanthoxin acid methyl ester zone	50

The incorporation into ABA appears low because a considerable proportion of the counts in the aqueous residue occurred at the same R_fs as metabolites of ABA and most of the labelled ABA formed from xanthoxin appears to have been degraded further.

Other experiments carried out with avocado fruit have shown that [^{14}C] labelled (+)-2-*cis*-xanthoxin acid is converted to ABA and that in a 'cold trap' consisting of a mixture of (±)-1′,4′-*cis*- and (±)-*trans*-diols only the *trans*-diol became heavily labelled. This probably occurred by reduction of the labelled ABA once formed since the same result was obtained

[15] MILBORROW, B. V. unpublished.

with (±)-[2-^{14}C]ABA as precursor.[13] The labelling of the *trans*-diol by reduction of ABA together with the spontaneous oxidation of both *cis*- and *trans*-diols in aqueous solutions in air renders the demonstration of the existence of the diols as intermediates between xanthoxin acid and ABA extremely difficult. The possibility of a diol being an intermediate in the conversion of xanthoxin acid into ABA has not been satisfactorily discounted. It is clear that whatever is the first step in the conversion of the epimeric 2-*cis*-xanthoxin acids into ABA the 1′,2′-*epi*-xanthoxin acid is not a substrate for the enzyme that effects this step.

There are two main possibilities. (a) A reaction, or series of reactions, causing the isomerization of the 1′,2′-epoxide group to a 1′-hydroxyl and a 2′-double bond (to produce a 1′,4′-diol of ABA); this would then be followed by oxidation of the 4′-hydroxyl to give ABA; (b) Oxidation of the 4′-hydroxyl group to give (+)-2-*cis*-4′-oxoxanthoxin acid X (or its enantiomer). This would be followed by the reaction(s) equivalent to isomerization of the 1′,2′-epoxide group, thereby forming ABA.

In chemical models the isomerization occurs much more easily in the presence of a 4′-oxo-group as in (b). Taylor and Burden[10] report that compound X, a postulated intermediate in pathway b, is highly labile and rearranges readily to ABA under mild conditions similar to those employed during our extraction procedure. It would seem likely, therefore, that once the 4′-hydroxyl group of xanthoxin acid has been oxidized, the product would rearrange and would be isolated as ABA; consequently any 1′(*R*),2′(*S*)-enantiomer of X present would be converted into (−)-ABA during extraction. The failure to detect any (−)-ABA in the ABA formed from I[4] suggests that the stereospecific block in the further conversion of (−)-2-*cis*-1′,2′-*epi*-xanthoxin acid to ABA is most likely to be the oxidation of the 4′(*S*)-hydroxyl group. As the hydroxyl group at C-4′ of IX has the same absolute configuration as the hydroxyl group at C-4′ of (+)-2-*cis*-xanthoxin acid the unnatural (*RR*) stereochemistry of the 1′,2′-epoxide group or some difference in shape induced by the presence of the 4′-hydroxyl and 1′,2′-epoxide group on the same side of the ring must be responsible for the failure of 4′(*S*) hydroxyl group to undergo oxidation. Whatever the cause it implies a high substrate specificity of the dehydrogenase enzyme system.

Metabolic Selection of Epimers

Since VI and VII are not enantiomers they would not be expected to have numerically equal specific rotations but the amplitude of the extrema at 289 nm would not be very different.

The closely similar specific rotation values (Fig. 1) of VI and VII at 289 nm and the failure of tomato plants to incorporate labelled IX into ABA, whereas they do this readily with VIII, suggest that the sample of (−)-2-*cis*-1′,2′-*epi*-xanthoxin acid IX isolated contains very little VIII. These specific rotation values are calculated on the basis that VIII and IX have the same extinction coefficient at 265 nm, consequently the ORD values may not be exactly comparable if these coefficients differ.

In previous experiments[4] the ABA formed from racemic I was found, within the limits of the method, to be 100% (+) so there is a high degree of selection at some stage for the enantiomer of I that gives rise to the (+)-ABA and against the one which would give (−)-ABA.

The presence in plant extracts of (−)-[2-^{14}C]2-*cis*,1′,2′-*epi*-xanthoxin acid and (+)-[^{14}C]ABA formed from (±)-[2-^{14}C] compound I show that the 1′(*R*),2′(*R*)-enantiomer of I can act as a substrate for a hydroxylating enzyme and suggests that the 1′(*S*),2′(*S*)-enantiomer (corresponding with (+)-ABA) is probably hydroxylated similarly.

The Occurrence of (+)-Xanthoxin Acid

There is no direct evidence for the formation of (+)-2-*cis*-xanthoxin acid from racemic I or for its being an intermediate in the metabolism of I to ABA. However, the formation of (−)-2-*cis*-1′,2′-*epi*-xanthoxin acid from racemic I (in tissues which bring about the formation of (+)-ABA from compound I), the rapid conversion of added (+)-2-*cis*-xanthoxin acid to ABA, the probable non-involvement of the diols in this process and the failure of the 1′(*S*),2′(*S*)-enantiomer of I to accumulate is strong indirect evidence suggesting that (+)-2-*cis*-xanthoxin acid is formed and rapidly metabolized.

Assuming this to be true, an experiment was devised to test whether 2-*cis*-xanthoxin acid is a natural intermediate in ABA synthesis: large amounts of (±)-I were fed to an avocado fruit that was synthesizing labelled ABA from [2-^{14}C]mevalonate. The product, presumably almost entirely 1′,2′-*epi*-xanthoxin acid, was separated as the *O*-acetyl methyl ester and counted. No significant radioactivity was found, indicating that either 2-*cis*-xanthoxin acid is not a natural intermediate in ABA biosynthesis, or that it was not being formed from I in sufficient quantity to act as a 'cold trap'.

The second method employed a conventional 'cold trap' of unlabelled (+)-2-*cis*-xanthoxin acid supplied to an avocado fruit during the synthesis of labelled ABA from [2-^{14}C]mevalonate. Both experiments were repeated and on all four occasions the counts found in the eluate of the *O*-acetyl-2-*cis*-xanthoxin acid methyl ester spot were not significantly different from zero. 2-*cis*-Xanthoxin acid, therefore, did not trap label from mevalonate and it appears from this that 2-*cis*-xanthoxin acid is probably not a normal intermediate in the biosynthesis of abscisic acid (Table 5) although it must be emphasized that this result does not exclude the possibility that a bound or conjugated form of 2-*cis*-xanthoxin acid occurs naturally as an intermediate.

TABLE 5. 'COLD TRAPS' OF 2-*cis*-XANTHOXIN ACID IN AVOCADO FRUIT SUPPLIED WITH (±)-[2-^{14}C]MEVALONOLACTONE (15 μg, 10·3 μCi/μM)

Expt.		dpm in total sample in excess of background (9·0 dpm)
1	*O*-acetyl-2-*cis*-xanthoxin aacid methyl ester	2·0
	ABA methyl ester	540 000
2	*O*-acetyl-2-*cis*-xanthoxin acid methyl ester	2·0
	ABA methyl ester	28 100
3	*O*-acetyl-2-*cis*-xanthoxin acid methyl ester	[0] (2·0 less than background)
	ABA methyl ester	4560
4	*O*-acetyl-2-*cis*-xanthoxin acid	0·4
	ABA methyl ester	61 000

In experiments 1 and 2 compound I (1·0 and 2·3 mg respectively) were added to a half of two avocado fruit (124 and 205 g total wt.) at the same time as the mevalonolactone. The fruit was harvested after 28 hr.

In experiment 3 (+)-2-*cis*-xanthoxin acid (1·0 mg) was added to 2 halves of 2 avocados (189 g total) after 7·5 hr after the addition of (±)-[2-^{14}C]mevalonolactone (55 μg, 10·3 μCi/μM). Half of each fruit was harvested after 10·5 hr and the remaining half after 25·5 hr when both extracts were combined.

In experiment 4 (±)-[2-^{14}C]mevalonolactone (15 μg, 10·3 μCi/μM) was added to two halves of an avocado fruit (118 g) and (+)-2-*cis*-xanthoxin acid (0·82 mg) was added 4 hr later. The fruit was harvested after a further 14 hr.

Formation of ABA Methyl Ester

The changes that have to be brought about to convert xanthoxin acid into ABA are confined to the ring so it was of interest to see whether the ring could be altered if the carboxyl group were blocked with a methyl ester group.

TABLE 6. CONVERSION OF (±)-[2-^{14}C]COMPOUND I METHYL ESTER AND ITS 2-*trans*-ISOMER INTO ABA

Expt. 1	
dpm in ABA methyl ester	16 360
dpm in ABA after hydrolysis and remethylation wt. of compound I converted into ABA = 27·6 μg = 5·5%	1262
Expt. 2	
dpm in ABA methyl ester	28 080
dpm in 2-*trans* ABA methyl ester	11 496
wt. of 2-*trans*-compound I converted into ABA = 1 μg = 1·1%	
dpm in ABA after hydrolysis and remethylation	870
dpm in 2-*trans*-ABA	958
Hydrolysed aqueous residue, ABA	4960
Hydrolysed aqueous residue, 2-*trans*-ABA	46 440

Experiment 1. 520 μg of compound I methyl ester were supplied to wheat shoots and the ABA methyl ester formed was isolated, hydrolysed and remethylated with TLC between each stage.

Experiment 2. 90 μg 2-*trans*-isomer of compound I methyl ester were supplied to wheat shoots which were treated as before.

(±)-[2-^{14}C]Labelled methyl ester of I was supplied to wheat plants which were later extracted and the ABA methyl ester isolated. Labelled ABA was also formed as was an alkali-hydrolysable conjugate. The formation of the latter and of the free acid could have occurred as the result of hydrolysis of the methyl ester of I followed by conversion, or conversion of I to ABA methyl ester followed by hydrolysis. The presence of [^{14}C] labelled abscisic acid methyl ester showed that the ring of compound I could undergo the reactions necessary to convert it into the ring of ABA even in the absence of a free carboxyl group at C-1, but the reaction takes place less rapidly. When the experiment was repeated using (±)-[2-^{14}C]-2-*trans*-compound I a small amount of radioactivity was found in the 2-*trans*-ABA methyl ester (Table 6). This result parallels the result obtained with the 2-*cis*- and 2-*trans*-isomers of the free acid[4] and shows that the presence of a 2-*trans*-double bond reduces the efficiency of I as a precursor of ABA (the amount of I converted fell from 5·5% to 1% on isomerization). Methylation of the C-1 carboxyl group does not completely prevent the metabolic changes which convert the molecule into methyl ABA. In contrast to this the biological activity of the methyl ester of ABA is negligible in short term (< 24 hr) experiments.[16,17] The 2-*trans*-isomer of ABA appears to be biologically inactive, and cannot be isomerised enzymatically.[18] It appears from this that the 2-*cis*-double bond exerts a major effect of biosynthesis and physiological action of ABA whereas the carboxyl group has little effect on the conversion of I into ABA but is essential for biological activity.

EXPERIMENTAL

Compounds used. The racemic abscisic acid used was synthesized by Cornforth *et al.*[5] the racemic 1′,4′-*cis*- and 1′,4′-*trans*-diols were prepared by the reduction of abscisic acid methyl ester with ice-cold $NaBH_4$ in

[16] MILBORROW, B. V. (1966) *Planta* **70**, 155.
[17] KRIEDEMANN, P. E., LOVEYS, B. R., FULLER, G. L. and LEOPOLD, A. C. (1972) *Plant Physiol.* **49**, 842.
[18] MILBORROW, B. V. (1970) *J. Exp. Botany* **21**, 17.

MeOH–H_2O (4:1) followed by hydrolysis in EtOH–10 M aq. KOH for 30 min and TLC. Racemic 5-(1,2-epoxy-2,6,6-trimethylcyclohexyl)-3-methylpenta-*cis*-2-*trans*-4-dienoic acid (I) was synthesized by Dr. M. Anderson[1] at Woodstock Agricultural Research Centre, Sittingbourne, Kent, U.K.; the [2-^{14}C] material was synthesized by Mr. R. Mallaby and Dr. G. Ryback in this laboratory and we thank them for providing this material. *RS*[2-^{14}C]Mevalonolactone (10·3 mCi/mmol) and *RS*[2-$^{2}H_2$]mevalonolactone (93 mCi/mmol) were purchased from The Radiochemical Centre, Amersham. 2,5-bis-(5-*t*-Butylbenzoxazol-2-yl) thiophen (BBOT) was obtained from CIBA (A.R.L.) Ltd, Duxford, Cambs.

Plant material. Tomato (*Lycopersicon esculentum*) cv. Ailsa Craig and wheat (*Triticum sativum*) cv. Kloker plants were grown in a glasshouse with supplementary lighting from mercury vapour lamps in short days. The shoots (150–200 mm) were cut at ground level and the ends trimmed square with a razor so that they would absorb all the liquid when placed in a beaker. Solutions were absorbed by the shoots, then the walls of the beaker and the stems were washed with dist. H_2O to ensure complete uptake of the solutions. Avocado fruits (*Persea gratissima*) of several varieties were purchased from an importer. They were used when they began to soften; the mesocarp was cut in half, the stone was removed, and the longitudinal and transverse cuts, 5–10 mm apart were made in the pulp without damaging the skin. The solutions, containing 0·2% Triton X100 as wetting agent (5 ml/fruit) were pipetted into the seed cavity and spread along the cuts.

Isolation of compounds. The tomato and wheat plants were chopped into small pieces and plunged into cold MeOH (2 l.) containing 2,6-di-*t*-butyl-4-methylphenol (BHT) (10 mg/l.) and soaked for 2–3 days; they were re-extracted 2× (2 × 1 l.). The avocado fruit were treated as described by Milborrow[19] and the Et_2O-soluble acid fractions of the two materials were also prepared as described before. In experiments in which unlabelled, racemic diols had been added to the fruit as 'cold traps' a further 0·5 mg of each as 'cold carrier' was added to the MeOH.

Chromatography. The acid fractions were applied to 2·5 mm thick pre-coated silica gel G_{254} thin-layer plates (200 × 200 mm) after the area of the plate that was to be the origin of the chromatogram had been anointed with an ethereal solution of BHT (1 mg/ml). The plates were usually given multiple developments in toluene–EtOAc–HOAc (25:15:2) but, where recorded, R_fs refer to one development only. The zones containing the relevant compounds were located by applying bands of marker on either side of the acid fraction. Part of the marker zone was covered by acid fraction because the presence of impurities often affected the R_f of the pure compound. The zone of silica gel carrying material to be isolated was cut from the plate and eluted in a glass sinter thimble with MeOH. If the first chromatography failed to give a suitable purification, the eluate of a zone was rechromatographed in the same solvent system. Free acids were then methylated and rechromatographed in hexane–EtOAc (2:1 to 4:1, all chromatographic solvents contained 200 μg BHT/ml). The methyl ester of each diol believed to contain label was chromatographed with unlabelled (±)-ABA (100 μg) just below, and the alternate diol applied just below (100 μg *trans*-diol), or above (100 μg *cis*-diol) the origin. This procedure ensures that any labelled impurity of ABA or the other diol was 'washed out' by chromatography of the cold, foreign material through the zone of the compound being purified. Altogether the diols were chromatographed 3× as free acids 2× as methyl esters, once after oxidation to methyl ABA and again after treatment of the ABA with Ac_2O in pyridine. ABA at the methyl ester stage of purification from avocados to which labelled mevalonate had been added was found to be radiochemically pure, however, the material was reduced with aq. methanolic $NaBH_4$ to give an approximately equal division of material between the 1′,4′-*cis*- and 1′,4′-*trans*-diols. The presence of label in both of the zones of the TLC plates occupied by these compounds is considered adequate evidence for the incorporation of added mevalonolactone into ABA.

Reactions of ABA and compounds I, VII *and* IX. Reduction of ABA to its isomeric diols has been described, I, VIII and IX are unaffected. All compounds were methylated in 0·1 ml MeOH by adding ethereal CH_2N_2. Acetylation was effected with Ac_2O–pyridine. Compounds I, VIII and IX were hydrolysed to their 1′,4′-dihydroxy derivatives by 0·5 M H_2SO_4 at 20°C for 30 min. ORD spectra and radioactivity were measured as described by Milborrow and Robinson (in press). Mass spectra were obtained on an A.E.I. M.S.9; NMR spectra in $CDCl_3$ with an internal TMS standard and IR spectra of thin films deposited on NaCl discs from $CHCl_3$ solution.

'Cold trap' experiments. (±)-[2-^{14}C]Mevalonolactone (10·3 μCi/μM) (15 μg) was added to the two halves of an avocado pear. 0·25 mg each of a mixture of 1′,4′-*cis*- or 1′,4′-*trans*-diols were added to one half-fruit initially and to the other after 8 hr incubation. The fruit pulp was homogenized after 24 hr in MeOH which contained a further 0·5 mg of each diol. The metabolically generated 'cold trap' of xanthoxin acid was formed by adding unlabelled, racemic compound I (2 mg) to an avocado fruit at the same time as the mevalonate. A conventional cold trap of unlabelled (+)-2-*cis*-xanthoxin acid (0·5 mg) was added initially and a further 0·4 mg after 8 hr. The trap material was isolated as a mixture of *O*-acetyl-2-*cis*-xanthoxin acid methyl ester and *O*-acetyl-2-*cis*-1′,2′-*epi*-xanthoxin acid methyl ester.

Formation of 2-cis-1′,2′-epi-*xanthoxin acid from* (±)-I. (±)-[2-^{14}C] I was converted into (−)-2-*cis*-1′,2′-*epi*-xanthoxin acid by tomato and wheat shoots and avocado fruit; it was separated by the same procedures as were used for ABA except that the eluate of the methylated compound was acetylated and then rechromato-

[19] MILBORROW, B. V. *Biochem. J.* (1972) **128,** 1135.

graphed. Most physical measurements were carried out on this *O*-acetylated, esterified material (λ_{max}^{EtOH} 265 nm). The ORD and UV measurements were calculated on the basis of 2-*cis*-1′,2′-*epi*-xanthoxin acid and 2-*cis*-xanthoxin acids' having the same molar extinction coefficient as compound I.[2] The free acid (IX) was formed by hydrolysis in ethanolic KOH.

Attempted conversion of (+)-2-cis-*xanthoxin acid and* (−)-2-cis-1′,2′-epi-*xanthoxin acid into ABA.* (−)-[2-^{14}C]-2-*cis*-1′,2′-*epi*-Xanthoxin acid (0·045 μCi/μM) was prepared by adding racemic compound I (1·94 mg) to an avocado fruit, isolating the (−)-*O*-acetyl-2-*cis*-1′,2′-*epi*-xanthoxin acid methyl ester and hydrolysing it to the free acid. The product (48 900 dpm) was divided and half was fed to 2 tomato plants and the remainder to a half avocado (137 g). The ABA (as methyl ester) isolated from the tomato plants was unlabelled. The methyl ABA fraction from the avocado was labelled but the methyl ABA diols formed from it were unlabelled showing that the methyl ABA had not contained [^{14}C]. (+)-[2-^{14}C]-2-*cis*-Xanthoxin acid (50 μg) (0·058 μCi/μM) was supplied to 2 tomato shoots which were analysed 60 hr later.

Formation of methyl ABA from I *methyl ester.* (±)-[2-^{14}C] I (0·072 μCi/μM) (0·52 mg) was esterified and an ethereal solution (100 ml) of the methyl ester was washed three times with 20 ml 1/10 satd. $NaHCO_3$ to remove any unesterified material. The ester was supplied to wheat seedlings (30 g) cut at ground level. After 28 hr (+)-ABA methyl ester was added to the extraction medium as cold carrier. An ethereal solution (300 ml) of the neutrals was washed with $NaHCO_3$ (3 × 20 ml) and then chromatographed in hexane–EtOAc (3:1) and the eluted ABA methyl ester hydrolysed to free acid. This fraction was chromatographed in toluene–EtOAc–HOAc and the ABA separated by elution, remethylation and a second chromatography as methyl ester. After this procedure the ABA methyl ester was reduced to the 1′,4′-diols. (±)-2-*trans*-[2-^{14}C] Compound I (90 μg) (1·9 μCi/μM) was prepared as radiochemically free of 2-*cis*-isomer as possible by adding an unlabaelled sample of the latter compound to the labelled 2-*trans*-material and separating them chromatographically. The remainder of the experiment was carried out as with the 2-*cis*-isomer except that the aqueous residue was hydrolysed by heating the solution to 60° for 30 min after adjusting the pH to 11. After acidification, extraction and chromatography both ABA and 2-*trans*-ABA were detected.

MS data. The cracking patterns of (−)-*O*-acetyl-2-*cis*-1′,2′-*epi*-xanthoxin and (+)-*O*-acetyl-2-*cis*-xanthoxin acid methyl esters introduced into the source of a GEC-AEI MS9 mass spectrometer on a probe (70 eV; 200°) showed different intensities of some peaks; in particular those at *m/e* 60 (30%), 81 (13%), 123 (30%), 128 (10%), 137 (12%), 149 (18%), 161 (18%), 196 (11%), 219 (13%), 247 (25%), 263 (8%), 290 (7%), 291 (5%) and the parent ion *m/e* 322 of VII (6%) were more intense than the same peaks in the spectrum of (+)-*O*-acetyl-2-*cis*-xanthoxin acid methyl ester (8, 5, 153, 1, 3, 7, 1, 2, 5, 3, 1, 0·5, 0·5, 1 and 1% respectively). Those peaks of the derivative of the *epi*-acid at *m/e* 119 (15%), 147 (14%), 148 (3%), 174 (5%), 179 (16%), 206 (6%) and 262 (8%) were weaker than the same peaks in the spectrum of the (+)-*O*-acetyl-2-*cis*-xanthoxin acid methyl ester (33, 40, 40, 33, 21, 85 and 12% respectively; base peaks *m/e* 43). Other prominent peaks at *m/e* 67 (9%), 69 (8%), 77 (9%), 91 (15%), 95 (8%), 115 (4%), 120 (9%), 125 (21%), 132 (14%), 133 (8%), 135 (18%), 145 (12%), 159 (16%), 187 (10%), 197 (4%), 203 (7%), 215 (9%), 221 (4%), 230 (7%), 231 (5%) were present in both.

Circular dichroism. The C.D. spectrum of *O*-acetyl-2-*cis*-*epi*-xanthoxin acid methyl ester shows a maximum negative value at 267 nm ($[\phi] = 2860°$, *c* 0·000016 in EtOH).

IR spectra. Strong absorption bands occur in the IR spectrum of *O*-acetyl-2-*cis*-*epi*-xanthoxin acid methyl ester at 2900, 2840, 1740, 1720, 1230 and 1160 cm^{-1}, weak bands at 2370, 2330, 1640, 1480, 1420, 1245, 1240, 1050, 920 and 840 cm^{-1} and bands of intermediate intensity at 1710, 1605, 1460, 1430, 1385, 1375, 1365, 1020 and 990 cm^{-1}. The compound VI synthesized from violaxanthin differs in that the 2900 band of the (−) occurs at 2940, the 1605 at 1600, 1640 at 1630, 990 at 993 and the triple peak at 1385, 1375 and 1365 cm^{-1} is replaced by a double peak at 1380 and 1365 cm^{-1}. In addition there are absorption bands at 1270, 1180, 1130, 868 and 848 cm^{-1} in the IR spectrum of VI.

NMR spectra. The NMR spectra of *O*-acetyl-2-*cis*-xanthoxin acid methyl ester and VII were determined in $CDCl_3$ solution at 100 MHz in a Varian HA 100. C-3 methyl *d* 2·013δ (*J* 1·6 Hz), C-ester methyl *s* 3·71δ, C-2 *s* 5·71δ, C-5 *d* 6·26δ (*J* 1·6 Hz), C-4 *d* 7·62δ (*J* 16 Hz) (attribution of C-4 and C-5 follows MacMillan and Pruce[20] for similar protons in ABA and phaseic acid; see also Table 2).

Acknowledgements—We thank Drs. Burden and Taylor of Wye College for their generous gift of (+)-*O*-acetyl-2-*cis*-xanthoxin acid methyl ester and (+)-[2-^{14}C]-2-*cis*-xanthoxin acid, Mr. R. Carrington for the NMR spectra and Mrs. Janet Tucker for the MS.

[20] MACMILLAN, J. and PRYCE, R. J. (1969) *Tetrahedron* **25**, 5891.