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METABOLISM OF ABSCISIC ACID AND THE OCCURRENCE OF *EPI*-DIHYDROPHASEIC ACID IN *PHASEOLUS VULGARIS*

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Key Word Index—*Phaseolus vulgaris*; Leguminosae; French bean; metabolism; abscisic acid; phaseic acid; dihydrophaseic acid; *epi*-dihydrophaseic acid; mass spectrometry; Kovats retention index.

Abstract—When (±)-abscisic acid-[2-^{14}C] or (±)-abscisic acid-[4′-^{18}O] was fed to bean (*Phaseolus vulgaris*) shoots, phaseic acid (PA) and dihydrophaseic acid (DPA) were the major metabolites, while *epi*-dihydrophaseic acid (*epi*-DPA) appeared as a minor metabolite. In the acidic fraction the amount of *epi*-DPA ranged from 18 to 42% of the DPA content, in the conjugated form from 50 to 200%. The content of endogenous *epi*-DPA amounted to only 1–2% of that of the DPA. These data indicate that the applied abscisic acid is not metabolised in a manner identical with that of the endogenous material. DPA and *epi*-DPA were shown to be formed separately from PA and could not be inter-converted either by the extraction conditions employed or when fed to bean shoots during short term experiments.

INTRODUCTION

Although the plant hormone (±)abscisic acid (ABA) [1] has been isolated from numerous species of higher plants, relatively little is known about its metabolic fate. Tomato shoots [1] convert (+)-ABA (**1**), but not (−)-ABA, into phaseic acid (PA) (**3**), and form the glucose ester of both (+)- and (−)-ABA. In embryonic bean axes [2,3] the major metabolites of **1** were **3** and dihydrophaseic acid (DPA) (**5**). The same two metabolites have also been isolated from ash seeds [4], but in addition a third unidentified metabolite, probably derived from **5**, was detected. The absolute configurations of **3** and of the 4′-epimeric dihydrophaseates (**5**, **9**) have been determined recently [5].

DPA (**5**) has been isolated from dry bean seeds [6], from immature pea seeds [7], and from endosperm of *Echinocystis macrocarpa* [8], but its occurrence in green plants has not been reported. The present paper describes the metabolism of ABA (**1**) in bean shoots and our evidence shows that PA (**3**) and DPA (**5**) are the major products of the metabolism of ABA, while *epi*-dihydrophaseic acid (*epi*-DPA) (**9**) occurs naturally and is a minor metabolite of ABA. A rapid and convenient method to separate and identify by TLC and GLC the methyl esters of ABA (**2**), PA (**4**), DPA (**6**), and *epi*-DPA (**10**), as well as their 2-*trans*-isomers, is also reported.

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RESULTS

Metabolism of (±)-*abscisic acid.* When (±)-ABA was applied to bean shoots and the acidic fraction was purified by TLC on silica gel F_{254}, three zones of heavily quenched fluorescence were detected under UV (Table 1). Of these the zone with the highest R_f contained residual ABA (**1**), as confirmed by GC–MS of the methyl

Table 1. Recovery of abscisic, phaseic, dihydrophaseic, and *epi*-dihydrophaseic acids from bean shoots after application of (±) abscisic acid

Compound	R_f*	In acidic fraction (μg)	Released by alkaline hydrolysis (μg)
ABA (**1**)	0·64–0·75	560 (ORD:285)†	640 (ORD:600)†
PA (**3**)	0·43–0·53	1250	175
DPA (**5**)	0·09–0·21	620	100
epi-DPA (**9**)	0·09–0·21	110	50

12 mg of (±)-ABA fed via transpiration stream to 30 bean shoots during 3 days. Fresh weight was 85 g. * System I, developed 2× to 120 mm. † Total wt was determined by GLC, the wt of the excess optically active (−)-ABA (negative extremum at 289 nm, positive extremum at 246 nm) was determined by ORD.

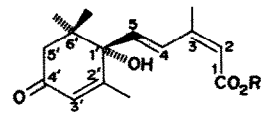
Table 2. Metabolism of (±)-abscisic acid-[2-^{14}C] by sterile bean plants

Compound present on chromatogram	[^{14}C]dpm in EtOAc fraction	[^{14}C]dpm in "released by hydrolysis" fraction	[^{14}C]dpm in ether-soluble neutral fraction (methyl esters of acids)
ABA (1)	83 900	3 898	26 109
2-*trans*-ABA	1492	0	398
PA (3)	4438	0	506
epi-DPA (9)	7538	331	828
DPA (5)	41 054	5811	2671

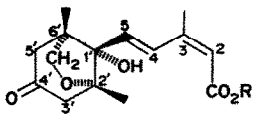
ester (9). The quantities of ABA (1), as determined by ORD and UV, indicated that the material remaining in the acidic fraction contained an excess of the (−)-enantiomer ABA (1) released by alkaline hydrolysis, presumably from the glucose ester, was predominantly (−)-ABA (Table 1). This is in accord with earlier observations that it is only the (+)-enantiomer of ABA (1) that is metabolised to PA (3).

In the second zone, at R_f 0·43–0·53 (Table 1), PA (3) was identified by GC–MS as methyl phaseate [10,11].

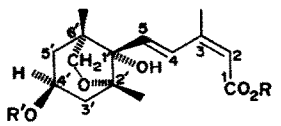
The most polar zone, at R_f 0·09–0·21 (Table 1), was methylated and re-chromatographed in solvent system III. Further analysis of the purified material by GLC with the stationary phase XE-60 gave two major peaks with retention times identical to those of the two products obtained by $NaBH_4$ reduction of Me-PA (4) [5], viz. Me-DPA (6) and *epi*-Me-DPA (10). In the acidic fraction the total amount of *epi*-DPA (9) (i.e. both the endogenous material and that formed from the ABA added) was 18% of the DPA (5), while in the conjugated form *epi*-DPA (9) occurred in 50% of the abundance of the DPA (5) (Table 1). That a higher proportion of the epimer was present in the bound form than as the free acid, was confirmed in several separate experiments, although the percentages varied from experiment to ex-



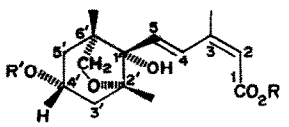
(1) R = H (revised absolute configuration)
(2) R = Me



(3) R = H
(4) R = Me



(5) R = H, R′ = H
(6) R = Me, R′ = H
(7) R = Me, R′ = MeCO
(8) R = Me, R′ = Si(Me)$_3$



(9) R = H, R′ = H
(10) R = Me, R′ = H
(11) R = Me, R′ = MeCO
(12) R = Me, R′ = Si(Me)$_3$

periment. The greatest amounts of *epi*-DPA (9) found, expressed as % of DPA (5), were 42% in the acidic fraction, and 200% in the bound form. Different solvents used for extraction (methanol or acetone) did not affect the ratio of *epi*-DPA (9) to DPA (5).

Formation of epi-*dihydrophaseic acid by sterile bean seedlings.* Of the 2·055 × 10^6 dpm of (±)-ABA-[2-^{14}C] added to the flasks some 184,712 dpm were present in the extracts of the sterile bean seedlings (Table 2). Autoradiography of the various plant extracts showed the presence of zones of labelled materials adjacent to markers although no labelled materials other than ABA and a trace of 2-*trans*-ABA were present in extracts of the agar; consequently the presence of other labelled materials can be attributed to metabolism in the plant. However, subsequent work [12] has shown that no ether-soluble neutral metabolites of (±)-ABA-[2-^{14}C] are formed. The methyl esters of **1**, **3**, **5** and **9** can be attributed to the methanolysis of the conjugated forms which occurs in neutral, and particularly in basic, methanol. The methanolysis of the ABA conjugate appears to proceed more readily than does methanolysis of conjugates of its metabolites because a higher proportion of the total ABA (1) is present as a methyl ester than was found with DPA (5) or *epi*-DPA (9).

Identification of metabolites. Me-DPA (6) and *epi*-Me-DPA (10) could be clearly separated on silica gel F_{254} by multiple development in solvent system III (see Experimental), compound **10** being less polar than **6**. Following acetylation of the methyl esters, yielding **7** and **11**, the relative polarities were reversed (see Experimental).

The MS of the epimeric methyl dihydrophaseates (**6**, **10**) isolated from bean shoots were compared with those of the products obtained by reduction of Me-PA (4) by $NaBH_4$ [5]. All four spectra were closely similar (Table 3). Neither the MS of the 4′-*O*-acetylated methyl dihydrophaseates (**7**, **11**) nor those of the trimethylsilyl ether of the methyl dihydrophaseates (**8**, **12**) showed significant differences (see Experimental). Initially it was not known whether the base peak at *m/e* 159 in the spectra of **8** and **12** contained the TMSi group, since the spectra of **7** and **11** did not show the expected fragment ion at *m/e* 129. In the meantime this question has been resolved by silylating Me-DPA (6) with trimethyl-d_9 chlorosilane [13], yielding Me-DPA-TMSi-d_9. The MS of this compound had its base peak at *m/e* 168, thus proving that the ion at *m/e* 159 in the MS of **8** does include the (Me)$_3$Si-group.

The epimeric dihydrophaseates (**6**, **10**) could, however, be very clearly differentiated by GLC using the stationary phase XE-60 (Table 4). They were also resolved from Me-ABA (2) and Me-PA (4). The 2-*trans*-isomers of the four compounds always had longer retention times than the respective 2-*cis*-compounds (Table 4). Thus TLC, followed by GLC on XE-60, provides an effective technique for characterising structural and stereochemical features of small quantities of ABA (1) and its metabolites. The data in Table 4 also show that the stationary phases SE-30 and OV-17 are less suitable for this purpose.

Both Me-DPA (6) and *epi*-Me-DPA (10) were converted into Me-PA (4) by oxidation with Jones reagent (see Experimental) as evaluated by GLC with XE-60. The

Table 3. MS of epimeric methyl dihydrophaseates isolated from bean plants, or prepared by reduction of methyl phaseate

	Me-DPA (6)		*epi*-Me-DPA (10)	
m/e	*Ex*bean	Me-PA + $NaBH_4$	*Ex*bean	Me-PA + $NaBH_4$
296	2	3	5	3
278	4	6	8	6
264	2	2	5	3
246	5	5	10	7
220	8	8	12	11
188	12	12	18	20
154	39	51	63	58
127	38	48	63	57
125	55	63	60	53
122	100	100	100	100
121	54	68	70	65
109	52	60	67	65
95	44	56	63	57
94	47	51	57	51
83	31	48	41	39
69	51	56	54	49
55	43	44	46	43

product of the two reactions was conclusively identified as Me-PA(4) by GC-MS. Since the proportion of *epi*-DPA (9) was always higher in the fraction released by alkaline hydrolysis than in the acid fraction (Table 1), the possibility was considered that the alkaline conditions employed caused epimerisation (cf. epimerisation of gibberellin A_1 at C-3 by treatment with dilute base [14], resulting in an equilibrium mixture containing GA_1 and 3-*epi*-GA_1 in the ratio 1:3). However, when pure DPA (5), or mixtures with *epi*-DPA (9) and DPA (5) were dissolved in alkaline solution and re-extracted after 2 days from acid, the ratio of the two epimers was unchanged so the possibility that *epi*-DPA was formed during the isolation procedure by these conditions was eliminated.

Metabolism of (±)-*abscisic acid*-[2-^{14}C]. Direct evidence for the conversion of ABA (1) into DPA (5) and *epi*-DPA (9) was obtained by using (±)-ABA-[2-^{14}C]. One bean shoot was kept on a solution containing $1{\cdot}25 \times 10^6$ dpm of (±)-ABA-[2-^{14}C] for 3 days. Following extraction and methylation 85 000 dpm (*ca* 14% of the (+)-ABA-[2-^{14}C] applied) were recovered in the Me-DPA zone. Analysis of the eluted material by GC–RC (see Experimental) showed that *ca* 8% of the total radioactivity was associated with the *epi*-Me-DPA peak. The remainder of the sample was used for separation of the two epimers by TLC. The result obtained by this method, that 9% of the radioactivity of the (+)-ABA was isolated as *epi*-Me-DPA (10), was in close agreement with the former measurement.

Metabolism of (±)-*abscisic acid*-[2-^{14}C,4′-^{18}O]. Although ^{14}C labelling had shown that the carbon skeleton of ABA had been converted into 5 and 9, it was considered that these compounds could arise in one of two ways. Either direct enzymatic reduction of PA (3) by two enzymes gives rise to two epimers, or one epimer is formed initially and then a part of this material could have the configuration of its 4′-hydroxyl group inverted

Table 4. Retention times and Kovats retention indices of derivatized abscisic acid and its metabolites on three different stationary phases

	XE-60		SE-30		OV-17	
Compound	Min	Retention index	Min	Retention index	Min	Retention index
Me-ABA (2)	5·0	2880	4·9	2049	4·4	2418
Me-*t*-ABA	8·3	3044	6·4	2122	5·6	2478
Me-PA (4)	6·7	2975	5·8	2094	5·7	2483
Me-*t*-PA	9·9	3104	7·2	2154	6·6	2523
Me-DPA (6)	7·3	3004	6·9	2143	7·0	2538
Me-*t*-DPA	10·2	3114	8·6	2202	8·0	2571
epi-Me-DPA (10)	5·4	2905	6·5	2126	5·7	2485
epi-Me-*t*-DPA	7·5	3011	8·0	2183	6·6	2521
Me-DPA-Ac (7)	6·3	2955	9·3	2225	9·0	2601
epi-Me-DPA-Ac (11)	8·1	3038	10·1	2247	9·8	2626
Me-DPA-TMSi (8)	5·5*	2625	7·1	2152	—	—
epi-Me-DPA-TMSi (12)	7·4*	2715	7·9	2177	—	—

1% XE-60 on Gas-Chrom Q 100/120 mesh; glass column 1·8 m × 3 mm; oven temp. 198°. 3% SE-30 on Gas-chrom Q 100/120 mesh; glass column 1·8 m × 3 mm; oven temp. 202°. 1% OV-17 on Gas-Chrom Q 100/120 mesh; glass column 1·5 m × 3 mm; oven temp. 185°. * Oven temp. 180°.

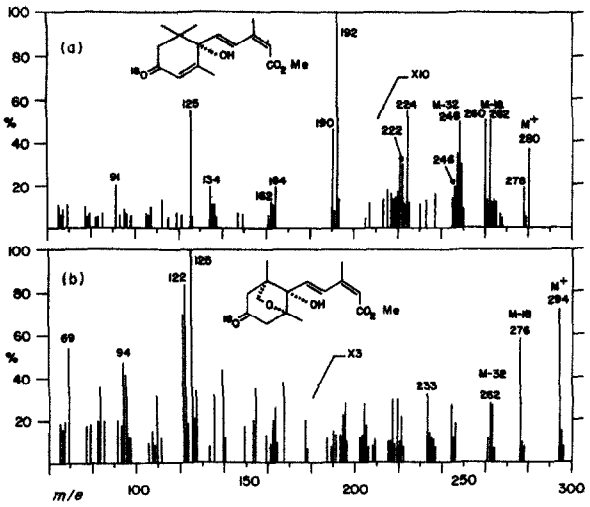


Fig. 1. Mass spectra of (a) the methyl ester of ABA-[4'-^{18}O], and (b) the methyl ester of PA-[4'-^{18}O].

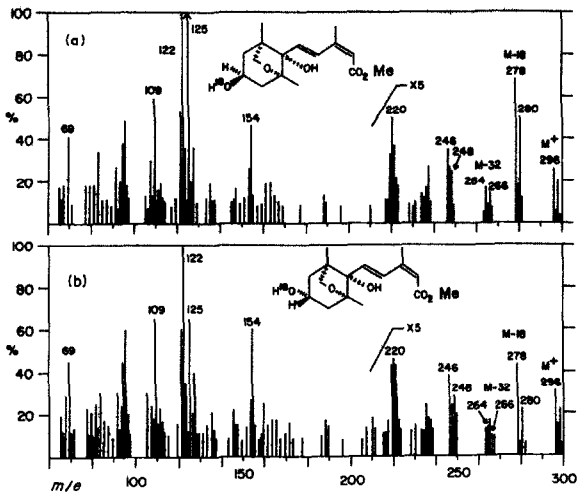


Fig. 2. Mass spectra of (a) the methyl ester of DPA-[4'-^{18}O], and (b) the methyl ester of *epi*-DPA-[4'-^{18}O].

by, for example, glucosylation followed by a hydrolytic cleavage of a 4'-glycosidic bond. This might replace the original 4'-ketone oxygen atom with an oxygen atom from the medium. It was of interest, therefore, to determine whether the 4'-oxygen of ABA was present as the 4'-hydroxyl oxygen atom in both **5** and **9**.

A sample of (±)-ABA-[2-^{14}C,4'-^{18}O] was fed to bean shoots. After isolation under acidic conditions, which would minimise the loss of [^{18}O] atoms by exchange (see Experimental), the fractions containing ABA (**1**) and its metabolites were analysed by GC-MS (Figs. 1 and 2, Table 5).

The data in Table 5 show that the [^{14}C]-labelled ABA (**1**) had been converted into PA (**3**), DPA (**5**) and *epi*-DPA (**9**) which were all diluted by the respective unlabelled endogenous materials. However, the GC-MS analysis shows that less [^{18}C] than expected on the basis of ^{14}C content is present (Table 5). ABA is known [9] to exchange its 4'-oxygen atom in an aqueous solution, but under the acidic conditions employed only 31% was lost.

The 4'-ketone oxygen atom of PA (**3**) appears to be more susceptible to exchange (82% of it was lost) than is the analogous atom of ABA (**1**). The higher proportion of [^{18}O] in the metabolites formed from PA (**3**) can be accounted for because [^{18}O] atoms in hydroxyl groups do not exchange readily with the medium and if PA (**3**) had been reduced soon after formation from ABA-[4'-^{18}O] (84·2% atoms [^{18}O]) then the [^{18}O] in DPA (**5**) and *epi*-DPA(**9**) would not be susceptible to loss thereafter. Any PA (**3**) not reduced would continue to lose [^{18}O] throughout the period of the experiment and during the early stages of isolation.

The results show that the [^{18}O] content of DPA (**5**) and *epi*-DPA (**9**) were quite similar at 43 and 41%, respectively. Taken together with the results of the ABA-[^{14}C] experiment, these data conclusively establish that bean shoots metabolise ABA (**1**) to both DPA (**5**) and *epi*-DPA (**9**) and the 4'-oxygen atom is not lost during a hypothetical conversion of **5** into **9**.

Epimerisation of dihydrophaseic-[^{14}C] and epi*-dihydrophaseic-[^{14}C] acids.* DPA-[^{14}C] (**5**) or *epi*-DPA-[^{14}C] (**9**), purified by TLC of the free acids and then of their methyl esters, was fed to two bean shoots. Unlike ABA (**1**) solutions, which tend to delay their own uptake, the shoots absorbed the solutions (10 ml) overnight. After 25 hr incubation the plants were analysed and negligible amounts of radioactivity were found in the areas of silica gel opposite the markers of the alternate epimers (Table 6). The 245 dpm in front of the DPA zone (R_f 0·16–0·21) may be attributable to the formation of a small amount of *epi*-DPA (**9**), but it could also be attributed to the formation of a trace of 2-*trans*-DPA which runs at an intermediate R_f. A similar proportion of the counts of

Table 5. Incorporation of abscisic acid into phaseic acid, dihydrophaseic acid and *epi*-dihydrophaseic acid

Compound	Amount recovered (μg)	% incorporation* of [^{14}C]ABA fed	dpm/μg	% of compound derived from ABA added: % based on [^{14}C] content	% based on [^{18}O] content†	% loss of [^{18}O]‡
ABA (**1**)	13	—	410	95	66	31
PA (**3**)	145	13·5	210	49	9	82
DPA (**5**)	79	11·0	310	72	43	40
epi-DPA (**9**)	12	3·8	(700)‖	—	41	—

33 μg (±)-ABA-[2-^{14}C] and 1000 μg (±)-ABA-[4'-^{18}O] dissolved in 0·01M KH_2PO_4 at pH 4·6 fed to 3 bean shoots for 3 days. Sp. act. 432 dpm ^{14}C/μg ABA. * Calculated on basis of (+)-ABA-[2-^{14}C]. † Determined by MS from the molecular ion region; see Figs. 1 and 2. ‡Figures calculated from the [^{18}O]% and [^{14}C]% contents. ‖ Value higher than theoretically possible due to radioactive contaminant.

Table 6. Absence of DPA/*epi*-DPA epimerase activity in bean shoots

53,817 dpm DPA-[^{14}C] taken up by bean shoots		4,705 dpm *epi*-DPA-[^{14}C] taken up by bean shoots	Position of markers
R_f of TLC zone	dpm [^{14}C]	dpm [^{14}C]	
Origin to 0·07	495	335	
0·07–0·16	4998	306	DPA (5)
0·16–0·21	245	1494	*epi*-DPA (9) and 2-*trans*-DPA
0·21–0·25	27	104	2-*trans*-*epi*-DPA
0·25–0·30	30	37	PA (3)
0·30–0·36	39	24	
0·36–0·42	17	30	
0·42–0·48	0	0	ABA (1)
0·48 to solvent front	0	0	

Autoradiograms showed that, apart from the compound supplied, no other discrete bands of labelled materials were present.

epi-DPA (104 dpm) occurred in the 2-*trans*-*epi*-DPA zone (R_f 0·21–0·25) and so a similar degree of isomerisation of the two epimers could heve occurred.

Metabolism of phaseic acid-[2-^{14}*C*]. Previous reports [2–6] and the results given above are in accordance with the sequence of conversions ABA (1)→PA (3)→DPA (5) established in embryonic bean axes [3]. However, it is also possible that an alternative pathway operates in green plants. This was investigated by feeding 140 μg PA-[2-^{14}C], containing 1·9 × 10^6 dpm, to 4 bean shoots for 4 days. After purification of the acidic fraction 32·5% of the total radioactivity applied was recovered which was distributed as follows: 6% in Me-PA (4), 25% in Me-DPA (6), and 1·5% in *epi*-Me-DPA (10). There was no radioactivity in the ABA zone. Alkaline hydrolysis released 3·3% of the total radioactivity applied which was distributed as follows: 1·1% in Me-PA, 0·3% in Me-DPA and 1·9% in *epi*-Me-DPA. All three radioactive compounds present in the acidic fraction were methylated and further analysed by GC–RC, and in each case the radioactivity was associated with the mass peak of the compound. Thus, PA (3) applied to bean shoots was converted with high efficiency into DPA (5) and *epi*-DPA (9).

Metabolites of endogenous abscisic acid. It is clear from the foregoing results that *epi*-DPA (9) occurs in bean shoots as a metabolite of applied ABA (1) but the formation of *epi*-DPA (9) from endogenous ABA (1) had not been established.

It has been reported [6] that dry bean seeds have a high DPA (5) content, and our results in Table 7 confirm this. In addition, a small amount of *epi*-DPA (9) was detected and conclusively identified by GC–MS of its methyl ester.

Table 7. Content of abscisic, phaseic, dihydrophaseic, and *epi*-dihydrophaseic acids in dry bean seeds

Compound	In acidic fraction (μg/kg)	Released by alkaline hydrolysis (μg/kg)
ABA (1)	45	40*
PA (3)	133*	<1*
DPA (5)	2900	102
epi-DPA (9)	30	<1

A total of 590 g of dry seeds extracted. * Determined by GLC with electron capture detector.

Wilting of bean plants results in a dramatic rise in the ABA concentration which then declines again upon removal of the stress condition [15]. Bean plants were therefore wilted and, following recovery, analysed for ABA (1) and its metabolites. As shown in Table 8 the ABA content had returned to normal after a 2-day recovery period, but the DPA (5) concentration greatly exceeded that of ABA (1) and of PA (3). A small amount of *epi*-DPA (9) was isolated and identified by GC–MS.

Thus, *epi*-DPA (9) was detected in beans at two stages of development. However, the *epi*-DPA (9) content was only 1–2% of the DPA content. This is much lower than when exogenously applied ABA had been metabolised by bean plants.

DISCUSSION

Previous work [3,4] on the metabolism of ABA (1) has established the pathway: ABA (1)→ PA (3)→ DPA (5). Our results demonstrate that this pathway also operates in bean shoots, but in addition to DPA (5), *epi*-DPA (9) is produced as a minor metabolite. It is unlikely that 9 is formed during extraction. Employing the same extraction and purification methods, the proportion of *epi*-DPA (9) formed from endogenous ABA (1) (Tables 7 and 8), was much lower than that after metabolism of exogenous ABA (Tables 1 and 5), and maintaining acidic conditions throughout the extraction procedure still yielded *epi*-DPA (9) in the proportion typical for metabolism of applied ABA (1) (Table 5). The experiment ABA-[4′-^{18}O] provides additional evidence that *epi*-DPA (9) is not an artefact. If DPA (5) formed a conjugate at C-4′ that was hydrolyzed during extraction with inver-

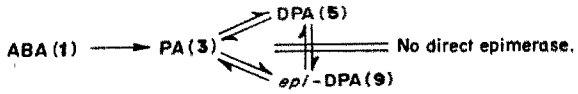
Table 8. Concentrations of abscisic, phaseic, dihydrophaseic, and *epi*-dihydrophaseic acids in bean plants following a period of water stress

Compound	In acidic fraction (μg/kg)	Released by alkaline hydrolysis (μg/kg)
ABA (1)	11	12
PA (3)	51	23
DPA (5)	857	15
epi-DPA (9)	18	6

Plants wilted and recovered 3× over an 8-day period. A total of 770 g of plant material harvested 2 days after last wilting.

sion this could result in loss of the 4'-oxygen atom. Since the two epimers (**5**, **9**) retained [4'-^{18}O] equally (Table 5), this mechanism can be ruled out.

Bean seedlings grown under aseptic conditions produced *epi*-DPA (**9**), indicating that it is not a microbial product (Table 2). In experiments in which either **5** or **9** was fed to bean shoots the respective compound could be recovered unchanged, so the existence of an epimerase catalysing the interconversion of the two compounds is unlikely. However, some interconversion of DPA (**5**) and *epi*-DPA (**9**) would be expected to occur eventually because the enzymes that catalyse the formation of hydroxyl from ketone groups are typically slightly reversible. If **5** and **9** were exchanged slowly with PA (**3**) by oxidation and reduction there would be some apparent epimerisation between DPA (**5**) and *epi*-DPA (**9**) without the participation of a direct epimerase.



This slow interconversion would also be expected to occur if one enzyme were capable of reducing the 4'-ketone group of PA (**3**) to give both epimers.

At present we have no indication whether there are two enzymes that reduce the 4'-ketone of PA (**3**) to hydroxyl groups of opposite configuration, or whether the PA (**3**) is reduced in two ways by one enzyme. If the latter possibility obtains then the 3',4' and 5' carbon atoms of the cyclohexyl ring of PA (**3**), which are non-chiral, could be expected to be accommodated in the enzyme's active site in two opposed orientations. Reduction of the ketone would then give C-4' hydroxyl groups in two configurations and the ratio of the products would reflect the efficiency of catalysis with the substrate in the two positions.

Thus, all the evidence indicates that *epi*-DPA (**9**) is enzymatically derived from ABA (**1**) in bean plants. A possible explanation for the quantitative difference between metabolism of endogenous and exogenously applied ABA (**1**) is that there are two different metabolic pools, each with a stereospecific enzyme for the reduction of PA (**3**) $\xrightarrow{\text{Enz. 1}}$ DPA (**5**), and PA (**3**) $\xrightarrow{\text{Enz. 2}}$ *epi*-DPA (**9**). Endogenous ABA (**1**) would be predominantly in one compartment while applied ABA (**1**) would be in both compartments. The pool with the exogenous ABA (**1**) would produce conjugates more readily than would the one with material that has been produced naturally (Tables 1 and 6). Other evidence suggesting the occurrence of ABA (**1**) in different cellular compartments has been reviewed recently [1].

One other major difference between the metabolism of endogenous and that of applied ABA (**1**) was that in the latter case the amount of PA (**3**) after 3 days always exceeded that of DPA (**5**) (Tables 1 and 5), although the solution with ABA (**1**) was taken up by the shoots within one day. In bean seeds (Table 7) and in plants recovered from wilting (Table 8) the quantities of DPA (**5**) were always far greater than those of PA (**3**).

Bean plants growing under sterile conditions were supplied with (±)-ABA-[^{14}C] through their cut ends. When harvested and analysed both DPA-[^{14}C] (**5**) and *epi*-DPA-[^{14}C] (**9**) were found. This result eliminated the possibility that **5** was formed by the enzymes of the bean while **9** was formed by micro-organisms.

The detection of *epi*-DPA (**9**) as a metabolite of ABA (**1**) in bean plants was due to the fortuitous use of the stationary phase XE-60 (Table 4). In the work of Sondheimer and co-workers [3,4] *epi*-DPA (**9**), if present, would have been included in the M-2 (=DPA) zone. It would seem that the faint zone just ahead of M-2 in their Fig. 1 [4] represents *epi*-DPA (**9**).

The MS of derivatised DPA and *epi*-DPA are quite similar but the two epimers can be readily separated by GLC. The same was found for epimeric sterols [16], and for the C-3 epimers of gibberellin A_1 [17].

The enzymic reactions of biosynthesis and degradation of ABA [1,11] that have been defined, are constrained stereochemically; for example, only the (+)-enantiomer of racemic ABA applied to plants is converted into PA (**3**). It is surprising, therefore, to find that this compound is reduced to form two epimeric products. The significance of this must await further investigation.

EXPERIMENTAL

Plant material. Plants of french bean (*Phaseolus vulgaris* L. cv. The Prince) were grown in a greenhouse until primary leaves were mature and the first trifoliate leaf half expanded. For feeding chemicals via the transpiration stream, the stems were cut under water and placed in the appropriate solutions.

Metabolism of (±)-ABA-[2-^{14}C] by sterile bean seedlings. Six bean seeds were surface sterilised in 50% saturated sodium hypochlorite soln, rinsed, and planted on sterile 1% agar (20 ml), in each of five 500 ml Erlenmeyer flasks. They were given weak illumination (500 lx) after 60 hr germination, to reduce etiolation during the subsequent 5 days growth, and the roots were cut with a scalpel and a soln of (±)-ABA-[2-^{14}C] ($4{\cdot}11 \times 10^5$ dpm, 1·6 μCi/μM) was injected onto the agar in each flask through a Millipore filter. After a further 5 days the plants were removed and extracted (see below). Flasks were kept for a further 4 days and no bacterial infection occurred. Agar was also extracted and found to contain ABA and 2-*trans*-ABA as the only labelled materials present. EtOAc soluble acid fraction was chromatographed in solvent system I and then an autoradiogram (Kodak Kodirex X-ray film) was made during 7 days exposure. The aq. residue was treated to give a "released by hydrolysis" fraction and this, and the ether-soluble neutral fraction, were analysed similarly. The presence of the labelled methyl esters has now been accounted for by methanolysis of the conjugates [12] which has been found to occur in neutral or alkaline MeOH.

Chemicals and their applications. The sample of (±)-ABA was the same as used in previous work [18]. (±)-ABA-[2-^{14}C] had a sp. act. of 1·6 μCi/μM, PA-[^{14}C] was isolated from plants fed with (±)-ABA-[2-^{14}C]. To increase the yield of PA, DPA-[^{14}C] was also isolated and converted into PA-[^{14}C] with Jones reagent. For application to bean shoots a chemical was dissolved in a small vol of Me_2CO (0·1 ml) with a drop of Tween 20, and H_2O was added to the required vol (20–50 ml). Bean shoots were placed in small tubes containing 2 or 3 ml of the soln. When the uptake was complete, distilled H_2O was added. The sample of (±)-ABA-[4'-^{18}O] (1 mg) synthesised by Gray *et al.* [9], was mixed with (±)-ABA-[2-^{14}C] (33 μg), its final [^{18}O] content was 84·2%. To prevent exchange of [^{18}O], it was dissolved in a small vol. of Et_2O with a drop of Tween 20 and 0·01M KH_2PO_4 at pH 4·6 (12 ml) was added and the Et_2O was blown off under a stream of N_2.

Extraction and purification. Plant samples were frozen with liquid N_2 and pulverised. MeOH, and in some experiments Me_2CO, was used for extraction. Solvents used for extraction contained between 1 and 10 μg/ml 2,6-di-*tert*-butyl-4-methylphenol (BHT). For preparation of the acidic fraction the same methods described before [19] were used, except that the buffer soln adjusted to pH 2·5 was partitioned 6× with equal

volumes of EtOAc. The aq. phase was then adjusted to pH 12 with 6 N KOH and left at room temp. for 2 days. After re-adjusting the pH to 2·5 with 6 N HCl, extraction with EtOAc was repeated. This fraction is referred to as "released by alkaline hydrolysis". In the experiment with ABA-[4'-^{18}O] glacial HOAc (1 ml) was added to the MeOH (100 ml) used for extraction. After evap of solvent *in vacuo*, the pH was immediately adjusted to 2·5, so that the acidic fraction also contained neutral materials. Dry bean seeds were pulverised in a Waring blender, and the resulting powder extracted with 80% MeOH. Further steps in the extraction and purification procedures were as described above. The acidic fraction was applied to the top of a silica acid–celite (1:1) column (170 × 1·5 mm), and eluted with 30, 60, and 80% EtOAc in $CHCl_3$, and finally with Me_2CO. ABA (**1**) was eluted in fraction 1, PA (**3**) in 2, and DPA (**5**) mainly in fraction 3. All 4 fractions were further purified by TLC.

TLC. Acidic fractions containing BHT were applied to Merck pre-coated Si gel F_{254} plates (200 × 200 × 0·25 mm), and developed 2× to 120 mm in solvent system I: Toluene–EtOAc–HOAc (50:30:4) [18]. R_f values of ABA (**1**) and its metabolites are recorded in Table 1. The zones of Si gel containing compounds of interest were scraped off and eluted with a mixture of EtOH–Me_2CO (1:1) [6]. Me-ABA (**2**) was further purified in solvent system II: EtOAc–hexane (1:1); system III: EtOAc–hexane (2:1) was used for **4**, **6** and **10**. After 5 developments in this system the R_f's of Me-DPA (**6**) and *epi*-Me-DPA (**10**) were 0·38 and 0·52, respectively. Acetylated methyl dihydrophaseates (**7** and **11**) were separated with system IV: EtOAc–hexane (1:2). After 5 developments the R_f was 0·20 for *epi*-Me-DPA-Ac (**11**) and 0·40 for Me-DPA-Ac (**7**).

Preparation of derivatives. Acids were methylated with ethereal CH_2N_2. Me-DPA (**6**) and *epi*-Me-DPA (**10**) were acetylated with Ac_2O in C_5H_5N (1:2) at 25° overnight. Me-DPA (**6**) and *epi*-Me-DPA (**10**) were silylated with hexamethyldisilazane and trimethylchlorosilane in C_5H_5N (2:1:5). For preparation of Me-DPA-TMSi-d_9 Me-DPA (**6**) was silylated with trimethyl-d_9 chlorosilane in C_5H_5N. Me-PA (**3**) was reduced with $NaBH_4$ in a mixture of MeOH–H_2O (2:1) at 0° for 1 hr [5]. The MeOH was removed under a stream of N_2 and the products were partitioned into EtOAc. The free acids (**5** and **9**) were obtained from their methyl esters, and the *O*-acetylated methyl esters, by hydrolysis in a drop of ethanolic NaOH (10 N aq. NaOH: EtOH, 1:2) at room temp. for 30 min. H_2O (1ml) was added and the pH adjusted to 2·5 before extraction with EtOAc. For oxidation of Me-DPA (**6**) and *epi*-Me-DPA (**10**) with Jones reagent [20], the sample was dissolved in 0·5 ml Me_2CO and reagent added until the mixture remained permanently brown. Excess reagent was destroyed by the addition of a little MeOH, H_2O was added and the product was extracted with EtOAc. The exposure of Me_2CO solns of the methyl esters of ABA (**2**), PA (**4**), DPA (**6**), and *epi*-DPA (**9**) to light from 2 Mazda white fluorescent tubes (intensity 9000 lx) for 4 days, isomerised each compound to a mixture of the respective 2-*cis* and 2-*trans* isomers. Under these conditions the two isomers of Me-ABA (**2**) reached a 1:1 equilibrium (determined by measuring the peak areas on the GLC chart) after 2 days; for the other 3 compounds it took 4 days to reach equilibrium. Isomerisation with visible light takes place more slowly than with UV, but it has the advantage that it is non-destructive.

GLC. Details on stationary phases, columns and temp are given in Table 4. For the determination of Kovats Retention Indices [21] $C_{22}H_{46}$, $C_{24}H_{50}$, $C_{28}H_{58}$ and Parafilm [22] were used as standards. GLC with a ^{3}H electron capture detector was carried out with a 4% XE-60 stationary phase and N_2 carrier gas at 230°. The sensitivity was such that less than 100 pg for Me-ABA (**2**), and 500 pg for Me-PA (**3**) and Me-DPA (**6**) could be detected.

GC–RC. 1% XE-60 stationary phase was used packed in a stainless steel column. The instrument was equipped with a splitter connected to a scintillation counter for the trapping of vapours and incremental measurement of radioactivity [23].

GC–MS. Mass spectra were obtained with an A.E.I. MS 30 or MS 902. For samples containing only minor impurities a column of 3% SE-30 (1·5 m × 1·5 mm) at 204° and a He flow of 50 ml/min was used. For samples with impurities a 1% XE-60 (1·5 m × 1·5 mm) was used at 200°. The spectra of the TMSi ethers of Me-DPA (**8**) and *epi*-Me-DPA (**12**) were obtained with an LKB-9000 and a column of 3% SP-2100 (1·8 m × 3 mm, the temp was programmed from 200° to 250° at 5°/min, and a He flow of 25 ml/min. All spectra were measured at 70 eV.

Measurement of radioactivity by scintillation spectrometry. Small aliquots of the solns containing radioactive materials were dried in vials to which were added 10 ml of a soln which contained naphthalene (80 mg) and 2,5 bis(5-*t*-butylbenzoxazol-2-yl) thiophen (BBOT) (6 mg) in methoxy ethanol (4 ml) and toluene (6 ml). Samples were counted at 50% counting efficiency for [^{14}C].

Quantitative determinations of compounds. Measurement of Me-ABA (**2**) by ORD was as described before [18]. UV absorption spectrometry was used for measuring purified chemicals. For calculation of the amounts of the various compounds the same values for λ_{max}'s and ε's were used as before [5,18]. When, as with small or impure samples, UV spectrometry was unsatisfactory, GLC with a flame detector was employed for quantitative determinations. For very small quantities (ng), GLC with an electron capture detector was used.

MS data. MS of the 4'-*O*-acetylated epimeric methyl dihydrophaseates (**7**, **11**), *m/e* (rel. int.): 338(3,2), 320(1,1), 278(5,6), 260(3,3), 246(5,5), 235(5,6), 220(15,18), 205(10,10), 188(30,30), 169(17,15), 163(19,24), 161(27,25), 154(48,43), 153(50,51), 125(76,66), 122(97,89), 121(97,93), 109(100,100), 95(43,46), 94(49,44), 69(48,44), 55(37,37). MS of the TMSi ethers of the epimeric methyl dihydrophaseates (**8**, **12**) *m/e* (rel. int.): 368(7,5), 350(2,2), 336(3,2), 295(3,4), 282(4,4), 278(15,15), 246(16,15), 237(9,8), 235(10,11), 226(12,10), 220(31,32), 188(54,45), 163(34,48), 161(48,37), 159(100,100), 154(46,44), 153(54,47), 135(39,36), 125(78,58), 122(82,57), 121(95,79), 117(80,69), 109(86,60), 95(46,34), 75(88,88), 63(174,155), 69(61,56), 55(34,35).

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