Plant Physiol. (1984) 75, 166–169
0032-0889/84/75/0166/04/$01.00/0

Incorporation of Oxygen into Abscisic Acid and Phaseic Acid from Molecular Oxygen[1]

Received for publication August 4, 1983 and in revised form December 5, 1983

ROBERT A. CREELMAN AND JAN A. D. ZEEVAART*
MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing, Michigan 48824

ABSTRACT

Abscisic acid accumulates in detached, wilted leaves of *Xanthium strumarium*. When these leaves are subsequently rehydrated, phaseic acid, a catabolite of abscisic acid, accumulates. Analysis by gas chromatography-mass spectrometry of phaseic acid isolated from stressed and subsequently rehydrated leaves placed in an atmosphere containing 20% $^{18}O_2$ and 80% N_2 indicates that one atom of ^{18}O is incorporated in the 6′-hydroxymethyl group of phaseic acid. This suggests that the enzyme that converts abscisic acid to phaseic acid is an oxygenase.

Analysis by gas chromatography-mass spectrometry of abscisic acid isolated from stressed leaves kept in an atmosphere containing $^{18}O_2$ indicates that one atom of ^{18}O is present in the carboxyl group of abscisic acid. Thus, when abscisic acid accumulates in water-stressed leaves, only one of the four oxygens present in the abscisic acid molecule is derived from molecular oxygen. This suggests that either (a) the oxygen present in the 1′-, 4′-, and one of the two oxygens at the 1-position of abscisic acid arise from water, or (b) there exists a stored precursor with oxygen atoms already present in the 1′- and 4′-positions of abscisic acid which is converted to abscisic acid under conditions of water stress.

Little is known about the biosynthetic pathway of ABA, except that as a sesquiterpenoid, ABA is ultimately derived from MVA.[2] When radioactive MVA was applied to higher plant tissues, the percentage of incorporation into ABA was always very low, and no intermediates have ever been isolated. Some controversy exists as to whether ABA is synthesized from a C-15 precursor, presumably farnesyl pyrophosphate (the direct pathway), or results from the degradation of a C-40 precursor (the indirect pathway), such as the xanthophyll violaxanthin (6). It is known that the stereochemistry of protons in ABA derived from MVA is identical to that found in carotenoids and it should be noted that the terminal ring structure of certain xanthophylls is similar to ABA (6).

Oxygen incorporation into carotenes to form xanthophylls is a late step in carotenoid biosynthesis occurring after ring formation. The oxygen atoms in the hydroxyl groups of lutein and the epoxide groups of antheraxanthin and violaxanthin are derived from molecular oxygen (12, 13). The keto group of spheroidenone also comes from molecular oxygen (8). By analogy with xanthophyll biosynthesis, if ABA is derived from farnesyl pyrophosphate, incorporation of oxygen at the 1′- and 4′-carbons of the ABA molecule should be a late step in the pathway, the oxygens being derived from molecular oxygen.

With respect to ABA catabolism, Gillard and Walton (2) have shown with a crude enzyme preparation from *Echinocystis lobata* that hydroxylation at the 6′-methyl group to give PA via the unstable intermediate 6′-hydroxymethyl-ABA, is inhibited by CO and anaerobic conditions. They concluded that the enzyme involved is very similar to Cyt P-450 monooxygenases found in animals, but it did not meet all the criteria necessary for calling ABA hydroxylating enzyme a Cyt P-450 monooxygenase. These criteria are: (a) inhibition of the reaction in the presence of CO, (b) presence in the reduced enzyme preparation of a CO-binding pigment with a maximum *A* at 450 nm in the CO difference spectrum, (c) reversal of CO inhibition by light with a maximum in the action spectrum at 450 nm, (d) demonstration of the expected reaction stoichiometry, and (e) incorporation of one oxygen atom from $^{18}O_2$ into each molecule of product (11).

We decided to study the origin of the oxygen atoms in ABA and PA by the use of $^{18}O_2$. The number of oxygen atoms present as well as their positions in the molecules can then be determined by MS of the purified compounds. It is essential that during incubation with $^{18}O_2$, large amounts of ABA and PA are synthesized by the experimental system under study. For this reason we chose detached leaves of *Xanthium*, since upon wilting their ABA content increases dramatically over a period of a few hours. If these leaves are subsequently rehydrated by immersing them in water, their ABA content decreases and PA, a catabolite of ABA, rapidly accumulates (15). *Xanthium* leaves are, therefore, an ideal system for rapidly inducing the accumulation of both ABA and PA, depending on how the leaves are manipulated.

MATERIALS AND METHODS

Plant Material. *Xanthium strumarium* L., Chicago strain, was grown as before (15). The youngest, fully expanded leaf blade, hereafter called leaf, was used in all experiments. For experiments involving ABA, PA, and ABA-GE, leaves were wilted until they had lost 13% of their fresh weight and then were stored in plastic bags for 6 h. Wilted leaves were rehydrated by immersing them in water for 5 min, and then were resealed in plastic bags, or in a 250-ml Erlenmeyer flask sealed with a serum stopper. Flasks were immediately evacuated until a final pressure of 7 to 13 Pa was reached, and then were backflushed with N_2. This procedure was repeated two more times. To test the effect of vacuum on PA accumulation, some flasks were unsealed to allow room air to enter and then were immediately resealed. If leaves were to be incubated in the presence of $^{18}O_2$, 50 ml of $^{18}O_2$ was added to the flask after 2 evacuation cycles and then the flask was filled with N_2. For experiments involving only ABA, a similar procedure was used except that after the leaves were wilted, they were immediately placed under N_2, or under a mixture of $^{18}O_2$ and N_2 as described above.

The primary leaves of *Phaseolus vulgaris* L., cv Redkloud were

[1] Supported by the United States Department of Energy under Contract DE-ACO2-76ERO-1338.

[2] Abbreviations: MVA, mevalonic acid; PA, phaseic acid; ABA-GE, β-D-glucopyranosyl abscisate; GC-SIM, gas chromatography-selected ion monitoring; m/z, mass/charge.

used in some experiments involving ABA accumulation. Leaves were harvested 10 d after planting and were treated as described above for *X. strumarium*.

Incubation of leaves in the presence of $^{18}O_2$ was carried out 2× in the case of the PA experiment. The ABA experiment was performed 3× with *Xanthium* leaves, and 1× with *Phaseolus* leaves. The experiments described in Tables I and II were performed twice with two replicates each time. Similar results were obtained in repetitions of all experiments.

Chemicals. $^{18}O_2$ was purchased from Stohler Isotope Chemicals Inc. (49 Jones Road, Waltham, MA). $H_2{}^{18}O$ was purchased from Kor, Inc. (56 Rogers Street, Cambridge, MA). One atom of ^{18}O was exchanged into the 4′-keto group of (±)–ABA (Sigma) by placing (±)–ABA in $H_2{}^{18}O$ with 1% (v/v) acetic acid for 2.5 d at room temperature.

Extraction, Purification, and Quantification Procedures. For experiments dealing with ABA, PA, and ABA-GE, the samples were purified and quantified according to Zeevaart (15, 16). For experiments involving only ABA, samples were extracted according to Zeevaart (15). ABA was further purified by semi-preparative reverse phase HPLC on a μBondapak C_{18} (10 μ-particle size), 30 × 0.78 cm column (Waters Associates, Milford, MA). The sample was eluted by means of a convex gradient (curve 5 on the Waters Associates Model 660 Solvent Programmer) from 0 to 50% Solvent B in Solvent A (Solvent A: water with 1% acetic acid; Solvent B: ethanol with 1% acetic acid) in 30 min at a flow rate of 2.5 ml/min. The fraction containing ABA was dried and further purified by analytical straight phase HPLC on a μBondapak NH_2 column (Waters Associates). ABA was eluted by means of a convex gradient (curve 5) from 50 to 100% ethyl acetate containing 1% acetic acid in hexane in 15 min at a flow rate of 2 ml/min. After elution from the analytical column, the fraction containing ABA was dried and methylated with ethereal diazomethane. Quantification of the methyl ester of ABA was performed with a Hewlett-Packard 5840A gas chromatograph equipped with a ^{63}Ni-electron capture detector (15). Samples were dissolved in ethyl acetate and analysis was done on a Durabond DB-1 (J & W Scientific, Inc., Rancho Cordova, CA) gas capillary column (30 m × 0.32 mm × 0.25 μm). GLC conditions were: oven temperature 165°C, H_2 carrier flow 10 ml/min, split ratio 5:1; argon-methane (95:5) was used as make-up gas and had a flow at the detector of 80 ml/min.

To determine if exchange from the 4′-keto group of ABA occurred during the extraction and purification process, a leaf sample with added 4′-^{18}O-ABA was extracted and purified as described above.

Mass Spectrometry. Mass spectra were obtained with a Hewlett-Packard 5985 quadrupole mass spectrometer connected to a 5840A gas chromatograph. GLC conditions were: 3% SE-30 on 100 to 200 mesh Gas Chrom Q in a silanized glass column (180 × 0.2 cm) held isothermally at 205°C for the methyl ester of PA, and temperature programmed from 185 to 240°C at 5°C/min for the methyl ester of ABA with a He flow rate of 30 ml/min. The ionizing potential was 70 ev.

The extent of exchange of the 4′-keto group during the extraction and purification procedure was determined by monitoring m/z 190 (base peak of ABA) and m/z 192 (base peak of 4′-^{18}O-ABA) by GC-SIM (dwell time for each ion was 400 ms). GLC conditions were as described above for ABA.

Analysis of air samples present around leaves during incubation in Erlenmeyer flasks was performed with a Varian-Mat GD-150 magnetic sector mass spectrometer.

Table I. *Effect of Anoxia on PA Accumulation in Xanthium Leaves*

Leaves were stressed, placed in a plastic bag for 6 h, and then rehydrated by immersing the leaves in water for 5 min. The leaves were then either frozen, or subjected to a vacuum-flush treatment to remove any oxygen present. Leaves subjected to a vacuum-flush treatment were then placed in an atmosphere of N_2, or room air was allowed to enter the flask and the flask was then resealed.

Treatment of Leaves	ABA	PA	ABA-GE
	$\mu g \cdot g^{-1}$ *fresh wt*		
Stressed 6 h → rehydrated → frozen	3.1	1.5	1.0
Stressed 6 h → rehydrated → vacuum → room air 5 h	0.9	4.5	1.2
Stressed 6 h → rehydrated → vacuum → 100% N_2 5 h	2.9	1.3	1.0

RESULTS AND DISCUSSION

The ABA level in stressed and subsequently rehydrated leaves incubated in room air declined and PA accumulated (Table I). By contrast, when stressed and subsequently rehydrated leaves were incubated in N_2, the ABA level remained high, and PA did not accumulate. The accumulation of ABA-GE did not appear to be greatly affected by anaerobic conditions. However, little ABA-GE accumulates in *Xanthium* leaves that have been stressed and subsequently rehydrated (16). The possibility remains that anoxia did not affect ABA catabolism directly, but rather indirectly via an effect on cell metabolism. GC-MS analysis of PA isolated from rehydrated leaves incubated in an atmosphere of 20% $^{18}O_2$ and 80% N_2 showed a new molecular ion at m/z 296 compared with a molecular ion of m/z 294 for PA from rehydrated leaves incubated in room air. This indicates the incorporation of one atom of ^{18}O into the PA molecules (Fig. 1). The presence of m/z 294 in the mass spectrum of PA isolated from leaves incubated with $^{18}O_2$ (Fig. 1B) is due to PA already present in turgid leaves and to PA synthesized during the stress portion of the experiment (15, 16).

From the fragmentation pattern derived from a high resolution mass spectrum of methylated PA (G. L. Boyer, R. A. Creelman, and J. A. D. Zeevaart, unpublished results), we conclude that the atom of ^{18}O in the PA molecule is located in the 6′-hydroxymethyl group for the following reasons: (a) m/z 125 (side chain containing carboxyl group) is not shifted, (b) m/z 276 (arising from the loss of the 1′-hydroxyl group as water from m/z 294) is shifted by 2 mass units, (c) m/z 139 (m/z 167 gives rise to m/z 139 with loss of $CO^{\cdot}$, the CO coming from the 4′-keto group) is shifted by 2 mass units, and (d) m/z 233 (m/z 263, which is shifted by 2 mass units, gives rise to m/z 233 with the loss of $CH_2O^{\cdot}$) is not shifted. These data support the conclusion (2) that ABA hydroxylating enzyme is an oxygenase and are consistent with Gillett's rule (3) that biological hydroxylation of a methyl group involves the direct participation of molecular oxygen.

ABA levels in stressed leaves of *X. strumarium* increased, and the vacuum-flush treatment had no effect on accumulation of ABA (Table II). On the other hand, accumulation of ABA in stressed leaves was inhibited by anoxia. However, as with the PA results (see above), one cannot rule out that anoxia had only an indirect effect on ABA biosynthesis. GC-MS analysis of ABA from leaves incubated in an atmosphere of 16% $^{18}O_2$, 4% $^{16}O_2$, and 80% N_2 shows a new molecular ion at m/z 280 compared with the molecular ion (m/z 278) found with ABA from leaves incubated in room air (Fig. 2). This indicates that only one atom of ^{18}O is incorporated into the ABA molecule. Similar results have been obtained with *P. vulgaris* cv Redkloud (data not shown). The atom of ^{18}O is located in the carboxyl group of ABA for the following reasons: (a) m/z 125 (side chain) is shifted by 2 mass units (4), and (b) the fragment derived entirely from the methylated carboxyl group, m/z 59 ($COOCH_3^+$) is shifted by 2 mass units to m/z 61.

The fragments m/z 125 and m/z 262 contain both oxygens of the 1-carboxyl group of ABA (4), and as expected, the ratios

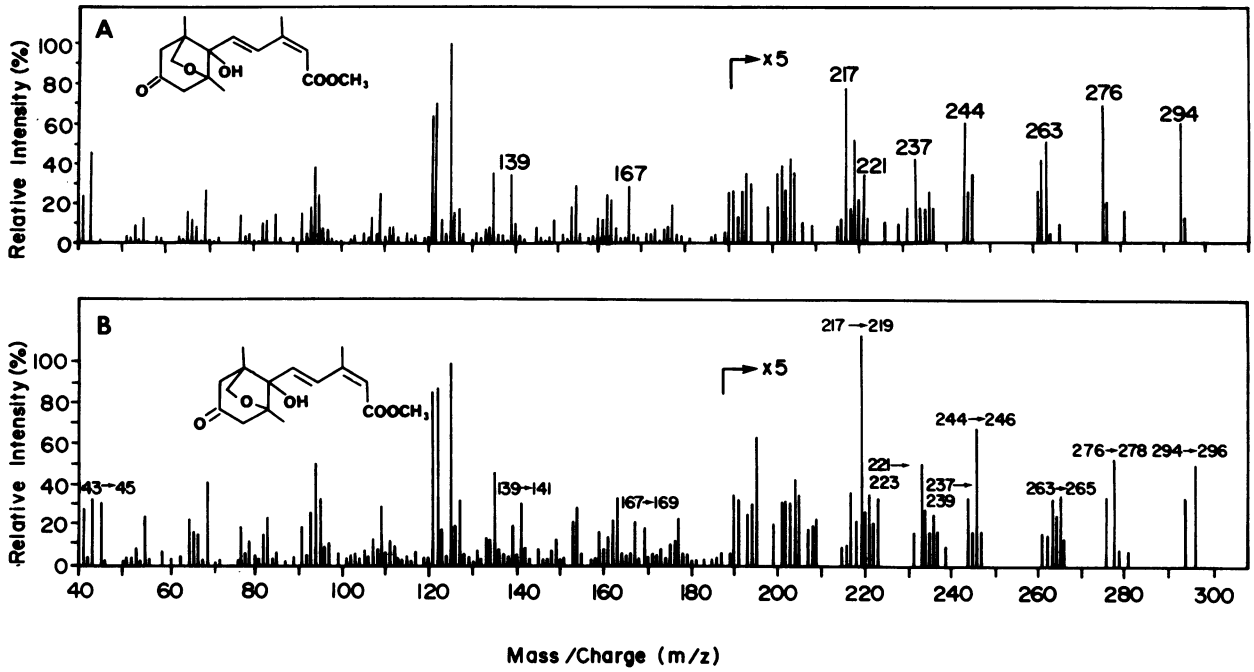

FIG. 1. Mass spectra of PA isolated from stressed and subsequently rehydrated *Xanthium* leaves incubated in room air (A), or $^{18}O_2$ (B).

Table II. *Effect of Anoxia on ABA Accumulation in Xanthium Leaves*

Leaves were stressed and then either placed in a plastic bag for 8 h, or in a 250-ml flask and subjected to a vacuum-flush treatment. Leaves subjected to a vacuum-flush treatment were then placed in an atmosphere of N_2, or room air was allowed to enter the flask and the flask was then resealed. Control indicates a turgid leaf placed in a plastic bag.

Treatment of Leaves	ABA
	μg g⁻¹ dry wt
Control 8 h	5.3
Stressed 8 h	24.5
Stressed → vacuum → room air 8 h	21.0
Stressed → vacuum → 100% N_2 8 h	3.3

m/z 125/127 and 262/264 are similar. On the other hand, the fragment m/z 190 contains only one of the two oxygens found in the carboxyl group (4). From a chemical viewpoint the two oxygens in the carboxyl group are equivalent. Thus, if there is only one ^{18}O present in the carboxyl group, as concluded above, only half the m/z 190 fragments will contain ^{18}O, resulting in a higher ratio of m/z 190/192 than found for m/z 125/127 and 262/264. This is indeed observed in Figure 2B.

Exchange of oxygen from the 1'-hydroxyl group of ABA with oxygen present in water during sample extraction is highly unlikely, since it was necessary to use synthetic methods to introduce ^{18}O in the 1'-hydroxyl group (4). It is also unlikely that the oxygen from the 4'-keto group or 1-carboxyl group exchanged with the oxygen in water, since synthesis of ABA containing ^{18}O in the carboxyl group or the keto group required strongly alkaline conditions and heat (4). Conditions used in the present experiment for the isolation of ABA were weakly acidic, and do not appear to cause exchange (17). However, when ABA was placed in $H_2{}^{18}O$ with 1% (v/v) acetic acid for 2.5 d one atom of ^{18}O was exchanged into the 4'-keto group of ABA, as determined by analysis of the fragmentation pattern (data not shown). Thus, it is possible that the keto group exchanged out during the extraction process. To determine if this was indeed the case, a small amount of 4'-^{18}O-ABA was added to a *Xanthium* leaf sample and the sample was extracted and purified as described above. At the same time, an equal amount of 4'-^{18}O-ABA was stored in a refrigerator, termed hereafter stored aliquot. After purification, the tissue sample was brought up to a final volume of 20 μl, as was the stored aliquot. Equal amounts of the extract and the stored aliquot were analyzed by GC-MS and m/z 192 was monitored by GC-SIM. The SIM response of the tissue and the stored aliquot were comparable, indicating that little or no exchange had occurred during extraction and purification. We conclude therefore that no ^{18}O was incorporated into the 4'-keto-group of ABA under our experimental conditions.

The fact that only one ^{18}O atom appeared in the carboxyl group of ABA that accumulates in water-stressed leaves is unexpected. Based on the strong similarities between ABA and carotenoids, we expected that the 4'-keto and 1'-hydroxyl groups would contain ^{18}O. Since they remain unlabeled, the oxygen atoms in the 4'-keto and 1'-hydroxyl groups must either (a) come from water, or (b) must already be present in a precursor, such as xanthoxin or certain xanthophylls (such as violaxanthin), that is converted to ABA under conditions of water stress. If this latter case is correct, it would be futile to search for intermediates in the ABA biosynthetic pathway by feeding radioactive MVA as a precursor.

There is no firm evidence to support either the direct or indirect pathway of ABA biosynthesis. Milborrow (see 6) rules out the indirect pathway on the basis of an experiment with [^{14}C] phytoene and [^{3}H]MVA fed to avocado fruit. ^{14}C and ^{3}H were both found in carotenoids, yet only ^{3}H was found in ABA. This work is not conclusive because phytoene would have had to penetrate to chloroplasts and then be converted to a xanthophyll. It is not known whether or not this occurred, since a detailed account of this work has never been published.

We believe that the data presented here are in accord with the indirect pathway, although the existence of two separate, independent pathways, one operating in turgid and a different one in water-stressed leaves, cannot be ruled out. Xanthoxin, a degradation product of violaxanthin (9), is endogenous to higher plants (1, 14). When labeled xanthoxin was fed to tomato and bean plants, it was converted to ABA and catabolites of ABA (10). Since xanthoxin is endogenous in higher plants, and is

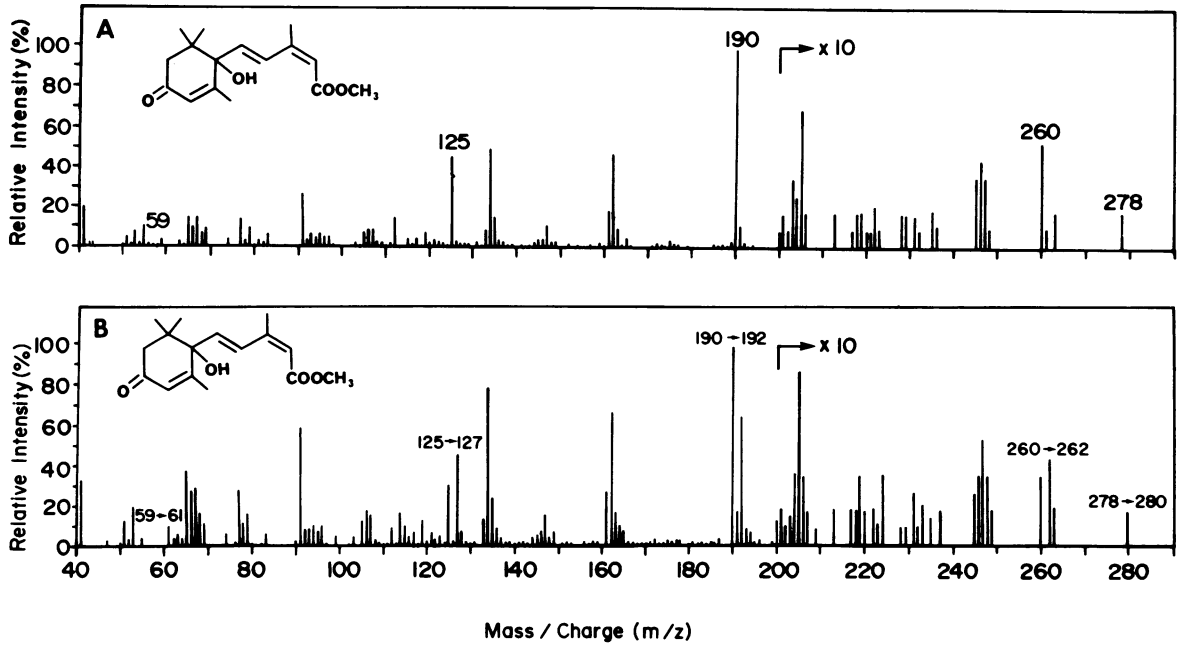

FIG. 2. Mass spectra of ABA isolated from stressed *Xanthium* leaves incubated in room air (A), or $^{18}O_2$ (B).

converted to ABA, it is a likely precursor of ABA in higher plants.

Neill *et al.* (7) have isolated 1′-deoxy-ABA from *Cercospora rosicola* and have shown that it is the immediate precursor of ABA in this fungus. There is no evidence, however, that 1′-deoxy-ABA is endogenous in higher plants. GC-SIM analysis of 1′-deoxy-ABA extracted from *Vicia faba* (the only plant tested which appears to convert 1′-deoxy-ABA to ABA) cuttings fed ^{2}H-α-ionylidene acetic acid showed that 1′-deoxy-ABA was 100% labeled, *i.e.* all the extracted 1′-deoxy-ABA was synthesized from the applied α-ionylidene acetic acid (7). This implies that either 1′-deoxy-ABA is not endogenous to *V. faba*, or that it is present in a small pool rapidly turning over. Furthermore, Lehmann and Schütte (5) observed that when α-ionylidene acetic acid was fed to barley plants, it was converted to 1′-deoxy-ABA and conjugates, but not to ABA. Our data also indicate that 1′-deoxy-ABA is not the immediate precursor of ABA in higher plants, unless the oxygen present in the 1′-hydroxyl group is derived from water rather than from molecular oxygen.

In conclusion, we have demonstrated that when stressed and subsequently rehydrated *Xanthium* leaves are incubated in an atmosphere containing $^{18}O_2$, one atom of ^{18}O is found in the 6′-hydroxymethyl group of PA, confirming that ABA hydroxylating enzyme is an oxygenase. When stressed *Xanthium* leaves are incubated in an atmosphere containing $^{18}O_2$, one atom of ^{18}O is found in the carboxyl group of ABA. This implies that either the oxygens in the 1′-hydroxyl group, 4′-keto group, and one of the two oxygens in the 1-carboxyl group come from water, or a stored precursor exists with oxygen atoms already present at the 1′- and 4′-positions, and possibly the 1-position.

Note Added in Proof. Since this work was completed, we have learned of similar $^{18}O_2$ labeling experiments with the fungus *Cercospora rosicola*. In this case, ABA contained four ^{18}O atoms when the fungus was cultured over a 48-h period under an atmosphere containing 20% $^{18}O_2$ (R. Horgan and D. C. Walton, personal communications).

Acknowledgments—We would like to thank J. Throck Watson and Brian Musselman for valuable discussions regarding the fragmentation pattern of PA and the Michigan State University-National Institutes of Health Mass Spectroscopy Facility (RR00480) for use of the GC-MS equipment. We also thank T. M. Shimei for MS analysis of gas samples.

LITERATURE CITED

1. FIRN RD, RS BURDEN, HF TAYLOR 1972 The detection and estimation of the growth inhibitor xanthoxin in plants. Planta 102: 115–126
2. GILLARD DF, DC WALTON 1976 Abscisic acid metabolism by a cell-free preparation from *Echinocystis lobata* liquid endosperm. Plant Physiol 58: 790–795
3. GILLETT JR 1959 Side chain oxidation of alkyl substituted ring compounds I. Enzymatic oxidation of *p*-nitrotoluene. J Biol Chem 234: 139–143
4. GRAY RT, R MALLABY, G RYBACK, VP WILLIAMS 1974 Mass spectra of methyl abscisate and isotopically labelled analogues. J Chem Soc Perkins Trans II: 919–924
5. LEHMANN H, HR SCHÜTTE 1976 Biochemistry of phytoeffectors 9. The metabolism of α-ionylideneacetic acid in *Hordeum distichon*. Biochem Physiol Pflanzen 169: 55–61
6. MILBORROW BV 1983 Pathways to and from abscisic acid. *In* FT Addicott, ed, Abscisic Acid. Praeger, New York, pp 79–111
7. NEILL SJ, R HORGAN, DC WALTON, D GRIFFIN 1982 Biosynthesis of abscisic acid. *In* PF Wareing, ed, Plant Growth Substances 1982. Academic Press, New York, pp 315–323
8. SHNEOUR EA 1962 The source of oxygen in *Rhodopseudomonas spheroides* carotenoid pigment conversion. Biochim Biophys Acta 65: 510–511
9. TAYLOR HF, RS BURDEN 1972 Xanthoxin, a recently discovered plant growth inhibitor. Proc R Soc Lond B 180: 317–346
10. TAYLOR HF, RS BURDEN 1973 Preparation and metabolism of 2-[^{14}C]-*cis trans*-xanthoxin. J Exp Bot 24: 873–880
11. WEST CA 1980 Hydroxylases, monooxygenases, and cytochrome P-450. *In* PK Stumpf, EE Conn, eds, The Biochemistry of Plants, Vol 2. Academic Press, New York, pp 317–364
12. YAMAMOTO HY, CO CHICHESTER 1965 Dark incorporation of ^{18}O into antheraxanthin by bean leaf. Biochim Biophys Acta 109: 303–305
13. YAMAMOTO HY, CO CHICHESTER, TOM NAKAYAMA 1962 Biosynthetic origin of oxygen in the leaf xanthophylls. Arch Biochem Biophys 96: 645–649
14. ZEEVAART JAD 1974 Levels of (+)–abscisic acid and xanthoxin in spinach under different environmental conditions. Plant Physiol 53: 644–648
15. ZEEVAART JAD 1980 Changes in the levels of abscisic acid and its metabolites in excised leaves of *Xanthium strumarium* during and after water stress. Plant Physiol 66: 672–678
16. ZEEVAART JAD 1983 Metabolism of abscisic acid and its regulation in *Xanthium* leaves during and after water stress. Plant Physiol 71: 477–481
17. ZEEVAART JAD, BV MILBORROW 1976 Metabolism of abscisic acid and the occurrence of *epi*-dihydrophaseic acid in *Phaseolus vulgaris*. Phytochemistry 15: 493–500