



3 1293 10757 8514

LIBRARY
Michigan State
University

This is to certify that the
dissertation entitled

"Stress-Induced Absciscic Acid Biosynthesis
in Higher Plants"

presented by

R. A. Creelman

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Botany and Plant Pathology


Major professor

Date June 11, 1986.



RETURNING MATERIALS:

Place in book drop to
remove this checkout from
your record. FINES will
be charged if book is
returned after the date
stamped below.

200-A330

163

STRESS-INDUCED ABSCISIC ACID BIOSYNTHESIS IN HIGHER PLANTS

By

Robert Arthur Creelman

A DISSERTATION

Submitted to

Michigan State University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

Department of Botany and Plant Pathology

1986

4276 70x

ABSTRACT

STRESS-INDUCED ABSCISIC ACID BIOSYNTHESIS IN HIGHER PLANTS

By

Robert Arthur Creelman

In water-stressed leaves, levels of abscisic acid (ABA) can increase 10- to 40-fold over those in turgid ones. Turgor loss is the crucial parameter of cell water relations governing the accumulation of ABA. Spinach leaf slices incubated in the presence of ethylene glycol, a penetrating solute, did not accumulate ABA, while slices incubated in non-penetrating solutes, such as mannitol or Aquacide III, did.

Two hypotheses have evolved concerning ABA biosynthesis: ABA is formed (i) directly from farnesyl pyrophosphate, or (ii) indirectly from a carotenoid, with xanthoxin as an intermediate in the pathway.

The immediate precursor(s) to ABA must be a C₁₅ compound. Three C₁₅ compounds with structures similar to ABA were synthesized. The biological activity of -ionylidene acetic acid was greater than that of ABA, indicating that it was active *per se* and not a precursor, while 1',2'-epoxy-ionylidene acetic acid had less biological activity than ABA. When the deuterated analogue of 1',2'-epoxy-ionylidene acetic acid was fed to *Xanthium* leaves, deuterium was incorporated into ABA. However, this compound is not endogenous in *Xanthium*. Radioactive 1',2'-epoxy-ionylidene acetaldehyde was metabolized by plant tissues (spinach leaves,

tomato shoots, and tomato fruits), but no incorporation into ABA or xanthoxin was detected.

Inhibitors of carotenoid biosynthesis decrease ABA accumulation. While levels of carbohydrates and organic acids were also changed, this result supports the hypothesis that ABA is derived from a carotenoid.

The biosynthesis of ABA requires molecular oxygen. When stressed leaves of *Xanthium* were incubated in the presence of $^{18}\text{O}_2$ for six hours, the majority of the ^{18}O present in ABA was found in the carboxyl group. With longer incubations (12 and 24 hours), increasing amounts of ^{18}O were found in the ring oxygen atoms of ABA. However, with stressed *Xanthium* roots, ^{18}O was already present in the ring oxygen atoms after only six hours. Incorporation of ^{18}O into phaseic acid, a catabolite of ABA, was also detected. These data are consistent with the indirect pathway for biosynthesis of ABA.

Knowledge is good (Emil Faber)

The struggles you survive make you stronger (F. Nietzsche)

It is by the solution of problems that the strength of the investigator is hardened; he finds new methods and new outlooks and gains a freer horizon (D. Hilbert)

The thing that hath been is that which shall be; and that which is done is that which shall be done; and there is no new thing under the sun (Ecclesiastes)

The more things change, the more they remain the same
(Montaigne)

I write my book, whether it be read by the present age or posterity imports little...has not God waited six thousand years for an observer of his works? (J. Kepler)

For we do not think that we know a thing until we are acquainted with its primary conditions or first principles and have carried our analysis as far as its simplest elements (Aristotle, Physics, book 1)

I have yet to see any problem, however complicated, which when you looked at it the right way did not become more complicated (P. Anderson)

Some day you will tax it (M. Faraday, on being asked by P.M. Gladstone what was the use of electricity)

Where there is a great deal of light, the shadows are deeper (Goethe)

It is the task of science to turn the impossible into the boring (R. Ornstein)

Wherever you go, there you are (Buckaroo Banzai)

Vegetables are organized bodies, which extract from the earth juices proper to their nature. Vegetable substances are much more compound than mineral. Their analysis is consequently more difficult; certain principles, of too great tenuity or volatility, escape us entirely (Beaume)

ACKNOWLEDGMENTS

There are many people I would like to thank for their help, but here I can only offer my gratitude to a few. Many thanks go to Jan Zeevaart, my major professor, for letting me do the experiments I wanted to do. I also wish to thank the members of my guidance committee, Ray Hammerschmidt, Hans Kende, and Frank Dennis, for their helpful comments and critical reviews. Much of this thesis consists of mass spectra; without the MSU-NIH Mass Spectroscopy Facility (RR00480) and the people who work there (among others, Brian Musselman, Betty Baltzer, and John Stults) I could not have accomplished what I did. Without a doubt, my interactions with other graduate students, post-docs, and faculty at MSU, the PRL, and the Botany and Plant Pathology Department (in particular Alice and Jennifer) have not been unrewarding or unpleasurable. Special thanks go to the Spuds/Citrus Cankers/Dynamo Buffuphna (past, present, and future) for all the fun, good times, and exercise. I would have never been able to get through this place without going insane if I had not known Ted John, Brian Parks, and Jim Smith. Thanks guys, this one is for you. I would also like my parents and family for all the support and encouragement through this period. And last but not least, SAK, who made these last few years so nice.

Without money, nothing can be done, so I would like to state that the research described in this thesis was supported by the United States Department of Energy under Contract DE-AC02-76ER0-1338.

TABLE OF CONTENTS

	Page
List of Tables	xi
List of Figures	xiii
List of Abbreviations	xvii
Chapter 1. INTRODUCTION	1
1.1. Isolation and Identification of Absciscic Acid	2
1.2. Occurrence in Plants	3
1.3. Chemical Properties	3
1.4. Biosynthesis in Fungi	5
1.5. Biosynthesis in Higher Plants	7
1.5.1. The Direct Pathway	8
1.5.2. The Indirect Pathway	10
1.5.3. Absciscic Acid Biosynthetic Mutants	15
1.6. Metabolism of Absciscic Acid	17
1.7. Physiology	20
1.8. Statement of Purpose	24
1.9. Literature Cited	25
Chapter 2. INHIBITION OF ABSCISCIC ACID ACCUMULATION IN WATER-STRESSED CORN SEEDLINGS IN THE PRESENCE OF CAROTENOID BIOSYNTHETIC INHIBITORS	34
2.1. Introduction	35
2.2. Materials and Methods	36
2.2.1. Plant Material	36
2.2.2. Absciscic Acid Analysis	36

	Page
2.2.3. Organic Acid Analysis	36
2.2.4. Soluble and Insoluble Sugar Analysis	38
2.2.5. Carotenoid Analysis	38
2.3. Results	39
2.3.1. Carotenoid, Sugar, and Organic Acid Levels in Inhibitor and Control Corn Seedlings	39
2.3.2. Effects of Carotenoid Biosynthetic In- hibitors on Absciscic Acid Accumulation	41
2.4. Discussion	41
2.4.1. Inhibition of Absciscic Acid Accumula- tion by Norflurazon and Fluridone	41
2.4.2. Non-specificity of Carotenoid Biosyn- thetic Inhibitors	44
2.5. Literature Cited	46
Chapter 3. BIOLOGICAL ACTIVITY AND METABOLISM OF COMPOUNDS STRUCTURALLY RELATED TO ABSCISIC ACID	48
3.1. Introduction	49
3.2. Materials and Methods	51
3.2.1. Synthesis of ^2H - α -Ionone	51
3.2.2. Synthesis of ^2H - and ^3H - β -Ionone	51
3.2.3. Synthesis of α -Ionylidene Acetic Acid	53
3.2.4. Synthesis of 1',2'-Epoxy-Ionylidene Acetic Acid	55
3.2.5. Synthesis of 1',2'-Epoxy-Ionylidene Acetaldehyde	61

	Page
3.2.6. Spectrometry	68
3.2.7. Plant Material	70
3.2.8. Extraction and Purification Procedures	70
3.2.9. Biological Activity Assay	72
3.3. Results	73
3.3.1. Biological Activity of α -Ionylidene Acetic Acid (X) and 1',2'-Epoxy-Ionyl- idene Acetic Acid (XV)	73
3.3.2. Incorporation of 1',2'-Epoxy-Ionylidene Acetic Acid (XV) into Absciscic Acid	73
3.3.3. Metabolism of 1',2'-Epoxy-Ionylidene Acetaldehyde (XVII) by Higher Plant Tissue	73
3.4. Discussion	80
3.5. Literature Cited	83
Chapter 4. INCORPORATION OF OXYGEN INTO ABSCISIC ACID AND PHASEIC ACID FROM MOLECULAR OXYGEN	86
4.1. Abstract	87
4.2. Introduction	87
4.3. Materials and Methods	87
4.3.1. Plant Material	87
4.3.2. Chemicals	88
4.3.3. Extraction and Purification Procedures	88
4.3.4. Mass Spectrometry	88
4.4. Results and Discussion	88
4.5. Literature Cited	90

	Page
Chapter 5. ACCUMULATION OF ABSCISIC AND PHASEIC ACID IN <i>XANTHIUM STRUMARIUM</i> LEAVES UNDER DIFFERENT OXYGEN TENSIONS	91
5.1. Introduction	92
5.2. Materials and Methods	92
5.2.1. Plant Material	92
5.2.2. Extraction and Purification Procedure	93
5.2.3. Oxygen measurements	93
5.3. Results	94
5.4. Discussion	94
5.5. Literature Cited	97
Chapter 6. INCORPORATION OF MOLECULAR OXYGEN INTO ABSCISIC AND PHASEIC ACID IN LEAVES AND ROOTS OF <i>XANTHIUM</i> <i>STRUMARIUM</i> DURING LONG TERM INCUBATIONS IN $^{18}O_2$	98
6.1. Introduction	99
6.2. Materials and Methods	100
6.2.1. Culture of Plant Material	100
6.2.2. Extraction and Purification of Absciscic and Phaseic Acid	101
6.2.3. Oxygen Measurements	101
6.2.4. Purification of Carotenoids	102
6.2.5. Mass Spectrometry	103
6.2.6. Chemicals	104
6.3. Results	104
6.3.1. Carotenoid levels in leaves and roots	104
6.3.2. Incorporation of ^{18}O into abscisic	

	Page
and phaseic acid during long term incubations in $^{18}O_2$	107
6.4. Discussion	115
6.5. Literature Cited	122
Chapter 7. INCORPORATION OF DEUTERIUM INTO ABSCISIC ACID, STEROLS, AND CAROTENOIDS FROM DEUTERIUM OXIDE . .	124
7.1 Introduction	125
7.2. Materials and Methods	127
7.2.1. Plant Material	127
7.2.2. Extraction and Purification of Absciscic Acid	127
7.2.3. Purification of Sterols	128
7.2.4. Purification of Carotenoids	129
7.2.5. Mass Spectrometry	130
7.3. Results and Discussion	131
7.4. Literature Cited	136
Chapter 8. THE ROLE OF XANTHOXIN IN ABSCISIC ACID BIOSYNTHESIS	137
8.1. Introduction	138
8.2. Materials and Methods	140
8.2.1. Plant Material	140
8.2.2. Chemicals	140
8.2.3. Extraction and Purification Procedures . .	140
8.2.4. Determination of Xanthoxin Stability . . .	143
8.2.5. Gas Chromatography	143
8.2.6. Mass Spectrometry	143

	Page
8.3. Results	144
8.3.1. Stability of Xanthoxin	144
8.3.2. Incorporation of $^{18}\text{O}_2$ into Xanthoxin	144
and Absciscic Acid	144
8.3.3. Xanthoxin Levels in Tissues Extracted in Air and Nitrogen	144
8.4. Discussion	148
8.5. Literature Cited	152
Chapter 9. ABSCISIC ACID ACCUMULATION IN SPINACH LEAF SLICES IN THE PRESENCE OF PENETRATING AND NON-PENETRATING SOLUTES	154
9.1. Abstract	155
9.2. Introduction	155
9.3. Materials and Methods	155
9.3.1. Plant Material	155
9.3.2. ABA Purification Scheme	155
9.3.3. [^{14}C]Mannitol Uptake and Catabolism	156
9.3.4. Water Potential Measurement	156
9.3.5. Mass Spectrometry	156
9.4. Results and Discussion	156
9.5. Literature Cited	158
Chapter 10. STRESS-INDUCED ABSCISIC ACID BIOSYNTHESIS IN HIGHER PLANTS -- A MODEL	159
10.1. Introduction	160
10.2. A Model Explaining Stress-Induced Absciscic Acid Biosynthesis	165

	Page
10.3. Literature Cited	168
Appendix. QUANTITATION OF ABSCISIC ACID USING INTERNAL STANDARDS	171

LIST OF TABLES

Table	Page
2.1. The effect of norflurazon and fluridone on levels of phytoene and total carotenoids in corn seedlings	42
2.2. The effect of norflurazon and fluridone on organic acid levels in corn seedlings	42
2.3. The effect of norflurazon and fluridone on levels of soluble and insoluble sugars in corn seedlings	43
2.4. The effect of norflurazon and fluridone on ABA levels in corn seedlings	43
3.1. Selected ion monitoring response of standard ABA and ABA isolated from leaves fed 1',2'-epoxy- ionylidene acetic acid	75
4.1. Effect of anoxia on PA accumulation in <i>Xanthium</i> leaves	88
4.2. Effect of anoxia on ABA accumulation in <i>Xanthium</i> leaves	89
6.1. Absorption maxima for some carotenoids found in roots of <i>Xanthium strumarium</i>	106
6.2. Levels of carotenoids and ABA in roots and leaves of <i>Xanthium strumarium</i>	108
6.3. Incorporation of ^{18}O into abscisic acid in stressed <i>Xanthium</i> leaves	109
6.4. Predicted and actual values for ^{18}O incorpor-	

	Page
Table	
ation into phaseic acid	120
7.1. Incorporation of deuterium from deuterium oxide	
into abscisic acid	134
8.1. Xanthoxin levels from spinach leaves extracted	
in air or N ₂	150
A.1. Example of abscisic acid quantitation by gas-	
liquid chromatography-electron capture detection	
with internal standards	175

LIST OF FIGURES

Figure	Page
1.1. Structure of abscisic acid	3
1.2. Biosynthesis of abscisic acid in <i>Cercospora rosicola</i>	6
1.3. Biosynthesis of abscisic acid in higher plants	9
1.4. Some xanthophylls with terminal ring structures similar to abscisic acid	11
1.5. Compounds produced when violaxanthin is photo-oxidized	12
1.6. Biosynthetic scheme for trisporic acid in the fungal order Mucorales	14
1.7. Catabolites of abscisic acid	18
2.1. Corn seedlings watered with either nutrient solution or 10^{-4} M norflurazon	40
3.1. Synthesis of deuterated α -ionone	52
3.2. Synthesis of deuterated α -ionylidene acetic acid (X)	54
3.3. Mass spectrum of deuterated methyl- α -io- nylidene acetic acid (X)	56
3.4. Synthesis of deuterated 1',2'-epoxy-io- nylidene acetic acid (XV)	57
3.5. Mass spectrum of deuterated ethyl- <i>cis</i> , <i>trans</i> -8-ionylidene acetate (XII)	58
3.6. Mass spectrum of deuterated methyl- <i>cis</i> ,	

Figure

<i>trans</i> -epoxy-ionylidene acetate (XV)	60
3.7. Synthesis of 1',2'-epoxy-ionylidene acet-	
aldehyde (XVII)	62
3.8. Mass spectrum of ethyl- <i>cis,trans</i> - β -io-	
nylidene acetate (XII)	63
3.9. Mass spectrum of ethyl- <i>cis,trans</i> -1',2'-	
epoxy-ionylidene acetate (XIV)	64
3.10. Mass spectrum of <i>cis,trans</i> -1',2'-epoxy-	
ionylidene ethanol (XVI)	66
3.11. Mass spectrum of <i>cis,trans</i> -1',2'-epoxy-	
ionylidene acetaldehyde (XVII)	67
3.12. Mass spectrum of deuterated <i>cis,trans</i> -1',	
2'-epoxy-ionylidene acetaldehyde (XVII)	69
3.13. Biological activity of α -ionylidene acetic	
acid (X) and 1',2'-epoxy-ionylidene acetic	
acid (XV)	74
3.14. Mass spectrum of a compound which co-	
chromatographed with standard methyl- <i>cis,</i>	
<i>trans</i> -1',2'-epoxy-ionylidene acetate (XV)	76
3.15. Metabolism of ^3H - <i>cis,trans</i> -1',2'-epoxy-	
ionylidene acetaldehyde (XVII) in spinach leaves	77
3.16. Metabolism of ^3H - <i>cis,trans</i> -1',2'-epoxy-	
ionylidene acetaldehyde (XVII) in tomato shoots	78
3.17. Metabolism of ^3H - <i>cis,trans</i> -1',2'-epoxy-	
ionylidene acetaldehyde (XVII) in immature	

Figure

(breaker stage) tomato fruits	79
4.1. Mass spectra of PA isolated from stressed and subsequently rehydrated <i>Xanthium</i> leaves incubated in room air (A), or $^{18}\text{O}_2$ (B)	89
4.2. Mass spectra of ABA isolated from stressed <i>Xanthium</i> leaves incubated in room air (A), or $^{18}\text{O}_2$ (B)	90
5.1. Accumulation of phaseic acid under different oxygen tensions	95
5.2. Accumulation of abscisic acid under different oxygen tensions	96
6.1. Purification of <i>Xanthium</i> root carotenoids by HPLC	105
6.2. Mass spectra of ABA obtained by DP-NCI	110
6.3. Mass spectrum of ABA isolated from stressed <i>Xanthium</i> roots incubated in $^{18}\text{O}_2$ for 6 h	112
6.4. Mass spectra of PA obtained by DP-NCI	113
6.5. Mass spectrum of ABA isolated from stressed <i>Xanthium</i> leaves incubated in $^{18}\text{O}_2$ for 24 h	116
7.1. Incorporation of deuterium from deuterium oxide into sitosterol and stigmasterol	132
8.1. Stability of xanthoxin under different environ- mental conditions	145
8.2. Mass spectrum of xanthoxin isolated from spinach leaves incubated in $^{18}\text{O}_2$ for 8 h	146

Figure

8.3. Mass spectrum of ABA isolated from spinach leaves incubated in $^{18}\text{O}_2$ for 8 h	147
8.4. Mass spectrum of the <i>O</i> -(2,3,4,5,6-penta- fluorobenzyl)hydroxylamine hydrochloride derivative of xanthoxin	149
9.1. Changes in ABA content of detached control (turgid), and in stressed (wilted) leaves	156
9.2. ABA accumulation in detached control (C), stressed (S) spinach leaves, and in spinach leaf slices and media	156
9.3. ABA accumulation in detached spinach leaves and in spinach leaf slices and media	156
9.4. Levels of ABA in detached spinach leaves and in spinach leaf slices and media	157
10.1. Representative sesquiterpenoids	161
10.2. Compounds with structures similar to abscisic acid	162
A.1. Electron capture detector response of abscisic acid and internal standards	174
A.2. Typical standard curve obtained by injecting internal standards with increasing concentrations of methyl abscisic acid	176

LIST OF ABBREVIATIONS

ABA-GE	β -D-glucopyranosyl abscisate
BHT	2,6-di- <i>tert</i> -butyl- <i>p</i> -cresol
B.R.	boiling range
BSA	<i>N,O</i> -bis-(trimethylsilyl)-acetamide
CD	circular dichroism
Ci	Curie(s)
cv	cultivar
d	day(s)
DPA	4'-dihydrophaseic acid
DP	direct probe
EtABA	ethyl ester of abscisic acid
FAB	fast atom bombardment
GC-NCI	gas chromatography-negative chemical ionization
GC-MS	gas chromatography-mass spectrometry
GC-SIM	gas chromatography-selected ion monitoring
GLC-ECD	gas-liquid chromatography-electron capture detection
GLC-FID	gas-liquid chromatography-flame ionization detection
GLC-TCD	gas-liquid chromatography-total conductivity detector
h	hour(s)

HMDS	hexamethyldisilazane
8'-OH-ABA	8'-hydroxy ABA
HPLC	high performance liquid chromatography
EI	electron impact
MeABA	methyl ester of abscisic acid
min	minute(s)
MVA	mevalonic acid
MVL	mevalonic acid lactone
m/z	mass/charge
NMR	nuclear magnetic resonance
ORD	optical rotary dispersion
PA	phaseic acid
PA-GE	β -D-glucopyranosyl phaseate
R _f	ratio of the distance traveled of a solute relative to solvent front
s	second(s)
SIM	selected ion monitoring
TLC	thin layer chromatography
TMCS	trimethylchlorosilane
TMS	tetramethylsilane
TMSi	trimethylsilyl
UV	ultraviolet

CHAPTER 1
INTRODUCTION

1.1. ISOLATION AND IDENTIFICATION OF ABSCISIC ACID

The discovery of abscisic acid (ABA, Figure 1.1) stemmed from investigations performed during the 1950's and early 1960's by three groups working on unrelated problems. One group, led by Addicott at the University of California at Davis, purified an active compound (abscisin II) from cotton bolls which accelerated petiole abscission in explants from young cotton seedlings (Ohkuma et al., 1963; Ohkuma et al., 1965).

At Aberystwyth, Wareing's group was investigating the cause of dormancy in trees. An active extract (termed dormin) was obtained from leaves of sycamore (*Acer pseudo-platanus*) which induced dormancy in buds of sycamore seedlings (Robinson and Wareing, 1964; Wareing et al., 1964). Cornforth et al. (1965) later showed that dormin was identical to abscisin II.

Rothwell and Wain (1964) isolated a compound which appeared to accelerate fruit and flower drop in yellow lupin, later identified as abscisin II (Cornforth et al., 1966b; Koshimizu et al., 1966; Porter and Van Steveninck, 1966). As a compromise, abscisin II (dormin) was renamed abscisic acid and is abbreviated ABA (Addicott et al., 1968). Recently, the numbering system of ABA was extended to include previously unnumbered methyl groups (Boyer et al., 1986).

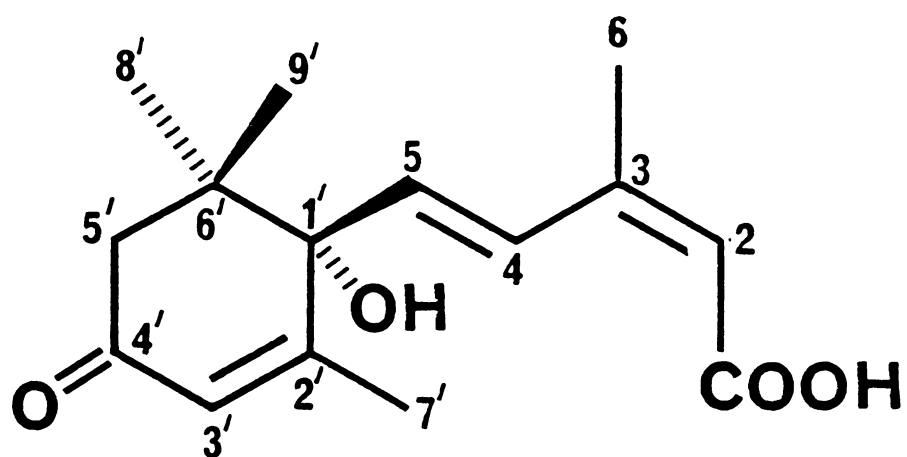


Figure 1.1. Structure of abscisic acid.

1.2. OCCURRENCE IN PLANTS

ABA has been identified in angiosperms (both monocots and dicots), gymnosperms, two ferns, a horsetail, and a moss (Milborrow, 1978; Bearder, 1980), and several genera of fungi (Assante *et al.*, 1977; Ichimura *et al.*, 1983; Dörffling and Peterson, 1984; Marumo *et al.*, 1984). ABA has been detected in every major (and minor) plant organ from shoot to root apices (Milborrow, 1978). ABA tends to occur in highest concentration in young leaves, buds, fruits, and seeds (Milborrow, 1978).

1.3. CHEMICAL PROPERTIES

ABA (MW 264 g/mole) is a sesquiterpenoid and contains a carboxyl, keto, and hydroxyl group (Figure 1.1). It is a weak acid (pK_a 4.8) and partitions into organic solvents, such as diethyl ether, dichloromethane, and ethyl acetate (but not hexane), at low pH. The molecule has one chiral center at C-1'. The naturally occurring enantiomer is dextro-rotary and has a sinister (S) configuration. ABA has high optical activity, with extrema at 289 nm (positive) and 246 nm (negative) (Cornforth *et al.*, 1966a). ABA absorbs in the UV, with its maximum varying with the pH of the solution (Dörffling and Tietz, 1983). UV, infrared, NMR, ORD, CD, and

mass spectra are presented in Dörffling and Tietz (1983).

The configuration of the side chain at C-2 can be either *cis* or *trans*. By convention, 2-*cis*,4-*trans*-ABA is ABA, and the 2-*trans*,4-*trans* isomer *t*-ABA (Addicott *et al.*, 1968). Strong light catalyzes the isomerization at the 2,3 double bond to establish an approximate 1:1 ratio of ABA:*t*-ABA in solution.

In several biological assays the unnatural (-) enantiomer was as active as natural (+)-ABA (Sondheimer *et al.*, 1971). However, (-)-ABA was much less active than (+)-ABA in closing stomata of barley leaves (Cummins and Sondheimer, 1973).

1.4. BIOSYNTHESIS IN FUNGI

The discovery that the fungus *Cercospora rosicola* produced large amounts of ABA (Assante *et al.*, 1977) initiated studies on the biosynthetic pathway in that organism. When ³H-MVA was applied to *C. rosicola* mycelia it was incorporated into two major fractions, ABA and 1'-deoxy-ABA (IV, Figure 1.2; Neill *et al.*, 1981; Neill *et al.*, 1982). Later it was shown that α -ionylidene ethanol (I), α -ionylidene acetic acid (II), and the epimeric 4'-hydroxy- α -ionylidene acetic acids (III) were also incorporated into ABA (Neill and Horgan, 1983; Horgan *et al.*, 1983). Of these compounds, only III and IV are endogenous in *C. rosicola* (Horgan *et al.*, 1983). Compounds with a β -ionylidene type structure are not converted to ABA (Neill and Horgan, 1983). Recently, Ichimura *et*

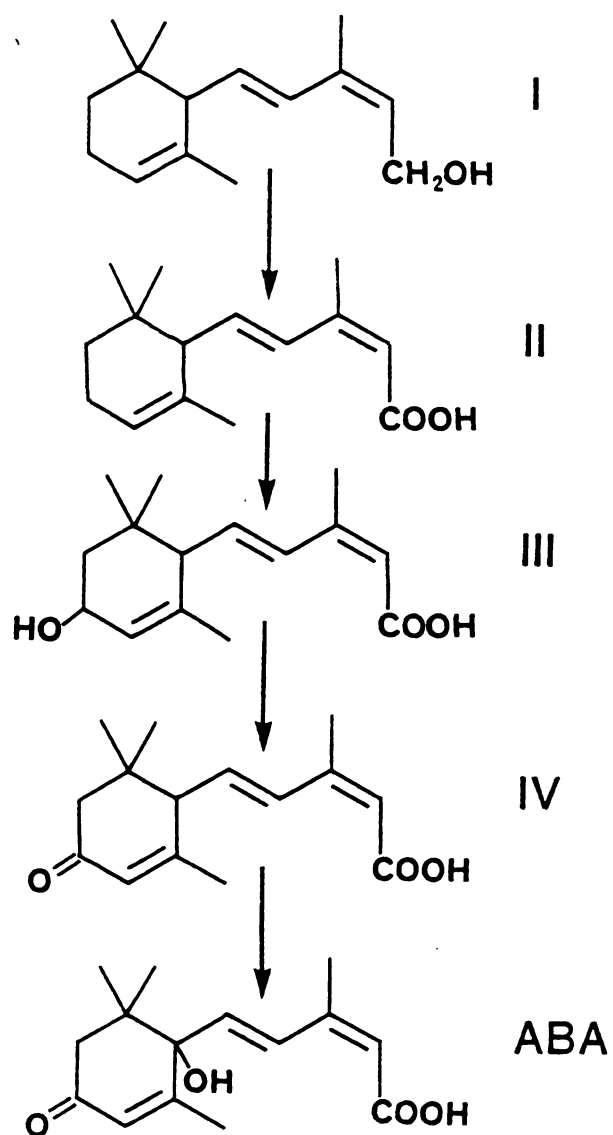


Figure 1.2. Biosynthesis of abscisic acid in *Cercospora rosicola*.

Only III, IV, and ABA are endogenous in *C. rosicola*. It is not known which epimer of III is the natural one.

a1. (1983) reported similar results with *C. cruenta*.

Interestingly, only the 2-*cis*,4-*trans* isomers of I and II were metabolized to ABA, while the 2-*trans*,4-*trans* isomers were converted only as far as 2-*trans*,4-*trans*-IV (Neill and Horgan, 1983). This implies that the early oxidizing enzymes are rather nonspecific, but the final hydroxylating enzyme has high specificity for the geometry of the side chain.

In summary, labeling studies with *C. rosicola* and *C. cruenta* suggest that ABA in these fungi is formed from α -ionylidene type compounds. Several possible intermediates are also converted to ABA, but only two of them (III and IV) appear to be endogenous. The final step in the biosynthetic pathway appears to be the hydroxylation of IV at the 1' carbon to give ABA. Nothing is known about the steps from MVA to the first cyclized intermediate.

1.5. BIOSYNTHESIS IN HIGHER PLANTS

In order to understand the physiology of ABA it is essential to understand how the ABA level is regulated. This requires information about how ABA is biosynthesized and catabolized. Surprisingly for this youngster of the plant growth substances, nothing is known about the biosynthetic precursors to ABA. Reasons for this are the low levels of ABA in plant tissues and lack of specific inhibitors of its formation. As ABA contains 15 carbons and fits the isoprene rule, it is considered to be a sesquiterpenoid. However, the

structure of ABA is radically different from any of the known sesquiterpenoids (Loomis and Croteau, 1980). Research has focused on two pathways: (a) the direct pathway involving a C₁₅ precursor derived from farnesyl pyrophosphate, and (b) the indirect pathway involving a precursor derived from a carotenoid (Figure 1.3). The relative contribution of each pathway is unknown, and the possibility exists that both may be operating in higher plants at the same time. In either case, MVA would be the ultimate precursor.

1.5.1. The Direct Pathway. Little evidence exists for the immediate biosynthetic precursors of ABA, yet there is evidence that MVA is a precursor. Label from MVA (or MVL) has been incorporated into ABA on several occasions (Noddle and Robinson, 1969; Milborrow and Robinson, 1973). However, even though large amounts of radioactivity were supplied, the amount of label found in ABA was a small fraction of the total fed. Possible reasons for low incorporation are competition for MVA by other terpenoid pathways (such as sterols), or that the applied radioactive MVA is extensively diluted by a (large) precursor pool to ABA.

Results from feeding experiments with labeled MVA indicate that three residues of the natural 3-R enantiomer are incorporated into ABA (Robinson and Ryback, 1969). In addition, the pro-4-R hydrogen of MVA is retained at carbons 2 and 5' of ABA (Robinson and Ryback, 1969), the pro-5-S hydrogen at carbon 5 (Milborrow, 1972), and a preponderance of the pro-2-R hydrogen at carbons 4 and 3' (Milborrow,

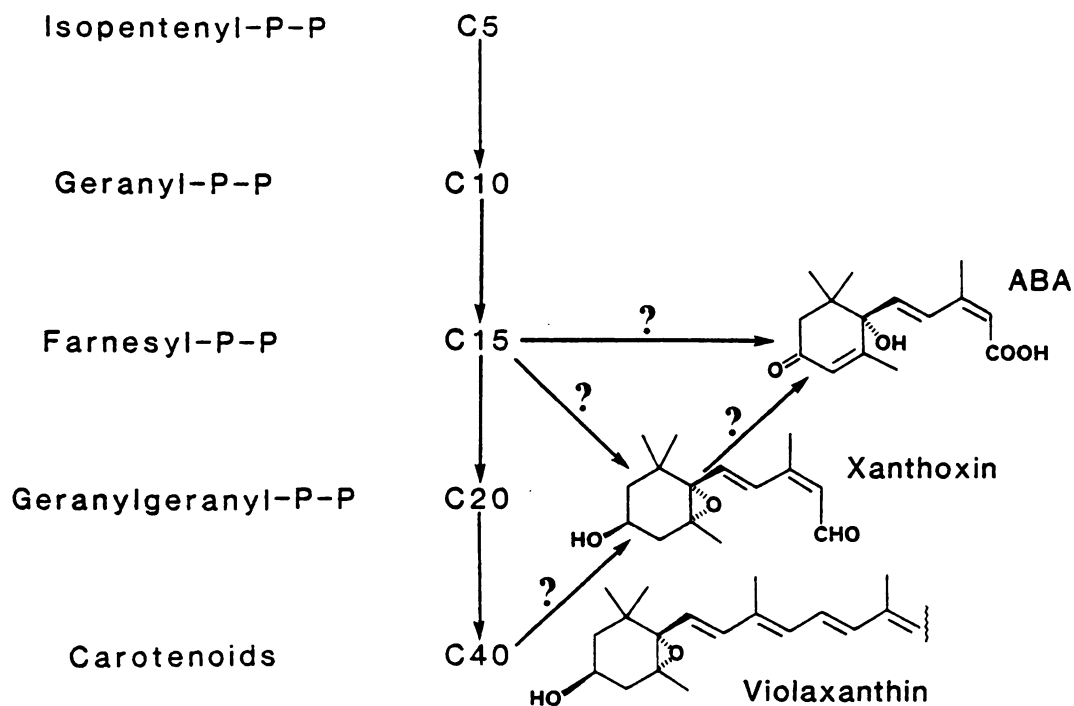


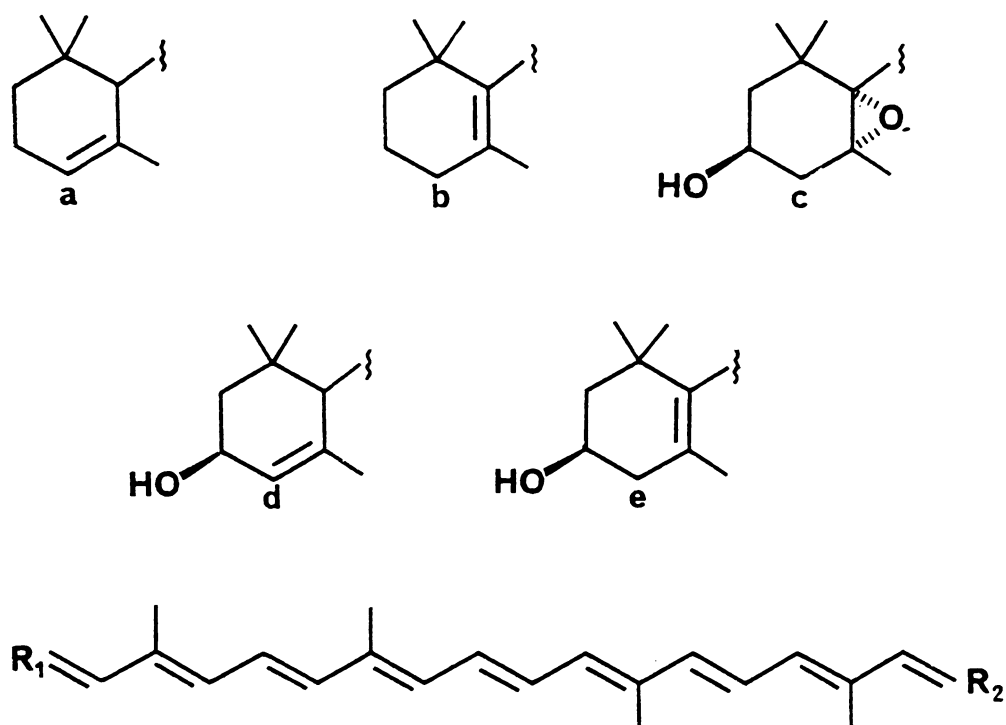
Figure 1.3. Biosynthesis of abscisic acid in higher plants. It is not known whether xanthoxin is a natural precursor to ABA, or if endogenous xanthoxin arises from a C₁₅ or C₄₀ compound (Adapted from Burden and Taylor, 1976).

1974b).

Similar retention of hydrogens from MVA has been observed with carotenoid biosynthesis. This similarity suggests that the same enzymes involved in carotenoid biosynthesis are also used in ABA biosynthesis, or that different enzymes (in different compartments?) operate with the same mechanism.

An *in vitro* plastid system from avocado fruit that synthesized ABA from MVL has been described (Milborrow, 1974a), although the amount of radioactivity recovered in ABA was very low. Recently, Hartung et al. (1981) presented data indicating that no biosynthesis of ABA occurred when isolated spinach chloroplasts were fed MVA. Incorporation into ABA was detected, however, when protoplasts or cytoplasmic fractions were incubated with MVA.

1.5.2. The Indirect Pathway. Once ABA had been identified, similarities between its structure and many xanthophylls, e.g. lutein, antheraxanthin, and violanxanthin, were noted (Figure 1.4). Taylor and Smith (1967) discovered that a mixture of carotenoids irradiated *in vitro* were degraded to an inhibitor with physiological properties similar to ABA. The greatest amount of inhibitor was obtained when violaxanthin was degraded. Later work showed that lolio- lide, butenone, and an isomeric mixture of xanthoxins (Figure 1.5) were produced when violaxanthin was photooxidized, and that 2-*cis*,4-*trans*-xanthoxin was the active ingredient causing growth inhibition in this mixture (Taylor and Burden, 1970; Burden and Taylor, 1970). Soon after this, Firn and



	R ₁	R ₂
β-carotene	b	b
violaxanthin	c	c
antheraxanthin	e	c
lutein	d	e

Figure 1.4. Some xanthophylls with terminal ring structures similar to abscisic acid.

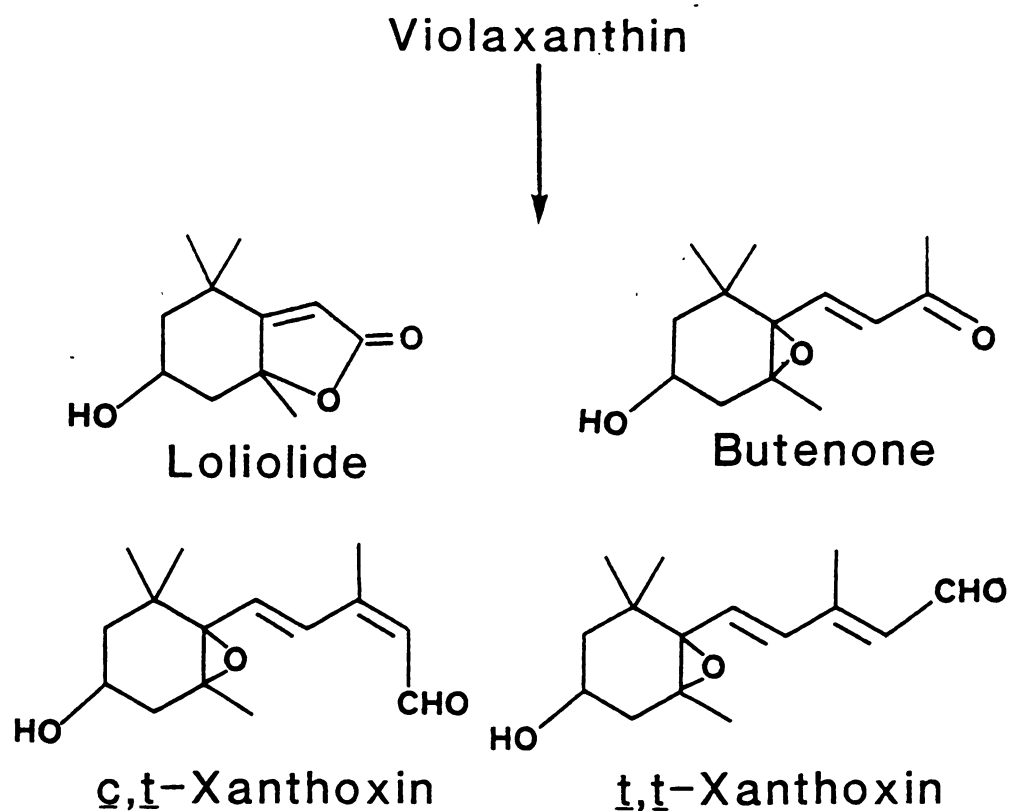


Figure 1.5. Compounds produced when violaxanthin is photo-oxidized.

These four compounds are also produced when violaxanthin is treated with zinc permanganate or soybean lipoxygenase. The *trans,trans* isomer is usually present in 2 X greater amount than the *cis,trans* isomer.

Friend (1972) showed that treatment of violaxanthin with soybean lipoxygenase could produce butenone and xanthoxin in yields similar to those when violaxanthin was photo-oxidized, indicating that light was not needed for xanthoxin production. In all instances more 2-*trans*,4-*trans*-xanthoxin was produced than the 2-*cis*,4-*trans* isomer. Interestingly, the pheromone trisporic acid, which regulates sexual reproduction in the Mucorales, has been shown to be a breakdown product of β -carotene (Figure 1.6; Gooday, 1974). However, it should be noted that endogenous xanthoxin has never been shown to be derived from violaxanthin *in vivo*. Furthermore, xanthoxin could also be an intermediate in the direct pathway.

Studies on xanthoxin indicate that 2-*cis*,4-*trans*-xanthoxin is more active in bioassays than 2-*trans*,4-*trans*-xanthoxin (Taylor and Burden, 1972). Only 2-*cis*,4-*trans*-xanthoxin was converted to ABA and PA when fed to tomato and dwarf bean plants (Taylor and Burden, 1973). When 2-*cis*,4-*trans*-xanthoxin was applied through the transpiration stream to leaves of several plants, stomata closed as fast as when ABA was used (Raschke *et al.*, 1975). However, when applied to isolated epidermal strips it was ineffective. Raschke *et al.* (1975) suggested that xanthoxin was converted to ABA during its passage to the epidermis when intact leaves were used. However, if xanthoxin was converted to ABA it should not act as fast as it did.

An attempt to differentiate between the indirect and direct pathways was performed by D.R. Robinson (cited in

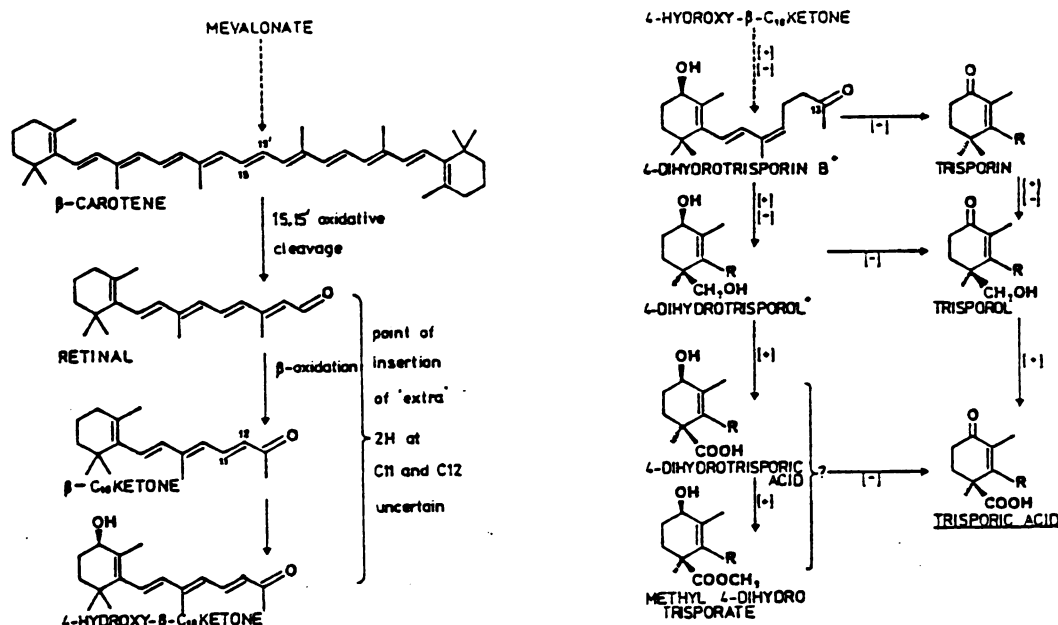


Figure 1.6. Biosynthetic scheme for trisporic acid in the fungal order Mucorales.

On the left are shown reactions common to both mating types. The compounds shown on the right are formed by the respective strains ([+] or [-]). Compounds marked with an asterisk are hypothetical and have not been isolated (From Gooday et al., 1978).

Milborrow, 1983). The first colorless carotenoid, phytoene (labeled with ^{14}C), was fed to avocado fruit slices along with ^3H -MVA. When the ABA isolated from this tissue was analyzed, only ^3H was found in ABA, yet both ^3H and ^{14}C were detected in carotenoids. This work is inconclusive because phytoene would have to penetrate to chloroplasts and then be further metabolized to a xanthophyll. It is not known whether this occurred, because a detailed account of this work has never been published.

1.5.3. Mutants Deficient in Abscissic Acid. Several mutants of higher plants have been found which appear to have an ABA minus phenotype. The recognition of an ABA deficient phenotype is based on excessive wilting or vivipary (premature germination). These mutants can be divided into two classes, those that are green and contain carotenoids, and those that are albino. It should be noted that there are no known mutants which produce no ABA at all. Perhaps this genotype is lethal.

Green ABA-deficient mutants are found in potato (Quarrie, 1982), pea (Wang *et al.*, 1984), tomato (Tal and Nevo, 1973), and *Arabidopsis* (Koornneef *et al.*, 1982). Of these, the only well documented mutants are the wilty mutants of tomato, *flacca* (*flc*), *sitiens* (*sit*), and *notabilis* (*not*). The tomato mutants are recessive and are located at three separate loci (Stubbe, 1957; Stubbe, 1958; Stubbe, 1959). ABA levels in the three mutants range from 12-15% of wild type for *sit*, 17-26% for *flc*, and 31-49% for *not* (Tal and Nevo,

1973; Neill and Horgan, 1985). Since the decreased ABA contents are not due to increased catabolism (Nevo and Tal, 1973), the lesions must be in enzymes involved in ABA biosynthesis.

Normal water stress induced accumulation of ABA does not occur in *droopy* (potato, Quarrie, 1982), *sit* and *flc* (tomato, Neill and Horgan, 1985) and *wilty* (pea, Wang et al., 1984). It is possible that these mutants are already stressed and have reached their maximum accumulation of ABA, or these mutants may not be able to accumulate stress-induced ABA. If the latter case is true, the implication is that in higher plants two pathways exist, one operating in turgid and the other operating in water-stressed leaves.

Examples of mutants in the second class are the maize viviparous mutants (viviparous 2, 5, and 9 [*vp2*, *vp5*, *vp9*], pink scutellum [*ps=vp9*], white seedling [*w3*], and yellow [*y9*]). All maize viviparous mutants are characterized by pale yellow endosperms and white or almost white seedlings (Robertson, 1975). The primary lesion in these mutants is defective carotenoid biosynthesis (Fong et al., 1983a). All carotenoid deficient mutants of maize have reduced levels of ABA in the embryos, from 7-70% of wild type (Brenner et al., 1977; Smith et al., 1978). It is possible that some of the ABA has a maternal origin. No ABA was detected in seedlings and roots of *w3*, *vp5*, and *vp7* (Moore and Smith, 1985), although the method used to detect ABA was rather insensitive.

Although not conclusive, the biochemistry of the viviparous maize mutants suggests the involvement of the indirect pathway. However, it is also possible that the decreased ABA levels in these mutants could simply be a secondary effect of carotenoid deficiency. If the indirect pathway is indeed operating, then the green wilted mutants represent blocks in the pathway after xanthophylls, since Nevo and Tal (1973) showed that *f1c* had slightly higher levels of carotenoids than the wild type 'Rheinlands Ruhm'.

1.6. CATABOLISM OF ABSCISIC ACID

While nothing is known about the intermediates in the ABA biosynthetic pathway in higher plants, much more is known about how ABA is degraded. ABA is rapidly catabolized in plants by either conjugation to water soluble catabolites or by oxidation to more polar compounds (Figure 1.7). This subject has recently been reviewed (Loveys and Milborrow, 1984). This section will deal with catabolites that have been fully characterized. See Loveys and Milborrow (1984) or Walton (1980) for a discussion of other less rigorously identified catabolites.

A major pathway in higher plants involves the hydroxylation of ABA at the 8'-geminal methyl group to give the unstable intermediate HM-ABA, which rearranges to form PA, and the subsequent reduction to DPA. This pathway has been shown to operate in several plants, including beans

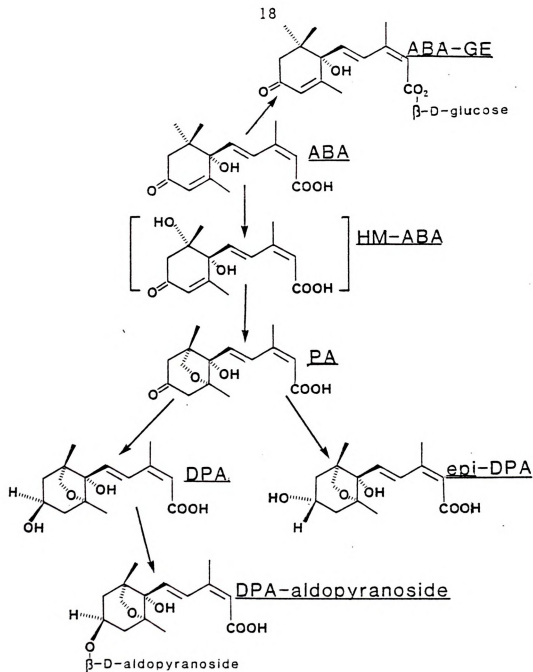


Figure 1.7. Catabolites of abscisic acid.

Only well characterized compounds are shown. PA-GE has also been demonstrated; its structure is similar to ABA-GE except with PA as the aglycone.

(Harrison and Walton, 1975), ash seeds (Sondheimer *et al.*, 1974), and castor bean (Zeevaart, 1977). In spite of many attempts to isolate it, 8'-OH-ABA has only been reported once (Milborrow, 1969). Its presence as an intermediate in the conversion of ABA to PA in a cell-free system was inferred from a product which was acetylated (Gillard and Walton, 1976). The epimer of DPA has also been reported (Zeevaart and Milborrow, 1976).

In addition to catabolism to PA and DPA, conjugation of ABA also occurs. ABA-GE appears to be widely distributed in plants, but has been unequivocally identified only a few times (Hirai *et al.*, 1978; Boyer and Zeevaart, 1982b). Other conjugates of PA (PA-GE; Boyer and Zeevaart, 1982a) and DPA (DPA aldopyranoside; Setter *et al.*, 1981) have been reported.

Many studies dealing with the catabolism of ABA have involved the use of radioactive ($\pm$)-ABA. Determination of whether the catabolites are natural or not is difficult unless the stereochemistry of the catabolite is reported. When ^{14}C -($\pm$)-ABA was fed to plants, only the (+) enantiomer was converted to PA and DPA, whereas hydrolysis of the ABA-GE gave predominantly (-)-ABA (Milborrow, 1978; Zeevaart and Milborrow, 1976). Another catabolite, 7'-hydroxy (-)-ABA has recently been reported (Boyer and Zeevaart, 1986). Thus, all studies dealing with ABA catabolism should state the stereochemistry of ABA fed and of the catabolites isolated.

1.7. PHYSIOLOGY

Numerous theories on the involvement of ABA as a plant growth regulator have been proposed, including, but not limited to, root gravitropism (Wilkins, 1978, 1984), seed and bud dormancy (Addicott and Lyon, 1969; Wareing, 1978), and stomatal closure (Raschke, 1975). However, Wareing (1978) and Walton (1980) have concluded that too little is known about the events occurring during any physiological process to state unequivocally that ABA is involved in its control.

Root gravitropism is thought to involve the asymmetric redistribution of a growth inhibiting substance. Although the identity of the growth inhibiting substance is unknown, ABA has been proposed to be the agent causing inhibition. However, recent evidence (Moore and Smith, 1984, 1985) argues against a role for ABA in gravitropism. Using carotenoid deficient mutants of maize (which have non-detectable levels of ABA), Moore and Smith (1985) demonstrated that gravitropic curvature still occurred. In addition, Moore and Smith (1984) showed that curvature also was present when ABA biosynthesis was inhibited using carotenoid biosynthetic inhibitors.

The best evidence, although still correlative, for the involvement of ABA in a physiological process has been obtained with experiments on water stress. Detached or attached leaves will accumulate ABA (usually 10 to 40 times that found in a turgid leaf) upon the imposition of a water

stress. When ABA is given to epidermal strips or fed to intact leaves through the transpiration stream, stomata close rapidly (Mittelheuser and Van Steveninck, 1969; Tucker and Mansfield, 1971). The stomatal response is specific for the naturally occurring (+) enantiomer and is reversible (Cummins and Sondheimer, 1973; Cummins *et al.*, 1971).

Thus, a role for ABA in controlling water deficit through regulating stomatal closure seems reasonable. ABA is clearly made during water stress, it will then travel to the guard cells *via* the transpiration stream and cause stomatal closure. However, stomata have been shown to close before a measurable increase in bulk leaf ABA occurs (Henson, 1981b). The results of Henson (1981a, 1981b) could be explained if ABA were compartmentalized as a large inactive pool and a small active one. Evidence exists for the compartmentation of ABA in leaves (Cummins and Sondheimer, 1973; Raschke *et al.*, 1976; Raschke and Zeevaart, 1976). In addition, following loss of turgor a sufficient amount of ABA moves into the apoplast to cause stomatal closure (Cornish and Zeevaart, 1985).

The effect of ABA on stomata is rather rapid compared to its effect on protein synthesis, as in the control of α -amylase production in barley aleurone layers (Higgins *et al.*, 1982; Mozer, 1980). In this tissue, ABA inhibits the gibberellin induced synthesis of α -amylase, which is needed for the hydrolysis of endosperm starch during germination

(Ho and Varner, 1976). This inhibition is not due to competition for a common site of action, because high concentrations of gibberellin do not overcome the effect (Ho and Varner, 1976). The effect of ABA can be prevented by cordycepin, indicating that continued protein or RNA synthesis is needed for the response to occur (Ho et al., 1985). ABA applied alone also appears to induce the synthesis of several proteins in barley aleurone layers (Higgins et al., 1982; Mozer, 1980). The function of these proteins is unknown.

ABA has been proposed to be an inhibitor of vivipary (precocious germination; see section 1.5.3) and a promoter of embryo maturation. Vivipary occurs normally in mangroves (*Rhizophora mangle*) and it has been shown that embryos of this species are insensitive to ABA (Sussex, 1975). The *vp* mutants of corn (see section 1.5.3) also have decreased levels of ABA. Addition of fluridone, a carotenoid biosynthetic inhibitor, induces vivipary in developing maize kernels (Fong et al., 1983a, Fong et al., 1983b).

In several other studies, it has been shown that exogenous ABA application to excised dicot embryos will inhibit both germination and the appearance of germination-specific proteins, and promotes embryo-specific proteins and mRNAs (Ihle and Dure, 1970; Dure et al., 1983; Crouch and Sussex, 1981; Crouch et al. 1984). Bray and Beachy (1985) showed that the B-subunit of B-conglycinin, a storage protein, accumulated when excised soybean cotyledons were treated with ABA. Addition of fluridone decreased B-subunit product-

ion. The accumulation of wheat germ agglutinin (a lectin found in wheat embryos, shoots, and roots) is also induced by ABA (Raikhel et al., 1986; Quatrano et al., 1983). Application of fluridone to wheat leaves decreases the amount of lectin present (Raikhel et al., 1986).

ABA appears to be necessary for other processes. An example is the biochemical, physiological and morphological changes that occur in the tomato mutants *f1c* and *sit* (see section 1.5.3). These mutants appear shorter, produce adventitious roots on stems, and the leaves show epinasty compared with wild type (Tal et al., 1979). In addition, levels of auxinlike substances and ethylene are higher than normal (Tal et al., 1979). Even though ABA is present (although in lower amounts than wild type) application of ABA will cause reversion to the wild phenotype (Imber and Tal, 1970; Bradford, 1983).

Thus, there appears to be several different roles for ABA in higher plants. One is a rapid response associated with stomatal closure. Here the site of action of ABA appears to be the plasmalemma (Hartung, 1983) where it appears to inhibit proton extrusion (Shimazaki, et al., 1986). Another role is associated with the regulation of protein accumulation, such as that found in barley aleurone layers with α -amylase or in wheat with wheat germ agglutinin. However, the effects of ABA on ion transport and gene expression cannot explain the phenotypes seen in the ABA deficient mutants *f1c* and *sit*.

1.8. STATEMENT OF PURPOSE

Earlier it was mentioned that ABA levels increased upon the imposition of water stress, and that ABA appears to ameliorate the stress by closing stomata. The increase in ABA is apparently regulated by loss of turgor (Pierce and Raschke, 1980). Environmental conditions other than water stress have also been reported to cause ABA accumulation, although to a lesser degree. Examples of this are low relative humidity (Wright, 1972), flooding of soil (Wright, 1972), salinity or osmotic stress of roots (Mizrahi et al., 1970), mineral deprivation (Mizrahi and Richmond, 1972), and chilling (Raschke et al., 1976). While the tissue did not appear to be visibly stressed in any of these cases, each of these conditions can be expected to change the water status or reduce translocation. Thus, the accumulation of ABA appears to be a situation where a physical phenomenon (loss of turgor) controls a biochemical pathway. I was interested in understanding how this occurs. Unfortunately, there is very little known about the intermediates of the ABA biosynthetic pathway. Therefore, I wanted to determine how ABA is made in higher plants. Specifically, I wanted to:

1. Determine which pathway, the indirect or direct one, operates during water stress;
2. Determine the role of xanthoxin in the pathway, and the intermediates between it and ABA and;

3. Confirm that loss of turgor regulates the accumulation of stress-induced ABA.

1.10. LITERATURE CITED

- Addicott, F.T., Lyon, J.L. (1969) Physiology of abscisic acid and related substances. *Ann. Rev. Plant Physiol.* 20:139-164.
- Addicott, F.T., Lyon, J.L., Ohkuma, K., Thiessen, W.E., Carns, H.R., Smith, O.E., Cornforth, J.W., Milborrow, B.V., Ryback, G., Wareing, P.F. (1968) Absciscic acid: a new name for abscisin II (dormin). *Science* 159:1493.
- Assante, G., Merlini, L., Nasini, G. (1977) (+)-Absciscic acid, a metabolite of the fungus *Cercospora rosicola*. *Experientia* 33:1556.
- Bearder, J.R. (1980) Plant hormones and other growth substances: their background, structures, and occurrence. In: *Hormonal regulation of development I. Molecular aspects of plant hormones*, pp. 9-112, MacMillan, J. ed., New York: Springer-Verlag.
- Boyer, G.L., Milborrow, B.V., Wareing, P.F., Zeevaart, J.A.D. (1986) The nomenclature of abscisic acid and its metabolites. In: *Plant growth substances 1985*, pp. Bopp, M., ed., New York: Academic Press.
- Boyer, G.L., Zeevaart, J.A.D. (1982a) Metabolism of abscisic acid in *Xanthium strumarium*. *Plant Physiol. Suppl.* 69:77.
- Boyer, G.L., Zeevaart, J.A.D. (1982b) Isolation and quantitation of β -D-glucopyranosyl abscisate from leaves of *Xanthium* and spinach. *Plant Physiol.* 70:227-231.
- Boyer, G.L., Zeevaart, J.A.D. (1986) 7'-hydroxy (-)-R-abscisic acid: a metabolite of feeding (-)-R-abscisic acid to *Xanthium strumarium*. *Phytochemistry* 25:1103-1105.
- Bradford, K.J. (1983) Water relations and growth of the *flacca* tomato mutant in relation to abscisic acid. *Plant Physiol.* 72:251-255.
- Bray, E.A., Beachy, R.N. (1985) Regulation by ABA of β -conglycinin expression in cultured developing soybean cotyledons. *Plant Physiol.* 79:746-750.

- Brenner, M.L., Burr, B., Burr, F. (1977) Correlation of genetic vivipary in corn with abscisic acid concentration. *Plant Physiol. Suppl.* 63:36.
- Burden, R.S., Taylor, H.F. (1970) The structure and chemical transformations of xanthoxin. *Tetrahedron Lett.* 47:4071-4074.
- Burden, R.S., Taylor, H.F. (1976) Xanthoxin and abscisic acid. *Pure Appl. Chem.* 47:203-209.
- Cornforth, J.W., Milborrow, B.V., Ryback, G. (1966a) Identification and estimation of (+)-abscisin II ('dormin') in plant extracts by spectropolarimetry. *Nature* 210: 627-628.
- Cornforth, J.W., Milborrow, B.V., Ryback, G., Rothwell, K., Wain, R.L. (1966b) Identification of the yellow lupin growth inhibitor as (+)-abscisin II ((+)-dormin). *Nature* 211:742-743.
- Cornforth, J.W., Milborrow, B.V., Ryback, G., Wareing, P.F. (1965) Identity of sycamore 'dormin' with abscisin II. *Nature* 205:1269-1270.
- Cornish, K., Zeevaart, J.A.D. (1985) Movement of abscisic acid into the apoplast in response to water stress in *Xanthium strumarium* L.. *Plant Physiol.* 78:623-626.
- Crouch, M.L., Sussex, I.M. (1981) Development and storage protein synthesis in *Brassica napus* L. *in vivo* and *in vitro*. *Planta* 153:64-74.
- Crouch, M.L., Tenbarger, K., Simon, A., Finkelstein, R., Scofield, S., Solberg, L. (1985) Storage protein mRNA levels can be regulated by abscisic acid in *Brassica* embryos. In: *Molecular form and function of the plant genome*, pp. 555-566, van Vloten-Doting, L., Groot, G.S.P., Hall, T.C., eds. New York: Plenum.
- Cummins, W.R., Kende, H., Raschke, K. (1971) Specificity and reversibility of the rapid stomatal response to abscisic acid. *Planta* 99:347-351.
- Cummins, W.R., Sondheimer, E. (1973) Activity of the asymmetric isomers of abscisic acid in a rapid bioassay. *Planta* 111:365-369.
- Dörffling, K., Peterson, W. (1984) Abscisic acid in phytopathogenic fungi of the genera *Botrytis*, *Ceratocystis*, *Fusarium*, and *Rhizoctonia*. *Z. Naturforsch.* 39c:683-684.
- Dörffling, K., Tietz, D. (1983) Methods for the detection and estimation of abscisic acid and related compounds.

- In: Absciscic acid, pp. 23-78, Addicott, F.T., ed. New York: Praeger Press.
- Dure, L., Galu, G., Chlan, C., Pyle, J. (1983) Developmentally regulated gene sets in cotton embryogenesis. In: Plant molecular biology, pp. 331-342, Goldberg, R., ed. New York: Liss.
- Firn, R.D., Friend, J. (1972) Enzymatic production of the plant growth inhibitor, xanthoxin. *Planta* 103:263-266.
- Fong, F., Koehler, D.E., Smith, J.D. (1983a) Fluridone induction of vivipary during maize seed development. In: Third international symposium on pre-harvest sprouting in cereals, pp. 186-196, Krueger, J.E., La Berge, D.E., eds. Boulder: Westview Press.
- Fong, F., Smith, J.D., Koehler, D.E. (1983b) Early events in maize seed development. 1-methyl-3-phenyl-5-(3-[trifluoromethyl]phenyl)-4-(1H)-pyridinone induction of vivipary. *Plant Physiol.* 73:899-901.
- Gillard, D.F., Walton, D.C. (1976) Absciscic acid metabolism by a cell-free preparation from *Echinocystis lobata* liquid endosperm. *Plant Physiol.* 58:790-795.
- Gooday, G.W. (1974) Fungal sex hormones. *Ann. Rev. Biochem.* 43:35-49
- Gooday, G.W., Jones, B.E., Leith, W.H. (1978) Trisporic acid and the differentiation in the Mucorales. In: Regulation of secondary product and plant hormone metabolism, pp. 221-229, Luckner, M., Schreiber, K., eds. New York: Pergamon Press.
- Harrison, M.A., Walton, D.C. (1975) Absciscic acid metabolism in water-stressed bean leaves. *Plant Physiol.* 56: 250-254.
- Hartung, W. (1983) The site of action of absciscic acid at the guard cell plasmalemma of *Valerianella locusta*. *Plant, Cell, and Environ.* 6:427-428.
- Hartung, W., Heilmann, B., Gimmler, H. (1981) Do chloroplasts play a role in absciscic acid synthesis? *Plant Sci. Lett.* 22:235-242.
- Henson, I.E. (1981a) Absciscic acid and after-effects of water stress in pearl millet (*Pennisetum americanum* (L.) Leeke). *Plant Sci. Lett.* 21:129-135.
- Henson, I.E. (1981b) Changes in absciscic acid content during stomatal closure in pearl millet (*Pennisetum americanum* (L.) Leeke). *Plant Sci. Lett.* 21:129-135.

- Higgins, T.J.V., Jacobsen, J.V., Zwar, J.A. (1982) Gibberellic acid and abscisic acid modulate protein synthesis and mRNA levels in barley aleurone layers. *Plant Mol. Biol.* 1:191-215.
- Hirai, N., Fukui, H., Koshimizu, K. (1978) A novel abscisic acid metabolite from seeds of *Robina pseudacacia*. *Phytochemistry* 17:1625-1627
- Ho, T.H.D., Varner, J.E. (1976) Response of barley aleurone layers to abscisic acid. *Plant Physiol.* 57:175-178.
- Ho, T.H.D., Nolan, R.C., Uknes, S.J. (1985) On the mode of action of abscisic acid in barley aleurone layers. In: Current topics in plant biochemistry and physiology 1985, pp. 118-125, vol. 4, Randall, D.D., Blevins, D.G., Larson, R.L., eds. Columbia, MO: University of Missouri.
- Horgan, R., Neill, S.J., Walton, D.C., Griffin, D. (1983) Biosynthesis of abscisic acid. *Biochem. Soc. Trans.* 11:553-557.
- Ichimura, M., Oritani, T., Yamashita, K. (1983) The metabolism of (2Z, 4E)- α -ionylideneacetic acid in *Cercospora cruenta*, a fungus producing (+)-abscisic acid. *Agr. Biol. Chem.* 47:1895-1900.
- Ihle, J.N., Dure, L. (1970) Hormonal regulation of translation requiring RNA synthesis. *Biochem. Biophys. Res. Comm.* 38:995-1001.
- Imber, D., Tal, M. (1970) Phenotypic reversion of *flacca*, a wilted mutant of tomato, by abscisic acid. *Science*: 169:592-593.
- Koornneef, M., Jorna, M.L., Brinkhorst-van der Swan, D.L.C., Karssen, C.M. (1982) The isolation of abscisic acid (ABA) deficient mutants by selection of induced revertants in non-germinating gibberellin sensitive lines of *Arabidopsis thaliana* (L.) Heynh. *Theor. Appl. Genet.* 61:385-393.
- Koshimizu, K., Fukui, H., Mitsui, T., Ogawa, Y. (1966) Identity of lupin inhibitor with abscisin II and its biological activity on growth of rice seedlings. *Agr. Biol. Chem.* 30:941-943.
- Loomis, W.D., Croteau, R. (1980) Biochemistry of terpenoids. In: The biochemistry of plants, a comprehensive treatise, pp. 363-418, vol 4, Stumpf, P.K., Conn, E.E., eds. New York: Academic Press.
- Loveys, B.R., Milborrow, B.V. (1984) Metabolism of abscisic

- acid. In: The biosynthesis and metabolism of plant hormones, pp. 71-104, Crozier, A., Hillman, J.R., eds. London: Cambridge University Press.
- Marumo, S., Katayama, M., Komori, E., Ozaki, Y., Natsume, M., Kondo, S. (1982) Microbial production of abscisic acid by *Botrytis cinera*. Agr. Biol. Chem. 46:1967-1968.
- Milborrow, B.V. (1969) Identification of 'metabolite C' from abscisic acid and a new structure for phaseic acid. Chem. Commun., pp. 966-967.
- Milborrow, B.V. (1972) Stereochemical aspects of the formation of double bonds in abscisic acid. Biochem. J. 128:1135-1146.
- Milborrow, B.V. (1974a) Biosynthesis of abscisic acid by a cell-free system. Phytochemistry. 13:131-136.
- Milborrow, B.V. (1974b) The chemistry and physiology of abscisic acid. Ann. Rev. Plant Physiol. 25:259-307.
- Milborrow, B.V. (1978) Abscisic acid. In: Phytohormones and related compounds-a comprehensive treatise, pp. 295-397, vol 1, Letham D.S., Goodwin, P.B., Higgins, T.J. V., eds. Amsterdam: Elsevier.
- Milborrow, B.V. (1983) Pathways to and from abscisic acid. In: Abscisic acid, pp.79-111, Addicott, F.T., ed. New York: Praeger Press.
- Milborrow, B.V., Robinson, D.R. (1973) Factors affecting the biosynthesis of abscisic acid. J. Exp. Bot. 24: 537-548.
- Mittelheuser, C.J., Van Steveninck, R.F.M. (1969) Stomatal closure and the inhibition of transpiration induced by (RS)-abscisic acid. Nature 221:281-282.
- Mizrahi, Y., Blumenfeld, A., Richmond, A.E. (1970) Abscisic acid and transpiration in relation to osmotic root stress. Plant Physiol. 46:169-171.
- Mizrahi, Y., Richmond, A.E. (1972) Abscisic acid in relation to mineral deprivation. Plant Physiol. 50:667-670.
- Moore, R., Smith, J.D. (1984) Growth, graviresponsiveness, and abscisic acid content of *Zea* seedlings treated with fluridone. Planta 162:342-344.
- Moore, R., Smith, J.D. (1985) Graviresponsiveness and abscisic acid content of roots of carotenoid-deficient mutants of *Zea mays* L. Planta 164:126-128.

- Mozer, T.J. (1980) Control of protein synthesis in barley aleurone layers by the plant hormones gibberellic acid and abscisic acid. *Cell* 20:447-455.
- Neill, S.J., Horgan, R. (1983) Incorporation of α -ionylidene ethanol and α -ionylidene acetic acid into abscisic acid by *Cercospora rosicola*. *Phytochemistry* 22:2469-2472.
- Neill, S.J., Horgan, R. (1985) Abscisic acid production and water relations in wilted tomato mutants subjected to water deficiency. *J. Exp. Bot.* 36:1222-1231.
- Neill, S.J., Horgan, R., Lee, T.S., Walton, D.C. (1981) 3-methyl-5-(4-oxo-2',6',6'-trimethylcyclohex-2'-enyl)-2,4-pentadienoic acid, a putative precursor of abscisic acid from *Cercospora rosicola*. *FEBS Lett.* 128:30-32.
- Neill, S.J., Horgan, R., Walton, D.C., Lee, T.S. (1982) The biosynthesis of abscisic acid in *Cercospora rosicola*. *Phytochemistry* 21:61-65.
- Nevo, Y., Tal, M. (1973) The metabolism of abscisic acid in *Flacca*, a wilted mutant of tomato. *Biochem. Genet.* 10:79-90.
- Noddle, R.C., Robinson, D.R. (1969) Biosynthesis of abscisic acid: incorporation of radioactivity from [2-¹⁴C]mevalonic acid by intact fruit. *Biochem. J.* 112:547-548.
- Ohkuma, K., Addicott, F.T., Smith, O.E., Thiessen, W.E. (1965) The structure of abscisin II. *Tetrahedron Lett.* 29:2529-2535.
- Ohkuma, K., Lyon, J.L., Addicott, F.T., Smith, O.E. (1963) Abscisin II, an abscission-accelerating substance from young cotton fruit. *Science* 142:1592-1593.
- Ortani, T., Ichimura, M., Yamashita, K. (1982) The metabolism of (2Z,4E)- α -ionylideneacetic acid in *Cercospora cruenta*, a fungus producing (+) abscisic acid. *Agr. Biol. Chem.* 46:1959-1962.
- Pierce, M., Raschke, K. (1980) Correlation between loss of turgor and accumulation of abscisic acid in detached leaves. *Planta* 148:174-182.
- Porter, N.G., Van Steveninck, R.F.M. (1966) An abscission-promoting factor in *Lupinus luteus* L. *Life Sci.* 5:2301-2308.
- Quarrie, S.A. (1982) Droopy: a wilted mutant of potato deficient in abscisic acid. *Plant Cell Environ.* 5:23-26.

- Raikhel, N.V., Palevitz, B.A., Haigler, C.H. (1986) Absciscic acid control of lectin accumulation in wheat seedlings and callus cultures. *Plant Physiol.* 80:167-171.
- Raschke, K. (1975) Stomatal action. *Ann. Rev. Plant Physiol.* 26:309-340.
- Raschke, K., Zeevaart, J.A.D. (1976) Absciscic acid content, transpiration, and stomatal conductance as related to leaf age in plants of *Xanthium strumarium* L.. *Plant Physiol.* 58:169-174.
- Raschke, K., Firn, R.D., Pierce, M. (1975) Stomatal closure in response to xanthoxin and absciscic acid. *Planta* 125:149-160.
- Raschke, K., Pierce, M., Popiela, C.C. (1976) Absciscic acid content and stomatal sensitivity to CO₂ in leaves of *Xanthium strumarium* L. after pretreatments in warm and cold growth chambers. *Plant Physiol.* 57:115-121.
- Robinson, D.R., Ryback, G. (1969) Incorporation of tritium from [(4)-4-³H] mevalonate into absciscic acid. *Biochem. J.* 113:895-897.
- Robinson, P.M., Wareing, P.F. (1964). Chemical nature and biological properties of the inhibitor varying with photoperiod in sycamore (*Acer pseudoplatanus*). *Physiol. Plant.* 17:314-323.
- Robertson, D.S. (1975) Survey of the albino and white-endosperm mutants of maize. *J. Heredity* 66:67-74.
- Rothwell, K., Wain, R.L. (1964) Studies on a growth inhibitor in yellow lupin (*Lupinus luteus* L.). In: *Régulateurs de la Croissance Végétale*, pp. 363-375, Nitsch, J.P., ed. Paris: Cent. Nat. Rech. Sci..
- Setter, T.L., Brenner, M.L., Brun, W.A., Krick, T.P. (1981) Identification of a dihydrophaseic acid aldopyranoside from soybean tissue. *Plant Physiol.* 68:93-95.
- Shimazaki, K., Iino, M., Zeiger, E. (1986) Blue light-dependent extrusion by guard-cell protoplasts of *Vicia faba*. *Nature* 319:324-326.
- Smith, J.D., McDaniel, S., Lively, S. (1978) Regulation of embryo growth by absciscic acid *in vitro*. *Maize Genet. Coop. News Lett.* 52:107-108.
- Sondheimer, E., Galson, E.C., Chang, P.P., Walton, D.C. (1971) Asymmetry, its importance to the action and metabolism of absciscic acid. *Science* 174:829-831.

- Sondheimer, E., Galson, E.C., Tinelli, E., Walton, D.
(1974) The metabolism of hormones during dormancy. IV.
The metabolism of (S)-2-¹⁴C-abscisic acid in ash seed.
Plant Physiol. 54:803-808.
- Stubbe, H. (1957) Mutanten der Kulturtomate, *Lycopersicon
esculentum* Miller I. Kulturpflanze 5:190-220.
- Stubbe, H. (1958) Mutanten der Kulturtomate, *Lycopersicon
esculentum* Miller II. Kulturpflanze 6:89-115.
- Stubbe, H. (1959) Mutanten der Kulturtomate, *Lycopersicon
esculentum* Miller III. Kulturpflanze 7:82-112.
- Sussex, I. (1975) Growth and metabolism of the embryo and
attached seedling of the viviparous mangrove *Rhizophora
mangle*. Am. J. Bot. 62:948-953.
- Tal, M., Imber, D., Erez, A., Epstein, E. (1979) Abnormal
stomatal behavior and hormonal imbalance in *flacca*, a
wilty mutant of tomato. V. Effect of abscisic acid on
indoleacetic acid metabolism and ethylene evolution.
Plant Physiol. 63:1044-1048.
- Tal, M., Nevo, Y. (1973) Abnormal stomatal behavior and root
resistance, and hormonal imbalance in three wilted mutants
of tomato. Biochem. Genet. 8:291-300.
- Tamura, S., Nagao, M. (1970) Synthesis and biological
activities of new plant growth inhibitors structurally
related to abscisic acid. Agr. Biol. Chem. 34:1393-1401.
- Taylor, H.F., Burden, R.S. (1970) Identification of plant
growth inhibitors produced by photolysis of violaxan-
thin. Phytochemistry 9:2217-2223.
- Taylor, H.F., Burden, R.S. (1972) Xanthoxin, a recently
discovered plant growth inhibitor. Proc. Roy. Soc.
B 180:317-346.
- Taylor, H.F., Burden, R.S. (1973) Preparation and metabol-
ism of 2-¹⁴C-*cis,trans* xanthoxin. J. Exp. Bot. 24:873-
880.
- Taylor, H.F., and Smith, T.A. (1967) Production of plant
growth inhibitors from xanthophylls: A possible source
of dormin. Nature 215:1513-1514.
- Tucker, D.J., Mansfield, T.A. (1971) A simple bioassay for
detecting "antitranspirant" activity of naturally
occurring compounds such as abscisic acid. Planta 98:157-
163.
- Walton, D.C. (1980) Biochemistry and physiology of abscisic

- acid. Ann. Rev. Plant Physiol. 31:453-489.
- Wang, T.L., Donkin, M.E., Martin, E.S. (1984) The physiology of wilted pea: abscisic acid production under water stress. J. Exp. Bot. 35:1222-1232.
- Wareing, P.F. (1978) Absciscic acid as a natural growth regulator. Phil. Trans. Roy. Soc. Lond. B 284:483-498.
- Wareing, P.F., Eagles, C.F., Robinson, P.M. (1964) Natural inhibitors as dormancy agents. In: Régulateurs de la Croissance Végétale, pp. 377-386, Nitsch, J.P., ed. Paris: Cent. Nat. Rech. Sci..
- Wilkins, M.B. (1978) Gravity sensing guidance mechanisms in roots and shoots. Bot. Mag. Tokyo Spec. Issue 1:255-277.
- Wilkins, M.B. (1984) Gravitropism. In: Advanced plant physiology, pp.163-185, Wilkins, M.B., ed. London: Pitman.
- Wright, S.T.C. (1972) Physiological and biochemical responses to wilting and other stress conditions. In: Crop processes in controlled environments, pp. 349-361, Rees, A.R., Cockshull, K.E., Hand, D.W., Hurd, R.G., eds. New York: Academic Press.
- Zeevaart, J.A.D. (1974) Levels of (+)-abscisic acid and xanthoxin in spinach under different environmental conditions. Plant Physiol. 53:644-648.
- Zeevaart, J.A.D. (1977) Sites of abscisic acid synthesis and metabolism in *Ricinus communis* L. Plant Physiol. 57:788-791.
- Zeevaart, J.A.D. (1980) Changes in the level of abscisic acid and its metabolites in excised leaf blades of *Xanthium strumarium* during and after water stress. Plant Physiol. 66:672-678.
- Zeevaart, J.A.D., Milborrow, B.V. (1976) Metabolism of abscisic acid and the occurrence of *epi*-dihydrophaseic acid in *Phaseolus vulgaris*. Phytochemistry 15:493-500.

CHAPTER 2
INHIBITION OF ABSCISIC ACID ACCUMULATION IN WATER-
STRESSED CORN SEEDLINGS IN THE PRESENCE OF CAROTENOID
BIOSYNTHETIC INHIBITORS

2.1. INTRODUCTION

If ABA is synthesized via the indirect pathway, then inhibition of carotenogenesis with specific inhibitors should also prevent the accumulation of ABA. Pyridinone (such as fluridone, 1-methyl-3-phenyl-5-[3-(trifluoromethyl)phenyl]-4(1*H*)pyridonone) and pyridazinone (such as norflurazon, also known as SAN-9789, 4-chloro-5-(methylamino)-2(α,α,α -trifluoro-*m*-tolyl)-3-(2*H*)pyridazinone) herbicides block the desaturation of phytoene to phytofluene, the first two colorless carotenoids (Bartels and Watson, 1978).

These inhibitors also have a number of effects aside from inhibiting carotenoid synthesis. When plant tissue lacking carotenoids is grown in light, chlorophyll, chloroplast proteins, and plastid ribosomal RNA do not accumulate (Bartels and Watson, 1978; Quarrie and Lister, 1984). In addition, alterations in the fatty acid composition of galactolipids also occur (St. John, 1976). How these alterations in plant metabolism might affect ABA accumulation is unknown. These non-specific effects require the measurement of primary metabolites to determine if they might be indirectly affecting ABA accumulation.

The effect of these inhibitors on ABA accumulation was determined using seedlings of *Zea mays*. Sugar (soluble and insoluble) and organic acid levels were also measured. This was done to determine if the inhibitors had a non-specific effect on primary cell metabolism.

2.2. MATERIALS AND METHODS

2.2.1. Plant Material. Seeds of corn (*Zea mays*, hybrid MS WFg x Bear 38, Custom Farm Seed, Decatur, IL, gift of Dr. K. Poff) were imbibed overnight in solutions of norflurazon, fluridone or distilled water. In most experiments the inhibitor concentration was 10^{-4} M. They were then planted in vermiculite and watered with the appropriate solutions. Flats were kept in darkness, except for a daily 2 h period when red light was given to inhibit mesocotyl growth. After 9 to 10 d, seedlings were harvested by clipping just above soil level. For experiments involving measurement of ABA, seedlings were either stressed until they lost 13% of their fresh weight, followed by storage in a plastic bag for 8 h, or placed immediately in a plastic bag. For analysis of primary cellular metabolites leaves were frozen immediately in liquid nitrogen (organic acids or sugars), or extracted in methanol (carotenoids). All experiments were repeated at least once, with two replicates per treatment.

2.2.2. Absciscic Acid Analysis. After the experimental period, the tissue was frozen and lyophilized. Extraction and quantitation were performed exactly as described in Zeevaart (1980).

2.2.3. Organic Acid Analysis. The procedure used was basically that of Stumpf and Burris (1979). Approximately 1 g of tissue was frozen, lyophilized, and powdered. This powder was placed in a 50 ml Erlenmyer flask to which 15 ml 95% ethanol

was added. The ethanolic solution was boiled for 10 min. The extract was centrifuged and the supernatant was taken to dryness. The residue was redissolved in one ml of distilled water.

The extract was then subjected to ion exchange chromatography. Dowex 1 and 50 (Dow Chemical Co.) were both washed with acetone, methanol, and toluene to remove colored contaminants. Dowex 1 was converted to the formate form by washing with 1 N formic acid until the pH of the eluate was less than 2. The resin was back-washed with distilled water until the pH was greater than 5. Dowex 50 was used as provided by the manufacturer.

An aliquot (0.3-0.5 ml) of the aqueous residue was applied to a Dowex 50 column (H^+ , bed volume 0.3 ml) on top of a Dowex 1 column (formate, bed volume 0.1 ml). The Dowex 50 column was washed with 2 ml of distilled water, removed, and the Dowex 1 column similarly treated. The organic acids were eluted from the Dowex 1 column with 4 ml 2 N HCl and dried. They were then redissolved in 400 μ l distilled water, transferred to a small vial, and dried.

The samples were resuspended in 50 μ l pyridine to which 25 μ l BSA was added 10 min prior to analysis. GLC-FID analysis was performed with a 3% SE-30 (2 m, 80-120 mesh, gas-chrom Q, 30 ml/min He flow) temperature programmed from 80 C to 240 C at 5 C per min. Quantitation was performed by comparison with known amounts of malonic, succinic, malic, and citric acids.

2.2.4. Soluble and Insoluble Sugar Analysis. The procedure used was basically that of McCready et al. (1950). Lyophilized leaves were finely powdered and extracted four times with 25 ml of hot 80% ethanol to give the soluble sugar fraction. Distilled water (5 ml) was added to the residues, followed with, after cooling in an ice bath, 6.5 ml of 53% perchloric acid. The mixture was stirred for 20 min after which 20 ml of distilled water was added. The mixture was next centrifuged. This was repeated once and the supernatants combined to give the insoluble carbohydrate fraction. Both fractions were lyophilized to dryness and known amounts of distilled water were added.

Aliquots were analyzed by the phenol-sulfuric acid method (Ashwell, 1966). To 2 ml of the sugar solution was added 50 μ l 80% phenol followed by 5 ml of sulfuric acid. After 15 min, the absorbance was read at 488 nm. Quantitation was performed by construction of a standard curve with known amounts of sucrose. To take into account water lost during the biosynthesis of starch, the value obtained from the standard curve was multiplied by 0.9.

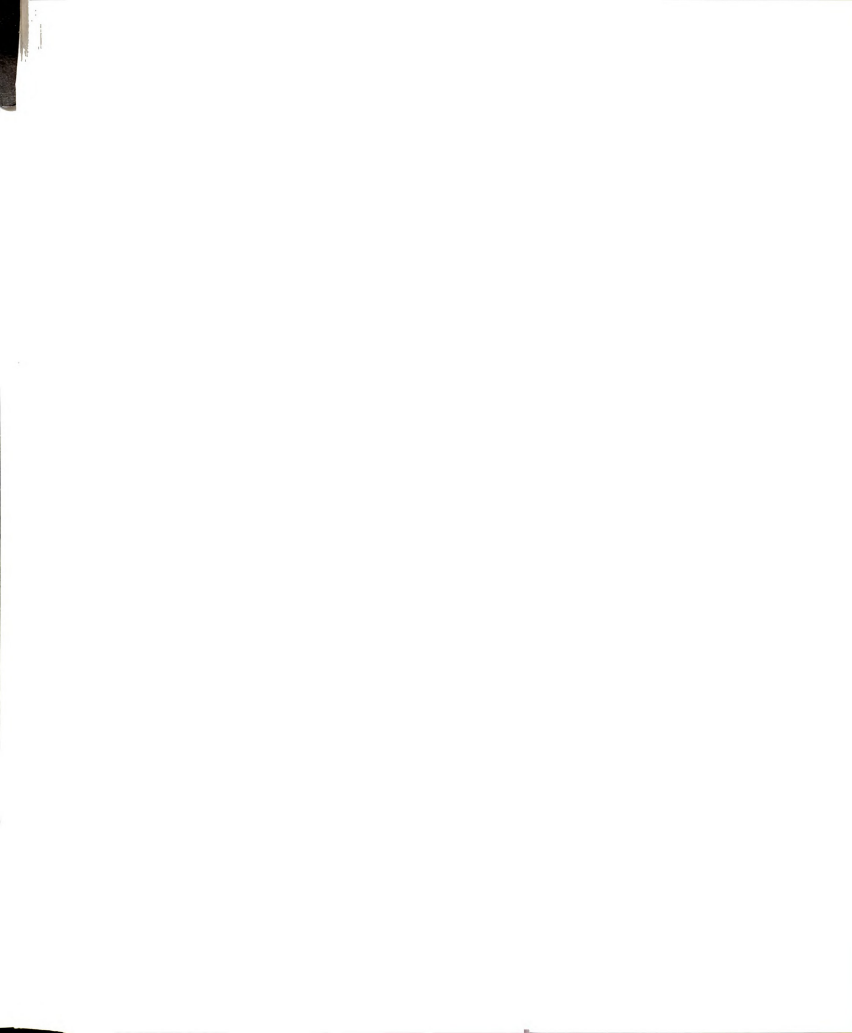
2.2.5. Carotenoid Analysis. The procedure used was that described in Davies (1976). After harvesting, leaves were immediately extracted in methanol. Following filtration, the filtrate was added to an equal volume of diethyl ether to which three volumes of aqueous 20% NaCl were added. The diethyl ether phase was washed three times with distilled water and dried over Na_2SO_4 . The ether was removed by

rotary evaporation and the residue dissolved in methanol to which a solution of 60% KOH in methanol was added to give a final concentration of 6% KOH in methanol. The solution was placed under nitrogen and stored in the dark at 4 C overnight. The saponified extract was added to an equal amount of diethyl ether to which three volumes of aqueous 20% NaCl was added. The ether phase (containing carotenoids) was washed four times with distilled water and the aqueous phase (containing degraded chlorophyll) was discarded.

If total carotenoids were to be determined, suitable aliquots were analyzed according to Davies (1976). If phytoene was to be measured, a small aliquot was subjected to silica gel TLC using hexane:acetone 9:1 as solvent. The zone containing phytoene (R_f 0.97), which fluoresces under UV as a weak violet zone, was eluted with hexane and quantified according to Davies (1976).

2.3. RESULTS

2.3.1. Carotenoid, Organic Acid, and Sugar Levels in Control and Inhibitor Treated Corn Seedlings. Seedlings grown in the presence of inhibitor concentrations greater than 10^{-3} M were stunted and generally had an abnormal appearance. At concentrations less than 10^{-6} M seedlings had the same appearance as control plants. At a concentration of 10^{-4} M seedlings were slightly shorter than control plants (Figure 2.1), but were suitable for experimental use. Both inhibitors caused a



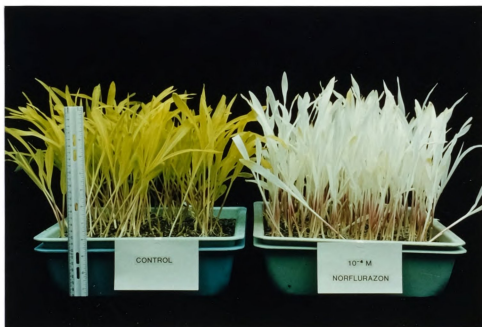


Figure 2.1. Corn seedlings watered with either nutrient solution or 10^{-4} M norflurazon.

decrease in total carotenoid content and increased phytoene levels at a concentration of 10^{-4} M compared with control seedlings (Table 2.1). Organic acid levels were increased in the inhibitor treated plants (Table 2.2), indicating that the tricarboxylic acid cycle was perturbed. Insoluble carbohydrates (starch) were slightly decreased, whereas soluble free sugars were slightly increased (Table 2.3) in seedlings grown in the presence of inhibitors.

2.3.2. Effects of Carotenoid Biosynthetic Inhibitors on Absciscic Acid Accumulation. ABA levels in turgid, inhibitor treated tissue, were decreased compared to green tissue (Table 2.4). In addition, the ability to accumulate water-stress-induced ABA was reduced in inhibitor-grown seedlings (Table 2.4).

2.4. DISCUSSION

2.4.1. Inhibition of Absciscic Acid Accumulation by Norflurazon and Fluridone. The results presented above show that the accumulation of ABA was drastically reduced when corn seedlings were grown in the presence of inhibitors of carotenoid biosynthesis. This result has been observed by other workers (pearl millet, Henson, 1984; barley, Quarrie and Lister, 1984; corn, Moore and Smith, 1984). Interestingly, fluridone induces vivipary when applied to developing maize seeds 11 d after pollination (Fong et al., 1983a; Fong et al., 1983b).

Table 2.1. The effect of norflurazon and fluridone on levels of phytoene and total carotenoids in corn seedlings. The data presented are from different experiments, and are the average of two replicates per treatment. The values in parenthesis are the per cent of control.

	Control	Norflurazon (10^{-4} M)	Fluridone (10^{-4} M)
$\mu\text{g/g}$ fresh wt.			
Phytoene	25(100)	63(252)	68(672)
Total carotenoids	62(100)	4.1(7)	0.8(2)

Table 2.2. The effect of norflurazon and fluridone on organic acid levels in corn seedlings.

The data presented are from different experiments, and are the average of two replicates per treatment.

Organic acid	Control	Norflurazon (10^{-4} M)	Fluridone (10^{-4} M)
$\mu\text{g/g}$ fresh wt.			
Malonic	21	nd*	63
Succinic	75	92	360
Malic	790	1890	2430
Citric	1000	1230	1520

* not detected

Table 2.3. The effect of norflurazon and fluridone on levels of soluble and insoluble sugars in corn seedlings. The data presented are from different experiments, and are the average of two replicates per treatment.

Sugar	Control	Norflurazon (10^{-4} M)	Fluridone (10^{-4} M)
mg/g fresh wt.			
Soluble	26	38	54
Insoluble	5.7	3.3	2.8

Table 2.4. The effect of norflurazon and fluridone on ABA levels in corn seedlings.

The data presented are from different experiments, and are the average of two replicates per treatment.

Treatment	ABA
$\mu\text{g/g fresh wt.}$	
Control	
Turgid	15
Stressed	307
Norflurazon (10^{-4} M)	
Turgid	1
Stressed	15
Fluridone (10^{-4} M)	
Turgid	0.5
Stressed	13

When green leaves (which already have a large carotenoid pool) were treated with norflurazon, stress induced ABA accumulation was not inhibited (Henson, 1984). In green bean leaves labeled with $^{14}\text{CO}_2$ and then water-stressed, the specific activities of ABA and xanthophylls were similar on a per carbon basis. However, when green leaves of bean were treated with norflurazon prior to $^{14}\text{CO}_2$ labeling, the specific activities of ABA and xanthophylls were reduced to the same extent, but total ABA biosynthesis was not affected (Walton *et al.*, 1985). Thus, the effect of norflurazon on ABA accumulation in green leaves must be on the biosynthesis of new carotenoids. These results (Henson, 1984; Walton *et al.*, 1985) are consistent with the idea that ABA arises from a pool of carotenoids.

2.4.2. Non-Specificity of Carotenoid Biosynthetic Inhibitors. Clearly, the experiments described in section 2.4.1 are strong evidence for the indirect pathway of ABA biosynthesis. However, the fact that these inhibitors have strong effects on primary metabolism imply that the conclusions should be interpreted cautiously with regard to ABA accumulation. In addition to being grown in the presence of inhibitors, the seedlings were also grown in darkness to avoid photo-bleaching. Because of these factors, the primary metabolism of corn seedlings grown under these conditions is different from plants grown under normal conditions. These facts lead to the conclusion, that while the inability to accumulate ABA is associated with carotenoid deficiency, it

cannot be separated from an indirect effect of altered primary metabolism on ABA biosynthesis.

Quarrie and Lister (1984) used a mutant of barley (*albostrians*) which has green, chimeric, and white leaves, and norflurazon-treated barley plants in studies dealing with ABA biosynthesis. These workers concluded that plastid ribosomes were needed for stress ABA and that the enzymes for ABA biosynthesis are encoded in nuclear DNA (Quarrie and Lister, 1984). They stated that the decreased ability to accumulate stress-induced ABA in the mutant and norflurazon treated plants was not due to decreased levels of NADPH, ATP, or carbohydrate because dark-grown etiolated plants accumulated ABA just as well as green plants. However, it should be noted that functional mitochondria are present in etiolated plants, and these organelles could substitute for chloroplasts with regard to energy production.

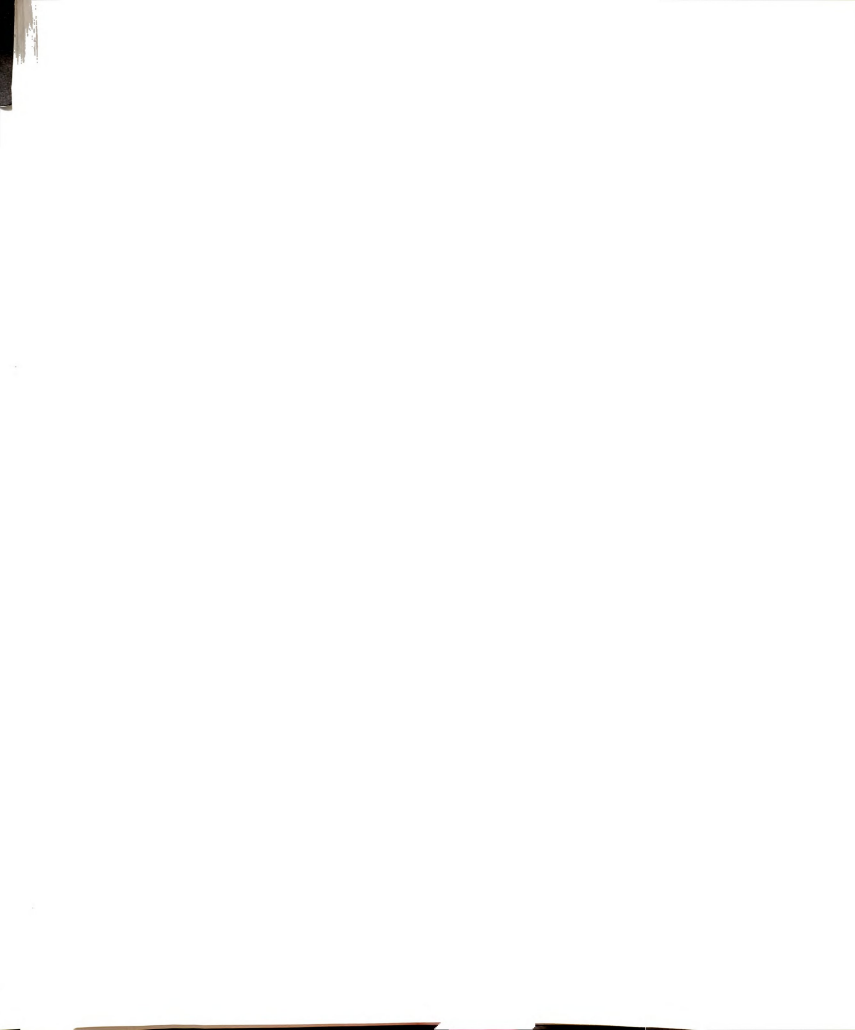
Quarrie and Lister (1984) also state that small amounts of ABA are produced during stress in the absence of plastid ribosomes, indicating that the nuclear genome codes for enzymes in ABA biosynthesis. However, the data they present are barely above the detection limit and they mention that there was a impurity which co-chromatographed with MeABA which made quantitation difficult. Since the data were obtained with the mutant *albostrians* the variability observed could simply represent ABA that was transported into the leaf before the stress period began.

2.5. LITERATURE CITED

- Ashwell, G. (1966) New colorimetric methods of sugar analysis. *Methods Enzymol* 8:85-95.
- Bartels, P.G., Watson, C.W. (1978) Inhibition of carotenoid synthesis by fluridone and norflurazon. *Weed Sci.* 26: 198-203.
- Davies, B.H. (1976) Carotenoids. In: *Chemistry and biochemistry of plant pigments*, pp. 38-165, vol. 2, Goodwin, T.W., ed. New York: Academic Press.
- Fong, F., Koehler, D.E., Smith, J.D. (1983a) Fluridone induction of vivipary during maize seed development. In: *Third international symposium on pre-harvest sprouting in cereals*, pp. 186-196, Krueger, J.E., La Berge, D.E., eds. Boulder: Westview Press.
- Fong, F., Smith, J.D., Koehler, D.E. (1983b) Early events in maize seed development. 1-methyl-3-phenyl-5-(3[trifluoromethyl]phenyl)-4-(1*H*)-pyridinone induction of vivipary. *Plant Physiol.* 73:899-901.
- Henson, I.E. (1984) Inhibition of abscisic acid accumulation in seedling shoots of pearl millet (*Pennisetum americanum* [L.] Leeke) following induction of chlorosis by norflurazon. *Z. Pflanzenphysiol.* 114:35-43.
- McCready, R.M., Guggolz, J., Silveira, V., Owens, H.S. (1950) Determination of starch and amylose in vegetables. *Anal. Chem.* 22:1156-1158.
- Moore, R., Smith, J.D. (1984) Growth, graviresponsiveness and abscisic-acid content of *Zea mays* seedlings treated with fluridone. *Planta* 162:342-344.
- Quarrie, S.A., Lister, P.G. (1984) Evidence of plastid control of abscisic acid accumulation in barley (*Hordeum vulgare* L.). *Z. Pflanzenphysiol.* 114:295-308.
- St. John, J.B. (1976) Manipulation of galactolipid fatty acid composition with substituted pyridazones. *Plant Physiol.* 57:38-40.
- Stumpf, D.K., Burris, R.H. (1979) A micromethod for the purification and quantification of organic acids of the TCA cycle. *Anal. Biochem.* 95:311-315.
- Walton, D.C., Li, Y., Neill, S.J., Horgan, R. (1985) Biosynthesis of abscisic acid: a progress report. In: *Current topics in plant biochemistry and physiology 1985*, pp. 111-117, vol. 4, Randall, D.D.,

Blevins, D.G., Larson, R.L., eds. Columbia, MO: University of Missouri.

- Zeevaart, J.A.D. (1980) Changes in the level of abscisic acid and its metabolites in excised leaf blades of *Xanthium strumarium* during and after water stress. Plant Physiol. 66:672-678.



CHAPTER 3
BIOLOGICAL ACTIVITY AND METABOLISM OF COMPOUNDS
STRUCTURALLY RELATED TO ABSCISIC ACID

3.1. INTRODUCTION

Regardless of whether ABA is synthesized *via* the direct or indirect pathway, the immediate precursor(s) to ABA must be a C₁₅ compound. I decided to synthesize three compounds structurally related to ABA, α -ionylidene acetic acid, 1',2'-epoxy-ionylidene acetic acid, and 1',2'-epoxy-ionylidene acetaldehyde, to determine if any of them would serve as a precursor to ABA.

One of these compounds, α -ionylidene acetic acid, has been shown to be a precursor to ABA in fungi (Neill and Horgan, 1983; Oritani *et al.*, 1982; Ichimura *et al.*, 1983). In several bioassays, this compound has greater biological activity than ABA (Walton, 1983). However, it has been shown that many plants can catabolize α -ionylidene acetic acid to 1'-deoxy-ABA and conjugates, but not to ABA (Lehmann and Schütte, 1976). Only with *Vicia faba* has any conversion to ABA been demonstrated (Walton *et al.*, 1985). Lack of conversion in many other plants could be due to a failure of the compounds to reach the proper compartment for metabolism to ABA. It could also be that the results with *V. faba* are anomalous because an enzyme is present in this plant that is lacking in others.

The only C₁₅ compounds known to be converted to ABA are 1',2'-epoxy ionylidene acetic acid (Milborrow and Noddle, 1970) and xanthoxin (Taylor and Burden, 1973). However, in both cases only ¹⁴C incorporation into ABA was

demonstrated instead of an unequivocal proof showing heavy-isotope incorporation by GC-MS.

In some bioassays (such as stomatal closure, rice seedling growth, and lettuce hypocotyl growth) 1',2'-epoxy-ionylidene acetic acid had less biological activity than ABA, whereas in others (lettuce seed germination and *Avena* coleoptile growth) it had the same or greater activity (Walton, 1983), depending on the concentration used. Xanthoxin was highly active in all biological assays, except when stomatal closure in epidermal strips was tested (Walton, 1983). Higher plants can convert 1',2'-epoxy ionylidene acetic acid to xanthoxin acid and ABA and other unknown catabolites (Milborrow and Noddle, 1970; Milborrow and Garms-ton, 1973). Similar results have been obtained with the fungus *C. cruenta* (Oritani and Yamashita, 1985). I decided to synthesize this compound and confirm that it is indeed converted to ABA.

Since the chemical synthesis of xanthoxin is very laborious and difficult (Kienzle et al., 1978), I decided to synthesize a compound very similar to both 1',2'-epoxy-ionylidene acetic acid and xanthoxin, namely 1',2'-epoxy-ionylidene acetaldehyde. This compound is identical to xanthoxin except that there is no hydroxyl group at C4'. Since it is known that higher plants can insert a hydroxyl group at this position with α -ionylidene type structures (Lehmann and Schütte, 1976; Walton et al., 1985), it seemed reasonable to assume that this would occur when 1',2'-epoxy-ionylidene

acetaldehyde was fed. If xanthoxin was made, further metabolism to ABA would occur. Incorporation of tritium and deuterium into this compound would allow for purification by HPLC and subsequent identification by GC-MS.

3.2. MATERIALS AND METHODS

3.2.1. Synthesis of ^2H Labelled α -Ionone (V, Figure 3.1).

Deuterium was exchanged into α -ionone by dissolving α -ionone (8.8 g, 25 mmol) and $^2\text{H}_2\text{O}$ (1 ml, 99% atom excess) in 12 ml of d_6 -acetone and adding about 1 mg of Na metal. After stirring at room temperature for 24 h the acetone was removed with the aid of a rotary evaporator. Chloroform (2 ml) was added and the mixture was partitioned against 3 ml $^2\text{H}_2\text{O}$ twice and 3 ml H_2O twice. Analysis by NMR and GC-MS indicated that the deuterium was located in the side chain methyl group of α -ionone (V) and had an isotopic composition, $[\text{}^2\text{H}_0]:[\text{}^2\text{H}_1]:[\text{}^2\text{H}_2]:[\text{}^2\text{H}_3]$, of 2:3:21:75 (2.69 deuterium/molecule).

3.2.2. Synthesis of ^2H and ^3H Labelled β -Ionone. Tritium was exchanged into β -ionone in a custom synthesis by Amersham Corporation by a method similar to that used by Walton et al. (1977). The exchange was performed by mixing β -ionone, alumina, anisole, and $^3\text{H}_2\text{O}$ (25 Ci) for 25 min at 120 C.

Deuterium was exchanged into β -ionone in a manner identical to that done with α -ionone. NMR and GC-MS analysis indicated that the deuterium was located in the side chain methyl group of β -ionone with an isotopic composition of

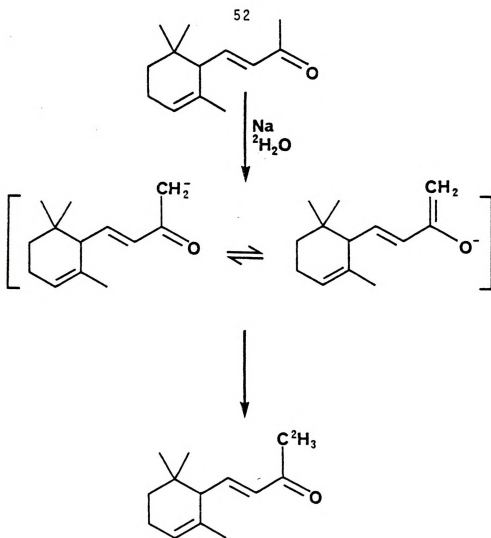


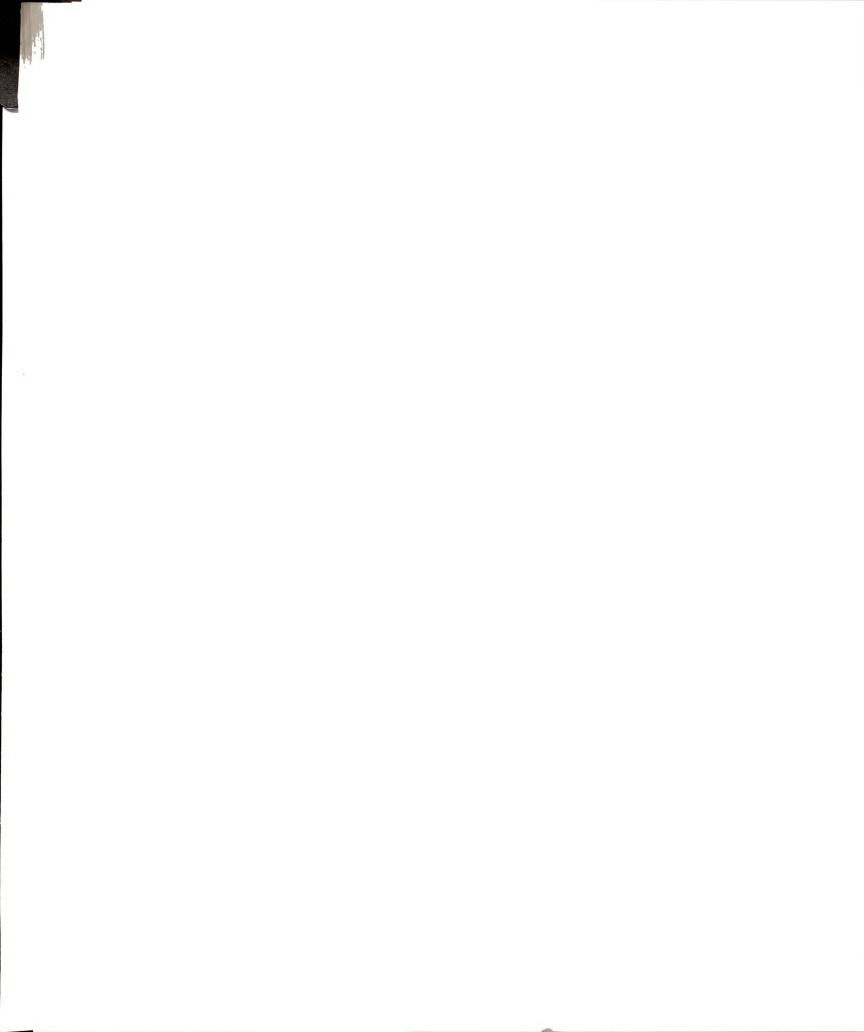
Figure 3.1. Synthesis of deuterated α -ionone.

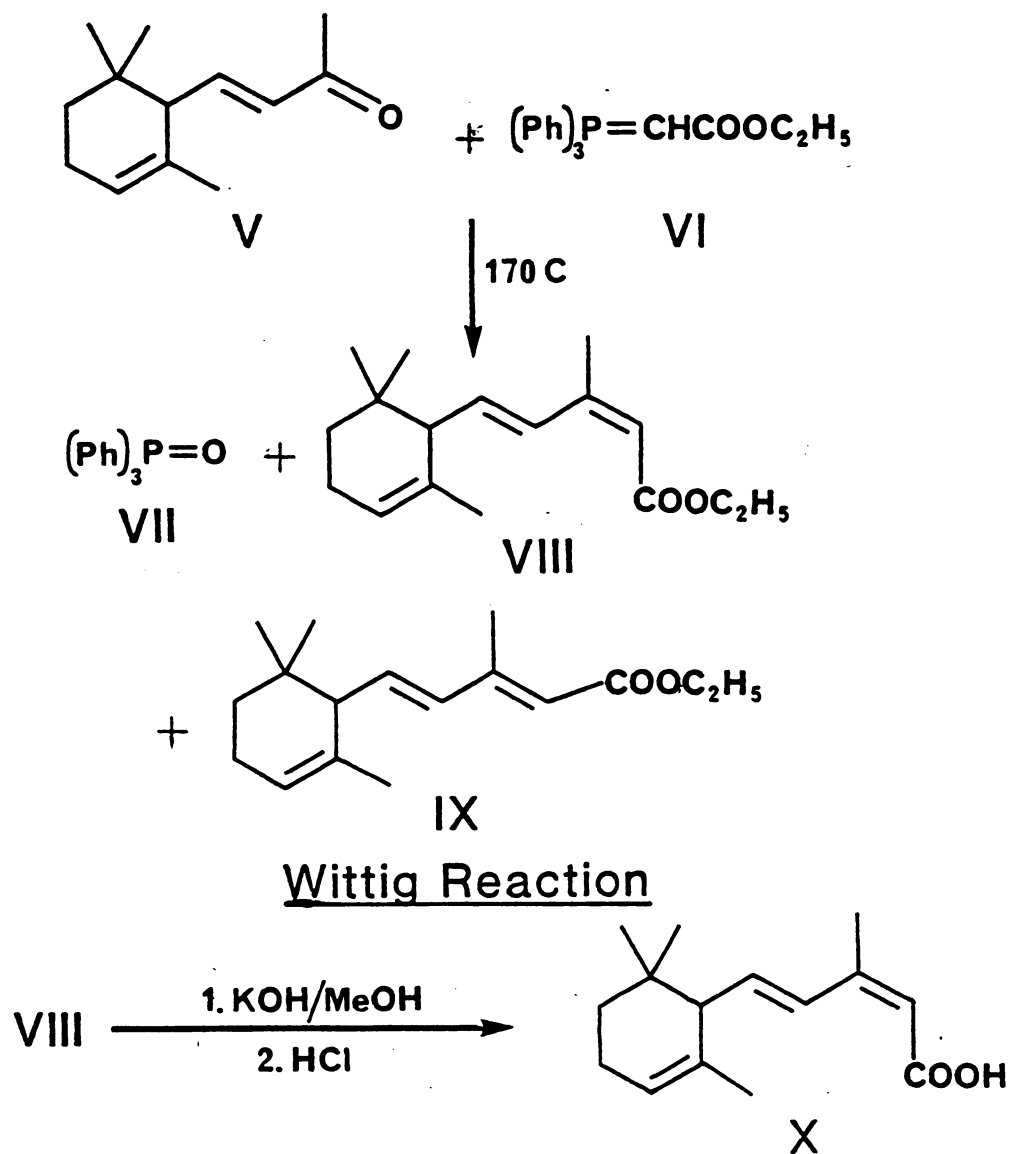
1:200:1800:8000 (2.77 deuterium/molecule).

3.2.3. Synthesis of α -Ionylidene Acetic Acid (X, Figure 3.2). A mixture of deuterated α -ionone (V, 2 g, 12.5 mmol) and carbethoxymethylenetriphenylphosphorane (VI, 8.8 g, 25 mmol) was heated at 170 C for 90 min. After cooling, the red viscous product was triturated with hexane and stored at 20 C overnight. Any triphenylphosphine oxide (VII) which precipitated was removed by filtration. The filtrate was concentrated to an oil by rotary evaporation. GLC-FID analysis (3% SP-2100, 140 C to 240 C at 5 C/min, 30 ml/min He flow) of the crude mixture indicated a 1:2 ratio of *cis,trans*- (VIII, retention time 4.96 min) and *trans,trans*-ethyl α -ionylidene acetate (IX, retention time 5.61 min).

The isomers were separated by silica gel chromatography yielding 110 mg pure *cis,trans*- (VIII) and 260 mg *trans,trans* ethyl α -ionylidene acetate (IX); both compounds were light yellow oils. The *cis,trans* isomer was saponified overnight in 1 ml 10% KOH/methanol. After acidification with 6 N HCl a white flocculate resulted which was partitioned into diethyl ether. The solvent was removed with a stream of nitrogen to give 96 mg of pure *cis,trans*- α -ionylidene acetic acid (X, 3-methyl-5-(2',6',6'-trimethyl-2'-cyclohexen-1'-yl)-*cis,trans*-2,4-pentadienoic acid).

Methylation of a small aliquot of X with ethereal diazomethane gave a single peak with GLC-FID. The fragmentation pattern of X obtained by GC-MS was similar to that of Neill and Horgan (1983), except that no molecular ion was present





Hydrolysis, Formation of Free Acid

Figure 3.2. Synthesis of deuterated α -ionylidene acetic acid (X).

because in this study a higher ionization potential was used. MS (GC-MS, Figure 3.3), methyl X, m/z (rel. int.): 252(M^+ ,0), 220(M^+ -32,3), 219(4), 195(4) (M^+ -56), 194(4), 193(3), 163(15), 162(20), 161(12), 160(8), 136(38), 135(55), 134(32), 133(18), 128(97), 127(100), 126(38), 125(6), 115(28), 114(19), 113(9), 112(5), 108(14), 107(31), 106(14), 105(16). The isotopic composition in the side chain methyl group was 2:16:41:40 (2.2. deuterium/molecule), indicating that some exchange occurred during the Wittig reaction [compare with 2.69 deuterium/molecule in α -ionone (section 3.2.1)].

3.2.4. Synthesis of 1',2'-Epoxy-Ionylidene Acetic Acid (XV, Figure 3.4). A mixture of deuterated β -ionone (XI, 0.7 g, 3.6 mmol) and carbethoxymethylenetriphenylphosphorane (VI, 1.5 g, 4.3 mmol) was heated at 155 C for 2.5 h. The product, a red oil, was triturated with hexane as described above in section 3.2.6. GLC-FID analysis indicated that 60% of the β -ionone present was used in the reaction. The crude mixture of isomers was purified as described above (section 3.2.4) to give pure ethyl *cis,trans*- β -ionylidene acetate (XII). UV XII λ max nm: 309, 260; MS (GC-MS, Figure 3.5), XII, m/z (rel. int.): 265(M^+ ,57), 264(42), 263(14), 262(2), 250(M^+ -15,13), 249(12), 248(5), 247(5), 236(8), 235(6), 234(2), 233(1), 220(34), 219(33), 218(15), 217(34), 204(32), 203(25), 202(9), 201(5), 181(11), 180(12), 179(12), 178(13), 177(26), 176(59), 175(54), 174(20), 173(9), 165(18), 164(38), 163(47), 162(68), 161(57),

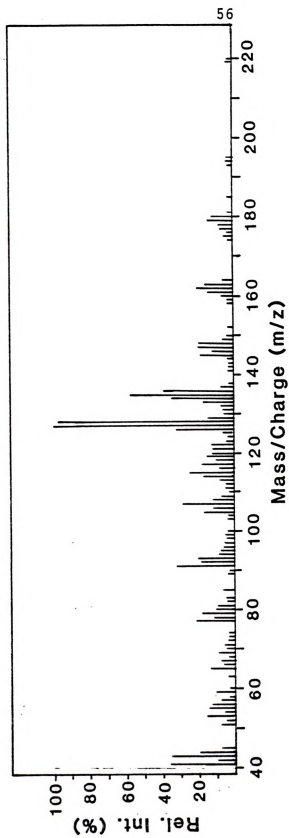
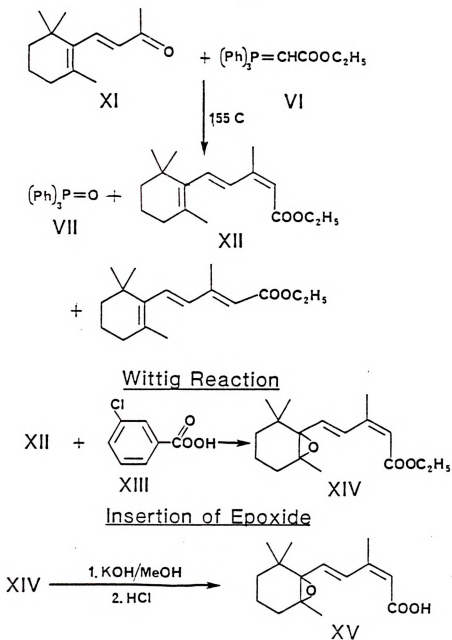


Figure 3.3. Mass spectrum of deuterated methyl- α -ionylidene acetic acid (X).





Hydrolysis. Formation of Free Acid

Figure 3.4. Synthesis of deuterated 1',2'-epoxy-ionylidene acetic acid (XV).

In both XI and XV the deuterium is located in the side chain methyl group.



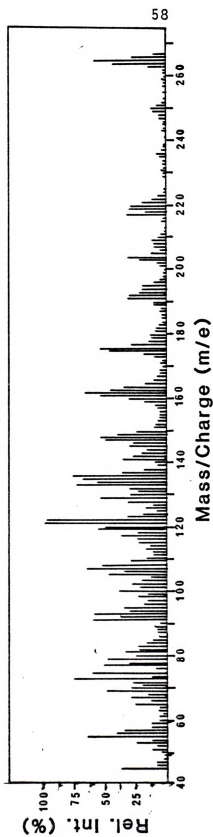


Figure 3.5. Mass spectrum of deuterated ethyl-*cis*,*trans*- β -ionylidene acetate (XII).

160(31), 159(16), 125(6), 124(18), 123(31), 122(100), 121(95), 120(49), 119(46). The side chain methyl group had an isotopic composition of 2:12:37:50 (2.3 deuterium/molecule), indicating that some exchange of deuterium present in the precursor β -ionone occurred during the Wittig reaction [compare with 2.77 deuterium/molecule in β -ionone (section 3.3.2)].

The epoxide was inserted by adding dropwise *m*-chloroperbenzoic acid (XIII, 322 mg in 1.5 ml CH_2Cl_2 , 1 M) to ethyl *cis,trans*- β -ionylidene acetate (XII, 396 mg in 1.5 ml CH_2Cl_2 , 1 M) cooled in an ice bath. After stirring at room temperature for 45 min the reaction mixture was washed sequentially with 10% sodium bisulfite, water, 10% sodium bicarbonate, and finally water. The solvent was removed with a stream of nitrogen. GLC-FID analysis indicated 100% conversion of the starting material into ethyl *cis,trans*-1',2'-epoxy-ionylidene acetate (XIV). The ester was saponified as described above in section 3.2.7 to give *cis,trans*-1',2'-epoxy-ionylidene acetic acid (XV; 3-methyl-5-(1',2'-epoxy-2',6',6'-trimethyl-1'-cyclohexyl)-*cis,trans*-2,4-pentadienoic acid). UV XV λ_{max} nm: 266; methyl XV: 266; ethyl XV: 266; MS (GC-MS, Figure 3.6), methyl XV, m/z (rel. int.): 267(M^+ ,6), 266(1), 252(M^+ -15,12), 251(5), 250(2), 249(2), 235(M^+ -32,18), 234(8), 233(3), 232(1), 224(35), 223(12), 222(4), 221(6), 192(20), 191(12), 190(6), 189(5), 183(15), 182(55), 181(23), 180(12), 179(13), 178(22), 177(27), 176(19), 150(53), 149(38), 148(22),



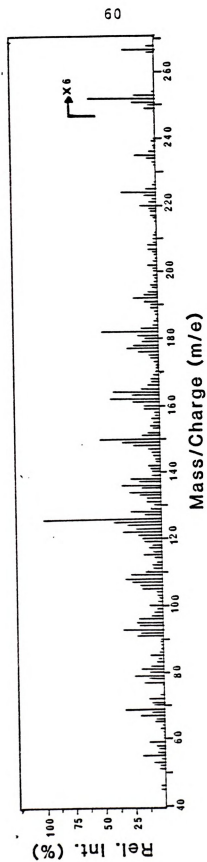


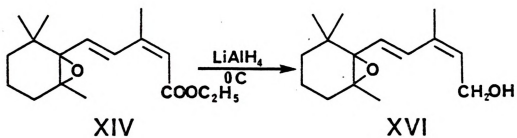
Figure 3.6. Mass spectrum of deuterated methyl-*cis,trans*-epoxy-ionylidene acetate (XV).

147(10), 126(100), 125(45), 124(29), 123(19), 122(26), 121(20), 120(14), 119(13). There was an isotopic composition of 10:10:17:63 (2.3 deuterium/molecule) in the side chain methyl group.

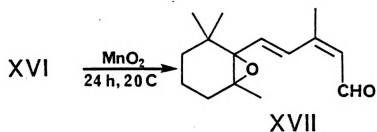
3.2.5. Synthesis of 1',2'-Epoxy-Ionylidene Acetaldehyde (XVII, Figure 3.7). This compound was first synthesized without deuterium to provide material for GC-MS and NMR analysis. A mixture of β -ionone (XI) and carbethoxymethylenetriphenylphosphorane (VI) was reacted and purified as described in section 3.2.7 to give ethyl *cis,trans*- β -ionylidene acetate (XII). UV XII $\lambda_{\max}$ nm: 311, 257; MS (GC-MS, Figure 3.8) XII, m/z (rel. int.): 262(M^+ ,15), 247(M^+-15 ,6), 233(2), 217(10), 189(8), 173(27), 161(14), 159(29), 145(20), 133(59), 119(100), 107(20), 105(54), 91(59), 77(48), 69(46), 67(22), 55(43).

The epoxide was inserted at the 1',2' double bond as described in section 3.2.7 to give ethyl *cis,trans*-1',2'-epoxy-ionylidene acetate (XIV). UV XIV $\lambda_{\max}$ nm: 266; MS (GC-MS Figure 3.9) XIV, m/z (rel. int.): 278(M^+ ,1), 263(M^+-15 ,4), 235(12), 217(6), 193(16), 174(13), 165(24), 161(31), 159(24), 147(32), 133(29), 123(100), 121(35), 119(33), 105(56), 95(27), 93(36), 91(77), 79(44), 77(65), 69(66), 67(35), 65(45), 55(59), 53(41); 1H NMR: 0.94(3H, s), 1.10(3H,s), 1.18(3H,s), 1.25(3H,s), 1.96(3H,s), 4.05(2H,q), 5.5(1H,s), 6.15(1H,d), 7.3(1H,d).

Ethyl *cis,trans*-1',2'-epoxy-ionylidene acetate (XIV) was reduced with $LiAlH_4$ in ether at 0 C for 30 min. After

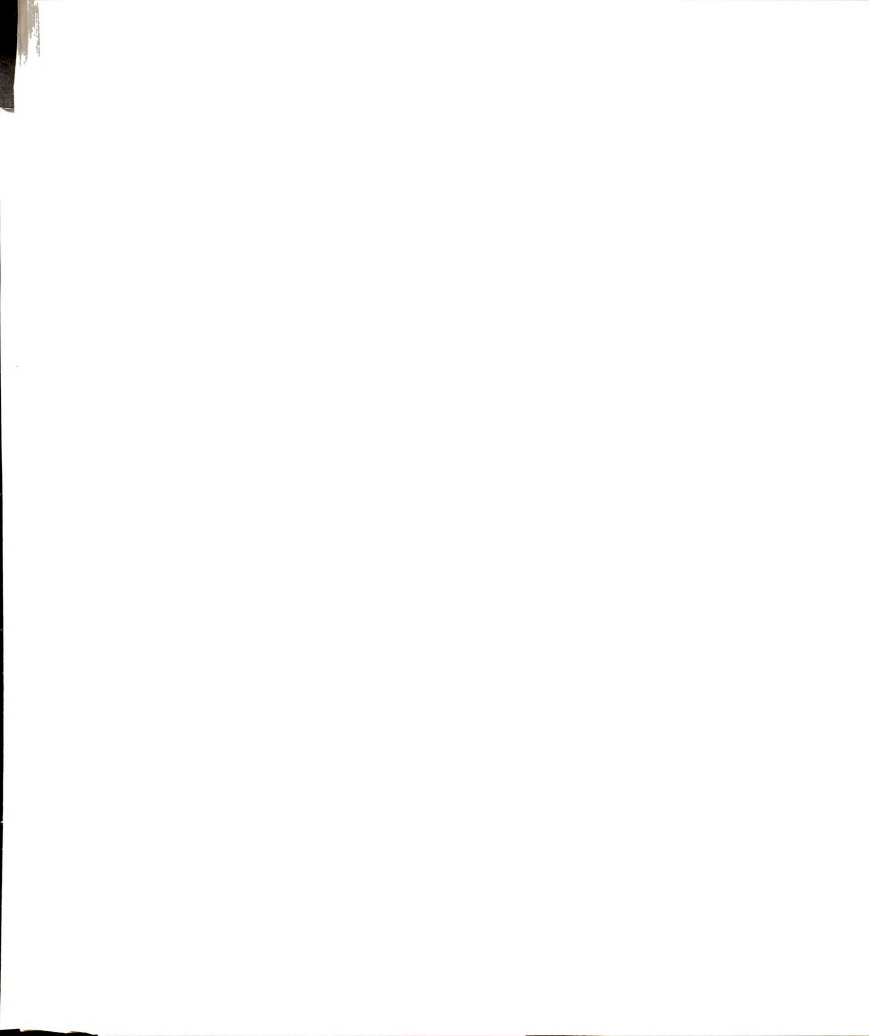


Reduction to Alcohol



Oxidation to Aldehyde

Figure 3.7. Synthesis of 1',2'-epoxy-ionylidene acet-aldehyde (XVII).



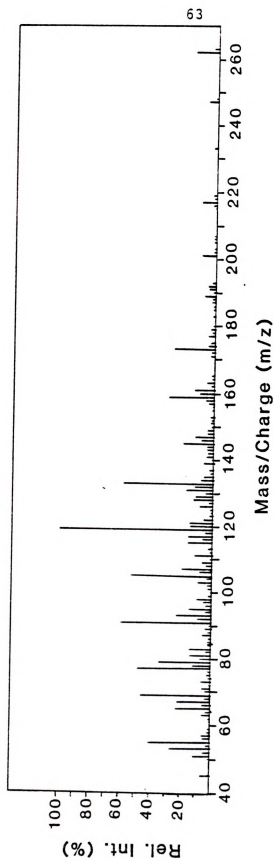


Figure 3.8. Mass spectrum of ethyl-*cis*,*trans*- β -ionylidene acetate (XII).

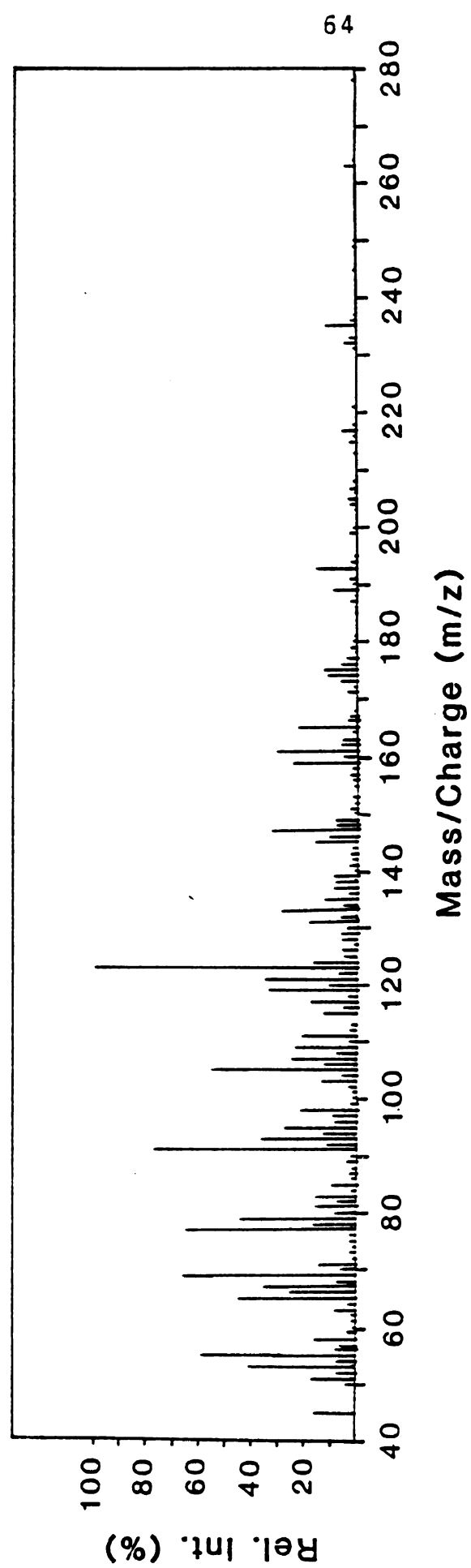
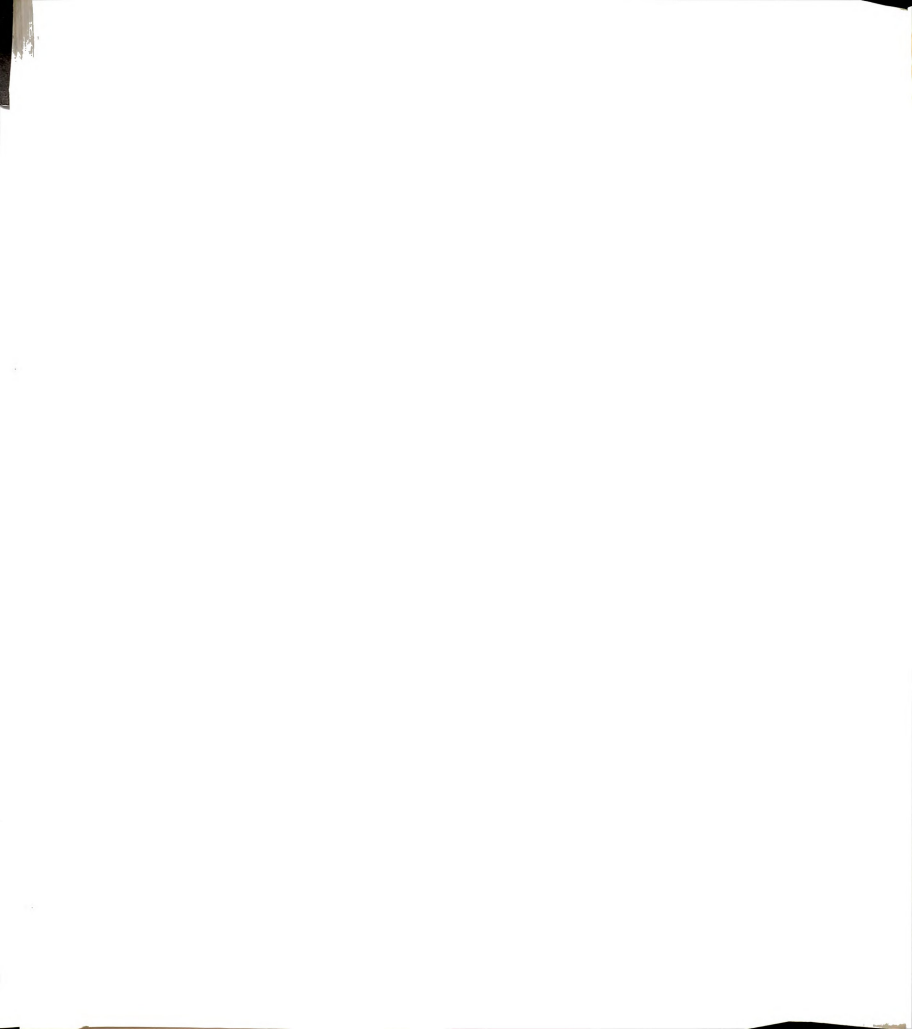


Figure 3.9. Mass spectrum of ethyl-cis,trans-1',2'-epoxy-ionylidene acetate (XIV).



decomposition of the excess LiAlH_4 with ethyl acetate and saturated ammonium chloride, the alcohol (XVI) was partitioned into ether. The starting material was converted 100% into *cis,trans*-1',2'-epoxy-ionylidene alcohol (XVI). UV XVII λ max nm: 239; MS (GC-MS, Figure 3.10) XVI, m/z (rel. int.): 218(M^+ -18,4), 203(M^+ -33,5), 193(2), 185(1), 175(5), 145(36), 133(23), 123(91), 121(32), 119(31), 109(44), 107(37), 105(57), 95(48), 93(37), 91(66), 85(22), 81(44), 79(58), 77(58), 71(38), 69(100), 67(43), 55(84), 53(51); ^1H NMR: 0.95(3H, s), 1.10(3H, s), 1.17(3H, s), 1.90(3H, s), 3.30(2H, d), 5.55(1H, s), 6.47(1H, s).

A solution of *cis,trans*-1',2'-epoxy-ionylidene alcohol (XVI) in chloroform was vigorously stirred with active MnO_2 (prepared by the method of Attenburrow et al., 1952) at room temperature for 24 h. Filtration and removal of solvent gave the expected product, *cis,trans*-1',2'-epoxy-ionylidene acetaldehyde (XVII, 3-methyl-5-(1',2'-epoxy-2',6',6'-trimethyl-1'-cyclohexyl)-*cis,trans*-2,4-pentadienal). XVII reacted positively with 2,4-dinitrophenyl hydrazine, giving a yellow spot indicative of an aldehyde or ketone function (Taylor and Burden, 1970). UV XVII λ max nm: 282; MS (GC-MS, Figure 3.11) XVI, m/z (rel. int.): 234(M^+ ,15), 219(M^+ -15,12), 205(M^+ -29,6), 201(M^+ -33,5), 191(8), 161(31), 149(66), 131(45), 105(56), 95(100), 93(37), 91(64), 82(46), 79(46), 77(49), 71(16), 69(57), 67(42), 65(29), 55(37), 53(24), 41(12); ^1H NMR: 1.00(3H, s), 1.15(3H, s), 1.25(3H, s), 2.05(3H, s), 4.00(2H, m), 5.70(1H, d), 6.30(1H, d), 7.20(1H, d), 9.90(1H,

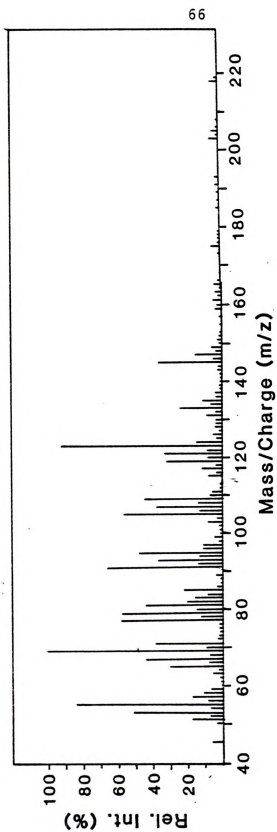


Figure 3.10. Mass spectrum of *cis,trans*-1,2'-epoxy-ionylidene ethanol (XVI).

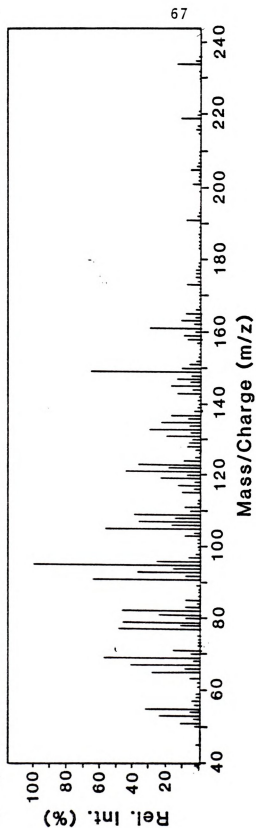
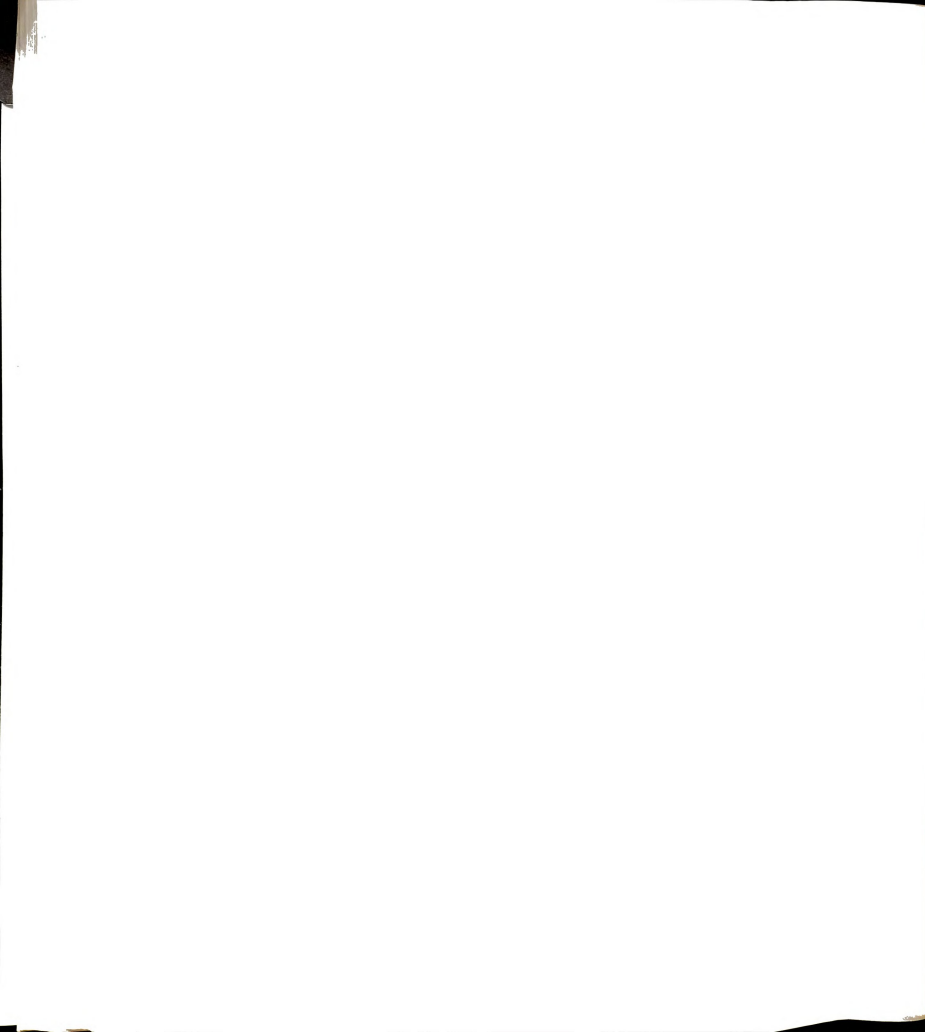


Figure 3.11. Mass spectrum of *cis,trans*-1',2'-epoxy-ionylidene acetaldehyde (XVII).



Deuterium labeled XVII was prepared from deuterium labeled β -ionone by identical methods as described above in this section. MS (GC-MS, Figure 3.12) deuterated XVII, m/z (rel. int.): 237(M^+ ,6), 236(7), 222(M^+-15 ,4), 221(5), 207(4), 199(9), 193(4), 192(4), 164(16), 163(13), 162(11), 161(10), 149(49), 151(36), 150(14), 149(12), 126(41), 125(26), 123(33), 123(23), 122(14), 121(14). The isotopic composition of the side chain methyl group was 1:11:38:50 (2.4 deuterium per molecule).

Tritium labeled XVII was prepared from tritium labeled β -ionone by identical methods as described above. The final specific activity of tritiated XVII was 1.6 mCi/mmol.

6. Spectrometry. Mass spectra were obtained with a Hewlett-Packard 5985 quadrupole mass spectrometer connected to a Hewlett Packard 5840A gas chromatograph. GLC conditions were: 3% SP-2100 on 100-200 mesh Gas Chrom Q in a stainless steel glass column (2 m x 0.2 cm) temperature programmed from 120 C to 240 C at 5 C/min. The ionizing potential was 70 eV. To detect incorporation of deuterated synthetic analogues the base peak of ABA (m/z 190) in addition to m/z 191, 192, and 193 was monitored by GC-MS. The dwell time for each ion was 140 ms. GLC conditions were as described above, except that the initial temperature was 190 C.

NMR spectra were obtained with a Varian EM-360 NMR (60 MHz) spectrometer. Sample concentration was 5% in $CDCl_3$.



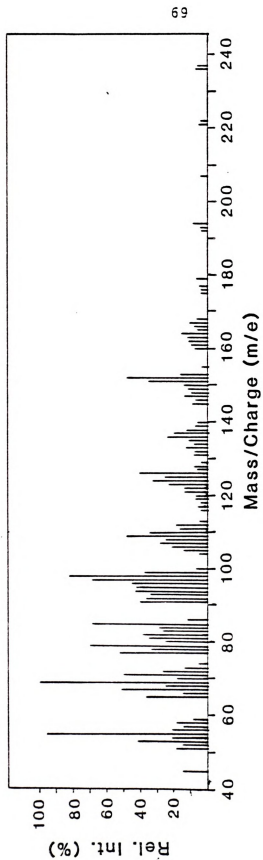


Figure 3.12. Mass spectrum of deuterated *cis,trans*-1',2'-epoxy-ionylidene acetaldehyde (XVII).

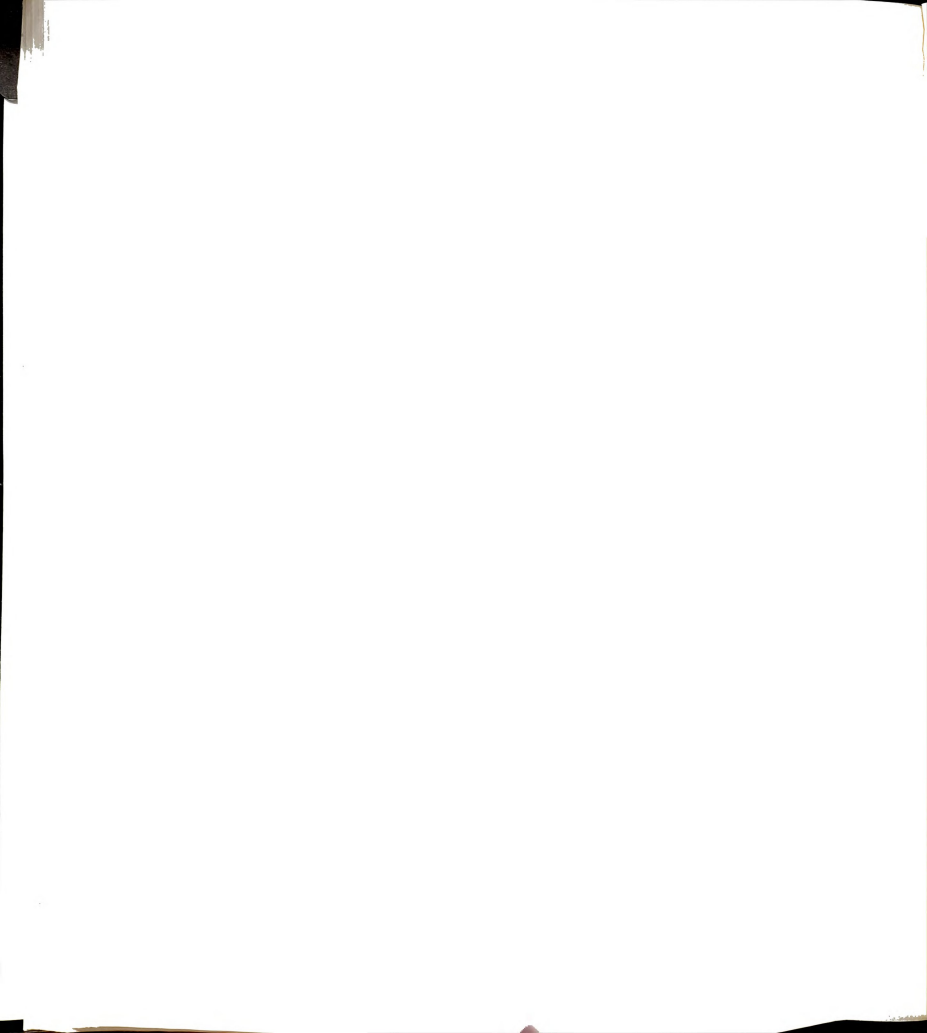
aining 1% TMS as internal standard.

UV spectra were obtained with a Perkin-Elmer Lambda 7
IS spectrophotometer. Compounds were dissolved in 95%
ol and scanned from 340 nm to 200 nm.

Plant Material. The metabolism of radioactive com-
s was investigated with either spinach leaves (*Spinacia*
cea L., cv Savoy Hybrid 612), young tomato shoots
persicon esculentum L., cv Moneymaker, 20-25 cm tall),
its of tomato (breaker stage). Compounds (usually 50 to
g of the deuterated analogues plus 10^6 dpm of the
active compound) were administered to leaves through the
iration stream or injected directly into fruits. Only
ated analogues of 1',2'-epoxy-ionylidene acetic acid
sed in feeds.

Extraction and Purification Procedures. After applica-
f radioactive compounds there was an incubation period
6 h. The tissue was extracted with methanol containing
L BHT. The extract was reduced to an aqueous residue
ary evaporation, frozen, and lyophilized. A small
was subjected to semi-preparative C₁₈ reverse phase
0% to 80% ethanol in aqueous 1% acetic acid in 1 h,
te 2.5 ml/min). Radioactivity in the column effluent
itored with a RadioAnalytic HP Flo-One radioactive
ector.

oactive peaks from tomato fruit were analyzed further
[Silica gel, 0.25 mm, hexane:ethyl acetate 1:1 (1X
ed), or hexane:ethyl acetate (3X), or hexane:ethyl



acetate:acetic acid 1:3:0.1, (1X)]. One cm zones were removed and radioactivity was measured by liquid scintillation counting. To determine if the catabolites were conjugates, small aliquots were subjected to either basic hydrolysis with 2 N H_4OH (2 h, 60 C), or they were treated with pectinase AC (Rohm and Haas, 0.1 M potassium phosphate, pH 4.7). These treatments distinguish between ester or glycosidic conjugates, respectively. To determine if a carboxylic acid group was present, the R_f before and after treatment with ethereal azomethane was compared.

To confirm that XV was converted to ABA, ABA from leaves and XV was purified as described in chapter 4. In an attempt to determine if XV was endogenous in *X. strumarium*, leaves (100 g fresh weight) were extracted in methanol. The methanol was removed with the aid of a rotary evaporator to an aqueous residue to which was added phosphate buffer (1 M, 50 ml, pH 5). The pH of the solution was lowered to 2.5 and partitioned four times against petroleum ether (B.R. 40-60 C). In this system, standard XV was partitioned into the petroleum ether. After removal of petroleum ether, the residue was dissolved in ethanol to which KOH was added to give a final concentration of 6% KOH. The saponified extract was kept at 4 C overnight. Distilled water was added, and after removal of the ethanol, the pH was lowered to 2.5. The aqueous residue was partitioned three times against petroleum ether (B.R. 40-60 C). The petroleum ether was removed by rotary evaporation and the residue applied to a silica gel column (hexane:ethyl

acetate:acetic acid 7:3:0.1). Using this solvent system standard XV eluted in the early fractions. Thus, after application of the residue, the identical fractions were pooled and dried. These combined fractions were dried and subjected to semi-preparative reverse phase C₁₈ HPLC (20% to 80% ethanol in 1% aqueous acetic acid, gradient time was 25 min). The fraction which eluted between 23 and 26 min (determined using standard XV) was collected, dried, and methylated with ethereal diazomethane. This fraction was then analyzed by GC-MS.

3.2.9. Biological Activity Assay. The biological activity of X and XV was determined using the IAA-induced *Avena* coleoptile elongation bioassay (Milborrow, 1978). In this bioassay ABA, or compounds structurally related to it, inhibit elongation (Milborrow, 1978). IAA must be added to cause significant elongation.

Oats (*Avena sativa* L., cv Korwood) were soaked in water for 2 h, exposed to red light for 4 h, and then sown, embryo side up, on moist Kimpack. After 3 d, coleoptiles were cut three mm below the tip into 10 mm sections and floated on 1 mg/L MnSO₄ for three h. Sections were then placed in one ml of the solution to be tested [IAA alone, IAA plus ABA (0.4 μ M), or IAA plus synthetic compound (0.4 μ M) containing 2% sucrose, 1.8 g/L K₂HPO₄ and 1.0 g/L citric acid, pH 5.0). Coleoptiles were measured to the nearest mm after an incubation period of 24 h at room temperature.

3.3. RESULTS

3.3.1. Biological Activity of α -Ionylidene Acetic Acid (X) and 1',2'-Epoxy-Ionylidene Acetic Acid (XV). The biological activity of X and XV was measured with the IAA *Avena* coleoptile elongation assay. In this assay, at the concentration used, X had greater biological activity than ABA and XV had less (Figure 3.13).

3.3.2. Incorporation of 1',2'-Epoxy-Ionylidene Acetic Acid (XV) into Abscissic Acid. After 24 h incubation, analysis of *Xanthium* leaves fed XV indicated that approximately 5% of the starting material was still present. No apparent degradation of XV occurred when standard material was subjected to the purification procedure used with plant tissue. The SIM response of ABA isolated from leaves fed XV showed that an isotope shift of the molecular ion had occurred (Table 3.1), confirming that higher plants can convert XV into ABA (Milborrow and Noddle, 1970).

However, it appears that XV is not endogenous in higher plants, at least not in *X. strumarium*. A compound which co-chromatographed with standard XV is clearly not identical to XV as determined by GC-MS (compare Figures 3.14 and 3.6).

3.3.3. Metabolism of 1',2'-Epoxy-Ionylidene Acetaldehyde (VII) by Higher Plant Tissue. Extensive metabolism of VII was observed, with little or no starting material remaining after 24 h (Figures 3.15, 3.16, and 3.17). However, only with immature tomato fruit was the majority of

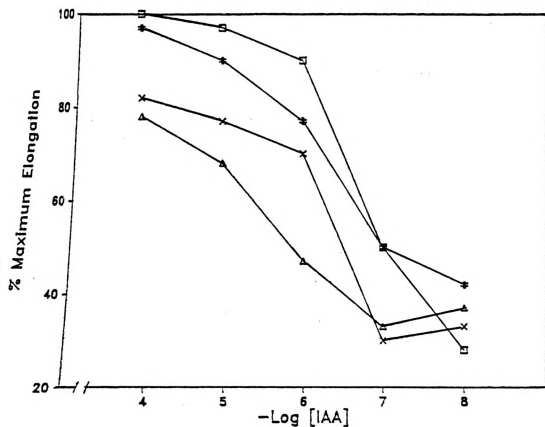


Figure 3.13. Biological activity of α -ionylidene acetic acid (X) and 1',2'-epoxy-ionylidene acetic acid (XV).

The biological response of X (Δ) and XV (*) are compared to ABA ($\times$) and IAA alone ($\square$). The standard deviation is 2%, 100% was 6 mm.

Table 3.1. Selected ion monitoring response of standard ABA and ABA isolated from leaves fed 1',2'-epoxy-ionylidene acetic acid (XV).

The data shown are the area (relative units) under the peak of the respective ion calculated by the GC-SIM. There is 2.5 deuterium per ABA molecule after taking into account endogenous ABA and natural abundance effects. This number was calculated by the weighted average method using the difference between the relative SIM response of ABA from standard material or that derived from fed XV.

m/z	SIM Response		Relative SIM Response	
	Standard	XV Fed	Standard	XV Fed
190.3	49890	41879	1.000	1.000
191.3	9928	9498	0.199	0.227
192.3	1422	4128	0.028	0.099
193.3	695	8058	0.014	0.192

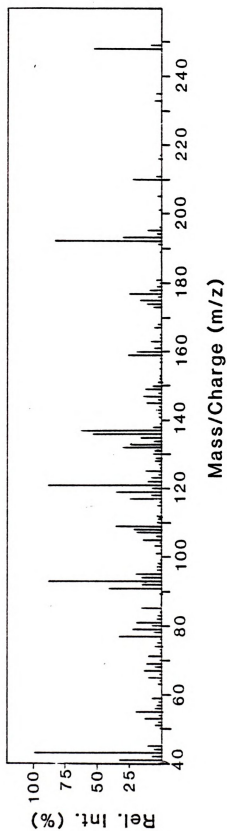
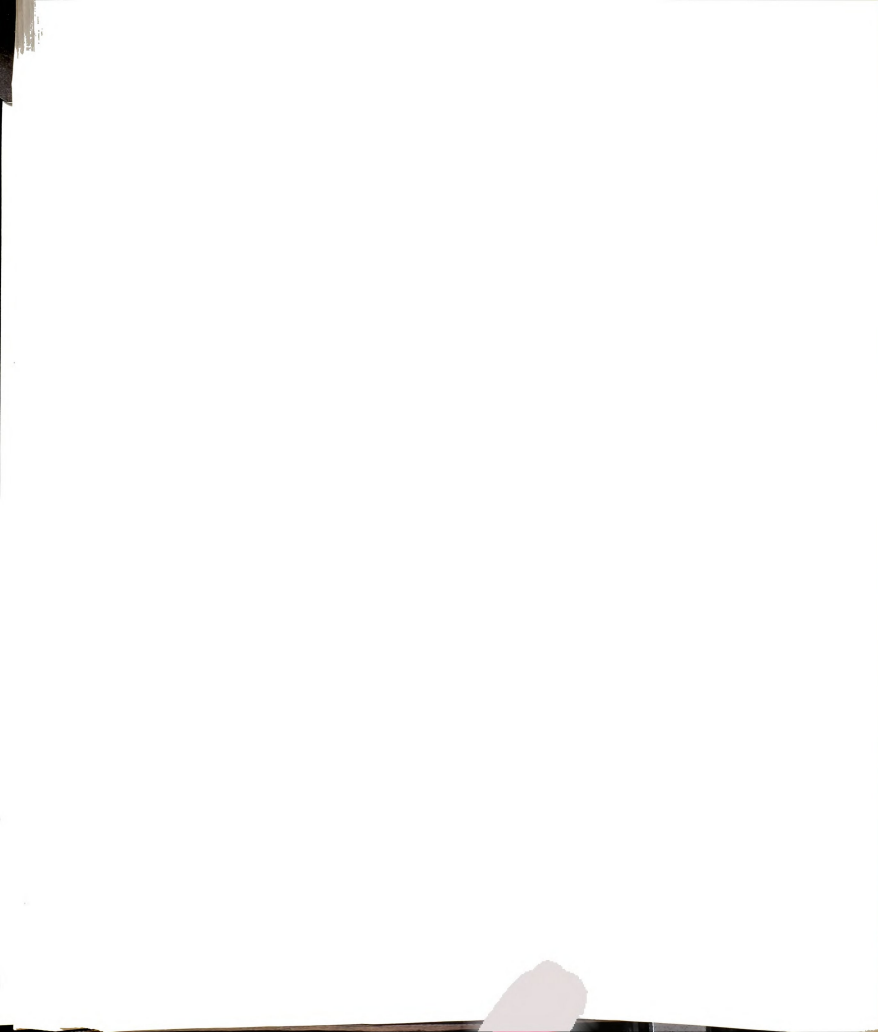


Figure 3.14. Mass spectrum of a compound which co-chromatographed with standard methyl-*cis,trans*-1,2'-epoxy-ionylidene acetate (XV).



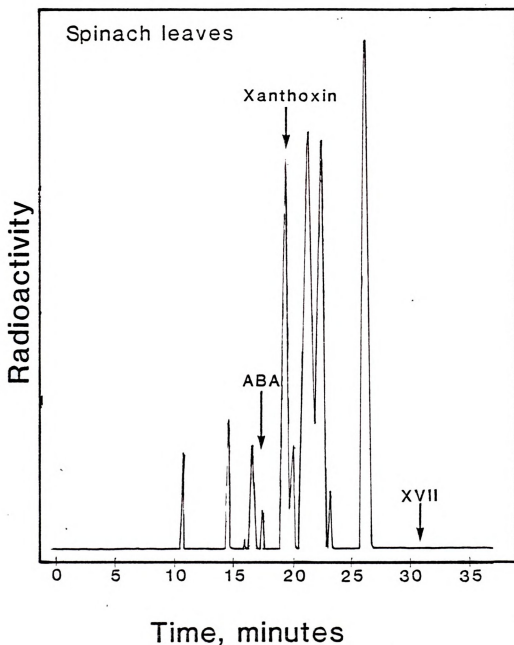
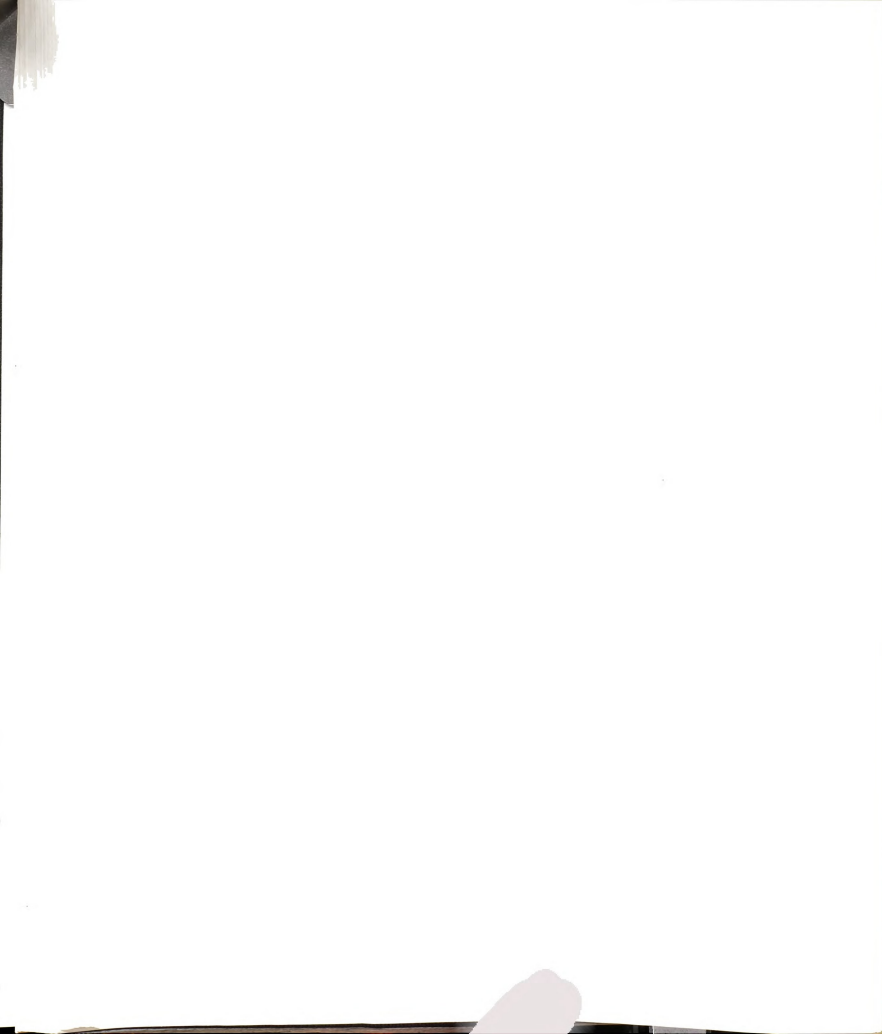


Figure 3.15. Radioactivity in HPLC effluent demonstrating metabolism of ^3H *cis,trans*-1',2'-epoxy-ionylidene acetaldehyde (XVII) in spinach leaves.

The arrows indicate the retention times of standards, and are not meant to indicate the actual presence of these compounds in the extract.



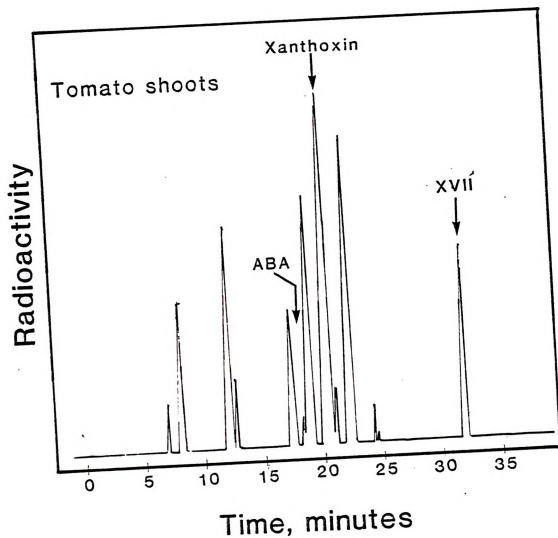


Figure 3.16. Radioactivity in HPLC effluent demonstrating metabolism of ^3H *cis,trans*-1',2'-epoxy-ionylidene acetaldehyde (XVII) in tomato shoots.

The arrows indicate the retention times of standards, and are not meant to indicate the actual presence of these compounds in the extract.



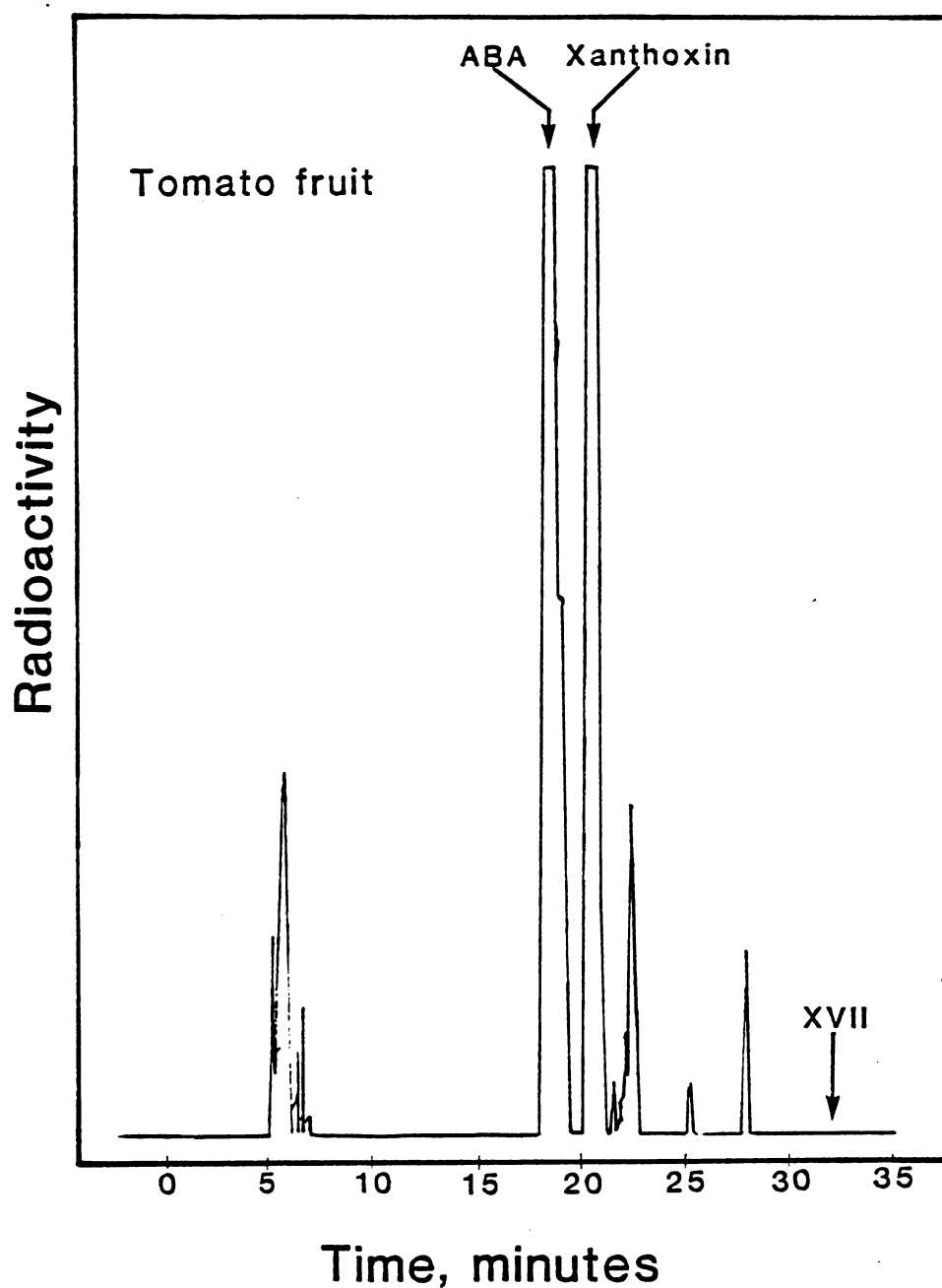


Figure 3.17. Radioactivity in HPLC effluent demonstrating metabolism of ^3H *cis,trans*-1',2'-epoxy-ionylidene acetaldehyde (XVII) in immature (breaker stage) tomato fruit.

The arrows indicate the retention times of standards, and are not meant to indicate the actual presence of these compounds in the extract.

XVII converted to compounds which co-chromatographed with ABA and xanthoxin. When these two peaks (termed fraction 23 for the ABA-like and fraction 27 for the xanthoxin-like) were analyzed by TLC, no radioactivity co-chromatographed with either xanthoxin or ABA. On the contrary, all radioactivity remained at the origin. This behavior is suggestive of either a highly hydroxylated metabolite, or a conjugate.

Basic hydrolysis of a small aliquot and subsequent TLC analysis indicated that the radioactivity still remained at the origin. Thus, the catabolites were not conjugates containing an ester linkage, such as that found in ABA-GE. Another aliquot was hydrolyzed with pectinase AC, which cleaves glycosidic linkages. After this treatment, radioactivity no longer remained at the origin on TLC plates. The major peak of radioactivity did not co-chromatograph with either xanthoxin or ABA, although a small minor peak did with xanthoxin. Both fractions had similar R_f values with TLC after hydrolysis with pectinase. The mobility was not changed when a small sample was methylated with ethereal diazomethane, indicating that no carboxyl group was present.

3.4. DISCUSSION

As shown in section 3.3.1, X had greater biological activity than ABA. Similar results have been obtained by Kumamoto *et al.* (1970). It is highly unlikely that a precursor

to ABA would have more biological activity than ABA. It appears that the structural similarity of X rather than biochemical conversion to ABA is responsible for the results obtained with the *Avena* coleoptile elongation bioassay.

In addition, the results described here with XV (section 3.3.2) and those with xanthoxin (Taylor and Burden, 1973) indicate that compounds that contain an epoxide at the 1',2' position can be converted to ABA. Thus it was unexpected that XVI I was not converted to ABA by immature tomato fruit (section 3.3.3).

Preliminary structural analysis of the catabolites formed in tomato fruit indicate that they may be diastereomers, i.e. compounds which contain two or more optically active centers. XV was synthesized as a racemic mixture at the epoxide group. Addition of a sugar via a glycosidic linkage would create another chiral center. Since diastereomers have different physical properties (such as melting points, solubilities in different solvents, etc.) it is possible to separate them on HPLC. Removal of the sugar linkage eliminates a center of optical activity and results in one and the same compound, although it should differ in the orientation of the epoxide group. When the fractions treated with pectinase were analyzed by TLC they had identical chromatographic behavior. The catabolites also appeared to retain the aldehyde, rather than it being converted to a carboxylic acid group, since treatment with diazomethane did not alter the R_f.

The fact that a glycosidic linkage is present in the tomato fruit catabolites implies that XVII was hydroxylated and then had a sugar attached to the hydroxyl group. The location of the hydroxyl group to which the sugar is conjugated is unknown, but it is not at C-4'. If this were the case, then the compound would be xanthoxin. This cannot be, since by TLC analysis the majority of radioactivity (after pectinase AC treatment) did not co-chromatograph with standard xanthoxin. It is possible that the epoxide opened up giving rise to a di-hydroxy compound. Another possibility is that IX was hydroxylated at positions in the ring other than at C-4'. This latter case seems unlikely given the relative non-polar nature (after pectinase AC treatment) of the catabolite.

It is apparent that C₁₅ compounds such as X, XV, and XVII are extensively metabolized (Lehmann and Schütte, 1976; Milborrow and Noddle, 1970; Milborrow and Garmston, 1973). In cases where incorporation into ABA (and ABA catabolites) was described, the percent incorporation was very low. This low amount of incorporation could be explained if the ABA biosynthetic pathway is highly compartmentalized and the fed compounds never reached the site of ABA biosynthesis. It is also possible that conjugation or oxidation of these C₁₅ compounds occurred rapidly as they entered the cytoplasm. Thus, the majority of the fed compounds would not have a chance to enter the ABA biosynthetic pathway.

Another possible explanation is that the ABA biosyn-



thetic pathway is catalyzed by a multi-enzyme complex (Stafford, 1981). In this case, due to metabolic channeling, the efficiency of incorporation would be very low, unless the correct intermediate in the pathway was fed. Penetration of the fed compound to the active sites in the complex would be low due to competition with endogenous material already bound.

3.5. LITERATURE CITED

- Attenburrow, J., Cameron, A.F.B., Chapman, J.H., Evams, R.M., Hems, B.A., Jansen, A.B.A., Walker, T. (1952) A synthesis of vitamin A from cyclohexanone. J. Chem. Soc. pp. 1094-1111.
- Ichimura, M., Oritani, T., Yamashita, K. (1983) The metabolism of (2Z, 4E)- α -ionylideneacetic acid in *Cercospora cruenta*, a fungus producing (+)-abscisic acid. Agr. Biol. Chem. 47:1895-1900.
- Kienzle, F., Mayer, H., Minder, R.E., and Thommen, H. (1978) Synthese von optisch aktiven, natürlichen Carotinoiden und strukturell verwandten Verbindungen. III. Synthese von (+)-Abscisinsäure, (-)-Xanthoxin, (-)-Loliolid, (-)-Actinidiolid und (-)-Dihydroactinidiolid. Helv. Chim. Acta 61:2616-2627.
- Kumamoto, J., Smith, O.E., Asmundson, C.M., Ingersoll, R.B., Sadri, H.A. (1970) *Cis,trans*- α -ionylideneacetic acid: a bioactive analog of abscisic acid. J. Agr. Food Chem. 18:531-533.
- Lehmann, H., Schütte, H.R. (1976) Biochemistry of phyto-effectors. 9. The metabolism of α -ionylideneacetic acid in *Hordeum distichon*. Biochem. Physiol. Pflanzen. 169:55-61.
- Milborrow, B.V. (1978) Abscisic acid. In: Phytohormones and related compounds-a comprehensive treatise, pp. 295-397, vol 1, Letham D.S., Goodwin, P.B., Higgins, T.J.V., eds. Amsterdam: Elsevier.
- Milborrow, B.V. (1983) Pathways to and from abscisic acid. In: Abscisic acid, pp.79-111, Addicott, F.T., ed. New York: Praeger Press.



- Milborrow, B.V., Garmston, M. (1973) Formation of (-)-1',2'-epi-2-cis-xanthoxin acid from a precursor of abscisic acid. *Phytochemistry* 12:1597-1608.
- Milborrow, B.V., Noddle, R.C. (1970) Conversion of 5-(1,2-epoxy-2,6,6-trimethylcyclohexyl)-3-methyl-penta-cis-2-trans-4-dienoic acid into abscisic acid in plants. *Biochem. J.* 119:727-734.
- Neill, S.J., Horgan, R. (1983) Incorporation of α -ionylidene ethanol and α -ionylidene acetic acid into abscisic acid by *Cercospora rosicola*. *Phytochemistry* 22:2469-2472.
- Neill, S.J., Horgan, R., Lee, T.S., Walton, D.C. (1981) 3-methyl-5-(4-oxo-2',6',6'-trimethylcyclohex-2'-enyl)-2,4-pentadienoic acid, a putative precursor of abscisic acid from *Cercospora rosicola*. *FEBS Lett.* 128:30-32.
- Oritani, T., Ichimura, M., Yamashita, K. (1982) The metabolism of (2Z,4E)- α -ionylideneacetic acid in *Cercospora cruenta*, a fungus producing (+) abscisic acid. *Agr. Biol. Chem.* 46:1959-1962.
- Oritani, T., Yamashita, K. (1985) Conversion of (2Z,4E)-5-(1',2'-epoxy-2',6',6'-trimethylcyclohexyl)-3-methyl-2,4-pentadienoic acid to xanthoxin acid by *Cercospora cruenta*, fungus producing (+)-abscisic acid. *Phytochemistry* 24:1957-1961.
- Stauffer, H.A. (1981) Compartmentation in natural product biosynthesis by multienzyme complexes. In: *The biochemistry of plants*, pp. 117-37, vol. 7, Stumpf, P.K., Conn, E.E., eds. New York: Academic Press.
- Taylor, H.F., Burden, R.S. (1970) Identification of plant growth inhibitors produced by photolysis of violaxanthin. *Phytochemistry* 9:2217-2223.
- Taylor, H.F., Burden, R.S. (1973) Preparation and metabolism of 2-¹⁴C-cis,trans xanthoxin. *J. Exp. Bot.* 24:873-880.
- Walton, D.C. (1983) Structure-activity relationships of abscisic acid analogs and metabolites. In: *Abscisic acid*, pp.113-146, Addicott, F.T., ed. New York: Praeger Press.
- Walton, D.C., Li, Y., Neill, S.J., Horgan, R. (1985) Biosynthesis of abscisic acid: a progress report. In: *Current topics in plant biochemistry and physiology* 1985, pp. 111-117, vol. 4, Randall, D.D., Blevins, D.G., Larson, R.L., eds. Columbia, MO: University of Missouri.



Walton, D., Wellner, R., Horgan, R. (1977) Synthesis of tritiated abscisic acid of high specific activity. *Phytochemistry* 16:1059-1061.



CHAPTER 4
INCORPORATION OF OXYGEN INTO ABSCISIC ACID
AND PHASEIC ACID FROM MOLECULAR OXYGEN

Incorporation of Oxygen into Absciscic Acid and Phaseic Acid from Molecular Oxygen¹

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

Absciscic acid accumulates in detached, wilted leaves² of *Xanthium strumarium*. When these leaves are subsequently rehydrated, phaseic acid, a catabolite of absciscic acid, accumulates. Analysis by gas chromatography-mass spectrometry of phaseic acid isolated from stressed and subsequently rehydrated leaves placed in an atmosphere containing 20% ¹⁸O₂ and 80% N₂ indicates that one atom of ¹⁸O is incorporated in the 6'-hydroxymethyl group of phaseic acid. This suggests that the enzyme that converts absciscic acid to phaseic acid is an oxygenase.

Analysis by gas chromatography-mass spectrometry of absciscic acid isolated from stressed leaves kept in an atmosphere containing ¹⁸O₂ indicates that one atom of ¹⁸O is present in the carboxyl group of absciscic acid. Thus, when absciscic acid accumulates in water-stressed leaves, only one of the four oxygens present in the absciscic 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 absciscic acid arise from water, or (b) there exists a stored precursor with oxygen atoms already present in the 1'- and 4'-positions of absciscic acid which is converted to absciscic 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'-car-

bons 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 ¹⁸O₂ into each molecule of product (11).

We decided to study the origin of the oxygen atoms in ABA and PA by the use of ¹⁸O₂. 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 ¹⁸O₂, 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 N₂. 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 ¹⁸O₂, 50 ml of ¹⁸O₂ was added to the flask after 2 evacuation cycles and then the flask was filled with N₂. For experiments involving only ABA, a similar procedure was used except that after the leaves were wilted, they were immediately placed under N₂, or under a mixture of ¹⁸O₂ and N₂ as described above.

The primary leaves of *Phaseolus vulgaris* L., cv Redcloud 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, *p*-D-glucopyranosyl abscisate; GC-SIM, gas chromatography-selected ion monitoring; m/z, mass/charge.

¹⁸O INCORPORATION INTO ABA AND PA

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 ¹⁸O₂ 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. ¹⁸O₂ was purchased from Stohler Isotope Chemicals Inc. (49 Jones Road, Waltham, MA). H₂¹⁸O was purchased from Kor. Inc. (56 Rogers Street, Cambridge, MA). One atom of ¹⁸O was exchanged into the 4'-keto group of (±)-ABA (Sigma) by placing (±)-ABA in H₂¹⁸O 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 Zeevaert (15, 16). For experiments involving only ABA, samples were extracted according to Zeevaert (15). ABA was further purified by semi-preparative reverse phase HPLC on a μ Bondapak C₁₈ (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 μ Bondapak NH₂ 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 ⁶³Ni-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 μ m). GLC conditions were: oven temperature 165°C, H₂ 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'-¹⁸O-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.

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 N₂, or room air was allowed to enter the flask and the flask was then resealed.

Treatment of Leaves	ABA	PA	ABA-GE
	$\mu\text{g} \cdot \text{g}^{-1}$ 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

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'-¹⁸O-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 Vanan-Mat GD-150 magnetic sector mass spectrometer.

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 N₂, 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% ¹⁸O₂ and 80% N₂ 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 ¹⁸O into the PA molecules (Fig. 1). The presence of m/z 294 in the mass spectrum of PA isolated from leaves incubated with ¹⁸O₂ (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. Zeevaert, unpublished results), we conclude that the atom of ¹⁸O 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, 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 CH₂O) 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% ¹⁸O₂, 4% ¹⁸O₂, and 80% N₂ 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 ¹⁸O is incorporated into the ABA molecule. Similar results have been obtained with *P. vulgaris* cv Redcloud (data not shown). The atom of ¹⁸O 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 (COOCH₃⁺) 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

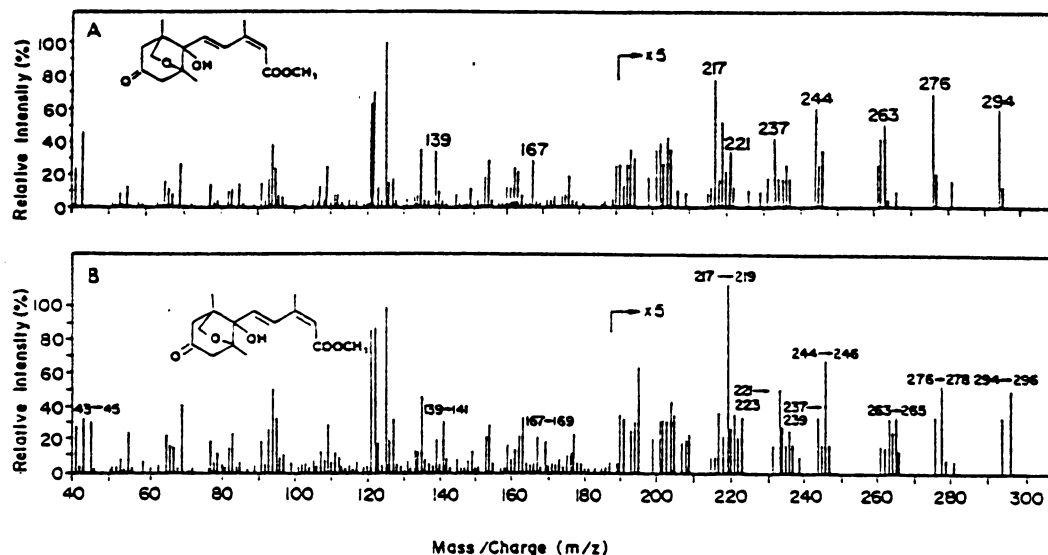


FIG. 1. Mass spectra of PA isolated from stressed and subsequently rehydrated *Xanthium* leaves incubated in room air (A), or $^{18}\text{O}_2$ (B).

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 $\mu\text{g g}^{-1}$ dry wt
Control 8 h	5.3
Stressed 8 h	24.5
Stressed $\rightarrow$ vacuum $\rightarrow$ room air 8 h	21.0
Stressed $\rightarrow$ vacuum $\rightarrow$ 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.

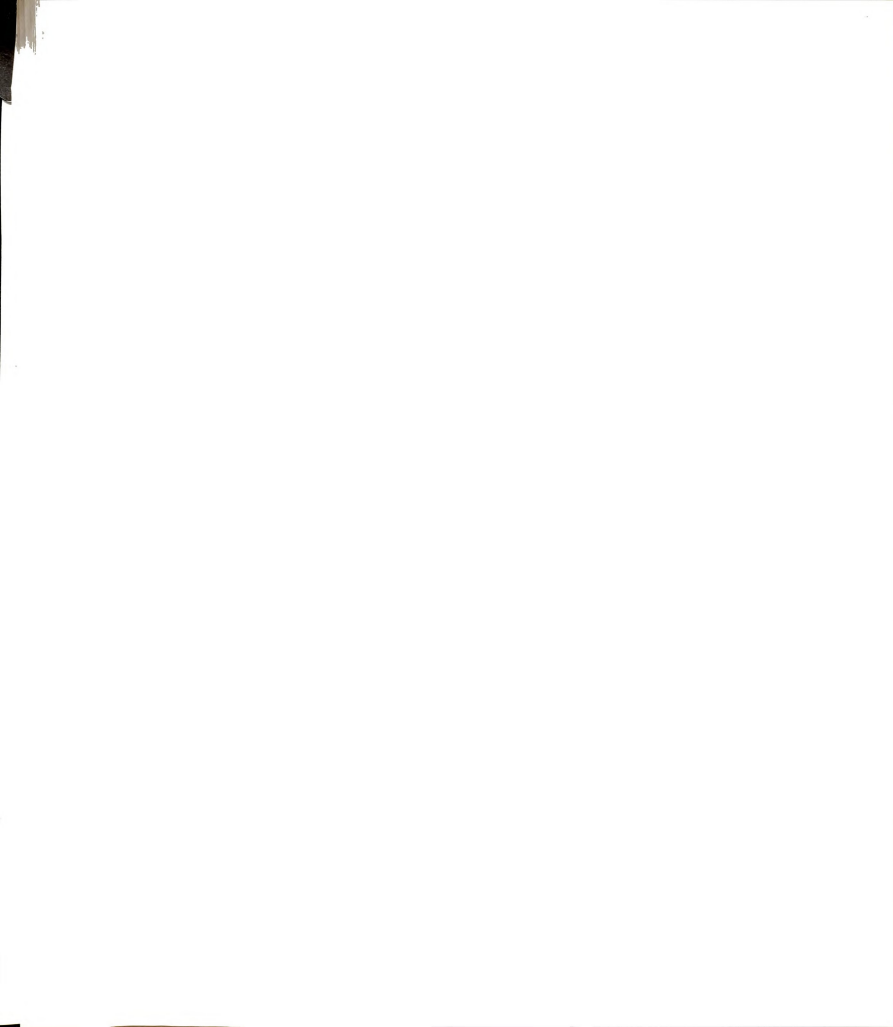
Exchange of oxygen from the 1'-hydroxyl group of ABA with oxygen present in water during sample extraction is highly unlikely, since it was necessary to use synthetic methods to introduce ^{18}O in the 1'-hydroxyl group (4). It is also unlikely that the oxygen from the 4'-keto group or 1-carboxyl group 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 ^3H MVA fed to avocado fruit. ^{14}C and ^3H were both found in carotenoids, yet only ^3H 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



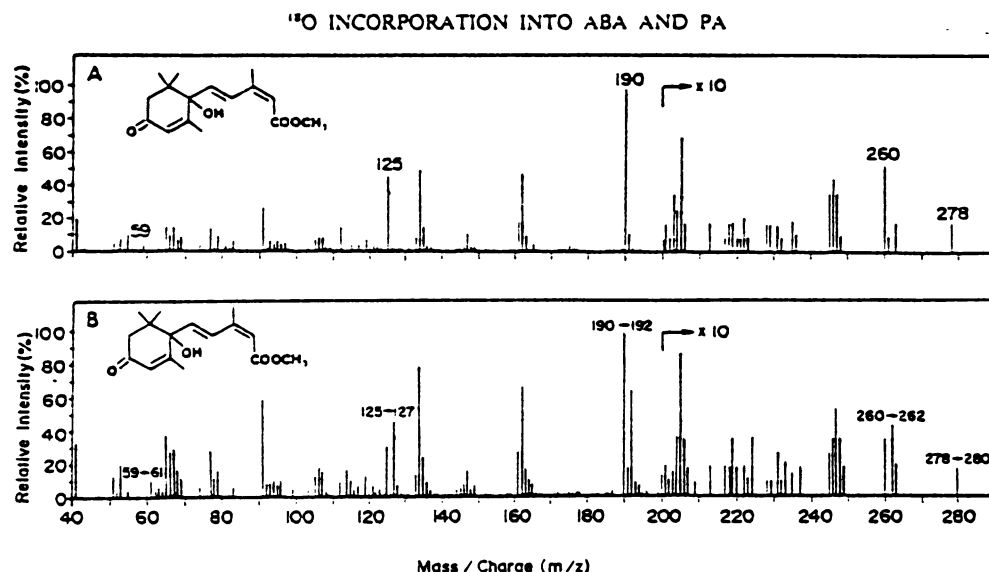


FIG. 2. Mass spectra of ABA isolated from stressed *Xanthium* leaves incubated in room air (A), or ¹⁸O₂ (B).

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 ²H- 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 ¹⁸O₂, one atom of ¹⁸O 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 ¹⁸O₂, one atom of ¹⁸O 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 ¹⁸O₂ labeling experiments with the fungus *Cercospora rosicola*. In this case, ABA contained four ¹⁸O atoms when the fungus was cultured over a 48-h period under an atmosphere containing 20% ¹⁸O₂ (R. Horgan and D. C. Walton, personal communications).

Acknowledgments—We would like to thank J. Throck Watson and Brian

Muselman 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. Shimer for MS analysis of gas samples.

LITERATURE CITED

1. FIEN 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 Absciscic acid metabolism by a cell-free preparation from *Echinocystis lobata* liquid endosperm. *Plant Physiol* 58: 790-795
3. GILLET 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 SCHUTTE 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. *Absciscic 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 *Rhodospseudomonas spheroides* carotenoid pigment conversion. *Biochim Biophys Acta* 63: 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-[¹⁴C]-*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 ¹⁸O 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 (+)-*isocisic* 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*-dihydrophenolic acid in *Phaseolus vulgaris*. *Phytochemistry* 15: 493-500

CHAPTER 5
ACCUMULATION OF ABSCISIC AND PHASEIC ACID IN
XANTHIUM STRUMARIUM LEAVES UNDER DIFFERENT
OXYGEN TENSIONS

5.1. INTRODUCTION

In chapter 4 it was shown that the formation of ABA and PA requires molecular oxygen. Hence, an enzyme(s) in the ABA biosynthetic pathway must at some step(s) require oxygen, as do mono-oxygenases and oxidases. The enzyme involved in the conversion of ABA to PA is presumed to be a mono-oxygenase (Gillard and Walton, 1976).

Some oxidases, such as ascorbic acid oxidase, are not saturated at a normal atmospheric oxygen tension of 20% (Thimann et al., 1954). With polyphenol oxidase, the K_m for oxygen was found to vary with substrate; with the exception of pyrocatechol, the enzyme-substrate complexes were saturated at room oxygen partial pressure (Butt, 1980).

We decided to determine the effect of different oxygen tensions on the accumulation of ABA and PA. *Xanthium* leaves are ideal for this study because they have the ability to accumulate rapidly either ABA or PA, depending on how the leaves are manipulated (Zeevaart, 1980).

5.2. MATERIALS AND METHODS

5.2.1. Plant material. *X. strumarium* was grown as previously described (Zeevaart, 1980). The youngest, fully expanded leaves were used in all experiments. For experiments involving ABA accumulation, leaves were excised and stressed by allowing them to lose 15% of their fresh weight. They were



then placed in 125 ml Erlenmyer flasks. For PA, the same procedure was followed as for ABA, except that after imposing stress they were placed in a plastic bag for six h. The leaves were rehydrated by submerging them in distilled water for five min and then placed in 125 ml Erlenmyer flasks. The flasks (closed with serum stoppers) were then subjected to a vacuum-flush treatment as described in chapter 4. A vacuum was created in the flasks with a vacuum pump. The flasks were then flushed with nitrogen. This was repeated three times. Suitable amounts of oxygen and nitrogen were then added to the flasks. For experiments dealing with ABA leaves were incubated under different oxygen concentrations for six h, and for PA five h.

All experiments were performed four times with two leaves per treatment. Similar results were obtained in all experiments.

5.2.2. Extraction and purification procedure. ABA and PA were extracted and purified as described in chapters 4 and 9 for ABA. Relevant HPLC fractions (determined with standard ABA and PA) were collected, dried, and methylated with ethereal diazomethane. ABA was analyzed by GLC-ECD as described in chapters 4 and 9. PA was analyzed in a similar manner except that the oven temperature was held isothermally at 187 C.

5.2.3. Oxygen measurements. Oxygen content in the flasks was measured at the beginning and end of the incubation period by GLC-TCD using a Varian 3700 gas chromatograph.

Analysis of the gas mixtures was performed with a molecular sieve column (5A, 45-60 mesh, 2 m x 1/8 inch stainless steel). Quantitation was performed using standards of 0, 2, 20 and 100% oxygen. The balance, if any, consisted of nitrogen. GLC conditions were: oven temperature 65 C, injector temperature 100 C, detector temperature 120 C, detector current 108 mA, He carrier flow 26 ml/min.

5.3. RESULTS

The oxygen tension in the flasks varied from 0 to 90% oxygen. Some respiration was noted in all flasks (except 0%); the amount of oxygen consumed was usually 2-3%. All data presented are related to the concentration of oxygen present at the beginning of the experiment.

The production of PA was not increased at tensions greater than 20% oxygen (Figure 5.1). On the other hand, ABA accumulation was not saturated until 60% oxygen was present (Figure 5.2).

5.4. DISCUSSION

The net accumulation of ABA depends on both its formation and degradation. Since the conversion of ABA to PA is not affected (Figure 5.1), the data described here imply that the oxygen requiring step(s) must be prior to ABA.

In addition, the enzyme responsible for the conversion

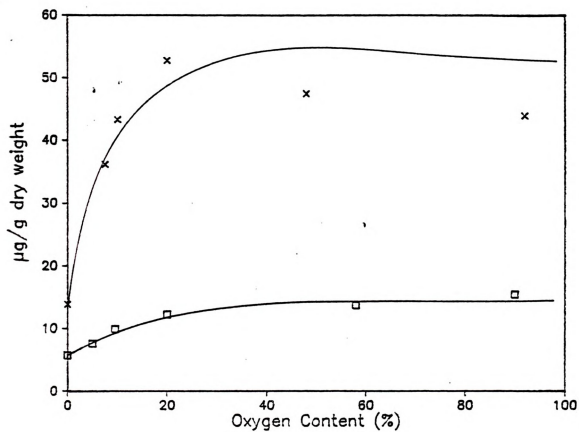


Figure 5.1. Accumulation of phaseic acid under different oxygen tensions.

The accumulation of PA in stressed ($\square$) and stressed and subsequently rehydrated leaves ($\times$) was saturated at 20% oxygen content.



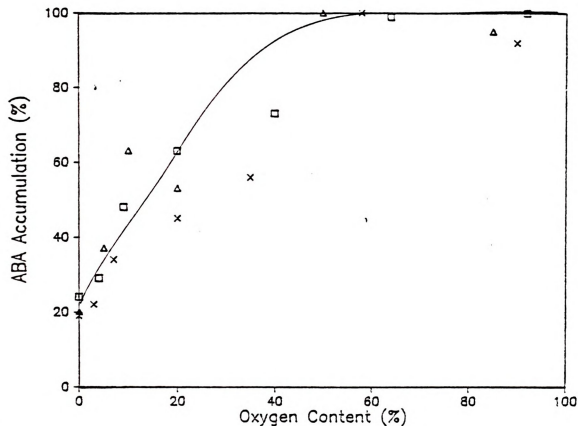


Figure 5.2. Accumulation of abscisic acid under different oxygen tensions.

The accumulation of ABA in stressed *Xanthium* leaves was not saturated until about 50-60% oxygen. Shown are the results from 3 experiments. The maximum accumulation in experiment 1 (□), 2 (Δ), and 3 (x) was 49.0, 59.5, and 43.0 g/μg dry weight, respectively.

of ABA to PA (ABA hydroxylase) appears to be similar to other mono-oxygenases in that ABA hydroxylase is saturated at atmospheric oxygen tensions. However, the enzyme(s) responsible for ABA biosynthesis appear to be different. The mechanism of the introduction of oxygen into ABA might be similar to the mechanism used by ascorbic acid or polyphenol oxidase. This is based on the observation that increasing the oxygen tension does not saturate these oxidases nor the ability to accumulate ABA.

5.5. LITERATURE CITED

- Butt, V.S. (1980) Direct oxidases and related enzymes. In: The biochemistry of plants, pp 81-123, vol 2, Stumpf, P.K., Conn, E.E., eds. New York: Academic Press.
- Gillard, D.F., Walton, D.C. (1976) Absciscic acid metabolism by a cell-free preparation from *Echinocystis lobata* liquid endosperm. Plant Physiol. 58:790-795.
- Thimann, K.V., Yocum, C.S., Hackett, D.P. (1954) Terminal oxidases and growth in plant tissues. III. Terminal oxidation in potato tuber tissue. Arch. Biochem. Biophys. 53:239-257.
- Zeevaart, J.A.D. (1980) Changes in the level of absciscic acid and its metabolites in excised leaf blades of *Xanthium strumarium* during and after water stress. Plant Physiol. 66:672-678.

Chapter 6

INCORPORATION OF MOLECULAR OXYGEN INTO ABSCISIC
AND PHASEIC ACID IN LEAVES AND ROOTS OF *XANTHIUM*
STRUMARIUM DURING LONG TERM INCUBATIONS IN $^{18}\text{O}_2$

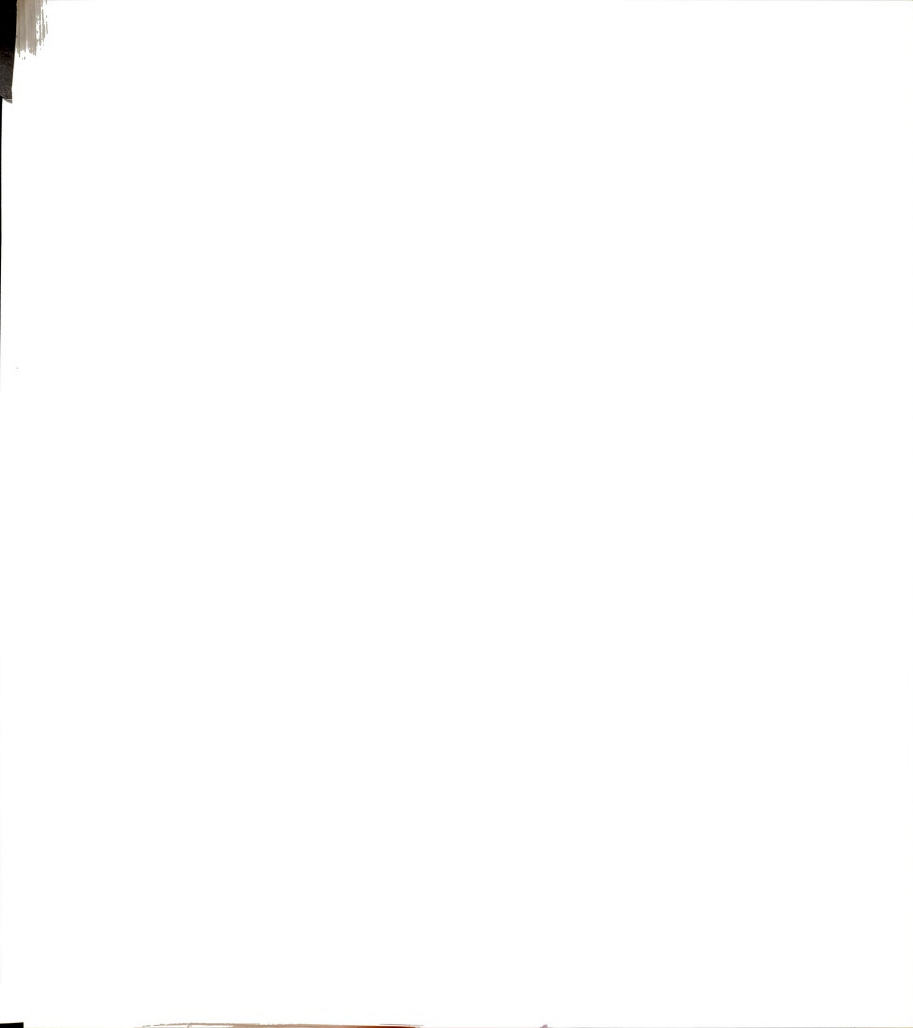


6.1. INTRODUCTION

Nothing is known about the biosynthetic pathway of ABA, except that as a sesquiterpenoid, ABA is ultimately derived from MVA. Research on ABA biosynthesis has focused primarily on two pathways: (a) the direct pathway involving farnesyl pyrophosphate, and (b) the indirect pathway involving a carotenoid, with xanthoxin as an intermediate in the pathway.

In chapter 4 evidence was presented indicating that one atom of ^{18}O was incorporated into the carboxyl group of ABA isolated from stressed *Xanthium* leaves incubated in the presence of $^{18}\text{O}_2$. This indicated that ABA is derived from a carotenoid precursor. In addition, in chapter 4 data were presented showing that the conversion of ABA to PA required molecular oxygen, with one atom of ^{18}O incorporated into what eventually forms the lactone oxygen of PA. Since these experiments were done using relatively short incubations (6 h), stressed *Xanthium* leaves were incubated with $^{18}\text{O}_2$ for 12 and 24 h to see if the incorporation of ^{18}O was changed under these conditions.

Detached roots of *Xanthium* also accumulate ABA, although the levels are lower than in stressed leaves. ABA levels increased up to 100-fold in stressed roots, while detached, stressed leaves accumulated only 15 times that found in turgid leaves (Cornish and Zeevaart, 1985). Thus, the possibility exists that the biosynthetic pathway for stress-induced ABA may be different in roots from the one operating



in leaves.

In order to measure low levels of ^{18}O incorporation, a highly sensitive mass spectrometer must be used. The procedure used must also measure the molecular ion as major ion. ABA has a small molecular ion, and analysis of the amount of incorporation in fragment ions is complicated. NCI has high sensitivity to electrophilic compounds (such as ABA) and causes little fragmentation (Watson, 1985). Furthermore, because of the low amount of energy given to the analyte, CI mass spectra contain fewer ions than the corresponding EI mass spectra, with the molecular ion (M^-) usually predominating.

We incubated stressed *Xanthium* leaves and roots in atmospheres containing 20% $^{18}\text{O}_2$ for various times and determined the amount of incorporation and location of ^{18}O in ABA and PA using NCI and EI. Levels of carotenoids were also measured in turgid leaves and roots because carotenoids may be important as precursors to ABA.

6.2. MATERIALS AND METHODS

6.2.1. Culture of Plant Material. *Xanthium strumarium* L., Chicago strain, was grown as before (Zeevaart, 1980). The youngest, fully expanded leaf was used in all experiments. Leaves were wilted until they had lost 13% of their fresh weight and were then placed in atmospheres of 80% N_2 and $^{18}\text{O}_2$ as described in chapter 4. Leaves were incubated for 6, 12,

and 24 h.

Roots of *Xanthium strumarium* were grown in solution culture as follows. Seeds were soaked in water for 2 d and embryos were removed and placed on moist filter paper in Petri dishes. After a further 2 d incubation, young seedlings were placed on a bed of soil and vermiculite was layered over them. After approximately 2 weeks of growth, the young plants were removed from the soil/vermiculite and suspended from perforated boards over trays containing half-strength Hoagland nutrient solution. The plants were then grown in a growth chamber for 2 weeks, with a change of the nutrient solution after 1 week.

In all experiments, roots were analyzed that had little secondary thickening. For stress experiments, the roots were detached from the plants, cut into approximately 2 cm lengths, and stressed by allowing them to lose 60-70% of their fresh weight (Cornish and Zeevaart, 1985). They were placed in an atmosphere of 80% N₂ and ¹⁸O₂ as described above for leaves. Roots were incubated in this mixture for 6 h.

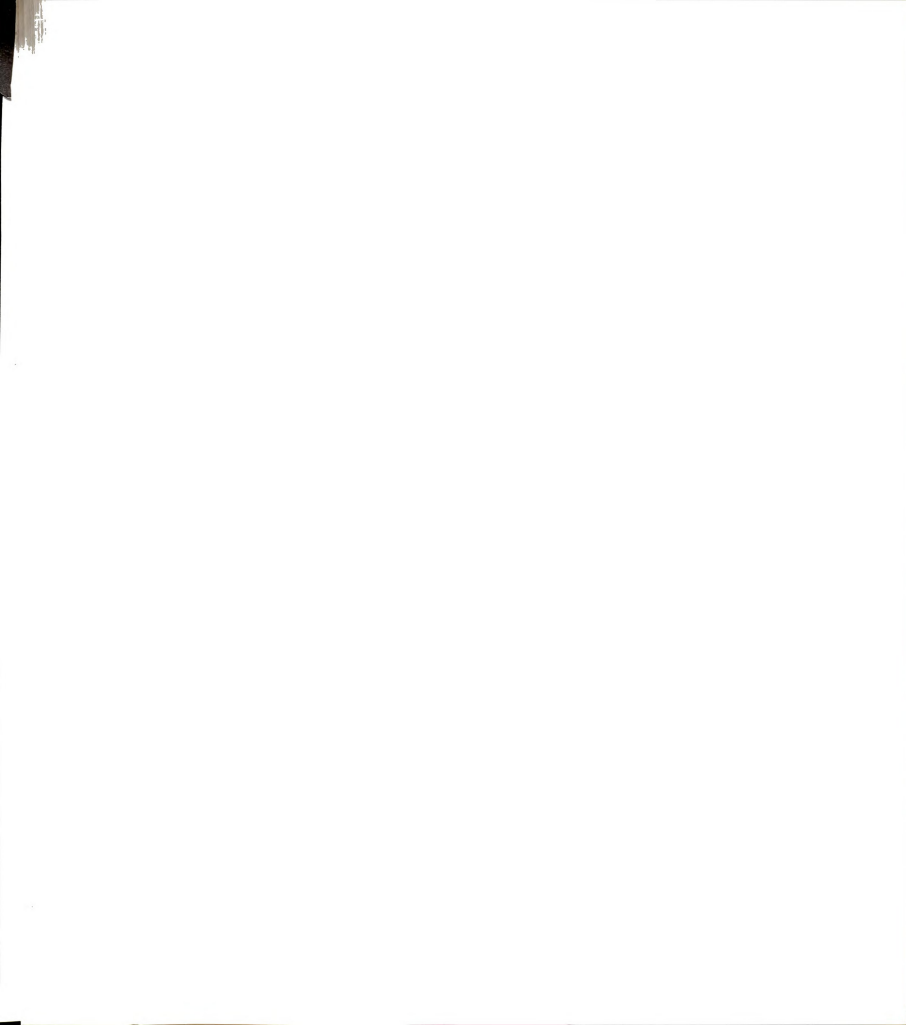
6.2.2. Extraction and Purification of Absciscic and Phaseic Acid. ABA and PA were purified as described in chapters 4, 9, and Cornish and Zeevaart (1984).

6.2.3. Oxygen measurements. To insure that depletion of oxygen did not occur during the incubations due to respiration, the oxygen concentration was monitored in all flasks every few h. Oxygen content in the flasks was measured by GLC-TCD using a Varian 3700 gas chromatograph. Analysis of

the gas mixtures was performed with a molecular sieve column (5A, 45-60 mesh, 2 m x 1/8 inch stainless steel). Quantitation was performed using standards of 0, 2, 20 and 100% oxygen. The balance, if any, consisted of nitrogen. GLC conditions were: oven temperature 65 C, injector temperature 100 C, detector temperature 120 C, detector current 108 mA, He carrier flow 26 ml/min.

6.2.4. Purification of Carotenoids. In separate experiments, leaves of *Xanthium* were extracted in methanol until a color-less residue was left. Equal portions of methanol and diethyl ether were mixed together to which three volumes of 10% sodium chloride were added. This had to be done carefully to prevent the formation of emulsions. The combined ether fractions were backwashed with water and dried over anhydrous sodium sulfate. The ether was removed and the residue dissolved in 6% KOH in methanol. After incubation overnight at 4 C, diethyl ether was added to the methanolic solution followed by water. By this procedure, chlorophyll was separated from carotenoids. The ether solution was backwashed with water and dried over anhydrous sodium sulfate. The ether was removed and the residue dissolved in methanol. Total carotenoids were determined according to Davies (1976).

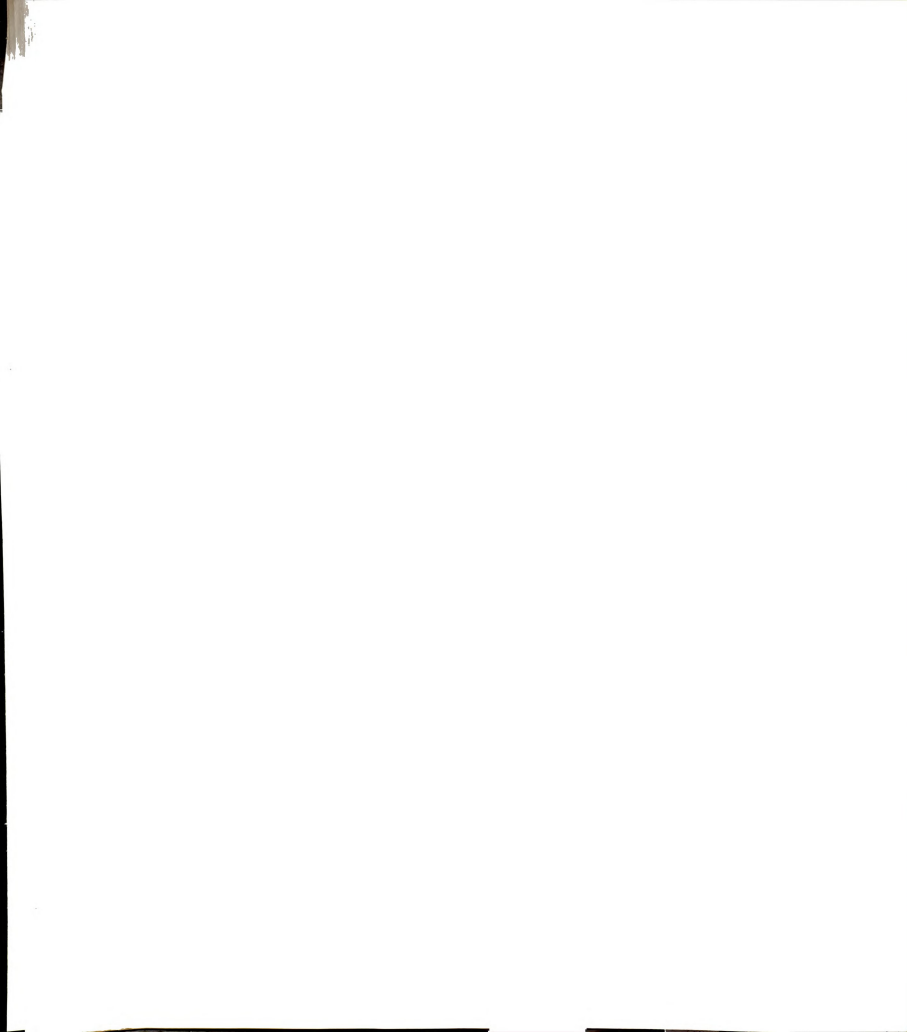
The extraction of carotenoids from roots was performed in a similar manner. Roots were homogenized in methanol containing 100 mg/L BHT and 10 g/L sodium bicarbonate. These were added to prevent oxidation and neutralize organic acids. After extraction in the methanolic solution for 4 to 6 h, the



root residue was extracted twice with diethyl ether. The methanolic solution was mixed with the ether extract and water was added as described above. The ether was dried over sodium sulfate, removed with a rotary evaporator, and the residue dissolved in hexane. This yellow solution was further purified by analytical normal phase HPLC using a Porasil column. Samples were purified by using a gradient of 0 to 66% ethyl acetate in hexane over 40 min. The solvent was delivered by two Waters Model 510 pumps, controlled by a Waters Automated Gradient Controller. Possible carotenoids were detected by a Waters Lambda Max Model 481 spectrophotometer at 450 nm and collected. After removal of HPLC solvent the samples were dissolved in hexane and their visible and UV spectra were recorded. Spectra were obtained with a Perkin-Elmer Lambda 7 UV-VIS spectrophotometer. Solutions were scanned from 550 to 210 nm.

6.2.5. Mass Spectrometry. GC-MS (EI) was performed with the methyl ester of ABA and PA as described in chapter 4 using a 3% SP-2100 column.

GC-NCI was performed using a modified Hewlett-Packard 5985A GC-MS (Her and Watson, 1985). The conditions were as follows: a capillary on-column injector (J & W, Inc.) and a GMCC/90 open split interface (Scientific Instrument, Inc.) were mounted on the 5985A GC-MS. A 55 m DB-1 capillary column (0.32 mm, J & W, Inc.) was used with temperature programming from 50 to 300 C at 10 C/min using He as carrier gas. The pressure of the reagent gas methane was 1.2×10^{-4} torr.



DP-NCI was performed using a JEOL JMS-HX-110HF double focusing mass spectrometer with a JMA-DA5000 data system using methane as the reagent gas (pressure in source 5×10^{-6} torr). The ion source temperature was 115 C with a filament emission current of 300 A and 70 eV. Approximately 2 μ l of each solution used was applied to a direct probe which was then inserted into the source and heated from 50 to 250 C at 32 C/min. The mass spectrum was scanned from 50 to 1000 daltons at 5 s/scan with a resolution (m/m) of 1000. Sensitivity of this procedure was 1 to 5 ng of ABA or PA.

6.2.6. Chemicals. $^{18}\text{O}_2$ (99%) was purchased from Stohler Isotope Chemicals, Inc. (Waltham, MA) or Cambridge Isotopes (Woburn, MA).

6.3. RESULTS

6.3.1. Carotenoid Levels in Leaves and Roots. It was relatively easy to purify carotenoids from leaves compared with roots. In roots, a compound which had absorption in the yellow portion of the visible spectrum, but was not a carotenoid, necessitated further purification of the extract by HPLC (Figure 6.1). This compound eluted in the void volume, and appeared to be present in levels about 100 times that of the carotenoids. Absorption maxima in the visible region of the spectrum (in hexane) for the carotenoids purified from roots are given in Table 6.1. None appeared to match with any of the known carotenoids (Davies, 1976), except for the compound which had maxima at 467, 441, and 419

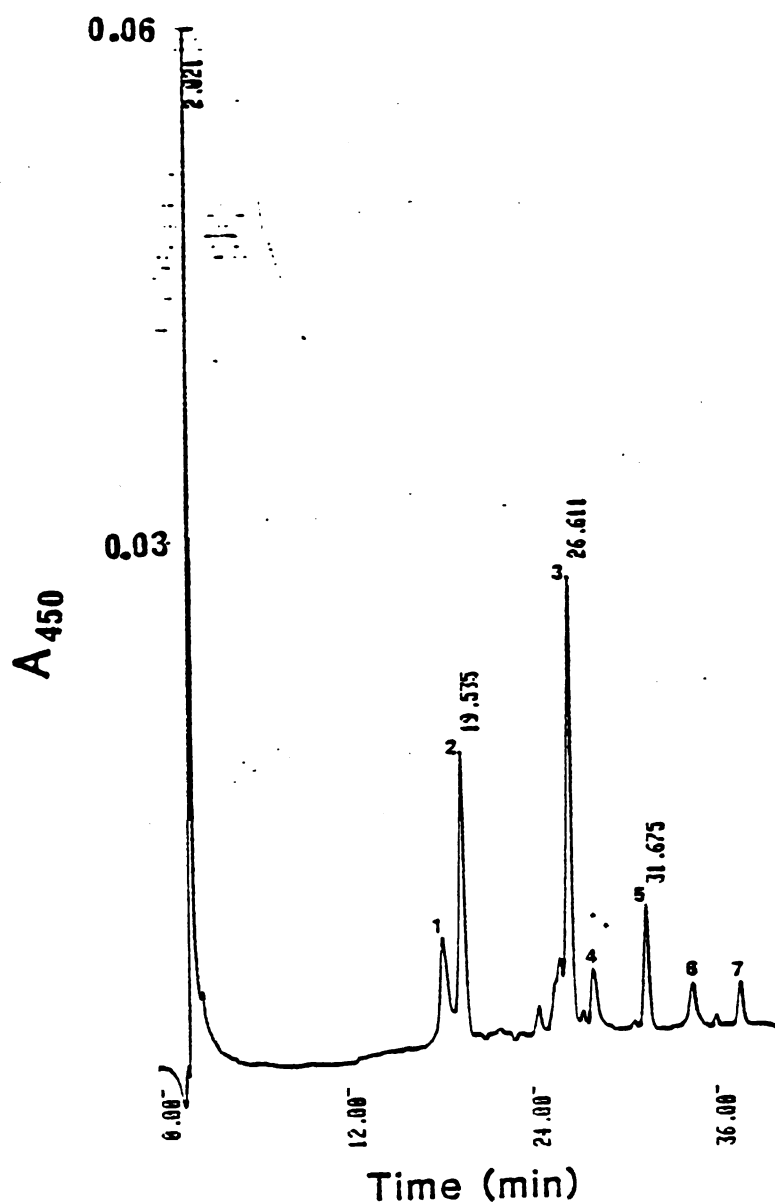


Figure 6.1. Purification of *Xanthium* root carotenoids by HPLC.

Eight compounds having absorbance at 450 nm were resolved using the HPLC system described in the text. The compound eluting in the void volume did not have a carotenoid type spectrum. This figure represents analysis of 1/200 of the total sample.

Table 6.1. Absorption maxima for some carotenoids found in roots of *Xanthium strumarium*.

Spectra were recorded in hexane. Numbers refer to peaks in Figure 6.1.

Peak	Visible maxima (nm)
1	463, 434, 410
2	443, 417, 392
3	463, 439, 416
4	463, 435, 411
5	467, 441, 419
6	447, 423, 400
7	468, 440, 417

nm. These maxima are similar to those found for violaxanthin or neoxanthin. The total amount of carotenoids present in roots and leaves are shown in Table 6.2. For comparison, ABA levels in turgid and stressed tissue are also shown.

6.3.2. Incorporation of ^{18}O into Abscissic and Phaseic Acid During Long Term Incubations in $^{18}\text{O}_2$. After 6 h, there was a prominent incorporation of an atom of ^{18}O into one of the carboxyl oxygen atoms. In addition, small amounts of ^{18}O were found in both ring oxygens of ABA (Table 6.3 and Figure 6.2). With longer incubations a steady decrease in the molecular ion (M^- 278) with an increase in M^- 284 and 286 (two and three ^{18}O atoms incorporated, respectively) was observed. The base peak (M^- 280) remained constant at all time points. The decrease in the molecular ion (M^- 278) is due to turnover of unlabeled ABA that was present at the beginning of the experiment. There was no indication at any time of four ^{18}O atoms incorporated into ABA. However, with *Xanthium* roots, there was a clear indication of incorporation of three atoms of ^{18}O incorporated after 6 h (Figure 6.3).

Incorporation of ^{18}O into PA was also detected during long term incubations (Figure 6.4). In the procedure used here, PA accumulation was not induced by a stress-rehydration cycle. Thus, any PA formed represents turnover of ABA during water-stress. It is for this reason that there is not a large M^-+2 .

The DP-NCI spectra of ABA and PA also show a large M^-+32 . Presumably, this is due to the formation of a methanol

Table 6.2. Levels of carotenoids and ABA in roots and leaves of *Xanthium strumarium*.

The ABA data are taken from Cornish and Zeevaart (1985).

	Total carotenoids	ABA	
		turgid	stress
	$\mu\text{g g}^{-1}$ dry wt	ng g^{-1} dry wt	
Root	9.4	50	1400
Leaf	2350	1300	17000

Table 6.3. Incorporation of ^{18}O into abscisic acid in stressed *Xanthium* leaves.

The values given were obtained by DP-NCI. A value of 100% indicates that it was the most prominent ion. All other ions are shown relative to the base peak.

Incubation time (h)	Per cent of base peak			
	m/e			
	278	280	282	284
6	78.6	100	5.5	1.1
12	35.3	100	7.0	2.0
24	27.3	100	10.5	5.4

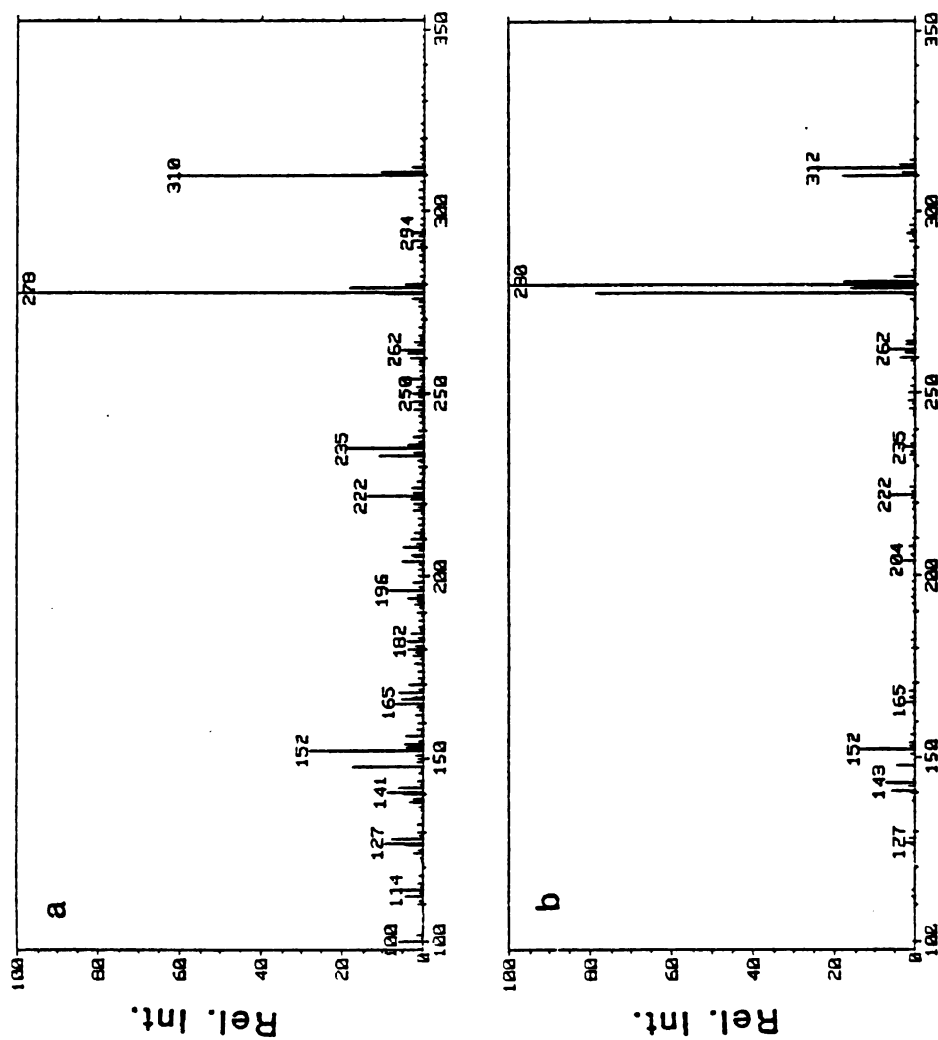


Figure 6.2. Mass spectra of ABA obtained by DP-NCI.

Shown are spectra for standard ABA (a) and ABA isolated from stressed *Xanthium* leaves incubated in $^{18}\text{O}_2$ for 6 h (b), 12 h (c), and 24 h (d). All spectra are of methylated compounds.



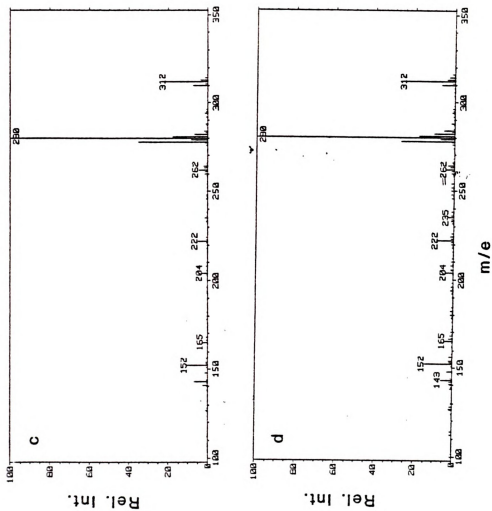


Figure 6.2 (continued)



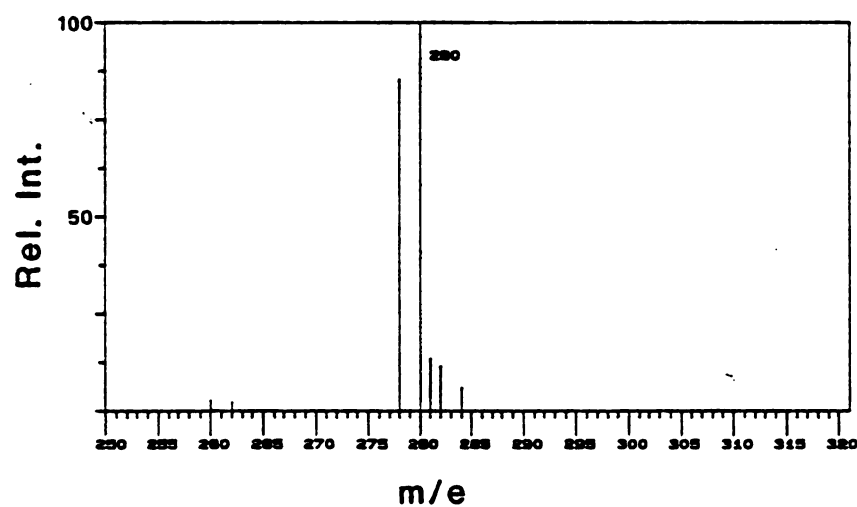


Figure 6.3. Mass spectrum of ABA isolated from stressed *Xanthium* roots incubated in $^{18}\text{O}_2$ for 6 h. The technique used here was GC-NCI.



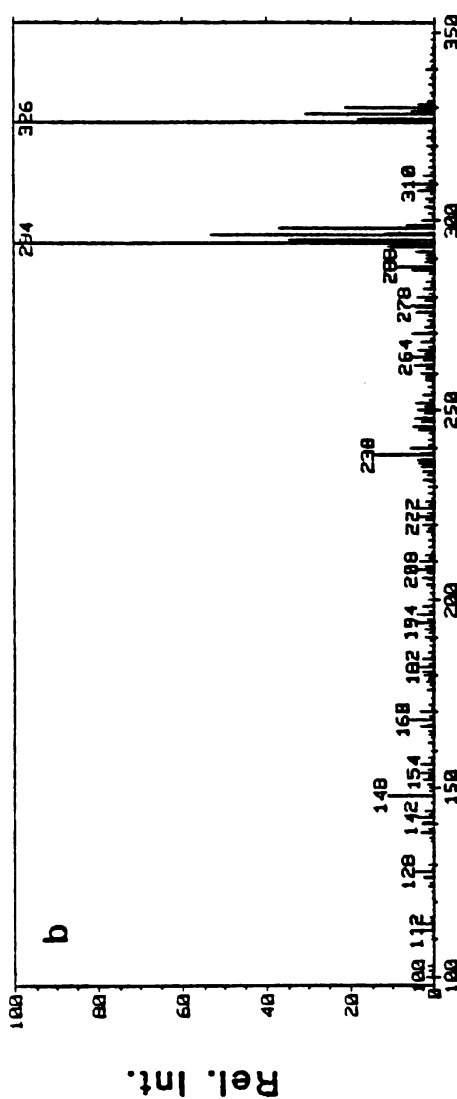
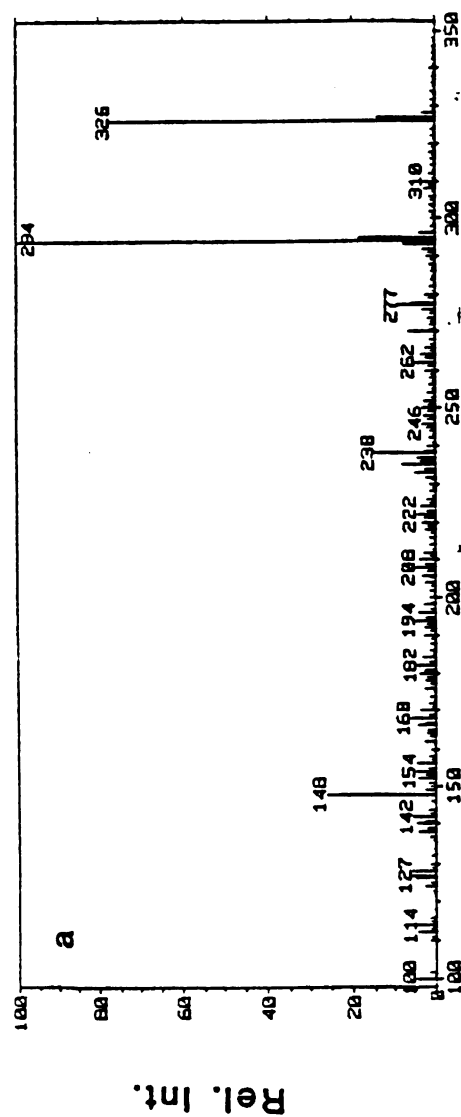


Figure 6.4. Mass spectra of PA obtained by DP-NCI.

Shown are spectra for standard PA (a) and PA isolated from stressed *Xanthium* leaves incubated in $^{18}\text{O}_2$ for 6 h (b), 12 h (c), and 24 h (d). All spectra are of methylated compounds.

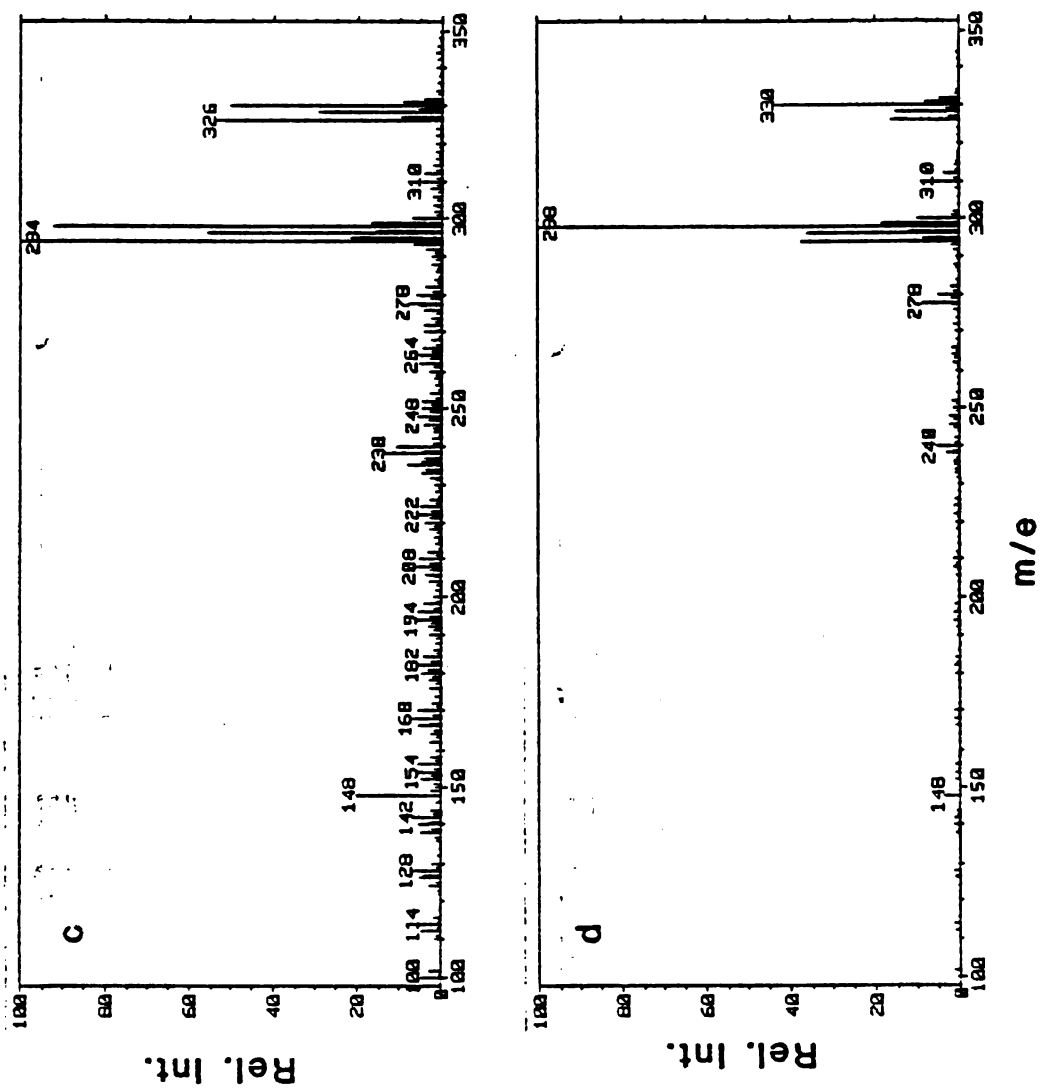


Figure 6.4 (continued)

adduct formed during ionization in the sample chamber. The adduct decreases as the amount of sample is lowered. The origin of the adduct is unknown; however, it is possible that both the methyl ester of ABA and PA could lose methanol in the source. This methanol could then form an adduct with another analyte in the source.

6.4. DISCUSSION

The results described are in agreement with those presented in chapter 4. However, the technique used to measure ^{18}O incorporation here is much more sensitive than the one used to obtain GC-MS (EI) spectra described in chapter 4. The present results indicate that, in addition to one atom of ^{18}O found in the carboxyl group, low amounts of ^{18}O incorporation into the ring oxygens occurred. The localization of the ^{18}O atoms in ABA is based on a GC-MS (EI) spectrum (Figure 6.5) of ABA isolated from stressed *Xanthium* leaves incubated in $^{18}\text{O}_2$ for 24 h. In this spectrum, the molecular ion indicates that three ^{18}O atoms were incorporated. The ion corresponding to the side chain which contains the carboxyl group (m/e 125) is shifted by 2 mass units, indicating one atom of ^{18}O is incorporated. On the other hand, m/e 190, which contains the two ring oxygens and one of the carboxyl oxygens, is shifted by up to six mass units (there are new ions at m/e 192, 194, and 196). This indicates that both ring oxygen atoms as well as one of

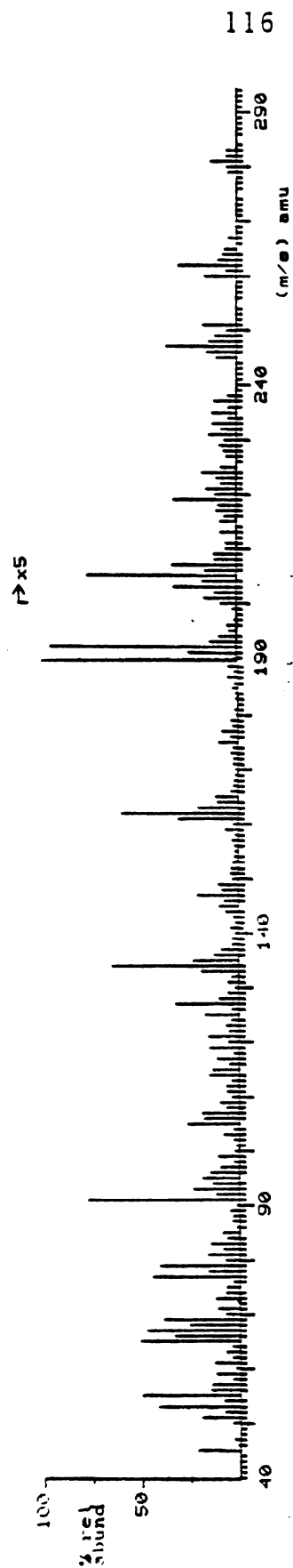
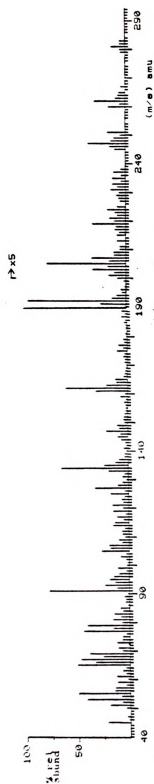


Figure 6.5. Mass spectrum of ABA isolated from stressed *Xanthium* leaves incubated in $^{18}\text{O}_2$ for 24 h.

This spectrum was obtained by GC-MS (EI). Note the difference in the fragmentation pattern (compare with Figure 6.2).



116

Figure 6.5. Mass spectrum of ABA isolated from stressed *Xanthium* leaves incubated in $^{18}\text{O}_2$ for 24 h.

This spectrum was obtained by GC-MS (EI). Note the difference in the fragmentation pattern (compare with Figure 6.2).

the carboxyl group oxygen atoms contain ^{18}O . While dehydration of the molecular ion (m/e 278 to 260) involves primarily the oxygen atom in the tertiary oxygen group of ABA, there is some evidence that the ketone oxygen atom (C-4') also plays a part in this elimination (Gray *et al.*, 1974). Because of this, it is not possible to determine which of the two oxygens in the ring contains the larger fraction of label.

The ^{18}O incorporation into ABA suggests that there is one large, primary precursor pool that forms ABA. This precursor gives rise to the large $M+2$. With incubation periods greater than 6 h, more ^{18}O starts to appear in the ring oxygen atoms. This incorporation implies that there may be other compounds feeding into this large precursor pool which, during the conversion to this pool, incorporate ^{18}O into positions which ultimately form the ring oxygens of ABA. On the other hand, there could be three separate pathways to ABA. In this case there would be three different precursors which incorporate one, two, or three atoms of ^{18}O respectively during their conversion to ABA. One compound (one atom of ^{18}O incorporated) would be rapidly and the other two slowly converted to ABA. However, in roots, this precursor appears to be depleted faster than in leaves because substantial amounts of ^{18}O were found in the ring oxygen atoms after 6 h. One would predict, therefore, that in ABA isolated from stressed roots more ^{18}O should be incorporated after 12 and 24 h of incubation in $^{18}\text{O}_2$ than with ABA from stressed leaves.

The oxygen incorporation data suggest that carotenoids are precursors to ABA. It is known that the hydroxyl groups of lutein and the epoxide groups of antheraxanthin and violaxanthin are derived from molecular oxygen (Yamamoto and Chichester, 1965; Yamamoto *et al.*, 1962). The turnover of carotenoids in green leaves is rather low (Goodwin, 1980). Hence, the low incorporation of ^{18}O into sites which ultimately form the keto and hydroxyl groups of ABA could represent the biosynthesis of lutein and antheraxanthin and subsequent conversion to violaxanthin. Since the pool of violaxanthin is so large, and turnover so small, the amount of incorporation of ^{18}O into the ring oxygens of ABA would be predicted to be small if ABA was derived from violaxanthin.

The amount of carotenoids found in *Xanthium* roots ($9.4\ \mu\text{g g}^{-1}$ dry wt) is similar to that found in corn root caps ($30\ \mu\text{g g}^{-1}$ dry wt; Maudinas and Lematre, 1979). Interestingly, the major carotenoid in corn root caps is violaxanthin. The fact that carotenoids are found in *Xanthium* roots indicates that, if ABA is derived from a carotenoid, then the level of total carotenoids in roots is not rate limiting. It is not unusual that roots contain carotenoids. There are several reports of carotenoids in roots in the literature (Goodwin, 1980; Karrer and Jucker, 1950). In commercial carrots β -carotene predominates while in wild carrots xanthophylls represent the majority of the small amount of carotenoids present (Goodwin, 1980). Since the majority of the root carotenoids described here eluted with increasing ethyl

acetate concentration, they are most likely xanthophylls, or are at the very least more polar than carotenes.

The incorporation of ^{18}O into PA was also demonstrated (Figure 6.4; see also chapter 4). If the pool of ABA for the conversion to PA is homogeneous, then the incorporation of ^{18}O into PA may be predicted by knowledge of the extent of ^{18}O incorporation into ABA and the percentage of PA that contains no ^{18}O . The calculation is based on the fact that ABA which contains zero, one, two, or three atoms of ^{18}O will give PA containing one, two, three, or four atoms of ^{18}O , respectively. Hence, one may calculate the distribution of ^{18}O in PA (Table 6.4) by multiplying the fraction of ABA by (1 minus fraction endogenous PA). While this calculation assumes that there is no discrimination between ABA molecules (*i.e.* homogeneous distribution of labeled ABA in the pool which forms PA), one may see that the predicted values are very close to those actually obtained.

ABA levels in stressed *Xanthium* leaves reach a new, steady state level after 6 h. This implies that the rate of formation of ABA is equal to its rate of degradation. One may distinguish between old (present before stress) and newly synthesized ABA (present after stress) because any ABA made after stress will contain ^{18}O . Hence, by following the disappearance of m/e 278 (ABA present before stress), one may calculate a half-life for stress ABA, assuming that the ABA pool for catabolism is homogenous. That this assumption is correct, at least for the conversion of ABA to PA, is

Table 6.4. Predicted and actual values for ^{18}O incorporation into phaseic acid.

The data shown were obtained from the 12 h incubation in $^{18}\text{O}_2$ and represent the contribution of each fraction to the total amount of ABA and PA injected into the mass spectrometer.

Ion	Actual per cent		Predicted per cent
	ABA	PA	PA
M^-	24.5	38.8	38.8
M^-+2	69.3	21.7	15.0
M^-+4	4.9	35.9	42.4
M^-+6	1.4	2.8	3.0
M^-+8	----	0.8	0.9

shown by the data in the last paragraph. Assuming a steady state level of 30 μg ABA/g dry weight (Zeevaart, 1980), the calculated (see below) turnover rate is 16.0 h (Aronoff, 1956).

$$\log M^1 - \log M^2 = - \frac{kt}{2.3M}$$

where M^1 = amount of ABA unlabeled at 24 h (5.7 $\mu\text{g/g}$ dry wt)
 M^2 = amount of ABA unlabeled at 6 h (12.7 $\mu\text{g/g}$ dry wt)
 M = steady state level of ABA (30 $\mu\text{g/g}$ dry wt)
 t = 18 h

$$k = \frac{0.35}{(18 \text{ h})} (2.3)(30 \mu\text{g/g dry wt})$$

$$= 1.3 \mu\text{g/g dry wt/h}$$

$$\text{Now } t_{\frac{1}{2}} = \frac{0.693M}{k} = \frac{0.693(30 \mu\text{g/g dry wt})}{1.3 \mu\text{g/g dry wt/h}}$$

$$= 16.0 \text{ h}$$

This value may be compared with that obtained from data found in Zeevaart (1980). Since during water stress ABA is primarily catabolized to PA, then a half-life may be calculated based on the rate of accumulation of PA. If one assumes that ABA has reached a new, steady state level and that the conversion to ABAGE is minimal during water stress (Zeevaart, 1980), then one obtains a value of 14.7 h for the half-life of ABA in stressed *Xanthium* leaves. The differences between the two methods is that the ^{18}O method contains data on the disappearance of ABA, while the PA method measures just one aspect of ABA catabolism and neglects further metabolism of PA and conversion of ABA to ABAGE.

In conclusion, it has been shown that during long term incubations (12 and 24 h) of stressed *Xanthium* leaves in $^{18}\text{O}_2$

incubations (12 and 24 h) of stressed *Xanthium* leaves in $^{18}\text{O}_2$ up to three atoms of ^{18}O are incorporated into ABA. One atom of ^{18}O is located in the carboxyl group of ABA and two atoms are found in the ring oxygen atoms and. ABA purified from stressed roots of *Xanthium* incubated in $^{18}\text{O}_2$ shows a similar pattern, but with more incorporation of ^{18}O into the ring positions within 6 h. Both roots and leaves of *Xanthium* contain sufficient total carotenoids to account for the amounts of ABA biosynthesized during water stress. However, if in roots only one carotenoid was converted to ABA, then the levels could be too low to support the accumulation of ABA.

6.5. LITERATURE CITED

- Aronoff, S. (1956) Techniques of Radiobiochemistry. New York: Hafner.
- Cornish, K., Zeevaart, J.A.D. (1984) Abscissic acid metabolism in relation to water stress and leaf age in *Xanthium strumarium*. Plant Physiol. 76:1029-1035.
- Cornish, K., Zeevaart, J.A.D. (1985) Abscissic acid accumulation by roots of *Xanthium strumarium* L. and *Lycopersicon esculentum* Mill. in relation to water stress. Plant Physiol. 79:653-658.
- Davies, B.H. (1976) Carotenoids. In: Chemistry and biochemistry of Plant Pigments, pp. 38-165, vol. 2, Goodwin, T.W., ed. New York: Academic Press.
- Goodwin, T.W. (1980) The biochemistry of carotenoids, vol. 1. New York: Chapman and Hall.
- Goodwin, T.W. (1980) Carotenoids. In: Secondary plant products, Encyclopedia of plant physiology NS, vol. 15, Bell, E.A., Charlwood, B.V., eds., New York: Springer Verlag.
- Gray, R.T., Mallaby, R., Ryback, G., Williams, V.P. (19

Mass spectra of methyl abscisate and isotopically labelled analogues. J.C.S. Perkins Trans. 919-924.

- Her, G. R., Watson, J. T. (1985) Quantitative methodology for corticosteroids based on chemical oxidation to electrophilic products for electron capture-negative chemical ionization using capillary gas chromatography-mass spectrometry. I. Assessment of feasibility in the analysis of horse urine for dexamethasone. Anal. Biochem. 151:292-298.
- Karrer, P., Jucker, E. (1950) Carotenoids. New York: Elsevier.
- Maudinas, B., Lematre, J. (1979) Violaxanthin, the major carotenoid pigment in *Zea mays* root cap during seed germination. Phytochemistry 18:1815-1817.
- Yamamoto, H.Y., Chichester, C.O. (1965) Dark incorporation of ^{18}O into antheraxanthin by bean leaf. Biochim. Biophys. Acta 109:303-305.
- Yamamoto, H.Y., Chichester, C.O., Nakayama, T.O.M. (1962) Biosynthetic origin of oxygen in the leaf xanthophylls. Arch. Biochem. Biophys. 96: 645-649.

Chapter 7.

INCORPORATION OF DEUTERIUM INTO

ABSCISIC ACID, STEROLS, AND CAROTENOIDS FROM DEUTERIUM OXIDE

7.1 INTRODUCTION

A major problem in investigating the biosynthesis of ABA is the inability to incorporate precursors, such as MVA, into ABA. A reason for this is the competition of compounds, such as carotenoids and sterols, for MVA and other intermediates in the terpenoid biosynthetic pathway. Another explanation for low MVA incorporation is that ABA is derived from a pool removed from the general terpenoid pathway (*i.e.*, the indirect pathway).

A way of testing this is to incubate leaves in the presence of deuterium oxide ($^2\text{H}_2\text{O}$). Deuterium oxide has the advantage as an *in vivo* label of quickly entering all subcellular compartments. Deuterium from $^2\text{H}_2\text{O}$ will be incorporated into intermediates in the terpenoid biosynthetic pathway at three points: (a) by exchange into acetoacetyl coenzyme A, (b) during the isomerization of isopentenyl pyrophosphate into dimethylallyl pyrophosphate, and (c) into the C-5 of MVA *via* NADPH⁺ (Lehninger, 1970). In addition, one deuterium should be incorporated during the cyclization of farnesyl pyrophosphate into what will be the 5' position of ABA (Britton, 1986). ABA synthesized from an acyclic precursor should, therefore, contain one deuterium at C-5'. ABA arising from a post-cyclized precursor (such as a carotenoid) would not contain any deuterium with short-term incubations, but rather would become slowly labeled with several deuterium atoms during long-term incubations (as would carotenoids).

In studies of *de novo* protein synthesis utilizing $^2\text{H}_2\text{O}$ it has been noted that if H_2O is present (at amounts of 10 to 20%) protons rather than deuteriums will be used preferentially because of isotope discrimination. Little or no deuterium is incorporated if less than 40% $^2\text{H}_2\text{O}$ is used (Chrispeels and Varner, 1973). Because of this, and the fact that $^2\text{H}_2\text{O}$ might be toxic at high concentrations, it is necessary to also measure deuterium incorporation into another compound known to be synthesized during the incubation period. Therefore, the presence of deuterium in the plant sterols stigmasterol and sitosterol was also determined by GC-MS.

To measure deuterium incorporation it is necessary to use a mass spectrometer. Because the levels of ABA will be quite low in turgid leaves, a requirement is that the mass spectrometer have high sensitivity. The procedure used must also measure the molecular ion as the major ion. ABA has a small molecular ion, and analysis of the amount of incorporation in fragment ions is complicated. A technique that meets these criteria is GC-NCI (Watson, 1985). This procedure affords high sensitivity towards electron withdrawing compounds such as ABA. Furthermore, a characteristic of CI mass spectra is that they contain fewer ions than corresponding EI mass spectra, with the molecular ion (M^+) predominating. However, GC-NCI (and most other mass spectroscopy techniques) cannot be used with structures of high molecular weight, low volatility, or poor electron withdrawing capability.

ity, such as carotenoids. For these compounds, FAB may be used to introduce the analyte into the ionization chamber (Watson, 1985). FAB utilizes an energetic beam of atoms to cause desorption/ionization of nonvolatile substances dissolved in a viscous, low vapor pressure matrix.

We decided to incubate half-expanded *Xanthium* leaves in 80% $^2\text{H}_2\text{O}$ for 6 h or 4d and determine if deuterium was incorporated into ABA, sterols, and carotenoids. *Xanthium* leaves at this age are ideal for this study because they have a high level of ABA and are synthesizing sterols.

7.2. MATERIALS AND METHODS

7.2.1. Plant Material. Half-expanded leaves of *Xanthium strumarium* were allowed to take up $^2\text{H}_2\text{O}$ (80% enriched) for 3 h via the transpiration stream. This allowed for complete replacement of tissue water with the mixture of $^2\text{H}_2\text{O}$ and H_2O . Leaves were then placed in a plastic bag for either 6 h or 4 d. Each experiment was performed twice.

7.2.2. Extraction and Purification of Absciscic Acid. The plant material was homogenized in methanol until a colorless residue was left. Water was added to the extract and the methanol removed to give an aqueous extract. This aqueous fraction had its pH adjusted to 7.0 and was partitioned 3 times against diethyl ether, giving free sterols in the ether fraction. The aqueous extract had its pH lowered to 2.5 and was partitioned against diethyl ether. This gave conjugated

sterols in the aqueous fraction and ABA in the diethyl ether fraction. The two sterol fractions were combined and further purified as described below. The ABA fraction was reduced to an aqueous residue which was frozen and lyophilized. It was then purified as described in chapters 9 and 4. After methylation with ethereal diazomethane, it was analyzed by GC-NCI.

7.2.3. Purification of Sterols. The combined fractions were reduced to an aqueous fraction, frozen, and lyophilized. The dry residue was dissolved in 100 ml of 10% KOH in 85% methanol and was refluxed for 1 h. After cooling, the hydrolysate was concentrated to a small volume and 40 ml water added. This aqueous solution was then extracted with 3 volumes of diethyl ether. The combined diethyl ether extracts were washed with water and dried over anhydrous sodium sulfate. The solvent was removed to give the crude non-saponifiable sterol fraction.

The free fraction was dissolved in 6 ml acetone:diethyl ether (1:1). To this solution was added 3 ml of an ethanolic digitonin solution (3 mg of digitonin in 1.4 ml water and 1.6 ml ethanol). The solution was shaken at room temperature for 6 h and stored at 4 C overnight. The solution was centrifuged and supernatant removed by pipetting. The resulting precipitate was washed 3X with cold diethyl ether.

The digitonides were dissolved in 3 ml pyridine and heated at 70 C for 1 h. The resulting solution was extracted with 3 10 ml portions of diethyl ether. The combined ether fractions were washed with 30 ml saturated cupric sulfate,



followed by 30 ml water, and were dried over anhydrous sodium sulfate.

After evaporation to dryness, the residue was fractionated by column chromatography. The residue was dissolved in a small amount of diethyl ether to which 1 g of celite was added. The ether was evaporated depositing the sterols onto the celite which was placed on top of a neutral aluminum oxide (grade III, 60 g) column. The column was eluted with increasing amounts of diethyl ether (0, 1, 2, 6, and 30%) in hexane; 4-demethylsterols (sitosterol and stigmasterol) eluted in the 30% ether fraction.

After reducing the column eluate to a small volume, the sterols were transferred to small vials and dried over P₂O₅ for 12 h. The sterols were dissolved in 20 μ l pyridine:HMDS:TMCS (9:3:1) and were heated at 60 C for 15 min. The resulting TMSi sterol ethers were then analyzed by GC-MS.

7.2.4. Purification of Carotenoids. In separate experiments, leaves of *Xanthium* were extracted in methanol until a colorless residue was left. Equal portions of methanol and diethyl ether were mixed together to which three volumes of 10% sodium chloride were added carefully to prevent emulsions. The combined ether fractions were backwashed with water and dried over anhydrous sodium sulfate. The ether was removed and the residue dissolved in 6% KOH in methanol. After incubation overnight at 4 C, diethyl ether was added to the methanolic solution followed by water. By this procedure, chlorophyll was separated from carotenoids. The ether

solution was backwashed with water and dried over anhydrous sodium sulfate. After removal of the ether, the carotenoid residue was dissolved in a small amount of hexane and applied to the top of an aluminum oxide column (neutral, grade III, 30 g). Carotenoids were eluted from the column with increasing amounts of diethyl ether in hexane. Identification of the carotenoid in each fraction was by absorbance maxima and order of elution from the column.

7.2.5. Mass Spectroscopy. GC-MS was performed as described in Chapter 4 using a 3% SP-2100 column, except the column temperature was 275 C.

GC-NCI was performed using a modified Hewlett-Packard 5985A GC-MS (Her and Watson, 1985). The conditions were as follows: a capillary on-column injector (J & W, Inc.) and a GMCC/90 open split interface (Scientific Instrument, Inc.) were mounted on the 5985A GC-MS. A 55 m DB-1 capillary column (0.32 mm, J & W, Inc.) was used with temperature programming from 50 to 300 C at 10 C/min using He as carrier gas. The pressure of the reagent gas methane was 1.2×10^{-4} torr.

FAB was performed with a Varian MAT CH5-DF double focusing mass spectrometer (Ackermann *et al.*, 1984) using a sample matrix of thiodiglycol:dithiothreitol:dithioerythritol (1:1:1). The fast atom gun was operated to give 6 keV Xe atoms with a pressure inside the ion source of 8×10^{-6} torr.

7.3. RESULTS AND DISCUSSION

In most vascular plants, sitosterol (stigmasta-5-en-3 β -ol) and stigmasterol (stigmasta-5,22-diene-3 β -ol) are the two major 4-demethylsterols. Both sterols have 29 carbon atoms and differ only in that stigmasterol has a double bond at C-22 of its C-17 side chain, whereas sitosterol has a saturated side chain. Sitosterol and stigmasterol were identified in purified *Xanthium* extracts by GC-MS both as free sterols and as their TMSi-ether derivatives. The derivatives gave better separation with GLC and were used for further analysis of deuterium content. Since the molecular ion is the best indicator of deuterium incorporation, samples containing sterols were scanned during GC-MS from m/e 290 to m/e 350. Analysis of sitosterol and stigmasterol from leaves incubated in the presence of $^2\text{H}_2\text{O}$ indicated that deuterium was indeed incorporated into both molecules (Figure 7.1). Although the amount of deuterium incorporation is low, more deuterium was found in both compounds after 4 d of incubation than after 6 h. Because of the structural similarities between the compounds, it has been postulated that a common biosynthetic pathway, sitosterol $\longrightarrow$ stigmasterol, exists. However, the evidence for this pathway is not unequivocal, and it has been proposed that two different pathways exist in dicots and monocots (Grunwald, 1986). The data here indicate that after a 6 h incubation in the presence of $^2\text{H}_2\text{O}$ more deuterium was present in sitosterol

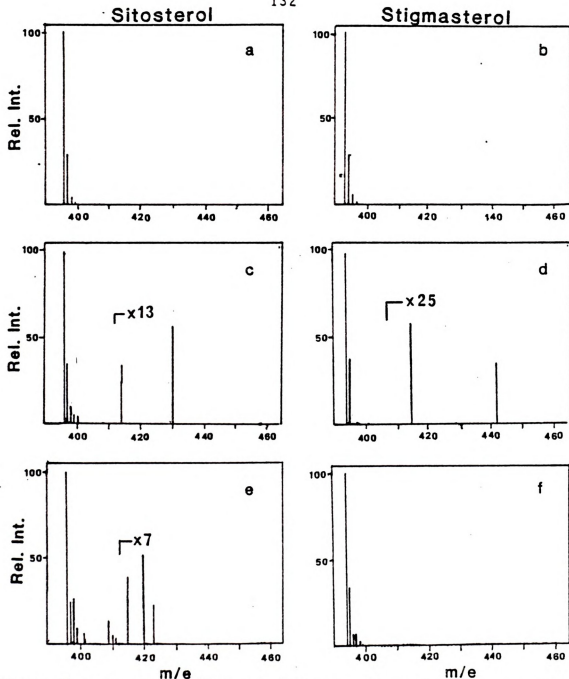


Figure 7.1. Incorporation of deuterium from deuterium oxide into sitosterol and stigmasterol.

Standard sitosterol (M^+ 396) and stigmasterol (M^+ 394) are shown in the top panels (a and b, respectively). Deuterium was incorporated into sitosterol (c and e) and stigmasterol (d and f) after 6 h (c and d) and 4 d (e and f).

than in stigmasterol. This would be in accord with the pathway described above. It is possible that the results described by Grunwald (1986) could represent metabolic channeling. The deuterium incorporation into sterols indicates that incubation in 80% $^2\text{H}_2\text{O}$ was not toxic to *Xanthium* leaves because the terpenoid biosynthetic pathway was operating.

Similar to the incorporation of deuterium into sterols, little deuterium was found in ABA analyzed by GC-NCI (Table 7.1). However, slightly more deuterium was incorporated into ABA after 4 d of incubation in $^2\text{H}_2\text{O}$ than after 6 h. There were two fractions, one (m/e 279) that had incorporated one deuterium, and another (m/e 284-290) that had 6-12 deuterium.

No deuterium was detected in β -carotene [λ_{max} , hexane, 470, 446, 422(shoulder) nm], a precursor to other carotenoids, such as lutein and violaxanthin. Analysis by FAB of other carotenoids was not successful, possibly because of matrix difficulties. Successful use of FAB depends on many parameters and is still in the development stage. Another problem associated with FAB is high background. However, β -carotene gave a clear m/e 535 (M^+-H). Because of the high background, it could be argued that some deuterium was incorporated; if this was true then the amount of incorporation was very low (at least less than 5%).

If one assumes that ABA is derived from carotenoids, then the fact that some deuterium was incorporated into ABA and none appeared to be in β -carotene can be explained in

Table 7.1. Incorporation of deuterium from deuterium oxide into abscisic acid.

The extent of deuterium incorporation after 6 h or 4 d was determined using GC-NCI. For comparison, standard ABA is also shown.

m/e	ABA		
	Standard	6 h	4 d
278	100.00	100.00	100.00
279	11.39	15.66	15.52
280	4.04	3.95	3.96
281	0.33	0.52	0.43
282	----	0.13	0.09
283	----	0.04	0.05
284	----	0.18	0.29
285	----	0.14	0.29
286	----	0.08	0.17
287	----	0.03	0.12
288	----	0.13	0.17
289	----	0.07	0.06
290	----	0.09	----
291	----	----	0.04
292	----	0.09	0.07
293	----	0.04	0.08
294	----	0.04	0.07
295	----	----	0.05
296	----	----	----
297	----	----	----
298	----	----	----



several ways. One explanation is that deuterium was incorporated into β -carotene (and not observed when analyzed by FAB). Another is that only a small fraction of the total β -carotene was labeled with deuterium (in amount, similar to that found in ABA) and that the bulk β -carotene (unlabeled) was observed by FAB. Another possibility is that the initial assumption (ABA arises from carotenoids) is incorrect.

Deuterium was incorporated into sterols, and the terpenoid pathway did not seem to be deleteriously affected by $^2\text{H}_2\text{O}$. However, no information was obtained on how fast ABA and sterols were being synthesized. Half-expanded leaves have a high rate of sterol biosynthesis. On the other hand, these leaves were not stressed, so the turnover of ABA was probably low. It was expected that more deuterium should have been incorporated into ABA. Since the time points were 6 h and 4 d, the possibility exists that the leaves became senescent in the course of the experiment. With these caveats, the data indicate that the majority of ABA does not arise from the cyclization of farnesyl pyrophosphate. If this had happened, then ABA would have a large M^+1 , and this was not observed. It appears that one ABA fraction is derived from precursors that are removed from the general terpenoid pathway (m/e 284 to m/e 290), and that the other fraction might arise from farnesyl pyrophosphate (m/e 279).

7.4. LITERATURE CITED

- Ackermann, B. L., Watson, J. T., Newton, J. F., Hook, J. B., Braselton, Jr., W. E. (1984) Application of fast atom bombardment mass spectrometry to biological samples: analysis of urinary metabolites of acetaminophen. *Biomed. Mass Spectrom.* 11:502-511.
- Britton, G. (1976) Later reactions of carotenoid biosynthesis. *Pure and Appl. Chem.* 47:223-236.
- Chrispeels, M. J., Varner, J. E. (1973) A test for *de novo* synthesis of enzymes in germinating seeds: density labeling with D₂O. In: *Molecular techniques and approaches in developmental biology*, pp 79-92, Chrispeels, M. J., ed. New York: John Wiley and Sons.
- Grunwald, C. (1986) *In vivo* synthesis of stigmasterol in *Nicotiana tabacum*. *Phytochemistry* 24:2915-2918.
- Her, G. R., Watson, J. T. (1985) Quantitative methodology for corticosteroids based on chemical oxidation to electrophilic products for electron capture-negative chemical ionization using capillary gas chromatography-mass spectrometry. I. Assessment of feasibility in the analysis of horse urine for dexamethasone. *Anal. Biochem.* 151:292-298.
- Lehninger, A.L. (1970) *Biochemistry*. New York: Worth.
- Watson, J. T. (1985) *Introduction to mass spectrometry*. New York: Raven.



Chapter 8

THE ROLE OF XANTHOXIN IN ABSCISIC ACID BIOSYNTHESIS



8.1. INTRODUCTION

ABA is a sesquiterpenoid and appears to be derived from three MVA residues. However, the structure of ABA is quite different from all other known sesquiterpenoids, and in fact resembles the terminal ring portions of many carotenoids.

Taylor and Smith (1967) showed that exposure of carotenoids to bright light on damp filter paper gave rise to a neutral compound which inhibited cress seed germination. Of the carotenoids tested, the greatest amount of inhibition occurred when violaxanthin and neoxanthin were photo-oxidized (Taylor, 1968). Extensive purification of the photo-oxidized products of violaxanthin indicated that the inhibitor was a mixture of *cis,trans*- and *trans,trans*-xanthoxin (Taylor and Burden, 1970; Burden and Taylor, 1970). In addition, butenone and loliolide were also identified.

It was later shown that *cis,trans*- and *trans,trans*-xanthoxin could be efficiently formed from violaxanthin, neoxanthin, antheraxanthin and lutein epoxide by zinc permanganate (Taylor and Burden, 1972). The chemical conversion of xanthoxin to ABA was also reported (Taylor and Burden, 1972). The biological activity of *cis,trans*-xanthoxin was greater than that of the *trans,trans* isomer in several assays (Taylor and Burden, 1972). Both isomers of xanthoxin have been detected in several plants, including ferns, a liverwort, and wheat (Firn et al., 1972). Firn and Friend (1972) showed that the isomers of xanthoxin, butenone, and loliolide could be produced by soybean lipoxygenase in the

presence of linoleate, indicating that these compounds could be produced without the action of oxidizing agents or light.

ABA levels showed a large increase after plants had been treated with solutions of *cis,trans*-xanthoxin (Taylor and Burden, 1972). To determine whether the increase was due to conversion to ABA rather than a stimulation of synthesis, ^{14}C -*cis,trans*-xanthoxin was prepared and fed to tomato shoots (Taylor and Burden, 1973; Taylor and Burden, 1974). After 8 h, ^{14}C -*cis,trans*-xanthoxin was converted to ABA (10.8%) and PA (4%). In addition, a small amount of conjugated *cis,trans*-xanthoxin acid was also formed (Taylor and Burden, 1974). When applied to tomato fruits and pea seeds, "small but significant" amounts of radioactivity were also found in ABA (Taylor and Burden, 1974).

Thus, xanthoxin appears to be an endogenous compound in many plants, and it is converted to ABA. Because of these considerations, xanthoxin is a likely precursor to ABA in higher plants and thus could be a key intermediate between carotenoids and ABA. However, the possibility exists that the levels of xanthoxin may be overestimated due to the lability of xanthophylls. We decided to determine if the extraction procedure (anaerobic vs. aerobic) has any effect on xanthoxin measurement. In addition, we wanted to determine if xanthoxin was as labile as described by Shen-Miller et al. (1982). These workers showed that xanthoxin was rapidly destroyed at room temperature or -2 C. Finally, if xanthoxin arises from an oxidative cleavage of a xanthophyll, the mechanism should

be similar to that operating in vitamin A biosynthesis (Britten, 1983). Leaves were therefore incubated in the presence of $^{18}\text{O}_2$ and the amount and location of ^{18}O incorporated into xanthoxin was determined.

8.2. MATERIALS AND METHODS

8.2.1. Plant Material. Spinach plants (*Spinacia oleracea* cv Savoy Hybrid 612) were grown under short day as described (Zeevaart, 1971) and transferred to long days and used after 10 to 12 d.

For experiments involving incorporation of ^{18}O , spinach leaves were detached from plants and incubated in an atmosphere containing 80% N_2 and 20% $^{18}\text{O}_2$ for 8 h as described in chapter 4.

8.2.2. Chemicals. Xanthoxin was initially purchased from Fluka. When this supply was depleted, xanthoxin was prepared according to Taylor and Burden (1970) from violaxanthin isolated from *Xanthium strumarium* L. leaves.

8.2.3. Extraction and Purification Procedures. In the ^{18}O incorporation experiment, spinach leaves were homogenized and extracted according to Zeevaart (1974). After partitioning against ethyl acetate to give neutral (xanthoxin) and acidic (ABA) fractions, the acidic fraction was further purified as described in chapters 4 and 9. The neutral fraction containing xanthoxin was further purified by semi-preparative reverse phase HPLC (20 to 60% ethanol in water in 30 min with

a 2 min hold at the beginning of the run, flow rate 2.5 ml/min). The fraction which eluted between 20.5 and 23.5 min was collected, reduced in volume, and lyophilized. If further purification was necessary, TLC was performed on the extract as described in Zeevaart (1974)

To determine if extractions done in atmospheres containing O_2 gave elevated levels of xanthoxin, some extractions were performed entirely under N_2 . In addition, unless otherwise noted, all solvents were purged of O_2 by vigorously bubbling N_2 through them. Leaf material was detached from plants and crushed in liquid N_2 to which 250 ml of methanol was added. After extraction overnight at 4 C in the dark, further purification was performed in a plexiglas chamber in an atmosphere of N_2 . The tissue was homogenized, filtered, and the residue washed with 150 ml methanol. To this was added sufficient water to make an 80% aqueous methanol solution. This solution was then rapidly applied to a preparative Bondapak C_{18} column (1 x 25 cm, particle size 37-75 μ) using a Beckman Accu-Flo pump. With this procedure, plant pigments and waxes are bound to the column packing and xanthoxin elutes from the column (Shen-Miller et al., 1982). After each sample the column is washed with 100% methanol and equilibrated with 80% methanol. The column eluate was reduced to a small volume by a rotary evaporator equipped such that the vacuum release was done with N_2 instead of air. This aqueous residue was then placed in a plexiglas chamber with a 100% N_2 atmosphere and partitioned 3



times against diethyl ether. Water was added to the ether phase and the ether removed leaving an aqueous fraction which was frozen and lyophilized. After lyophilization, all further operations were performed in room air. The dry residue was then subjected to reverse phase HPLC using a Waters Radial Pak 4 μ C₁₈ cartridge (10 to 70% methanol in water in 45 min with a 2.5 minute hold at the beginning of the run, flow rate 2.0 ml/min). Two Waters Model 510 pumps were controlled by a Waters Automated Gradient Controller with the absorbance at 282 nm monitored using a Waters Lambda Max Model 481 spectrophotometer. This column was able to resolve the isomers of xanthoxin (retention *t*,*t*-xanthoxin 31.00 to 32.00 min, *c*,*t*-xanthoxin 32.75 to 33.75 min). The isomers were collected separately, reduced in volume, frozen, and lyophilized. As a control, the same procedure was followed as described above except that all procedures were carried out in room air. This experiment was performed three times with two replicates per treatment.

To increase the sensitivity of xanthoxin to GC-ECD, it was derivatized with *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride (Koshy et al., 1975). This reagent reacts with the aldehyde group of xanthoxin to give an oxime. To the dry residue was added 100 μ l of the derivatization reagent in pyridine (2 mg/ml). Vials were heated at 65 C for 30 min and the pyridine removed with a stream of N₂ at 45 C. One ml of hexane was added which was washed with 500 μ l water. The water was removed and the hexane dried over sodium

sulfate.

8.2.4. Determination of Xanthoxin Stability. To determine the stability of xanthoxin, identical aliquots were stored at room temperature (24 C), cold room temperature (4 C), refrigerator temperature (4 C), -20 C, and -80 C. MeABA was also present in this solution as an internal standard to correct for evaporation. Xanthoxin was not derivatized in this procedure.

8.2.5. Gas Chromatography. Underivatized xanthoxin was detected by GC-ECD using a Hewlett-Packard 5840A gas chromatograph equipped with a 10 m Hewlett-Packard methyl silicon wide bore column (inner diameter 0.53 mm) in the splitless mode. Column temperature was 175 C, column pressure 9 kg/cm², Ar-methane (make up gas) flow 60 ml/min.

The oxime derivative of xanthoxin was also detected by GC-ECD. However, due to the large, bulky nature of the derivative, it had a much longer retention time, so that the analysis was performed with a 1.5% OV-1 column operated isothermally at 225 C (2 m, 80-120 mesh, Gas chrom Q, 30 ml/min Ar-methane flow).

8.2.6. Mass Spectrometry. To detect ¹⁸O incorporation in ABA (as the methylated derivative) and underivatized xanthoxin, mass spectra were obtained by direct injection on a modified Hewlett-Packard 5895A mass spectrometer (Her and Watson, 1985) in the positive EI mode. Samples were injected with the column (55 m DB-1) initially at 50 C. Following injection, the column was rapidly heated to 150 C at 30 C/min after



which it was slowly heated to 240 C at 10 C/min.

The EI mass spectra (GC-MS) of the oxime derivative of xanthoxin were obtained on a Hewlett-Packard 5895A mass spectrometer. Gas chromatography was performed as described above in section 8.2.5.

8.3. RESULTS

8.3.1. Stability of Xanthoxin. As seen in Figure 8.1, under all conditions tested, xanthoxin was stable. No isomerization of *c,t*-xanthoxin to *t,t*-xanthoxin occurred.

8.3.2. Incorporation of ^{18}O into Xanthoxin and Absciscic Acid. GC-MS (EI) analysis of xanthoxin isolated from spinach leaves incubated in an atmosphere containing $^{18}\text{O}_2$ indicated that no incorporation of ^{18}O had occurred (Figure 8.2). On the other hand, analysis of ABA isolated from the same leaves indicated that incorporation into this molecule had occurred (Figure 8.3).

8.3.3. Xanthoxin Levels in Tissues Extracted in Air and Nitrogen. As is seen when making the acylated derivative of xanthoxin, derivatization with *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride gave two isomers. However, since the two isomers were separated by HPLC before derivatization, it is possible to quantitate the levels of the isomers actually present in the extract, not those created during derivatization. The *cis,trans* oxime derivative of xanthoxin gave a mass spectrum with a molecular ion (M^+

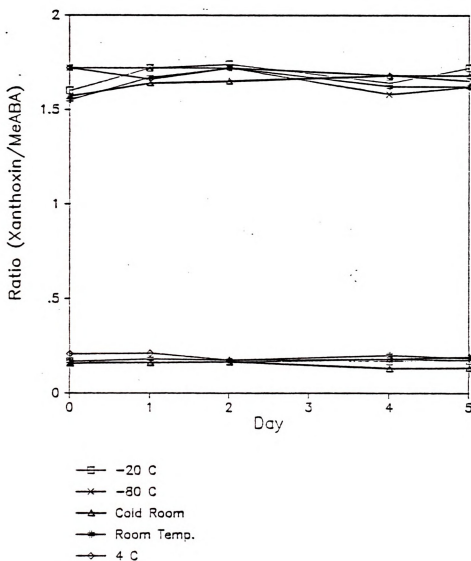


Figure 8.1. Stability of xanthoxin under different environmental conditions.

Plotted are the ratio of *cis,trans*-xanthoxin (upper lines) and *trans,trans*-xanthoxin (lower lines) to MeABA.

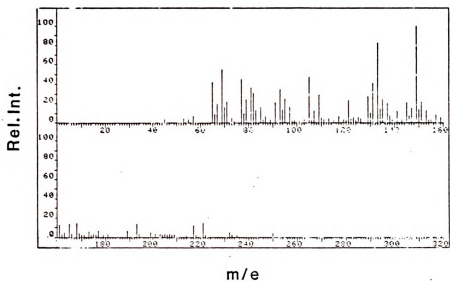


Figure 8.2. Mass spectrum of xanthoxin isolated from spinach leaves incubated in $^{18}\text{O}_2$ for 8 h. Standard xanthoxin has a molecular ion of 250, and is identical to the spectrum shown here. There is no incorporation of ^{18}O .

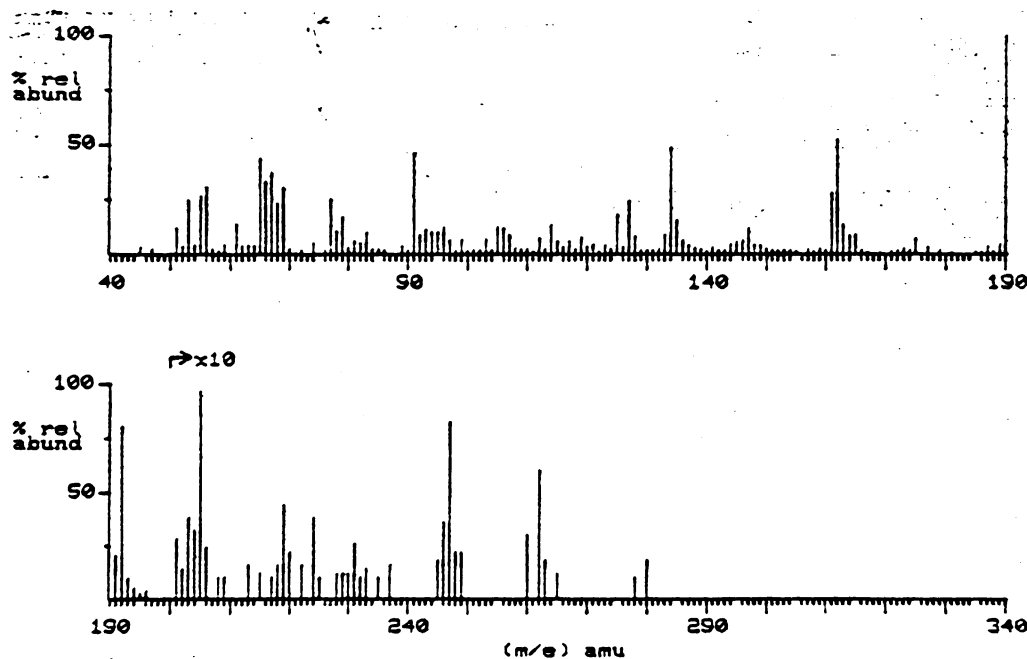


Figure 8.3. Mass spectrum of ABA isolated from spinach leaves incubated $^{18}\text{O}_2$ for 8 h. ABA was purified from the same extract from which xanthoxin was purified in Figure 8.2. Contrary to that seen with xanthoxin, there is incorporation of ^{18}O .

445) and fragmentation pattern consistent with the presumed structure of the derivative (Figure 8.4). Absorption in the UV region gave a maximum at 280 nm (xanthoxin has a maximum at 282 nm). A standard series was created using the extinction coefficient for xanthoxin at 282 nm. Since the addition of the derivative did not change the absorption maximum, it probably would not interfere with the extinction coefficient. The detection limit on GC-ECD was 100 pg/ μ l.

Using this derivative, values were obtained for xanthoxin from spinach leaves incubated in room air or N₂ (Table 8.1). As can be seen, the levels of xanthoxin in spinach leaves extracted under N₂ were about 13X lower than those found in extracts from leaves prepared in air.

8.4. DISCUSSION

Contrary to the results of Shen-Miller et al. (1982), both isomers of xanthoxin were highly stable under all conditions tested. The only differences between the procedure used here and that in Shen-Miller et al. (1982) is that these workers stored xanthoxin in methanol, whereas in the present work it was stored in ethyl acetate. However, xanthoxin was stored in methanol at both 4 C and -20 C with no apparent degradation for several months.

If xanthoxin is a degradation product of violaxanthin, it should have one atom of ¹⁸O inserted into the aldehyde group, similar to what happens in the formation of vitamin A from

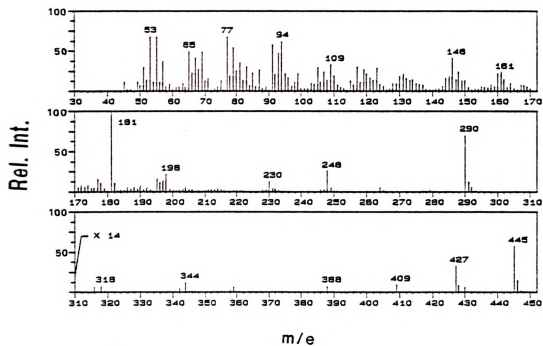


Figure 8.4. Mass spectrum of the 0-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride derivative of xanthoxin. Shown is the mass spectrum for the *cis,trans* derivative.

Table 8.1. Xanthoxin levels from spinach leaves extracted in air or N₂.

This experiment was performed 3 times with 2 replicates per treatment. Similar results were obtained each time.

Treatment	Isomer	
	ng g ⁻¹ fresh wt	
	<i>cis,trans</i>	<i>trans,trans</i>
Air	29.2	47.5
N ₂	2.2	3.5

β -carotene (Britton, 1983). However, as evident from the mass spectrum of xanthoxin isolated from leaves extracted in air, this did not occur (Figure 8.2). As a control, the incorporation of ^{18}O into ABA was detected (Figure 8.3). In other words, ^{18}O incorporation was detected in ABA, but not in its putative precursor xanthoxin. There could be several reasons for this. One is that, in spite of all the evidence that xanthoxin is a precursor, in reality xanthoxin is not a true endogenous precursor to ABA. This would seem unlikely with all the data that implicate carotenoids as precursors to ABA (i.e. carotenoid-less mutants, inhibitors, and the ^{18}O incorporation patterns in ABA). Another possible explanation is that xanthoxin containing ^{18}O is diluted by xanthoxin produced from violaxanthin (or other xanthophylls) during extraction and purification. Because the purification and extraction was done in room air, any xanthoxin produced by violaxanthin degradation would contain ^{16}O instead of ^{18}O in its aldehyde group. Due to the relative lack of sensitivity and large fragmentation that occurs with GC-MS (EI), low amounts of incorporation would not be detected. This result suggests that much of the xanthoxin seen in plants could be an artifact caused by the purification procedure.

This was tested by extracting xanthoxin in room air and N_2 (Table 8.1). As can be seen, the levels of xanthoxin are lower in the leaves extracted under N_2 . The values obtained for air extraction are similar to those reported by Zeevaart (1974). This same situation is seen in bean leaves extracted

under conditions which degrade violaxanthin, yet do not degrade xanthoxin (Walton, pers. com.). When bean leaves incubated in $^{18}O_2$ were extracted carefully to prevent violaxanthin breakdown, no ^{18}O was found in xanthoxin (Walton, pers. com.). However, their method of detection might not be sensitive to low levels of incorporation.

A better chance to detect ^{18}O incorporation would be to use GC-NCI (or DP-NCI). Xanthoxin is an electrophilic compound, although not as good as ABA. With the higher sensitivity and low fragmentation that occurs with NCI, a low amount of incorporation would still be detected because, assuming that the molecular ion was the base peak, incorporation of 1 to 2% would still be detected as the $M^+ + 2$.

8.5. LITERATURE CITED

- Britten, G. (1983) The biochemistry of natural pigments. Cambridge: Cambridge University Press.
- Burden, R.S., Taylor, H.F. (1970) The structure and chemical transformations of xanthoxin. Tetrahedron Lett. 47:4071-4074.
- Firn, R.D., Burden, R.S., Taylor, H.F. (1972) The detection and estimation of the growth inhibitor xanthoxin in plants. Planta 102:115-126.
- Firn, R.D., Friend, J. (1972) Enzymatic production of the plant growth inhibitor, xanthoxin. Planta 103:263-266.
- Her, G. R., Watson, J. T. (1985) Quantitative methodology for corticosteroids based on chemical oxidation to electrophilic products for electron capture-negative chemical ionization using capillary gas chromatography-mass spectrometry. I. Assessment of feasibility in the analysis of horse urine for dexamethasone. Anal. Biochem. 151:292-298.

- Koshy, K.T., Kaiser, D.G., VanDerSlik, A.L. (1975) O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride as a sensitive derivatizing agent for the electron capture gas liquid chromatographic analysis of keto steroids. *J. Chromatogr. Sci.* 13:97-104.
- Raschke, K., Firn, R.D., Pierce, M. (1975) Stomatal closure in response to xanthoxin and abscisic acid. *Planta* 125:149-160.
- Shen-Miller, J., Knecht, E., Vermeer, E., Bruinsma, J. (1982) Purification and lability of *cis*-xanthoxin and its occurrence in phototropically stimulated hypocotyls of *Helianthus annuus* L.. *Z. Pflanzenphysiol.* 108:289-294.
- Taylor, H.F. (1968) Carotenoids as possible precursors of abscisic acid in plants. In: *Plant growth regulators*, S.C.I. Monograph Number 31, pp. 22-35, London: Society of Chemical Industry.
- Taylor, H.F., Burden, R.S. (1970) Identification of plant growth inhibitors produced by photolysis of violaxanthin. *Phytochemistry* 9:2217-2223.
- Taylor, H.F., Burden, R.S. (1970) Xanthoxin, a new naturally occurring plant growth inhibitor. *Nature* 227:302-303.
- Taylor, H.F., Burden, R.S. (1972) Xanthoxin, a recently discovered plant growth inhibitor. *Proc. R. Soc. Lond. B* 180:317-346.
- Taylor, H.F., Burden, R.S. (1973) Preparation and metabolism of 2-[¹⁴C]-*cis,trans*-xanthoxin. *J. Exp. Bot.* 24:873-880.
- Taylor, H.F., Burden, R.S. (1974) The biochemistry of xanthoxin and its relationship to abscisic acid. In: *Biochemistry and chemistry of plant growth regulators*, pp. 187-196, Schreiber, K., Schütte, H.R., Semadeni, G., eds., Halle (Saale): Academy of Sciences of the German Democratic Republic.
- Taylor, H.F., Smith, T.A. (1967) Production of plant growth inhibitors from xanthophylls: a possible source of dormin. *Nature* 215:1513-1514.
- Zeevaart, J.A.D. (1971) Effects of photoperiod and endogenous gibberellins in the long day rosette plant spinach. *Plant Physiol.* 71:477-481.
- Zeevaart, J.A.D. (1974) Levels of (+)-abscisic acid and xanthoxin in spinach under different environmental conditions. *Plant Physiol.* 53:644-648.

Chapter 9.

ABSCISIC ACID ACCUMULATION IN SPINACH LEAF
SLICES IN THE PRESENCE OF PENETRATING AND NON-
PENETRATING SOLUTES



Abscisic Acid Accumulation in Spinach Leaf Slices in the Presence of Penetrating and Nonpenetrating Solutes¹

Received for publication July 6, 1984 and in revised form September 14, 1984

ROBERT A. CREELMAN AND JAN A. D. ZEEVAART*

MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing, Michigan 48824

ABSTRACT

Abscisic acid (ABA) accumulated in detached, wilted leaves of spinach (*Spinacia oleracea* L. cv Savoy Hybrid 612) and reached a maximum level within 3 to 4 hours. The increase in ABA over that found in detached turgid leaves was approximately 10-fold. The effects of water stress could be mimicked by the use of thin slices of spinach leaves incubated in the presence of 0.6 molar mannitol, a compound which causes plasmolysis (loss of turgor). About equal amounts of ABA were found both in the leaf slices and in detached leaves, whereas 2 to 4 times more ABA accumulated in the medium than in the slices. When spinach leaf slices were incubated with ethylene glycol, a compound which rapidly penetrates the cell membrane causing a decrease in the osmotic potential of the tissue and only transient loss of turgor, no ABA accumulated. Ethylene glycol was not inhibitory with respect to ABA accumulation. Spinach leaf slices incubated in both ethylene glycol and mannitol had ABA levels similar to those found when slices were incubated with mannitol alone. Increases similar to those found with mannitol also occurred when Aquacide III, a highly purified form of polyethylene glycol, was used. Aquacide III causes cytorrhysis, a situation similar to that found in wilted leaves. Thus, it appears that loss of turgor is essential for ABA accumulation.

When spinach leaf slices were incubated with solutes which are supposed to disturb membrane integrity (KHSO₃, 2-propanol, or KCl) no increase in ABA was observed. These data indicate that, with respect to the accumulation of ABA, mannitol caused a physical stress (loss of turgor) rather than a chemical stress (membrane damage).

ABA levels in water-stressed tissues are usually 10 to 40 times greater than those found in turgid tissues (21). Several workers (1, 2, 8, 22) have noted that ABA accumulation is dependent on leaf water potential declining below a certain 'threshold' level, usually around -1.0 to -1.2 MPa. Pierce and Raschke (16) concluded that turgor is the critical component of plant cell water relations that controls ABA levels, i.e., loss of turgor is the signal that causes ABA accumulation.

We decided to use leaf slices of spinach incubated in various solutes as a way of studying ABA accumulation. Several workers have triggered ABA accumulation by incubating plant tissues in hyperosmotic solutions of mannitol or sorbitol (7, 12, 13, 15, 18, 20). These compounds will cause plasmolysis (loss of turgor). Greenway and Leahy (6) have shown that ethylene glycol, since it rapidly penetrates the cell membrane, will decrease a cell's osmotic potential and cause only a transient loss of turgor. With the use of penetrating and nonpenetrating solutes, one should be

able to determine whether or not loss of turgor is important in causing ABA accumulation.

For those cases where mannitol caused increased ABA levels (7, 12, 13, 15, 18, 20), the possibility exists that mannitol may have a perturbing effect on the cell membrane. Rivov and Yang (17) used 100 mM mannitol to stimulate ethylene production in *Citrus* leaf discs. They concluded that mannitol exerted a chemical stress on the membranes since the mannitol concentration used was too low to induce water stress. Other workers (5) have observed that when cucurbit leaf discs were incubated in the presence of 2-propanol, KHSO₃, or KCl, the production of ethane increased. Since ethane production is a result of membrane damage, addition of these compounds to the medium with spinach leaf slices should indicate if ABA production is caused by a chemical stress to the membranes.

MATERIALS AND METHODS

Plant Material. Spinach plants (*Spinacia oleracea* cv Savoy Hybrid 612) were grown under SD as described (23) and were transferred to LD for 8 to 14 d. For experiments involving detached leaves, just fully expanded (light green in color) leaves were excised and either placed immediately in plastic bags ('control' leaves), or were allowed to lose 15% of their fresh weight and then placed in plastic bags ('stressed' leaves).

Leaf slices were prepared in the following manner. Just fully expanded leaves were detached, midribs removed, and leaf blades were sliced 1 to 2 mm in width with a sharp razor blade. Slices were placed in BM² (10 mM Hepes, pH 6.5 with KOH) containing 2.5 g·l⁻¹ PVP-40 for 10 min, then rinsed and placed in BM for 10 min. Next, slices were blotted dry and placed in the appropriate incubation medium (10 ml total volume). Total incubation time in most experiments was 4 h. Slices were incubated in 60-ml test tubes under light from a General Electric H400RDX33-1 mercury-vapor lamp (50 w/m²) which was filtered through a 5-cm layer of distilled H₂O. To insure that the slices did not experience anaerobic conditions, air was bubbled into the solution throughout the rinse and incubation period at a rate of approximately 25 ml/min.

All experiments were performed at least twice with two replicates per treatment.

ABA Purification Scheme. After the incubation period, slices and medium were separated. Slices were washed for 10 min with glass-distilled H₂O, and the wash was then combined with the incubation medium. Slices were frozen and lyophilized for dry weight determinations, and then extracted as described (4). ABA was purified by HPLC with a C₁₈ μ Bondapak column (4), using a convex gradient from 0 to 50% ethanol containing 1% acetic acid in aqueous 1% acetic acid. The increase in ethanol with

¹ Supported by the United States Department of Energy under Contract DE-AC02-76ER0-1338 and the National Science Foundation under Grant PCM78-07653.

² Abbreviations: BM, basal medium; GC-SIM, gas chromatography-selected ion monitoring; m/z, mass/charge; ECD, electron capture detector; MeABA, methyl ester of ABA.

respect to time (t , mins) may be described as: % ethanol = $50[(1 - (1/20)^t)]$. The fraction containing ABA, which eluted between 18 and 21 min, was dried, methylated, and quantified on a Hewlett-Packard 5840A gas chromatograph (4) equipped with a Hewlett-Packard 7672A automatic sampler. The ethyl ester of ABA was used as an internal standard.

The medium plus wash was acidified with glacial acetic acid to give 1% acetic acid and applied to a C_{18} Sep-Pak cartridge (Waters Associates) equilibrated with 1% acetic acid. A small amount of ($\pm$)-[3H]ABA ($16.4 \text{ Ci} \cdot \text{mmol}^{-1}$) was added before acidification of the medium to determine losses during the purification procedure. The Sep-Pak was washed sequentially with 2 ml of 1% aqueous acetic acid and 20% ethanol in 1% acetic acid. ABA was eluted with 5 ml of 40% ethanol in 1% acetic acid. The eluate was dried, methylated, and quantified as described above for the extracts from leaf slices.

The percentage recovery of [3H]ABA from tissue was 60% to 80%, while for medium it was 70% to 90%. All data were corrected for losses.

[^{14}C]Mannitol Uptake and Catabolism. Spinach leaf slices (2.1 g fresh weight) were incubated with 0.6 M mannitol plus $1 \mu\text{Ci D-[1-}^{14}C\text{]mannitol}$ ($45 \text{ mCi} \cdot \text{mmol}^{-1}$, New England Nuclear) for 4 h. The tissue was treated as described above and the sugars extracted three times with boiling 80% ethanol. After removal of the ethanol with a stream of N_2 , an aliquot of the aqueous solution was applied in a narrow band to a 22-cm strip of Whatman 3MM paper and subjected to descending chromatography for 18 h with methyl ethyl ketone:acetic acid:water saturated with boric acid (9:1:1) (10) as solvent. Standards ($10 \mu\text{g}$ each) of sucrose, glucose, fructose, and mannitol were also applied to the origin. Sugars were visualized by spraying with $\text{Cr}_2\text{O}_7\text{-KMnO}_4$ spray (25). Peaks of radioactivity were detected with a Packard model 7220/21 radiochromatogram scanner.

Water Potential Measurement. Leaf water and osmotic potential were measured with a Wescor HR-33T dew point microvoltmeter equipped with 6 C-52 sample chambers as described (24). Turgor potential was determined by subtraction. The osmotic potential of the incubation medium was measured by placing one drop of medium on a cellulose disc in a C-52 sample chamber for 2 h.

Mass Spectrometry. The GC-SIM response at m/z 190 (dwell time, 100 ms) was monitored as described (4).

RESULTS AND DISCUSSION

Maximum accumulation of ABA occurred when detached spinach leaves were allowed to lose 13% or more of their fresh weight and were then placed in a plastic bag for 4 h (data not

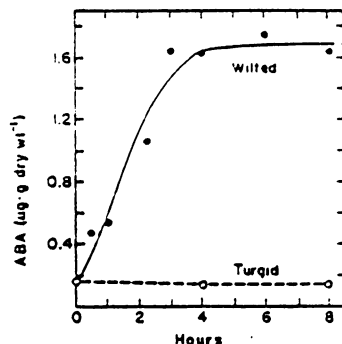


FIG. 1. Changes in ABA content of detached control (turgid), and in stressed (wilted) spinach leaves. The fresh weight of wilted leaves was reduced by 15%.

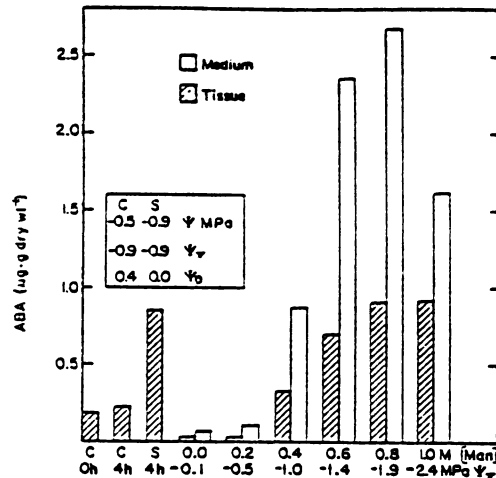


FIG. 2. ABA accumulation in detached control (C), stressed (S) spinach leaves, and in spinach leaf slices and media. Leaf slices were incubated in increasing concentrations of mannitol (Man). Parameters for cell water relations of detached leaves (insert) and osmotic potentials of the incubation media (ordinate) are indicated.

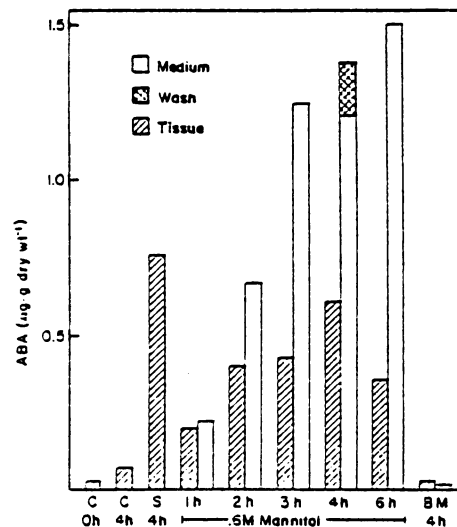


FIG. 3. ABA accumulation in detached spinach leaves, and in spinach leaf slices and media. The leaf slices were incubated in 0.6 mannitol (Man) for the times indicated. In this experiment, the wash (see "Materials and Methods") was analyzed separately from the medium.

shown). A maximum level of ABA (about a 10-fold increase) was reached within 3 to 4 h when such leaves had lost 15% of their fresh weight (Fig. 1). Therefore, in all experiments described here, leaves to be stressed were detached and allowed to lose 15% of their fresh weight. At this point, turgor was zero (Fig. 2).

In a similar manner, ABA accumulation in spinach leaf slices began only when mannitol concentrations greater than or equal to 0.4 M were used, and reached a maximum at 0.6 to 1.0 M (Fig. 2). Concentrations greater than 1.0 M could not be used due to solubility problems. In all further experiments 0.6 M mannitol



LOSS OF TURGOR CAUSES ABA ACCUMULATION

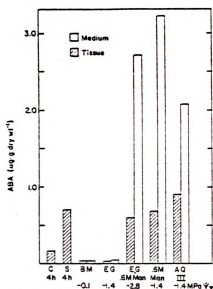


FIG. 4. Levels of ABA in detached spinach leaves, and in spinach leaf slices and media. The leaf slices were incubated in ethylene glycol (EG), EG plus 0.6 M mannitol (Man), 0.6 M mannitol, or Aquacide III (AQ III).

was used. When slices were incubated in 0.6 M mannitol, maximum accumulation of ABA in the tissue occurred within 3 h (Fig. 3), while ABA levels continued to increase somewhat in the medium. Some ABA was also found in the wash. This ABA was probably due to efflux from the slices. In addition, if slices were incubated in 0.6 M mannitol for 4 h and were then placed in BM for 4 h, ABA levels in the tissue declined to those found in control leaves or slices incubated in BM for 3 h (data not shown). Thus, with regard to ABA accumulation, spinach leaf slices incubated in mannitol solutions act in a manner similar to a stressed, detached *Xanthium* leaf (24).

To insure that the measured ECD response of ABA from tissue and medium was due solely to MeABA, equal amounts of ABA (as determined by ECD) extracted from tissue and medium, along with standard (ze)-ABA (Sigma) were analyzed by GC-SIM. If the MeABA peak from tissue or medium contained a contaminant, its SIM response would be less than that of the standard. The SIM response at m/z 190 (base peak) of MeABA from tissue, medium, and standard were identical within experimental error (data not shown), indicating that no contaminating peaks cochromatographed with MeABA during the measurement of ABA.

Some variation in the ABA level in turgid leaves and the accumulation of ABA in stressed leaves occurred between experiments. Possible sources of variation include leaf age (leaves were harvested 8 to 14 d after transfer to LD) and the stress history of the plant. To minimize these effects, uniform leaves were selected from groups of plants placed in trays containing deionized H₂O for 12 to 16 h before the onset of the experiment.

When leaf slices were incubated in 0.6 M ethylene glycol, no ABA accumulated in either the medium or the tissue (Fig. 4). Similar results have been obtained with thiourea (data not shown), another solute which is able to rapidly penetrate the cell membrane (9). However, when slices were incubated in 0.6 M mannitol or Aquacide III, ABA levels rose in both the medium and tissue. Aquacide III, a highly purified form of polyethylene

glycol, average mol wt 18,000, is unable to penetrate the cell wall and causes cytorrhysis (14). Thus, treatment with Aquacide III mimicks the situation found in water-stressed leaves (14). When slices were incubated with ethylene glycol plus mannitol, ABA accumulation was similar to that with mannitol alone. Thus, accumulation with regard to ABA accumulation, ethylene glycol was not toxic or injurious to the leaf tissue. Since ABA accumulation occurred only when solutes were used which caused plasmolysis or cytorrhysis, we conclude that ABA accumulation is dependent on the loss of turgor.

Since Riv and Yang (17) observed uptake and metabolism of mannitol in *Citrus* leaf discs, we were concerned that mannitol could penetrate the cell membrane in our system and cause osmotic adjustment, or mannitol could be metabolized. Based on radioactivity found in the tissue, 0.73% of the total mannitol found in the medium penetrated the cell membrane. The concentration of mannitol in the tissue was approximately 20 mM, or contributed about -0.05 MPa to the osmotic pressure (assuming a 1.0 M ideal solution is equivalent to -2.4 MPa). This is not enough to cause osmotic adjustment. In addition, contrary to the results that Riv and Yang obtained with *Citrus* (17), mannitol was not metabolized to sucrose in spinach (data not shown).

No significant accumulation of ABA occurred when three different concentrations of KHSO₅ (5, 15, and 25 mM), 2-pyridinol (1, 5, and 10%), or KCl (3, 50, and 100 mM) were used (data not shown). These compounds caused the production of ethane (a measure of membrane damage) when incubated with cucurbit leaf discs (5). We conclude, therefore, that the effect of mannitol on leaf slices is a physical one involving water stress, and not a chemical one involving membrane damage.

Of considerable interest is the large accumulation of ABA in the medium (Figs. 2-4; see also Ref. 12). In most experiments, approximately 400 mg fresh weight of slices were used per 10 ml incubation medium, presenting a relatively 'infinite' free space to the tissue. A likely explanation is that ABA crosses the plasma membrane and accumulates in the medium where it cannot be catabolized. On the basis of the model for cellular compartmentation of ABA (3), and appropriate values for the relative volumes of free space, tissue, chloroplasts, and vacuoles, one can calculate that most of the ABA will be found in the medium.

Attempts to develop similar systems with slices of bean (*Phaseolus vulgaris* cv Redcloud) and *Xanthium strumarium* failed. Accumulation of ABA in slices of these tissues was erratic and irreproducible. Interestingly, Loves (11) reported higher levels of ABA in *Vicia faba* mesophyll tissue infiltrated with 0.88 M mannitol than with buffer, but the total ABA in the tissue plus medium was lower than the value reported for nonstressed turgid leaves. *Xanthium* mesophyll cells have high photosynthetic activity (19), yet do not appear to accumulate ABA when stressed with mannitol (ML Pierce, EA Bray, personal communications) even though stressed, detached leaves of *Xanthium* accumulate large amounts of ABA (24). When spinach leaf slices were incubated in 0.6 M mannitol along with *Xanthium* mesophyll cells (19), the amount of ABA that accumulated was reduced (EA Bray, RA Creelman, JAD Zeevaert, unpublished results), indicating the presence of a diffusible inhibitor. This inhibitor is probably nonspecific in action and most likely a result of tissue wounding and cellular leakage. It is also possible that the *Xanthium* mesophyll cell system was not fully optimized for ABA production.

Pierce and Raschke (16) observed that ABA accumulation occurred when turgor was zero using data obtained from pressure-volume curves. Using spinach leaf slices incubated with plasmolyzing or nonplasmolyzing solutes, we obtained results that support the hypothesis of Pierce and Raschke (16). We conclude, therefore, that turgor is the critical parameter of cell water relations governing stress-induced ABA accumulation.

Acknowledgments.—We would like to thank the Michigan State University-National Institute of Health Mass Spectroscopy Facility (R000440) for use of the GC-MS equipment.

LITERATURE CITED

- BEARDSLEY MF, D COHEN 1975 Relationship between leaf water status, abscisic acid levels, and stomatal resistance in maize and sorghum. *Plant Physiol* 56: 207-212
- BLAKE J, WK FERRELL 1977 The association between soil and xylem water potential, leaf resistance, and abscisic acid content in droughted seedlings of *Coultasifera (Pennisetum purpureum)*. *Physiol Plant* 39: 106-109
- COWAN JR, JA RAYEN, W HARTUNG, GD FARQUHAR 1982 A possible role for abscisic acid in coupling stomatal conductance and photosynthetic carbon metabolism in leaves. *Aust J Plant Physiol* 9: 489-498
- CREELMAN RA, JAD ZEEVAART 1984 Incorporation of oxygen into abscisic acid and phaseic acid from molecular oxygen. *Plant Physiol* 75: 166-169
- FILVER P, H RENNENBERG, J SEXTA, RA BRESMAN, LG WILSON, L LACROIX, T SIEGEL 1983 Bioynthesis and emission of hydrogen sulfide by higher plants. In MJ Kozol, FR Whalley, eds. *Gaseous Air Pollutants and Plant Metabolism*. Butterworths, London, pp 391-412
- GREENWAY H, M LEAHY 1970 Effects of rapidly and slowly permeating osmoticum on metabolism. *Plant Physiol* 46: 259-262
- HARTUNG W, H CHUMLER, B HELLMANN 1982 The compartmentation of abscisic acid (ABA), of ABA-bioynthesis, ABA-metabolism and ABA-conjugation. In PF Wareing, ed. *Plant Growth Substances 1982*. Academic Press, New York, pp 325-333
- HENFELD DD, JK HS TUCKER JR 1975 Effect of intermittent mist on abscisic acid levels in *Eucalyptus alatus* Sieb: leaching vs. moisture stress. *Hortscience* 10: 369-370
- LEVITT J 1983 Plasmolysis shade in relation to freeze-hardening of cabbage plants and to the effect of penetrating solutes. *Plant Cell Environ* 6: 465-470
- LEWIS DH, DC SWITH 1967 Sugar alcohols (polyols) in fungi and green plants II. Methods of detection and quantitative estimation in plant extracts. *New Phytol* 66: 185-204
- LOVETTS BR 1977 The intracellular location of abscisic acid in stressed and non-stressed leaf tissue. *Physiol Plant* 40: 9-10
- LOVETTS BR, CJ BAREN, PE KÄRDEMANN 1975 Bioynthesis of abscisic acid under osmotic stress studied based on a dual labeling technique. *Physiol Plant* 33: 166-170
- MAVISON BT, B COLMAN, WR CLIMMING 1981 Abscisic acid and photosynthesis in isolated leaf mesophyll cells. *Plant Physiol* 67: 233-236
- OERTLI JJ 1976 The uses of water in the plant. In OL Lange, L Kaepfen, E-D Schulze, eds. *Water and Plant Life*. Springer-Verlag, New York, pp 19-31
- PERCE ML 1981 Turpore dependence of bioynthesis and metabolism of abscisic acid. PhD thesis, Michigan State University, East Lansing
- PERCE ML, K RASCHKE 1980 Correlation between loss of turpore and accumulation of abscisic acid in detached leaves. *Planta* 148: 174-182
- ROY J, SF YANG 1982 Stimulation of enzyme production in citrus leaf discs by mannitol. *Plant Physiol* 70: 142-146
- RIVIER L, J-F LEONARD, J-P COTTER 1983 Rapid effect of osmotic stress on the content and exo-diffusion of abscisic acid in *Zea mays* roots. *Plant Sci Lett* 31: 133-137
- SHARKEY TD, K RASCHKE 1980 Effects of phasic and dihydrophasic acid on stomata and photosynthetic apparatus. *Plant Physiol* 65: 291-297
- WONG JR, LM SUBER 1980 Isolation of abscisic acid-resistant variants from tobacco cell cultures. *Planta* 148: 97-102
- WRIGHT STC 1978 Phytohormones and stress phenomena. In DS Latham, PB Goodwin, TIV Higgins, eds. *Phytohormones and Related Compounds, A Comprehensive Treatise*, Vol 2. Elsevier/North-Holland, New York, pp 495-516
- ZAMADAL TJ 1974 A water potential threshold for the increase of abscisic acid in leaves. *Plant Physiol* 53: 125-127
- ZEEVAART JAD 1971 Effects of photoperiod on growth rate and endogenous gibberellins in the long day rose plant species. *Plant Physiol* 47: 821-827
- ZEEVAART JAD 1983 Metabolism of abscisic acid and its regulation in *Xanthium* during and after water stress. *Plant Physiol* 71: 477-481
- ZWIG G, JR WHITTAKER 1967 Paper Chromatography and Electrophoresis. Vol 1. Academic Press, New York, p 241



Chapter 10
STRESS-INDUCED ABSCISIC ACID BIOSYNTHESIS IN
HIGHER PLANTS -- A MODEL



10.1. INTRODUCTION

As mentioned in chapter 1, nothing is known about intermediates in the biosynthetic pathway of ABA, except that as a sesquiterpenoid, ABA is ultimately derived from MVA. Research on the ABA biosynthesis centers around two pathways: the direct pathway involving a C₁₅ precursor derived from farnesyl pyrophosphate and the indirect pathway involving a precursor derived from carotenoids.

Sesquiterpenoids are characterized as compounds that have a basic skeleton of fifteen carbon atoms formed by a regular repetition of isoprene units. Almost 200 different carbon skeletons containing up to four carbocyclic rings are found in this group (Loomis and Croteau, 1980). Representative sesquiterpenoids are illustrated in Figure 10.1. The carbon skeleton of ABA can easily be derived from farnesyl pyrophosphate by cyclization on paper. However, the abscisane skeleton is very unique, with ABA (and its derivatives, such as PA) and xanthoxin being the only members of this class. There are many compounds, however, with a ring structure and side chain similar to ABA, such as "allenic sesquiterpenoid", and a component of the aroma of tea, theaspirone (Ina et al., 1968; Figure 10.2). "Allenic sesquiterpenoid" was found in the flightless grasshopper *Romalea microptera* and is considered to be a breakdown product of neoxanthin (Meinwald et al., 1968). Other similar compounds are loliolide, butenone (Figure 1.5), α -ionone, β -ionone, and dihydroactinidiolide

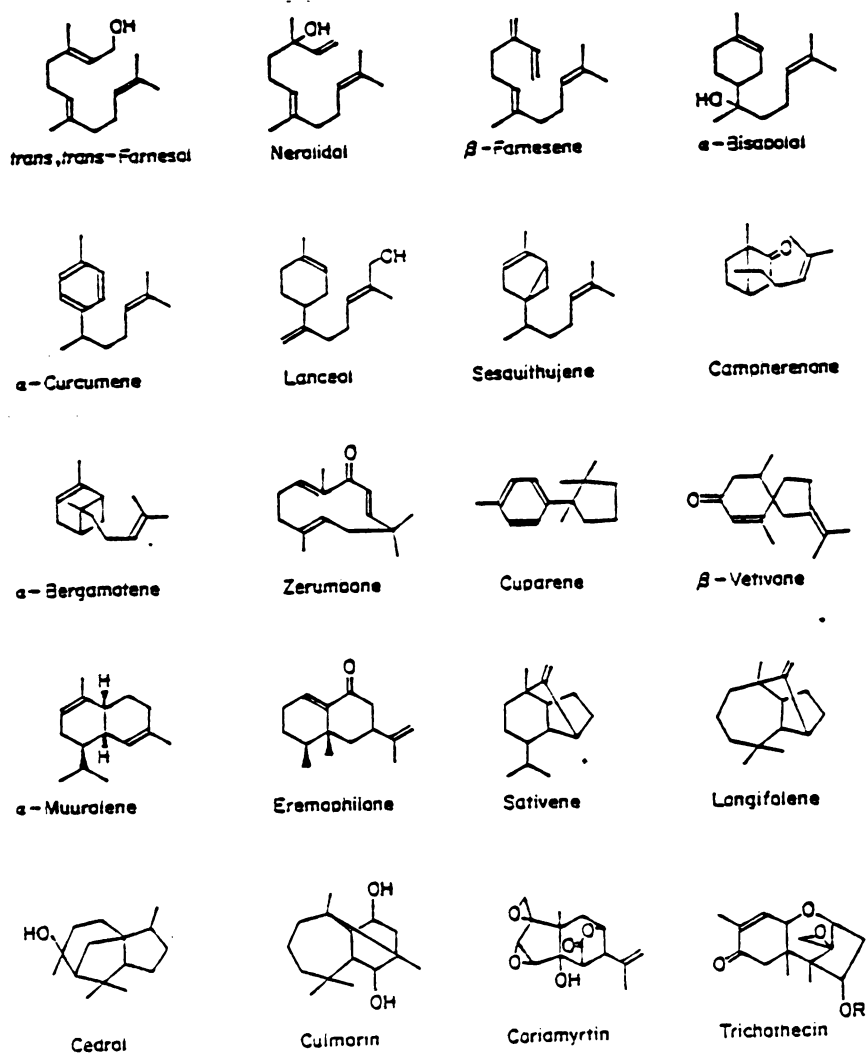


Figure 10.1. Representative sesquiterpenoids.

Note the large diversity of structures. From Loomis and Croteau (1980).



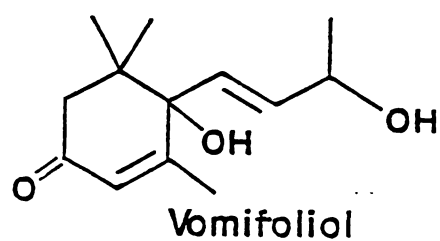
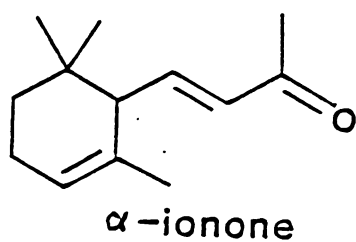
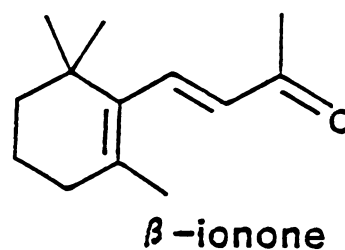
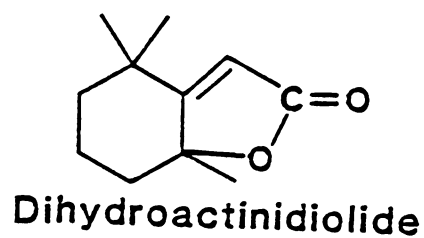
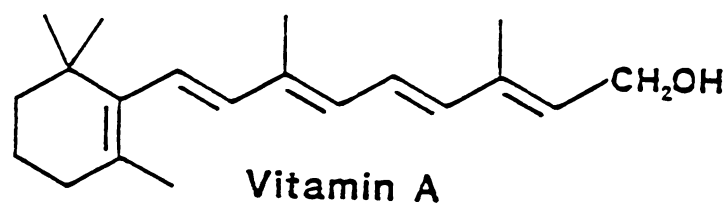
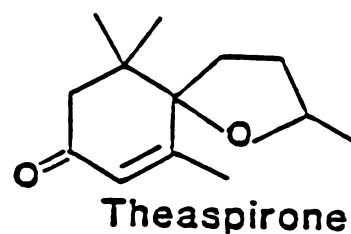
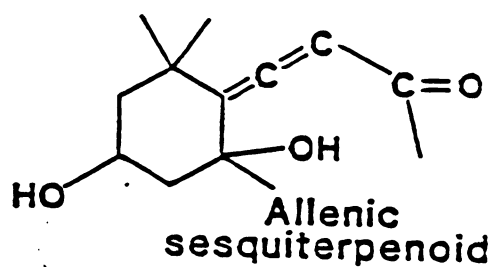


Figure 10.2. Compounds with structures similar to abscisic acid.

These compounds have a ring skeleton similar to ABA, and are though to be derived from carotenoids.

(Figure 10.2). All of these compounds are found in plants (except "allenic sesquiterpenoid"), and are considered to be derived from carotenoids. However, it is not known whether these compounds are endogenous or are formed by oxidative destruction of carotenoids during extraction. Both β -ionone and dihydroactinidiolide can be formed by an oxidative attack on β -carotene (Isoe et al., 1969). The only known cases where compounds have been shown to be derived from a carotenoid are vitamin A (in animal intestine; Figure 10.2; Britten, 1983), and trisporic acid (in fungi; Figure 1.6).

The best evidence for a compound being derived from a carotenoid and being further catabolized to a physiologically active substance is the production of trisporic acids from β -carotene (Bu'Lock et al., 1976; Gooday et al., 1979; Figure 1.6). It should be noted that some uncertainty exists as to whether the first compound formed is indeed a C_{20} (retinal) derivative. Trisporic acids are produced by, and act only on, fungi of the order Mucorales. The observed effect of trisporic acid is to induce zygospores in both mating types of a particular species. Enzymatic conversion of β -carotene to trisporic acids is believed to occur for the following reasons: (a) radioactive retinol, β - C_{18} -ketone, and 4-hydroxy- β - C_{18} -ketone are converted to trisporic acids (Bu'Lock et al., 1974); (b) the distribution of radioactive label from MVA is correlated in both β -carotene and trisporic acid (Austin et al., 1970); (c) in mated *Blakeslea trispora*, inhibition of β -carotene biosynthesis by diphenylamine

prevents trisporic acid production (Austin *et al.*, 1969); (d) *Phycomyces blakesleeenanus* mutants blocked in β -carotene synthesis are also blocked in trisporic acid production (Bergman *et al.*, 1969). As described in chapter 1, a similar situation exists with regard to ABA production (carotenoid mutants and biosynthetic inhibitors, conversion of radioactive xanthoxin to ABA).

Thus, there are many compounds which appear to be carotenoid degradation products. With regard to carotenoid biosynthesis, much more is known about the anabolism of carotenoids than their catabolism. The pathway of carotene biosynthesis from isopentenyl pyrophosphate through the cyclic carotenes has been established (Spurgeon and Porter, 1980). An enzyme complex from tomato fruit plastids that converts isopentenyl pyrophosphate to phytoene (Porter and Spurgeon, 1979) has been described. This complex is apparently located in the chloroplast envelope, as is phytoene dehydrogenase (Lütke-Brinkhaus *et al.*, 1982). In view of the extensive knowledge concerning the enzymatic formation of carotenoids, surprisingly little is known about the rate of carotenoid catabolism, the nature of the catabolites, or which enzymes are involved. Several enzyme systems, such as peroxidase (Matile and Martinoia, 1982), or lipoxygenase (Gardner, 1980) are known which will convert carotenoids to colorless products; however, it is not known if these enzymes perform the same role *in vivo*.

In conclusion, there are many compounds which can be

derived from carotenoids by paper chemistry. However, in many cases it is not known if these compounds are artifacts of extraction, or are endogenous. Only two compounds have been shown to result from carotenoid degradation *in vivo*, vitamin A and trisporic acid. Enzymes have been described which will degrade carotenoids, however, their role *in vivo* is unknown.

10.2. A MODEL EXPLAINING STRESS-INDUCED ABSCISIC ACID BIOSYNTHESIS

Any model proposing to explain the water stress induced biosynthetic pathway in higher plants must explain the following observations: (a) the lag time of 20 to 50 min before the accumulation of ABA (Guerrero and Mullet, 1986; Henson, 1981; Henson and Quarrie, 1981; Zeevaart, 1980), (b) the fact that much more ABA appears to be made during water stress than is needed for stomatal closure (Raschke, 1975), and (c) the heavy oxygen incorporation data described in chapters 4 and 6.

The lag in the accumulation of ABA after imposition of water stress is consistent with the notion that an enzyme is induced. This enzyme appears to be encoded in the nucleus because ABA accumulation is inhibited by cycloheximide, an inhibitor of cytosolic protein synthesis, and not with chloramphenicol, lincomycin, or spectinomycin, inhibitors of plastid protein synthesis (Quarrie and Lister, 1984; Guerrero



and Mullet, 1986). Accumulation of ABA was also inhibited with actinomycin D and cordycepin, nuclear transcription inhibitors (Guerrero and Mullet, 1986). The signal created by water stress causing the accumulation of ABA appears to be the loss of turgor (chapter 9; Pierce and Raschke, 1980).

The induced enzyme (or another enzyme in the ABA biosynthetic pathway) appears to be sensitive to the oxygen tension surrounding the leaf. The accumulation of ABA is not saturated until approximately 60% oxygen. While it is difficult to extrapolate from data generated from a whole leaf system to a specific enzyme, this result suggests that an enzyme in the biosynthetic pathway of ABA has a low affinity for oxygen, and may be a mono-oxygenase (or oxygenase).

If ABA is derived from a carotenoid(s), then there is a huge excess of potential precursor in a green leaf. This could explain why more ABA is made than is needed for stomatal closure. Water stress could change the topology of chloroplasts membranes, exposing carotenoids to degradative enzymes. There are also enough total carotenoids present in roots to account for the amount of ABA made.

However, there are certain lines of evidence that indicate that if leaf ABA is derived from carotenoids, it is from a small fraction of the total carotenoid pool. Walton *et al.* (1985) introduced ^{18}O into violaxanthin via the xanthophyll cycle. In this cycle, violaxanthin loses its epoxide groups, ultimately forming zeaxanthin when leaves are incubated in light under nitrogen. If leaves are then placed



in the dark, the level of violaxanthin increases with the incorporation of oxygen from molecular oxygen into the epoxide group. When this was done in an atmosphere containing $^{18}O_2$, between 40 and 45% of the total violaxanthin contained ^{18}O ; no ^{18}O was found in lutein or neoxanthin. When these leaves were stressed, the ABA which accumulated contained 10-15% ^{18}O in the ring oxygens. It was not possible to determine the position of incorporation in the ABA molecule. This result suggests that a portion of the ABA which was made during the stress period came from violaxanthin. An explanation for the low amount of incorporation into ABA is that ABA is produced from other xanthophylls or from a non-xanthophyll precursor. Another possibility is that ABA is derived from the violaxanthin pool which was not labeled with ^{18}O . There exist two violaxanthin pools, with the majority of violaxanthin found in the chloroplast envelope and the rest in thylakoids (Douce *et al.*, 1973). An enzyme has been isolated from spinach chloroplasts which will convert violaxanthin to zeaxanthin (Hager and Perz, 1970). This enzyme is apparently located in the grana and stroma thylakoids (Siefermann and Yamamoto, 1976). Thus, if in the experiment of Walton *et al.* (1985), ABA came primarily from violaxanthin in the outer envelope, then it might not have much ^{18}O present in the ring positions. The ^{18}O incorporation data described in chapters 4 and 6 support the hypothesis that ABA is derived from a carotenoid.

Most of the data described in this thesis suggest that

carotenoids are precursors to ABA. What is needed, however, is a definitive experiment to prove that a carotenoid does break down to form ABA. Since stressed *Xanthium* roots contain low amounts of carotenoids, it might be possible to deplete the tissue of carotenoids. A time course study of changes in carotenoid levels and ABA accumulation in roots needs to be done. If ABA came from a particular carotenoid in roots, then the amount of that carotenoid should decline with water stress.

Another question that needs to be answered is the role of xanthoxin. Labeled xanthoxin needs to be synthesized and fed to the three tomato mutants that do not accumulate stress-induced ABA (*flc*, *sit*, and *not*; see chapter 1). If ABA does arise from xanthoxin, it is possible that intermediates might accumulate in these mutants. Possible intermediates between xanthoxin and ABA are xanthoxin acid and abscisic aldehyde. These compounds can also be synthesized from intermediates used in the biosynthesis of labeled xanthoxin and used in feeding studies or as standards.

10.3. LITERATURE CITED

- Austin, D.J., Bu'Lock, J.D., Drake, D. (1970) The biosynthesis of trisporic acids from β -carotene via retinal and trisporal. *Experientia* 26:348-349.
- Austin, D.J., Bu'Lock, J.D., Winstanley, D.J. (1969) Trisporic acid biosynthesis and carotenogenesis in *Blakeslea trispora*. *Biochem. J.* 113:34P.
- Bergman, K., Burke, P.V., Cerda-Olmedo, E., David, C.N., Delbrück, M., Foster, K.W., Goodell, E.W., Heisenberg,

- M., Meissner, G., Zalocar, M., Dennison, D.S., Shropshire, W. (1969) Phycomyces. Bacteriol. Rev. 33:100-157.
- Britton, G. (1983) The biochemistry of natural pigments. Cambridge: Cambridge University Press.
- Bu'Lock, J.D., Jones, B.E., Taylor, D., Winskill, N., Quarrie, S.A. (1974) Sex hormones in Mucorales. The incorporation of C₂₀ and C₁₈ precursors into trisporic acids. J. Gen. Microbiol. 80:304-306.
- Bu'Lock, J.D., Jones, B.E., Winskill, N. (1976) The apocarotenoid system of sex hormones and prohormones in Mucorales. Pure and Appl. Chem. 47:191-202.
- Douce, R., Holtz, R.B., Benson, A.A. (1973) Isolation and properties of the envelope of spinach chloroplasts. J. Biol. Chem. 248:7215-7222.
- Gardner, H.W. (1980) Lipid enzymes: lipases, lipoxygenases, and "hydroperoxidases". In: Autoxidation in food and biological systems, pp 447-504, Simic, M.G., Karel, M., eds. New York: Plenum.
- Gooday, G.W., Jones, B.E., Leith, W.H. (1978) Trisporic acid and the differentiation in the Mucorales. In: Regulation of secondary product and plant hormone metabolism, pp. 221-229, Luckner, M., Schreiber, K., eds. New York: Pergamon Press.
- Guerrero, F., Mullet, J.E. (1986) Increased abscisic acid biosynthesis during plant dehydration requires transcription. Plant Physiol. 80:588-591.
- Hager, A., Perz, H. (1970) Veränderung der Lichtabsorption eines Carotenoids im Enzym (De-epoxidase)-Substrat (Violaxanthin)-Komplex. Planta 93:314-322.
- Henson, I.E. (1981) Changes in abscisic acid content during stomatal closure in pearl millet [*Pennisetum americanum* (L.) Leeke]. Plant Sci. Lett. 21:121-127.
- Ina, K., Sakato, Y., Fukami, H. (1968) Isolation and structural elucidation of theaspirone, a compound of tea essential oil. Tetrahedron Lett. 2777-2780.
- Isoe, S., Hyeon, S.B., Sakan, T. (1969) Photo-oxygenation of carotenoids. I. The formation of dihydroactinidiolide and B-ionone from B-carotene. Tetrahedron Lett. 279-281.
- Loomis, W.D., Croteau, R. (1980) Biochemistry of terpenoids. In: The biochemistry of plants: a comprehensive treatise, pp. 363-418, vol. 4, Stumpf, P.K., Conn, E.E., eds. New York: Academic Press.



- Lütke-Brinkhaus, F., Liedvogel, B., Kreuz, K., Klenig, H. (1982) Phytoene synthase and phytoene dehydrogenase associated with envelope membranes from spinach chloroplasts. *Planta* 156:176-180.
- Matile, P., Martinoia, E. (1982) Catabolism of carotenoids: involvement of peroxidase? *Plant Cell Reports* 1:244-246.
- Meinwald, J., Erikson, K., Hartshorn, M., Meinwald, Y.C., Eisner, T. (1968) Defensive mechanisms of arthropods. XXIII. An allenic sesquiterpenoid from the grasshopper *Romalea microptera*. *Tetrahedron Lett.* 2959-2962.
- Pierce, M.L., Raschke, K. (1980) Correlation between loss of turgor and accumulation of abscisic acid in detached leaves. *Planta* 148:174-182.
- Porter, J.W., Spurgeon, S.L. (1979) Enzymatic synthesis of carotenes. *Pure and Appl. Chem.* 51:609-622.
- Spurgeon, S.L., Porter, J.W. (1980) Carotenoids. In: *The biochemistry of plants*, pp 420-484, vol 4, Stumpf, P.K., Conn, E.E., eds. New York: Academic Press.
- Quarrie, S.A., Lister, P.G. (1984) Effects of inhibitors of protein synthesis on abscisic acid accumulation in wheat. *Z. Pflanzenphysiol.* 114:309-314.
- Walton, D.C., Li, Y., Neill, S.J., Horgan, R. (1985) Biosynthesis of abscisic acid: a progress report. In: *Current topics in plant biochemistry and physiology 1985*, pp. 111-117, vol. 4, Randall, D.D., Blevins, D.G., Larson, R.L., eds. Columbia, MO: University of Missouri.
- Zeevaart, J.A.D. (1980) Changes in the levels of abscisic acid and its metabolites in excised leaf blades of *Xanthium strumarium* during and after water stress. *Plant Physiol.* 66:672-678.



APPENDIX
QUANTITATION OF ABSCISIC ACID USING INTERNAL STANDARDS



Quantitation of ABA in large numbers of samples requires the use of automated equipment in order to give the experimenter time to perform other tasks than injecting samples into a gas chromatograph. In our laboratory, ABA samples are routinely analyzed with a Hewlett-Packard 7672A automatic injector coupled to a Hewlett-Packard 5840 gas chromatograph.

The use of such equipment requires that an internal standard be used to take into account two possible events that may occur during analysis: (a) evaporation of solvent from vials as they wait for their turn to be sampled, and (b) injections are not exactly reproducible from one sample to the next. Evaporation occurs because the seal on the sample vials is not airtight; this is unlikely to occur given the time scale of analysis. The latter event (b) is inherent in any machine-operated analysis and is due to the fact that an exact syringe volume cannot be obtained with each injection.

Certain criteria must be met for a compound to be used as an internal standard: it must have (a) the ability to capture electrons, and (b) a retention time different from contaminating peaks and MeABA. We commonly use EtABA and/or enderin, a chlorinated pesticide.

The procedure is as follows. Once the samples have been purified by HPLC, they are dried, and methylated with ethereal diazomethane. The manner in which the samples are further analyzed depends on the amount of ABA expected to be present. For small amounts of ABA the sample may be

dissolved in a small volume of a stock solution containing the internal standard(s) in ethyl acetate. The dissolved sample is then transferred to an injection vial.

For samples containing large amounts of ABA dilution is necessary. This is commonly done by dissolving the sample in ethyl acetate (for instance 10 to 25 ml). A small aliquot is transferred to an injection vial and the solvent is allowed to evaporate. The stock solution containing the internal standard is added and the samples run on the automatic injector (see Figure A.1 for a standard run).

Standard amounts of ABA are also dissolved in the same stock solution. Because MeABA, EtABA, and enderin are stable, standards can be used several times. For a given set of standards, the stock solution (containing the internal standards) may be used for dissolving samples as long as evaporation does not significantly increase the peak area of the internal standards. If this occurs, then a new set of standards must be made.

If solvent evaporation or an injection error occurs, the peak areas of both MeABA and the internal standards will increase to the same extent. This occurs because the detector response is proportional to the amount of compound present in the injected solution (sample). Thus, comparison of the ratio of the peak areas of MeABA and the internal standard in samples and in standards (see Table A.1 for typical data and Figure A.2 for typical standard curves) will give an estimation of the concentration of ABA in the



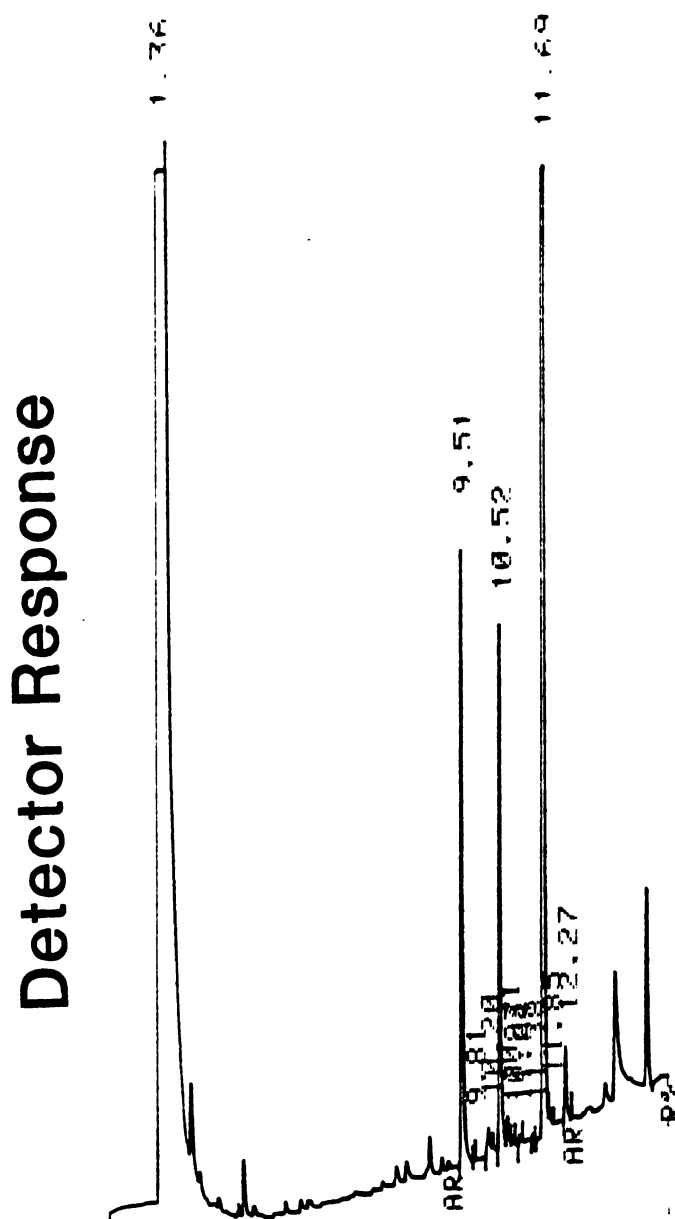


Figure A.1. Electron capture detector response of abscisic acid and internal standards.

The retention times of MeABA, EtABA, and enderin are 9.51, 10.52, and 11.68 min, respectively. The peak corresponding to MeABA represents an injection of 200 pg in 1 μ l.

Table A.1. Example of abscisic acid quantitation by gas-liquid chromatography electron capture detection with internal standards.

The data presented in (A) are typical of those obtained for standards by GLC-ECD, and are the same as those presented in figure A.2. For the ratio MeABA/EtABA the regression line is: $\text{Ratio} = 0.00613(\text{pg}/\mu\text{l}) + 0.0414$ ($r^2 = 0.999$). For the ratio MeABA/enderin the regression line is: $\text{Ratio} = 0.00070(\text{pg}/\mu\text{l}) + 0.0100$ ($r^2 = 0.997$). Shown in (B) are data obtained from a Xanthium tissue extract. The samples were dissolved in 15 ml ethyl acetate. From this, 100 μl were removed, placed in a sample vial, and dried. The stock solution (500 μl) containing the internal standards was then added to the vial and 1 μl injected into the gas chromatograph (dilution factor is 75000).

175

(A) MeABA		Area		Ratio		
pg/ l		MeABA(a)	EtABA(b)	Enderin(c)	a/b	a/c
10		292	5970	48780	0.049	0.006
20		628	5164	43860	0.122	0.014
50		1579	5192	40460	0.304	0.039
100		3552	5684	43450	0.625	0.082
200		7412	5243	43860	1.414	0.169
(B)		Area		Ratio		
MeABA(a)		EtABA(b)	Enderin(c)	a/b	a/c	a/c
3480		5770	45074	0.60	0.077	91 95

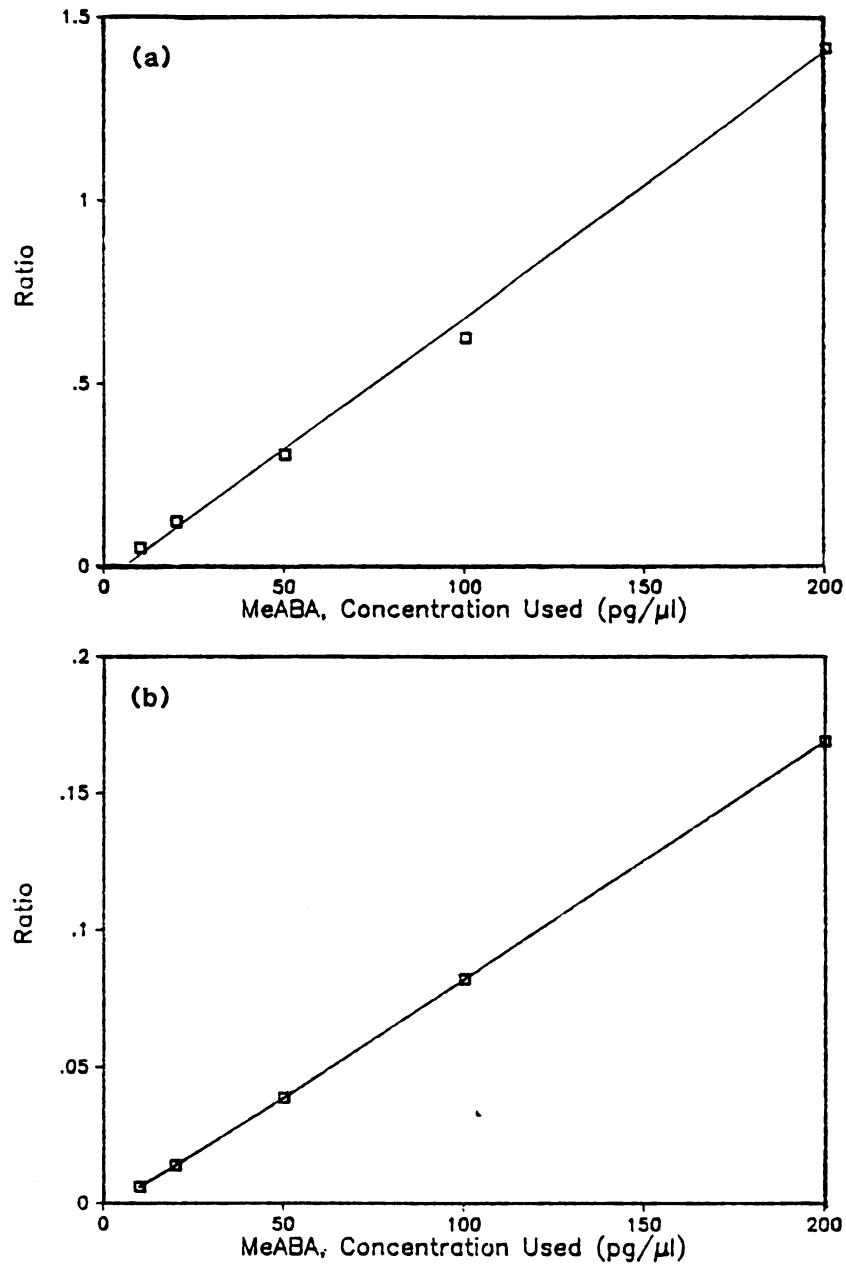


Figure A.2. Typical standard curve obtained by injecting internal standards with increasing concentrations of methyl abscisic acid.

In figure (a) MeABA/EtABA vs. the concentration of MeABA used is plotted; in (b) MeABA/enderin vs. the concentration of MeABA used is plotted.



sample vial. The amount of ABA present in sample is then calculated knowing the volume injected (usually 1 μ l), per cent recovery, dilution, and amount of tissue, using the formula below:

$$\text{ABA/g dry weight} = \frac{(\text{concentration})(\text{dilution factor})}{(\text{recovery})(\text{g dry weight})}$$

Using values obtained from Table A.1 the amount of ABA present in the tissue extract was:

$$\frac{(91 \text{ pg}/\mu\text{l})(75000)}{(0.75)(0.4 \text{ g dry weight})} = 22.7 \text{ } \mu\text{g/g dry weight.}$$





MICHIGAN STATE UNIV. LIBRARIES



31293107578514