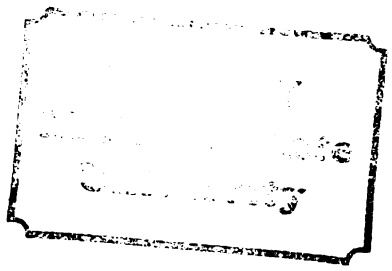

TURGOR DEPENDENCE OF BIOSYNTHESIS AND METABOLISM OF ABSCISIC ACID

By

Margaret Lee Pierce

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ABSTRACT

TURGOR DEPENDENCE OF BIOSYNTHESIS
AND METABOLISM OF ABSCISIC ACID

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MARGARET LEE PIERCE

The relation between leaf water potential (ψ_{leaf}) and steady-state level of ABA was determined for mature leaves of Phaseolus vulgaris L., Xanthium strumarium L., and Gossypium hirsutum L. Leaf sections from any one leaf covered a range of known ψ_{leaf} values, turgors (p), and osmotic pressures (π). At p=1 bar, ABA content averaged 4 times the level found in unstressed samples. Below p=1 bar, ABA content increased sharply to as much as 40 times the level found in turgid samples. Leaves from stress-conditioned cotton plants, having higher π, required lower ψ_{leaf} in order to accumulate ABA than did leaves from previously unstressed cotton plants. Thus, turgor, rather than ψ_{leaf} or π, appears to be the critical parameter of plant water relations which controls ABA production in stressed leaves.

Stomatal closure occurred in leaves of Commelina communis L. before an increase in ABA was obvious in the leaf as a whole, but a gradual increase, of 50-100%, in the level of ABA in the epidermis paralleled stomatal closure. The hypothesis that ABA helps cause stress-induced stomatal

closure is tenable, based on a comparison of these results with estimates of stomatal sensitivity to exogenous ABA.

Loss of turgor in detached leaves of P. vulgaris was correlated with accumulation, not only of ABA, but also of its metabolites, predominantly phaseic acid (PA). Upon rehydration the rate of synthesis of ABA dropped to zero within about 3 h, while conversion to PA accelerated; it reached a rate sufficient to convert almost ½ of the ABA present in the tissue to PA within 1 h. In contrast, the alternate route of metabolism of ABA, synthesis of conjugated ABA, was not stimulated by rehydration. Conversion of ABA to PA was accelerated at the slightest indication of a regain of turgor. Thus, the opposing processes of synthesis and metabolic removal of ABA appear to be linked to loss of turgor on the one hand and recovery of turgor on the other.

In memory of Mr. Karl N. Pierce, my grandfather

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ABA	Abscisic acid
(±)-ABA	Racemic mixture of abscisic acid
Me-ABA	Abscisic acid methyl ester
DPA	Dihydrophaseic acid
epi-DPA	epi-Dihydrophaseic acid
PA	Phaseic acid
HEPES	N-2-hydroxyethylepiperazine-N^1-2-ethanesulfonic acid
MES	2-(N-morpholino)ethanesulfonic acid
PVP-40	Polyvinylpyrrolidone
Bq	Becquerel, one disintegration per second
DW	Dry weight
FW	Fresh weight
MPa	Megapascal, 10 bar
GLC-EC	Gas liquid chromatography with electron capture detector
HPLC	High performance liquid chromatography
ψ	Water potential
ψ_{leaf}	Leaf water potential
π	Osmotic pressure
p	Volume-averaged turgor
ε	Volumetric modulus of elasticity

Chapter 1

General Introduction

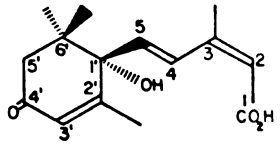
1.1. INTRODUCTORY REMARKS

Abscisic acid (ABA) has become established over the past 15 to 20 years as one of the major classes of plant growth substances (see reviews by Addicott and Lyon 1969, Milborrow 1974a, Wareing 1978, Zeevaart 1979, Walton 1980). Usually, ABA is classified as a growth inhibitor because it behaves as one in a number of bioassays, counteracting the effect of growth-promoting substances: auxins, gibberellins, and cytokinins (Wareing 1978). Numerous physiological roles have been postulated for ABA, including, but not limited to, (1) a contribution to geotropism in roots, (2) a contribution to seed dormancy, especially in cases where the embryo is innately dormant (Wareing, 1978), (3) induction and maintenance of bud dormancy, (4) promotion of tuber formation, and (5) promotion of stomatal closure during water stress. Recently Wareing (1978) and Walton (1980) have critically reviewed the evidence concerning the role of ABA in these processes and concluded that we do not know enough about the effects of ABA on plant cells or the nature of the individual processes to state unequivocally in any case the degree of involvement of ABA. Possibly the most compelling evidence of a functional role for ABA comes from studies on the physiology of water-stressed plants; this evidence is summarized in Section 1.4. This thesis is an account of a further investigation of the regulation of biosynthesis and metabolism of ABA in water-stressed leaves and the regulation

of stomatal aperture by ABA.

1.2. BIOSYNTHESIS AND METABOLISM OF ABA

1.2.1. Precursors and products. The immediate precursors of ABA (I), a sesquiterpenoid, are unknown.

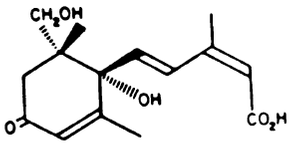
I. 

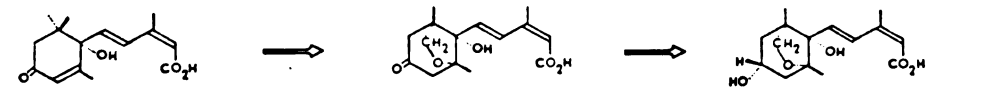
(+)-Abscisic acid

Milborrow and Robinson (1973) demonstrated the incorporation of label from mevalonate into ABA in different parts of plants, including seeds, stems, leaves, and fruits. There are basically two pathways which can account for the derivation of ABA from mevalonate: 1) direct incorporation of three isoprene units, derived from mevalonate, into ABA and 2) indirect incorporation of isoprene units into ABA via carotenoids. The bulk of the evidence, mostly from experiments on avocado fruit, has favored the first of the two pathways (Milborrow 1974a).

Abscisic acid is metabolized predominantly via 6'-hydroxymethyl-ABA, an unstable intermediate, to phaseic acid (PA). In many plant tissues, PA is further metabolized to 4'-dihydrophaseic acid (DPA), which appears to accumulate as an end product. Evidence has been presented that ABA is metabolized by this route (see Figure 1-1) in ash seeds (Sondheimer et al. 1974), in endosperm of immature fruits of Echinocystis (Gillard and Walton 1976), in excised bean roots

Figure 1-1. Pathways of ABA metabolism. The conversion of ABA to PA occurs via the unstable intermediate 6'-hydroxymethylabscisic acid:

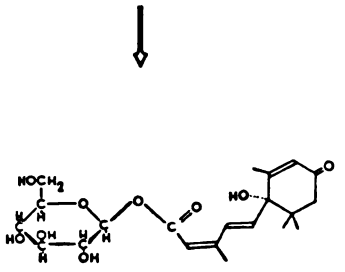




ABSCISIC ACID
(ABA)

PHASEIC ACID
(PA)

DIHYDROPHASEIC ACID
(DPA)



(+)-ABA-β-GLUCOSYL
ESTER

Figure 1-1

(Walton et al. 1976), and in the shoots or leaves of a number of species (Harrison and Walton 1975, Zeevaart 1977, Tietz et al. 1979, Sivakumaran et al. 1980). However, the leaves of Xanthium strumarium contain only trace amounts of DPA; the metabolic fate of PA in this tissue is not yet known (Zeevaart 1980).

The liquid endosperm from Echinocystis provided a cell-free system for the metabolism of ABA. After centrifugation, ABA-hydroxylating activity was found in the particulate fraction and PA-reducing activity was associated with the supernatant fraction (Gillard and Walton 1976). The intermediate 6'-hydroxymethyl-ABA spontaneously rearranges to PA, and it is not known whether that step is enzymatic in vivo or not.

As indicated in Figure 1-1, ABA can also be metabolized to a conjugate with glucose, abscisyl-β-D-glucopyranoside (Milborrow 1978), but conjugation appears normally to occur to a lesser extent than conversion to PA, at least in bean leaves (Harrison and Walton 1975, Zeevaart and Milborrow 1976). Whereas the naturally-occurring (+)-enantiomer of ABA can be converted to PA or conjugated with glucose, it appears that (-)-ABA can only be conjugated and when (±)-ABA is fed to plants, hydrolysis of the resulting glucose ester yields predominantly (-)-ABA (Milborrow 1970, Zeevaart and Milborrow 1976). It is not known whether leaves produce other conjugates of ABA (but see Weiler 1980). The conjugate β-hydroxy-β-methylglutarylhydroxy-abscisic acid was isolated

from seeds of Robina pseudacacia (Hirai et al. 1978). Zeevaart and Milborrow (1976) isolated epi-dihydrophaseic acid (epi-DPA) and alkaline-hydrolyzable conjugates of PA, DPA, and epi-DPA as minor metabolites of ABA when several days were allowed for the metabolism of exogenous (±)-ABA by bean shoots; PA and DPA were still the major metabolites.

1.2.2. Sites of synthesis and metabolism. Milborrow (1974b) obtained preparations of lysed chloroplasts from bean and avocado leaves and from both white and green portions of ripening avocado fruit, preparations which synthesized ABA from labelled mevalonate. Chloroplasts are the presumed site of biosynthesis of ABA, but extrachloroplastic synthesis of ABA has not been ruled out.

In contrast, the enzymes for metabolizing ABA appear to be located outside the chloroplast. As noted in Section 1.2.1, liquid endosperm of Echinocystis has been found to metabolize ABA. Hartung et al. (1980), using non-aqueous isolation procedures, separated mesophyll cells of spinach into a fraction largely devoid of chloroplasts and two chloroplasts fractions, one containing intact and the other containing broken chloroplasts. Only the fraction not containing chloroplasts converted ^{14}C-labelled ABA into other labelled compounds.

1.2.3. Distribution. Abscisic acid is common, perhaps ubiquitous, in all families of angiosperms, gymnosperms, and ferns. It has also been isolated from horsetails, lycopods, and mosses, but not from liverworts, or a species

of blue-green alga, or several species of fungi (Weiler 1979, Milborrow 1978). However, Assante et al. (1977) found that the fungus Cercospora rosicola produced copious amounts of ABA.

Abscisic acid readily moves throughout the plant via the xylem and phloem (Walton 1980), and it is found in all parts of plants. Nevertheless, the ability to synthesize ABA is not restricted to mature, photosynthesizing tissue; detached roots (Walton et al., 1976) and young leaves that have just unfolded (Zeevaart 1977) can also make ABA. Buds, fruits, and young seeds generally have a high level of ABA (Milborrow 1978). Young leaves have been reported to contain a higher concentration of ABA than mature leaves (Raschke and Zeevaart 1976).

Zeevaart (1977) showed that ABA and its metabolites PA and DPA are translocated in the phloem of castor beans. On the other hand, conjugated ABA could not be detected in either the xylem or phloem sap of Xanthium plants, so conjugated ABA does not appear to be exported from its region of synthesis (Zeevaart 1981).

Evidence that ABA readily moves from the cells which produce it comes from the finding of Hemphill and Tukey (1973) that ABA is leached from Euonymous plants by intermittent mist. Nearly all of the ABA of mesophyll cells from turgid spinach leaves was found in the chloroplasts (Loveys 1977). Heilmann et al. (1980) found in uptake studies that ABA became distributed between chloroplasts and

the medium depending on the difference in pH between the two compartments, in accordance with the behavior of a weak acid, presuming that the chloroplast membrane is permeable to undissociated ABA but relatively impermeable to the anion. On this basis, in illuminated leaves where the chloroplast stroma would be more alkaline than the cytoplasm, it is possible to account for the presence in the chloroplasts of more than 80% of the total leaf ABA. In accordance with this view, vacuoles, with their low pH, have been found to contain relatively little ABA (Milborrow 1979, Heilmann et al. 1980).

1.3. FACTORS WHICH CAUSE ACCUMULATION OF ABA IN LEAVES

1.3.1. Wilting. Water deficit, sufficient to cause leaves to wilt, generally results in increases on the order of 10- to 40-fold in ABA content of the leaves during a period of a few hours (see review by Wright 1978). When water is withheld from plants, ABA levels can increase several-fold by the time early-wilting symptoms are visible (Most 1971, Wright 1972). Little ABA accumulates in leaves at high ψ_{leaf}; ABA appears to accumulate below a "threshhold ψ_{leaf}" (Zabadal 1974). Section 2.1 provides further information from the literature on the accumulation of ABA as a function of ψ_{leaf}. Water-stressed leaves accumulate ABA whether they are attached or detached and whether they are kept in light or in darkness (Wright 1978). Detached leaves can accumulate ABA due to wilting, recover pre-stress levels of ABA after rehydration, and can repeat the accumulation of ABA when wilted again on the following day (Zeevaart 1980). Leaves are not alone in their ability to respond to partial dehydration by making more ABA. Some accumulation of ABA was found in excised apices, stems, roots, immature pods, and immature seeds after partial dehydration (Wright 1978).

Several theories have been proposed to account for effects of water stress on metabolism in plants. One, specifically proposed to account for accumulation of ABA, was outlined by Mansfield et al. (1978) and by Milborrow

(1979): If chloroplasts are the site of synthesis of ABA (Section 1.2.2) and if they retain, in turgid leaves, most of the ABA which they produce (Section 1.2.3.), then perhaps wilting increases the permeability of the chloroplast envelope to ABA, allowing ABA to leak into cytoplasm, relieving presumed feed-back inhibition by ABA on its synthesis in the chloroplasts, causing the total level of ABA in the leaf to rise. Rehydration would effectively "re-seal" the chloroplasts, restore inhibition of ABA synthesis, and allow ABA to return to its pre-stress level as it is metabolized in the cytoplasm (Section 1.2.2). More data (the identification and sub-cellular distribution of ABA-synthesizing enzymes, for example) are needed before this hypothesis can be evaluated. It is not certain that chloroplasts (plastids) are the only site of ABA synthesis, and there is only slight evidence that ABA inhibits its own synthesis (Milborrow 1978). According to the hypothesis, the level of ABA in chloroplasts of wilted leaves could be equal to or less than, but not greater than, the level in chloroplasts of turgid leaves. However, Loveys (1977) found that, while chloroplasts of wilted spinach leaves contained 15% rather than 96% of the total ABA of the leaf, the actual concentration of ABA in chloroplasts of wilted leaves was higher than in those from turgid leaves. Davies *et al.* (1980) have proposed that compounds like farnesol and fatty acids, which build up in cells during water-stress (Ogunkanmi *et al.* 1974, Willmer *et al.* 1978) and are known

to disrupt chloroplasts (Fenton et al. 1976), may naturally play a role in regulating the permeability of chloroplast membranes. Exogenous farnesol induced accumulation of ABA in spinach leaves, but failed to do so in tomato or silverbeet leaves (Milborrow 1979). This hypothesis still leaves open the more basic question of how water stress is linked to an effect on farnesol or fatty acid metabolism.

Levitt and BenZaken (1975) suggested that accumulation of ABA, decrease in CO_2 fixation, and other metabolic responses of wilting leaves might be due to a secondary O_2-deficit stress arising from reduced intercellular space during wilting. The percentage of intercellular space in wilted sunflower leaves was reduced to half or less of what it was in turgid leaves. Wilted and turgid orange leaves had the same percentage of intercellular space, but this was interpreted as evidence of the sclerophyll nature of orange leaves and of an adaptation to prevent water-stress-induced O_2-deficit.

Levitt and BenZaken (1975) suggested that another possible link between water stress and metabolic responses might be effects from a change in lateral compression of membrane lipids or proteins accompanying loss of turgor. This hypothesis has been amplified and examined experimentally by Zimmermann and colleagues (Zimmermann 1978). Section 2-4 contains more information on the subject of sensing turgor via membrane compression.

1.3.2. Interruption of translocation in the phloem.

Fruit removal and stem or petiole girdling caused the level of ABA to increase in grape vine leaves (Loveys and Kriedemann 1974) or soybean leaves (Setter et al. 1980). The effect appears to be reproducible but variable: Setter et al. (1980) found that depodding of soybean plants resulted in a 2-fold increase in the level of ABA in 3 h and almost a 10-fold increase by 48 h, whereas Setter et al. (1981) found no increase in 3 h and less than a 2-fold increase in 24 h. The response is considerably less than that which occurs in response to wilting. Both Loveys and Kriedemann (1974) and Setter et al. (1980) mentioned that ψ_{leaf} was as high or higher in leaves after girdling treatments than before. Setter et al. (1981) attributed the increased level of ABA in soybean leaves after depodding or girdling treatments to obstruction of export of ABA from the leaves rather than to enhanced synthesis. If obstruction of export of ABA from leaves can cause the level of ABA in leaves to rise, why do turgid, detached leaves, not accumulate ABA? Detached, turgid Xanthium leaves can be kept for at least 24 h without any increase in their ABA content (Zeevaart 1980). It is possible to reconcile the apparently contradictory results. In studies of accumulation of ABA by wilted, detached leaves, the controls are normally kept turgid by preventing transpiration. If, in the depodded or girdled soybean plants, the transpiration stream sweeps ABA from the mesophyll cells to the sites of transpiration, presuming ABA does exhibit significant feedback inhibition of its synthesis, then ABA

would build up a higher steady-state level. By implication, preventing transpiration from depodded soybean plants should reduce accumulation of ABA in the leaves, and allowing detached leaves to transpire while keeping the petiole in water should result in some build up of ABA in those leaves (see Figure 2 in Raschke and Zeevaart 1976).

1.3.3. Other factors. A variety of environmental conditions have been reported to cause accumulation of ABA in leaves: low relative humidity (2-fold increase reported by Wright 1972), long photoperiod (2-fold to 3-fold increase reported by Zeevaart 1974), flooding the soil (5-fold increase reported by Wright 1972), salinity or osmotic stress of the roots (Mizrahi 1970), mineral deprivation of the roots (5-fold increase reported by Mizrahi and Richmond 1972), and chilling (2-fold increase reported by Raschke et al. 1976). These environmental conditions cause considerably less accumulation of ABA than does withholding water until leaves wilt. In none of these cases were the leaves visibly wilted or else wilting was transient. However, each of these conditions can be expected to either lower the water status of the leaves or reduce the ability of leaves to export materials in the phloem, or both. It is likely that these conditions result in accumulation of ABA via a secondary stress, either the approach of incipient wilting or reduced translocation in the phloem.

1.4. THE POSSIBLE INVOLVEMENT OF ABA IN RESISTING WATER STRESS

1.4.1. Regulation of stomatal aperture. Stomata are viewed as the focal point of a complicated biological control system, operating to optimize CO_2 uptake and water vapor loss by leaves (Raschke 1979). This section summarizes the evidence for the proposal that ABA functions in the plant as a signal in the control of water loss: appearing at the stomata when the water status of the leaves declines, promoting closure of the stomata, and disappearing when leaf-water status rises again.

First of all, there is need for a signal. According to what is known about the mechanics of stomatal movement, a significant decline in ψ_{leaf} of 10 bar would be expected to produce an insignificant decline in stomatal aperture of about 10% due to water loss from the guard cells (Raschke 1979). In order for stomata to close further for the same 10-bar decline in ψ_{leaf}, the guard cells would have to lose solutes. Water-stress-induced stomatal closure has been reported to be paralleled by loss of K^+ from the guard cells (Hsiao 1973a, Ehret and Boyer 1979). It is known that stomata are relatively insensitive to reductions in leaf-water content until a threshold value has been reached, below which significant stomatal closure sets in (summarized in Hsiao 1973b, Turner 1974). A closing stimulus should appear at the threshold ψ_{leaf}.

Experiments with exogenous (±)-ABA have shown that ABA

would make a suitable regulator of stomatal aperture (see review by Wright 1978). ABA is a powerful antitranspirant (Little and Eidt 1968) by causing stomata of leaves to close (Mittelheuser and Van Steveninck 1969). Stomata in epidermal strips also close in response to ABA (Horton 1971, Tucker and Mansfield 1971). When stomatal opening is suppressed by ABA, accumulation of K^+ by guard cells is prevented (Mansfield and Jones 1971, Horton and Moran 1972), and when ABA causes stomatal closure, loss of K^+ from the guard cells results (Ehret and Boyer 1979). Abscisic acid, added to the water supply of detached leaves, is active in closing stomata at concentrations of 10^{-5}M (±)-ABA or less, concentrations which are normal for the activity of phytohormones. Kriedemann *et al.* (1972) estimated from supplying (±)-ABA to detached leaves of bean and rose, that during stomatal closure, the leaves had taken up an amount of (+)-ABA approximately equal to their normal endogenous content. Raschke (1975a) found that as lower concentrations of ABA were supplied, leaves had taken up less ABA by the time significant stomatal closure had occurred. When 10^{-7}M (±)-ABA was fed to detached leaves of *Xanthium strumarium*, an amount of exogenous ABA equal to 1-2% of the original content of ABA in the leaf was enough to produce a stomatal response. The time between addition of ABA to the water supply of leaves and observation of a stomatal response can be as short as 3 to 10 min (Cummins *et al.* 1971, Kriedemann *et al.* 1972, Raschke 1975a). The stomatal response is

specific for the (+)-enantiomer of ABA (Cummins and Sondheimer 1973). There is no evidence of toxicity in the response; stomata begin to reopen within minutes of removing the supply of ABA (Cummins et al. 1971). The effect of ABA on stomata appears to be specific, rapid and reversible.

Measurements of endogenous ABA content also support the idea that ABA is involved in regulation of water loss through stomata. As presented in Section 1.3.1, wilting induces accumulation of ABA. The response appears to be ubiquitous in vascular plants and it is particularly pronounced in mesophytes (Dörffling et al. 1977, Wright 1978). The wilty mutant of tomato flacca contains a low level of ABA compared to the normal variety Rheinlands Ruhm (Tal and Imber 1970). The stomata of flacca are sluggish in response to all closing stimuli (Tal 1966), so a specific role of ABA cannot be discerned, nevertheless the abnormally low level of ABA in the mutant is quite suggestive of an involvement of ABA in the normal behavior of stomata.

Accumulation of ABA in response to water stress demonstrates a similar sensitivity to changes in ψ_{leaf} as was mentioned for stomatal closure: both processes are insensitive to changes in ψ_{leaf} at high ψ_{leaf}, and both abruptly increase in sensitivity below an apparent threshold ψ_{leaf} (Zabadal 1974, Turner 1974). The threshold ψ_{leaf} values for the two processes were found to be the same in maize and sorghum (Beardsell and Cohen 1975). When ABA accumulates in response to water stress, it becomes available

to the rest of the plant. Increases in the concentration of ABA in the xylem sap of sunflower plants were observed in less than 1 h after wilting was induced in the leaves by osmotic stress to the roots (Hoad 1975). Water stress also causes the level of ABA in phloem sap to increase (Zeevaart 1977, Hoad 1978).

From the information presented on the response of stomata to exogenous ABA, it is clear that, if ABA appears in the vicinity of the guard cells during water stress, the stomata will close. From the information presented on accumulation of ABA and its movement throughout the plant during water stress, it is clear that ABA will appear in the vicinity of the guard cells. Abscisic acid undoubtedly acts as a natural closing stimulus to the stomata during periods of water stress.

Some ambiguity remains. Hiron and Wright (1973) observed a 50% increase in the level of ABA in bean leaves within 10 min of exposing the seedlings to a stream of warm air, and increases in stomatal resistance paralleled the increases in level of ABA. Such a good correlation has never again been reported. Considerable variation has been found in the coordination between onset of stomatal closure and noticeable accumulation of ABA in the leaves. Section 3.1 presents details of these studies. If ABA is to trigger stomatal closure as well as maintain the stomata closed, then the active pool of ABA must be small relative to the total ABA in the leaf.[1] That is a reasonable possibility

[1] See Cummins (1973), Raschke and Zeevaart (1976) and Raschke et al. (1976) for evidence of an inactive pool of ABA in the leaf.

based on the sensitivity of stomata to exogenous ABA. Either rapid production of a small amount of ABA near the guard cells or a rapid redistribution of some of the original ABA to the vicinity of the guard cells (see Raschke (1975b) could trigger stomatal closure and account for the observation of stomatal closure prior to a measurable increase in over-all level of ABA in the leaf.

A similar discrepancy in timing has been observed between reopening the stomata and re-establishment of a low level of ABA in the leaf following rehydration. During recovery from water stress, stomatal reopening is delayed beyond the time required for recovery of pre-stress ψ_{leaf} values ("aftereffect on stomata", reviewed in Hsiao 1973b). This phenomenon provided early evidence of the involvement of a stress-induced factor in the regulation of stomatal movement. Attempts have been made to discern the relationship between the aftereffect of water stress on stomata and changes in the level of endogenous ABA (Dörffling et al. 1977, Bengtson et al. 1977, Henson 1981a). In general, stomata begin to reopen when ABA is at its maximum level or even while the level is still rising. In detached shoots, pre-stress levels of stomatal conductance and ABA are reached at about the same time, while in intact plants, complete reopening of stomata takes longer than recovery of pre-stress levels of ABA. Solution to the discrepancy, between expecting the level of ABA to drop before stomata start to reopen and the observed response,

may rest in how ABA is distributed between the mesophyll tissue and the epidermis, and, once again, in postulating a relatively small active pool of ABA.

1.4.2. Other possible functions. A number of responses of plants to water stress, in addition to stomatal closure, are thought to be of positive, adaptive, value to the plant in resisting drought (see Hanson and Nelsen 1980). Some of these responses may be mediated by ABA, especially the ones that concern processes which depend on H^+/K^+ transport, since ABA is known to affect H^+/K^+ exchange in causing stomatal closure (Raschke 1977). (1) Davies et al. (1980) have pointed out that the ability of ABA to inhibit H^+/sucrose co-transport into the phloem of Rincinus leaves (Malek and Baker 1978) may provide a means by which osmotic adjustment (increasing π) in leaves takes place in response to water deficit. (2) Whereas water stress reduces the growth of leaves, the root/shoot ratio has been found to increase in water-stressed plants, and root growth may even be stimulated during mild stress (Sharp and Davies 1979). Does ABA increase the roots' sink strength for carbohydrates? (3) Considerable effort has been made to study the effect of ABA on water and ion movement in the xylem (review by Van Steveninck 1976). Tal and Imber (1971) found that exogenous ABA increased the exudation rate of decapitated tomato plants, especially in the wilty mutant flacca. The experiments by Glinka (1980) with decapitated sunflower plants indicated that ABA promoted, probably independently,

both sap flow and release of ions to the xylem. Transitory stimulation by ABA of guttation and ion flux from passive hydathodes of intact barley plants was measured by (Dieffenbach et al. 1980). If water-stress-induced accumulation of ABA by leaves of intact plants normally stimulates water uptake by the roots, that would increase the rate at which ABA-induced stomatal closure would improve the water status of the shoots.

Experiments by Morgan (1980) implicated ABA as the factor responsible for reducing seed set in water-stressed wheat plants. Morgan suggested that limiting seed set could offer survival advantage for the seeds which did develop, since the amount of carbohydrate available for seed development would be reduced during water stress. Likewise, the suspected ability of ABA to inhibit germination of seeds of some species, until it has been leached from the seeds (Wareing 1978), can also be viewed as a mechanism for surviving drought.

1.5. STATEMENT OF PURPOSE

Substantial evidence that the accumulation of ABA during water stress has adaptive value for the plant in resisting water stress was presented in the previous section. Such evidence warrants further attempts to increase our knowledge of the role of ABA as a phytohormone. Additional information would have practical value, one hopes, in defining a metabolic response that would be an appropriate criterion in breeding crop plants for adaptation to dry environments as well as helping to define appropriate irrigation practices.

The study of accumulation of ABA by plants also has intrinsic value in increasing our understanding of a biological control system: how water deficit results in the appearance of ABA as a signal mediating the plant's response to an environmental stimulus and how correction of the deficit results in disappearance of the accumulated ABA. This thesis describes investigation of three specific aspects of this system.

1) Which parameter of plant water status serves as a stimulus for inducing accumulation of ABA?

2) Based on measurements of the accumulation of ABA in epidermis during stress-induced stomatal closure, how sound is the hypothesis that ABA regulates stomatal behavior during water stress?

(3) How do effects on biosynthesis and metabolism of

ABA contribute to the accumulation of ABA during water stress and to the disappearance of ABA after rehydration?

Chapter 2

Correlation between loss of turgor and accumulation of abscisic acid in detached leaves

2.1. INTRODUCTION

This investigation of water-stress-induced accumulation of ABA in leaves began with an attempt to characterize the response as a function of the parameters of leaf water status, notably leaf water potential (ψ_{leaf}), osmotic pressure (π), and turgor (p).[1] Previous studies have, for the most part, dealt with the relationship between ABA content and ψ_{leaf}. Zabadal (1974) studied accumulation of ABA in leaves of Ambrosia plants which had been placed in a desiccating environment. As the plants depleted the soil of water, ABA content increased only after ψ_{leaf} declined to -10 to -12 bar. Zabadal introduced, therefore, the concept of a threshold ψ for the accumulation of ABA in water-stressed leaves. Similar results were obtained by Hemphill and Tukey (1975) for leaves of Euonymus alatus and by Blake and Ferrell (1977) for needles of Douglas-fir seedlings. ABA content increased steadily after ψ_{leaf} reached -8 to -10 bar in maize and sorghum (Beardsell and Cohen 1975). Wright (1977) used detached leaves in order to allow the same amount of time for production of ABA at each different ψ_{leaf}. He found that the ABA content of wheat leaves progressively increased with decreasing ψ_{leaf}.

The response curve which Wright found for wheat leaves became very steep below -9.3 bar, the ψ of plants showing early wilting symptoms. Beardsell and Cohen (1975)

[1] π is used here as a positive quantity, and hence, $\psi_{leaf} = p-\pi$.

suggested that loss of turgor might correspond to the critical ψ_{leaf} at which ABA levels begin to increase. Davies and Lakso (1978) found in apple seedlings that declining turgor was better correlated with increasing ABA content than was declining ψ_{leaf}. How leaf turgor was related to the pattern of accumulation of ABA during water stress became an intriguing question.

Other work indicated that zero turgor might be important for ABA production. In 1974 Turner provided evidence that threshold ψ's which had been observed for stomatal closure in a variety of species were all associated with turgor close to zero. ABA has been strongly implicated in causing stomatal closure during water stress (Little and Eidt 1968, Mittelheuser and Van Steveninck, 1969, Wright and Hiron 1969, Hiron and Wright 1973), so one would expect stomatal closure to indicate that ABA was accumulating.

The following experiments were designed to test the prediction that species should differ in the ψ_{leaf} required for accumulation of ABA, according to the reduction of ψ_{leaf} required for elimination of turgor. Excised leaves of Phaseolus vulgaris, Xanthium strumarium, and Gossypium hirsutum were used to study the relationship between ψ_{leaf}, π, p, and abscisic acid content. Another objective was to find how gradual or abrupt the onset of accumulation of ABA was in the approach to zero turgor. Uncertainty because of variation between leaves could be eliminated by cutting a series of samples from individual leaves as they lost water.

Monitoring the same leaves for decreasing ψ_{leaf} and fresh weight permitted calculation of π and p from a plot of $-\psi_{leaf}^{-1}$ versus fresh weight loss (Scholander *et al.* 1965, Tyree and Hammel 1972, Talbot *et al.* 1975).

2.2. MATERIALS AND METHODS

2.2.1. Plants. Plants of Xanthium strumarium L. (cocklebur) (from a strain collected near Chicago, Ill., USA and propagated in California and later in Michigan) and plants of Phaseolus vulgaris L. cv. Mecosta (red kidney bean; seeds from Foundation Seed Co., East Lansing, Mich., USA) were grown in a potting mixture in a greenhouse. Air temperature maxima were between 23 and 29°C, and the relative humidity was generally 70-80%. The X. strumarium plants were kept pruned to the top five or six leaves and did not flower under the long-day conditions provided by extending the natural light period to 20 h/d by Sylvania (Danvers, Mass., USA) Gro-lux fluorescent tubes giving an irradiance of about 0.3 W m^{-2} at plant level. Fully developed leaves of X. strumarium were taken for the experiments when the plants were 10-14 weeks old. Plants of P. vulgaris were also cultivated in a growth chamber, which had a 16-h photoperiod at 85 W m^{-2} of light from General Electric (Cleveland, O., USA) cool-white fluorescent lamps. Day temperature was 27°C; night temperature was 21°; relative humidity was 80%. The terminal leaflets of fully developed P. vulgaris leaves were used for the experiments when the plants were 4.5-6 weeks old. Both X. strumarium and P. vulgaris plants were kept well-supplied with water.

Plants of Gossypium hirsutum L. (cotton) (cv. Acala SJ-1; seeds from C. A. Beasley, University of California,

Riverside) were cultivated in two groups under different conditions. For 7 weeks both groups were exposed to a 13.5-h photoperiod, composed of 12 h of 60 W m^{-2} of light from General Electric cool-white fluorescent tubes plus 1.5 h of light from incandescent lamps only (4.5 W m^{-2}). Day temperature was 32°C; night temperature was 22°; relative humidity was 85%. In this growth chamber the plants were watered daily. After 7 weeks one group was transferred to another growth chamber where the plants were exposed to a 13.5-h photoperiod with a peak irradiance of 230 W m^{-2} of light from General Electric lamps H 400 DX 33-1 (mercury vapor) and LU 400 (high temperature sodium vapor). Day temperature was 27°C; night temperature was 21°; relative humidity was 67%. Water was withheld from these plants until the mature leaves were visibly wilted. After 8 stress/recovery cycles of 2-3 days duration each, the plants were watered regularly for 3 days before an experiment was performed. The 10th and 11th oldest leaves were used from plants 10 weeks old in both the unstressed and stress-conditioned groups of G. hirsutum. All measurements of irradiance were made with an Epply pyranometer (Newport, R. I., USA) behind a Corning (Corning, NY., USA) no. 4600 infrared-absorbing glass filter.

2.2.2. Extraction and purification of abscisic acid. Samples were cut from leaves with a razor blade to have an area between 4 and 8 cm^2. The samples were frozen and

lyophilized. They ranged in dry weight from 10 to 50 mg. For these small samples the extraction procedure of Zeevaart (1974) was simplified. Each lyophilized sample was homogenized at room temperature in 15 ml methanol. The methanol extract was separated from the debris by vacuum filtration and the debris was re-extracted by shaking overnight in another 15 ml of methanol. The second methanol fraction was combined with the first. A 250 Bq-aliquot of (±)-[^{3}H]ABA (hereafter designated [^{3}H]ABA) was added to the methanol extract for monitoring recovery. In some experiments chlorophyll concentration was determined in the methanol solution according to Holden (1965). Five ml H_2O were added to the methanol extract, and the methanol was evaporated under reduced pressure. Material insoluble in H_2O was removed by filtration through a Millipore (Bedford, Mass., USA) AP prefilter. The aqueous phase was acidified to pH 2.5 with HCl and extracted 3 times with equal volumes of ethyl acetate. ABA in the ethyl acetate extract was further purified by TLC and methylated as described by Zeevaart (1977). Recovery of ABA from the original methanol extract averaged 70-75%.

The amounts of Me-ABA in the samples were determined with a Varian (Palo Alto, Cal., USA) 3700 gas chromatograph equipped with a ^{63}Ni electron capture detector. A 1.8 m glass column, 2 mm internal diameter, packed with 3% SE-30 on 80/100 Supelcoport (Supelco, Bellefonte, Pa., USA) was used. Carrier gas was N_2, flowing at a rate of 20 ml min^{-1}.

The column oven temperature was 200°C; the detector temperature was 300°C. Measurements of ABA by GLC-EC was not difficult even in the leaf sample which contained the least amount of ABA (2.7 ng). In that case, when one fiftieth of the sample was injected, the detector response at the retention time for Me-ABA produced a peak 30 mm high and well-resolved from any other peaks. Calibration curves were prepared by injecting known amounts of standards. The concentration of (±)-Me-ABA in a stock solution was measured by spectrophotometry in methanol using an extinction coefficient of 20900 M^{-1} cm^{-1} at λ_{max} = 265 nm (Milborrow and Robinson 1973).

The [^{3}H]ABA, specific activity 8.3 · 10^{14} Bq mol^{-1}, was purchased from Amersham, Arlington Heights, Ill., prepared according to Walton et al. (1977). The starting material for the synthesis of [^{3}H]ABA, 1-hydroxy-4-keto-α-ionone, was a gift from R. J. Reynolds Tobacco Company, Winston-Salem, N.C., USA.

2.2.3. Methods of measuring leaf water status. (i) Pressure-bomb method. The pressure-bomb technique developed by Dixon (1914) and by Scholander et al. (1965) was used to determine ψ_{leaf}. The chamber (PMS Instrument, Corvallis, Ore., USA models 600L and 1000) was lined with moist filter paper to reduce water loss from the leaf during measurements. Chamber pressure was increased by adding compressed nitrogen at a rate approximately 0.05 bar s^{-1} until xylem sap appeared at the cut surface of the petiole. At this point the chamber

pressure is equal in magnitude but opposite in sign to the xylem tension and is called the "balancing pressure" (p_{bomb}). When cell and apoplastic water potential are in equilibrium, $\psi_{leaf} = - p_{bomb} - \pi_{xylem}$. The π_{xylem} was determined to be negligible for the leaves used in our experiments: all tests of turgid and wilted leaves gave π_{xylem} readings of less than 0.5 bar in a dew point hygrometer (Wescor, Logan, Ut., USA; model HR-33(T) Dew Point Microvoltmeter with a C-52 sample chamber) used as a psychrometer. Therefore, balancing pressures were taken as measurements of $-\psi_{leaf}$.

Leaf osmotic and turgor pressures were determined from "pressure-volume curves" (Scholander et al. 1965). These were prepared by plotting inverse balancing pressure ($= -\psi_{leaf}^{-1}$) versus decreasing leaf fresh weight (Talbot et al. 1975) as in Figure 2-1. The theory relating balancing pressures as a function of tissue volume has been developed by Tyree and Hammel (1972) and by Tyree et al. (1973). The reciprocal bulk leaf osmotic pressure can be read from the linear portion of the curve or its extrapolation to the ordinate. Leaf turgor can be derived from the curve as the difference between osmotic and balancing pressures.

(ii) Dew-point method. The theoretical basis of the Wescor dew-point microvoltmeter for measurements of ψ has been described by Campbell et al. (1973). Samples consisted of 1-cm leaf discs. Repeating the readings after freezing and thawing the leaf discs three times provided measurements of osmotic pressure.

Figure 2-1. A "pressure-volume curve" determined for a Gossypium hirsutum leaf. The original fresh weight of the leaf was 3.41 g; the leaf weighed 89% of its original fresh weight at p = 0. The same leaf provided the samples for ABA analysis, the results of which are shown in Figure 2-4, G. hirsutum, watered daily. ($1/\pi_o$ = inverse original bulk osmotic pressure.)

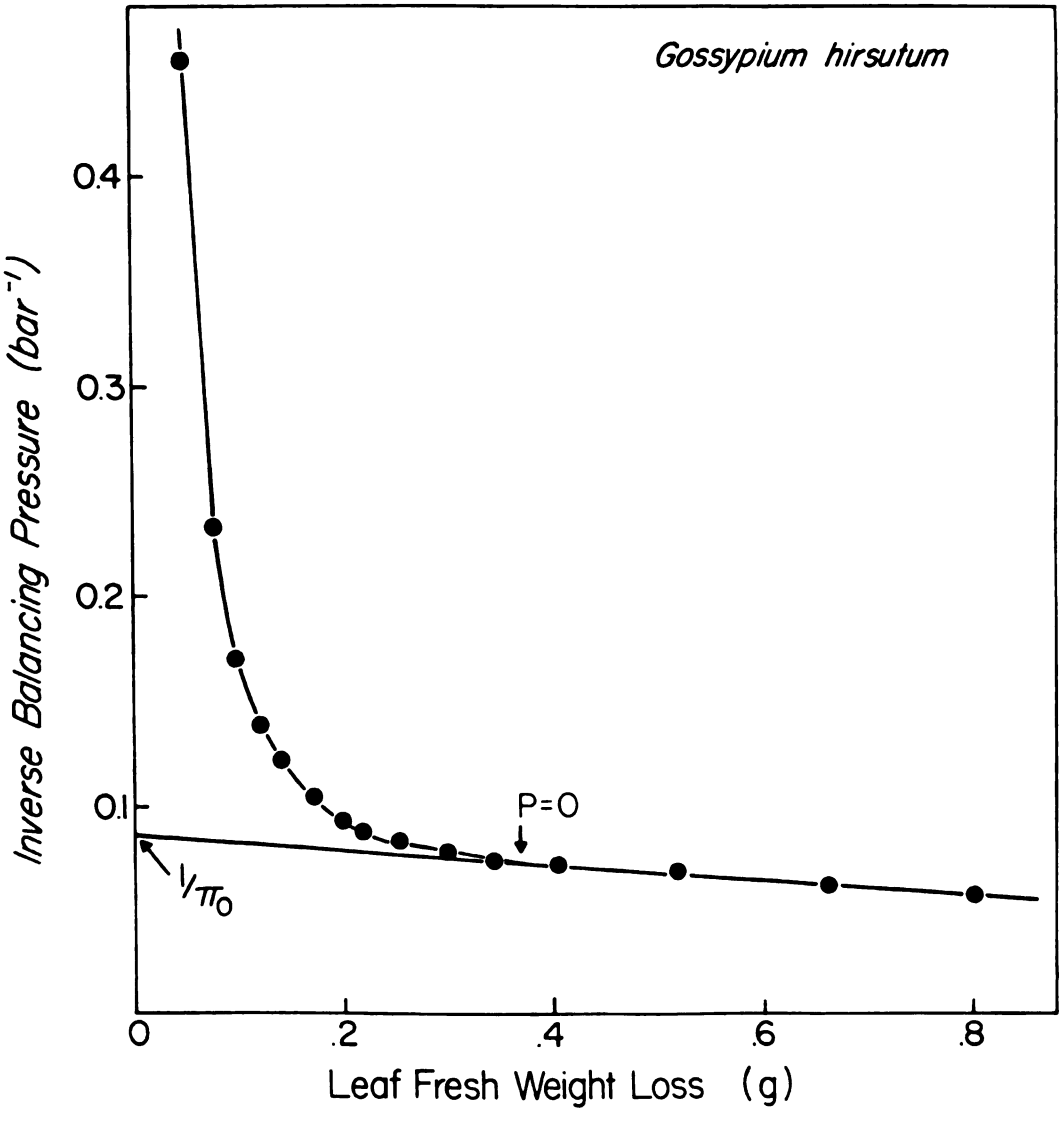

Gossypium hirsutum
Inverse Balancing Pressure (bar⁻¹)
0.4
0.3
0.2
0.1
P=0
1/π0
0
.2
.4
.6
.8
Leaf Fresh Weight Loss (g)

Figure 2-1

2.2.4. Preparation of samples of known ψ_{leaf}, π, and p. Plants were watered and placed in a dark cabinet in the evening before an experiment was performed. A mature leaf was cut under water and allowed to take up water for about 1 h still in darkness. Such turgid G. hirsutum leaves were obtained by applying 0.5-1 bar positive pressure for 10 min to the water in which the cut petiole was placed. To enable pressure-bomb work with P. vulgaris the groove in the petiole was filled with silicone rubber (not acetate-cured) (Wacker-Chemi, Munich, Germany) several hours before the leaf was cut.

The dehydration procedure consisted of the following sequence. The leaf was weighed. A balancing pressure was determined. The bomb pressure was released at less than 0.1 bar s^{-1}. Two more minutes were allowed for ψ equilibration within the leaf. Then the leaf was taken from the chamber, and a sample was quickly excised and wrapped in pre-weighed foil. Sample plus foil were weighed. The rest of the leaf was left on the laboratory bench to lose water, and its weight was monitored until the leaf was judged to be at appropriate water content for a new measurement of ψ_{leaf} with the pressure bomb. The cycle was repeated until enough samples had been taken to span the range from full turgor to several bars beyond the wilting point. At the beginning of the experiment samples had to be taken one after another as quickly as possible, but the rate of water loss dropped and the water loss requirement (per bar decrease in ψ_{leaf})

increased as the leaves wilted. To speed up the sampling rate, when necessary, toward the end of the experiment, the pressure bomb was used to force water from the cut end of the petiole (as in the original description by Scholander *et al.* 1965, for preparing pressure-volume curves).

Cutting samples from the leaf meant that the running total of fresh-weight loss could not be used directly in plotting $-\psi_{leaf}^{-1}$ versus fresh weight. The weight of the samples and the fact that the amount of leaf left to lose water was continuously decreasing had to be taken into account. The weight losses between sampling were calculated according to the following formula:

$$\frac{(\text{wt before sampling} - \text{sample wt}) - \text{wt after water loss}}{\text{fraction of leaf remaining}}$$

The fraction of leaf which a sample represented was estimated from dry-weight measurements.

Curves prepared as described were indistinguishable from those of control leaves from which no pieces were cut. Therefore, it was concluded that no significant error was introduced, either by unavoidable cutting of vascular tissue during sampling or by calculating whole-leaf-equivalent weight loss. Cutting major veins during sampling was avoided.

Twelve experiments were performed which provided samples for analysis of ABA as a function of ψ_{leaf}, π, and p in individual leaves. Six examples are presented here.

2.2.5. Considerations for sampling. In the procedure

described above, samples for analysis of ABA were cut from random positions around the leaf. Variability would be introduced into the results if there were gradients of ψ or π along the leaf.

The positional destribution of ψ and π was checked on control leaves (no samples cut for analysis of ABA) which were close to wilting. The dew-point method was used to measure ψ and π in discs cut from various positions on the leaf blades. Figure 2-2 shows the range of values obtained. In no case was there a discernable pattern in the variability in terms of the location on the leaf from which a sample was taken. It was concluded that taking all samples for analysis of ABA from one leaf was justified and superior to using different leaves for each point.

2.2.6. Sample incubation. When samples for analysis of ABA were excised from a leaf they were at known ψ's. After weighing, the foil covered samples were placed in Petri dishes lined with wet filter paper for incubation to allow ABA to accumulate or not. After the incubation period the samples were unwrapped, reweighed, then frozen with liquid nitrogen. Samples were lyophilized for dry weight determination. An unavoidable but small loss of weight occurred during sample incubation. In general the change in weight was around 1%, and samples generally weighed between 100 and 200 mg. Respiration during 10 h of incubation could account for roughly 1/3 of the weight loss, and perhaps another 1/3 of the loss happened during the post-incubation

Figure 2-2. Evidence for uniform ψ_{leaf}, π, and p for different positions in leaf blades. Measurements of leaf water potential (ψ_{leaf}, upper number of each pair) and osmotic pressure (π, lower number) were made with a dew-point microvoltmeter on discs cut from leaves of Xanthium strumarium, Phaseolus vulgaris, and Gossypium hirsutum. Each value is the mean from measurements made on three leaves of similar ψ_{leaf}.

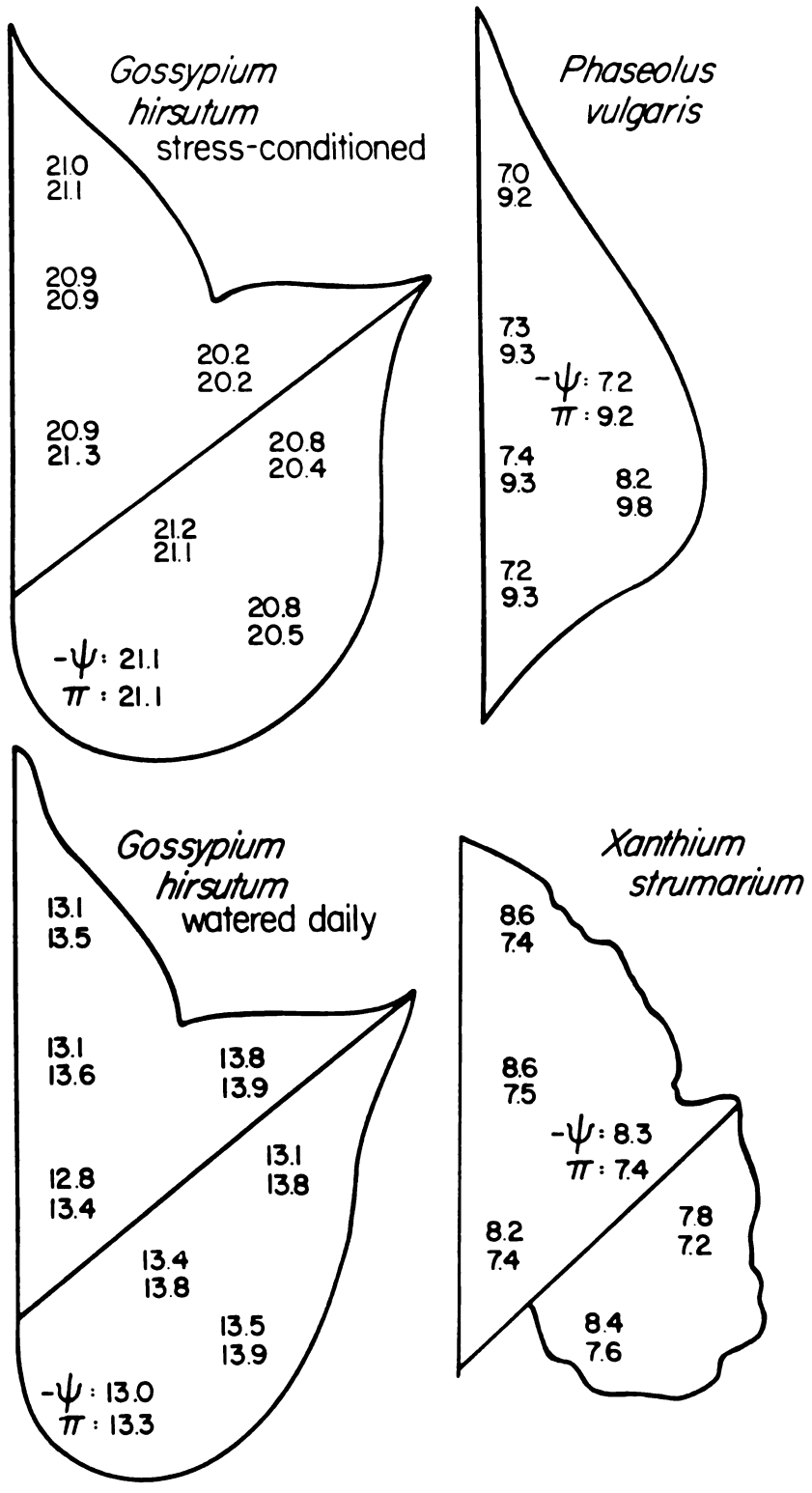

Gossypium hirsutum
stress-conditioned
21.0
21.1
20.9
20.9
20.2
20.2
20.9
21.3
20.8
20.4
21.2
21.1
20.8
20.5
-ψ: 21.1
π: 21.1
Phaseolus vulgaris
7.0
9.2
7.3
9.3
-ψ: 7.2
π: 9.2
7.4
9.3
8.2
9.8
7.2
9.3
Gossypium hirsutum
watered daily
13.1
13.5
13.1
13.6
13.8
13.9
13.1
13.8
12.8
13.4
13.4
13.8
13.5
13.9
-ψ: 13.0
π: 13.3
Xanthium strumarium
8.6
7.4
8.6
7.5
-ψ: 8.3
π: 7.4
8.2
7.4
7.8
7.2
8.4
7.6

Figure 2-2

weighing. For fully turgid samples, loss in weight no matter how small represents a significant change in ψ_{leaf} and such turgid samples may have declined in ψ during the incubation by as much as 1 bar. For most of the samples, however, the water status was nearly constant during the incubation period.

Choice of the sample incubation period was based on the time course of accumulation of ABA for the three species (Figure 2-3). The time courses were prepared as follows. After cutting a turgid control sample, a leaf was dehydrated until turgor was zero. The leaf was removed from the bomb and divided into about 10 pieces, which were wrapped individually in foil and incubated. At various times a piece was frozen and later extracted for ABA. The control sample was incubated until the last wilted sample was frozen (Figure 2-3, at the left), affirming that excision and long incubation by themselves do not result in accumulation of ABA. In Figure 2-3 ABA content was plotted versus time from when zero turgor was reached. Considerable ABA had accumulated by 6 h, and there was a tendency for the rate of accumulation to decrease after that time; and at least 8 h were required for a new steady-state level to be reached.

On the basis of the time courses all samples for a dehydration series were incubated until 8.5 h had elapsed from the time zero turgor was reached. It took about 1.5 h to reach zero turgor and a total of about 4 or 5 h to take a leaf through the entire dehydration procedure. By

Figure 2-3. Time course of accumulation of abscisic acid in detached, water-stressed leaves of 3 species. Leaves of Xanthium strumarium (a: ψ_{leaf} = -10.1 bar, b: ψ_{leaf} = -11.1 bar), Phaseolus vulgaris--greenhouse culture (ψ_{leaf} = -8.4 bar), and Gossypium hirsutum--culture watered daily (ψ_{leaf} = -13.4 bar) were used for the measurements.

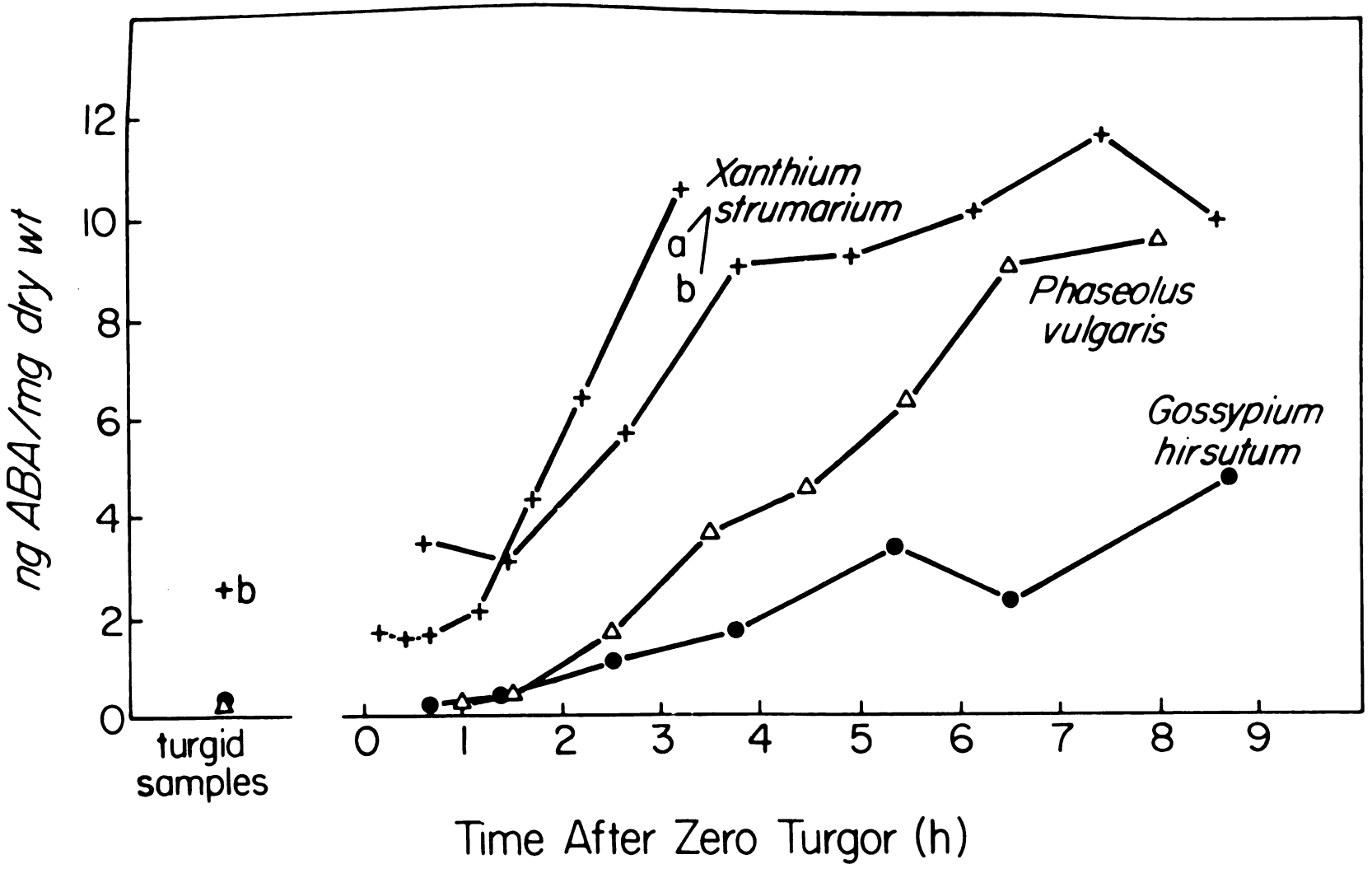

ng ABA/mg dry wt
12
10
8
6
4
2
0
Xanthium strumarium
a
b
Phaseolus vulgaris
Gossypium hirsutum
+b
turgid samples
0
1
2
3
4
5
6
7
8
9
Time After Zero Turgor (h)

Figure 2-3

freezing samples all at the same time the bias of having samples with lower ψ_{leaf} also having experienced water stress for a longer time (as occurs in soil-drying experiments) was eliminated. The samples should all have had close to a new steady-state level of ABA for each respective ψ_{leaf}.

2.3. RESULTS

2.3.1. The relationship between ψ_{leaf}, π, p and accumulation of ABA. These experiments all followed the same procedure. Individual leaves were allowed to wilt slowly. Samples for ABA analysis were cut from a leaf as it lost known amounts of water and incubated at constant water content to allow accumulation of ABA to a new steady-state level. Just before a sample was cut, ψ_{leaf} was determined with a pressure bomb. Curves were prepared from the ψ_{leaf} and weight-loss measurements (e.g. Figure 2-1). These were used to determine π and p.

The results of leaf-dehydration series are shown in Figure 2-4 for leaves of Xanthium strumarium, Phaseolus vulgaris, Gossypium hirsutum, and G. hirsutum which had been forced into osmotic adjustment by periodic withholding of water. Values for ABA content were plotted as ng ABA mg^{-1} dry weight. Lines of the same shape were obtained when ABA content was expressed on the basis of chlorophyll content. The curves of ABA content versus ψ_{leaf} all show a region of high ψ_{leaf} (right side of Figure 2-4) over which little ABA accumulated, followed by gradual transition to a region of ψ_{leaf} over which ABA content rose steeply. The curves differed in the ψ_{leaf} at which the steepest slope occurred. The responses became very steep below -8 bar for these examples of X. strumarium and P. vulgaris, below -13.5 bar for a G. hirsutum leaf, and below -18.5 bar for a leaf

Figure 2-4. The effect of leaf water potential on abscisic acid content in single, detached leaves of Xanthium strumarium, Phaseolus vulgaris, and Gossypium hirsutum.

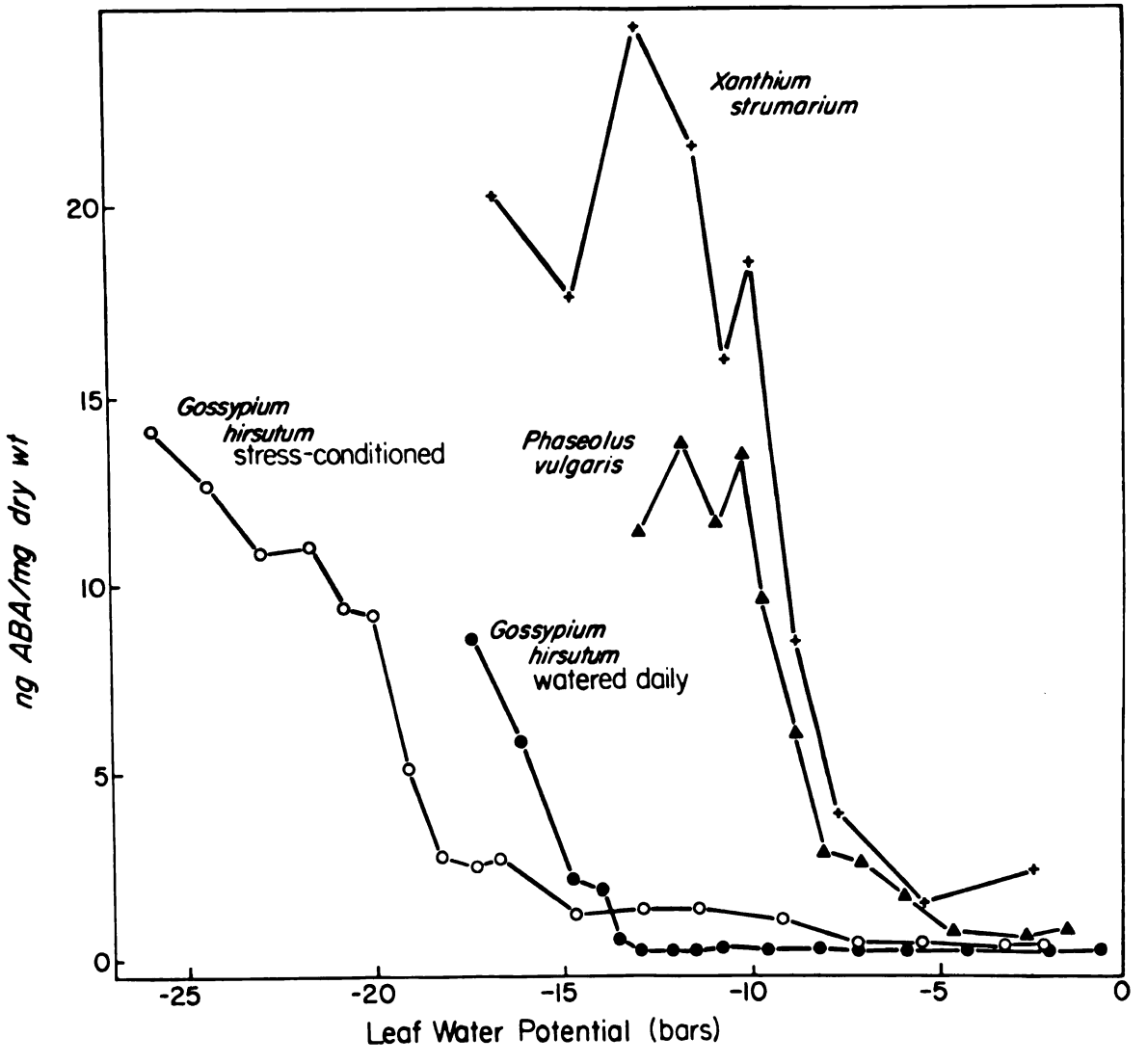

Xanthium strumarium
Gossypium hirsutum stress-conditioned
Phaseolus vulgaris
Gossypium hirsutum watered daily
ng ABA/mg dry wt
Leaf Water Potential (bars)
0
5
10
15
20
-25
-20
-15
-10
-5
0

Figure 2-4

from a stress-conditioned G. hirsutum plant. The variation appeared to arise from the leaves having different π's and, hence, different ψ's at which loss of turgor occurred. At full hydration ($\psi_{leaf} = 0$) these leaves had bulk osmotic pressures of 8.3 bar for P. vulgaris, 8.6 bar for X. strumarium, 11.6 bar for G. hirsutum and 17.2 bar for stress-conditioned G. hirsutum. At zero turgor (when ψ_{leaf} reached $-\pi$) the leaves had π's of 9.6 bar for P. vulgaris, 9.7 bar for X. strumarium, 13.7 bar for G. hirsutum, and 21.5 bar for stress-conditioned G. hirsutum. Figure 2-4 shows that leaves with similar original bulk osmotic pressures had almost overlapping curves of accumulation of ABA in response to decreasing ψ_{leaf}. Leaves with different π's, even of the same species, differed with respect to the ψ_{leaf} (and π) at which accumulation of ABA took place.

Data for π and p were added to a graph of ABA content versus ψ_{leaf} for another X. strumarium leaf in Figure 2-5. Figure 2-5 illustrates the correspondence between loss of turgor and accumulation of ABA. Samples with low but still positive turgor developed levels of ABA which were up to three times the level of ABA in fully turgid samples. Zero turgor strongly affected the accumulation of ABA; the steepest increase in ABA content occurred between the first two samples having zero turgor.

Greenhouse and growth chamber conditions produced P. vulgaris plants with slightly different π's. Figure 2-6 shows that the small difference in π was reflected in an

Figure 2-5. The relationship between osmotic pressure (π), turgor (p), and leaf water potential, and the effect of ψ_{leaf} on abscisic acid content in samples from a single, detached leaf of Xanthium strumarium.

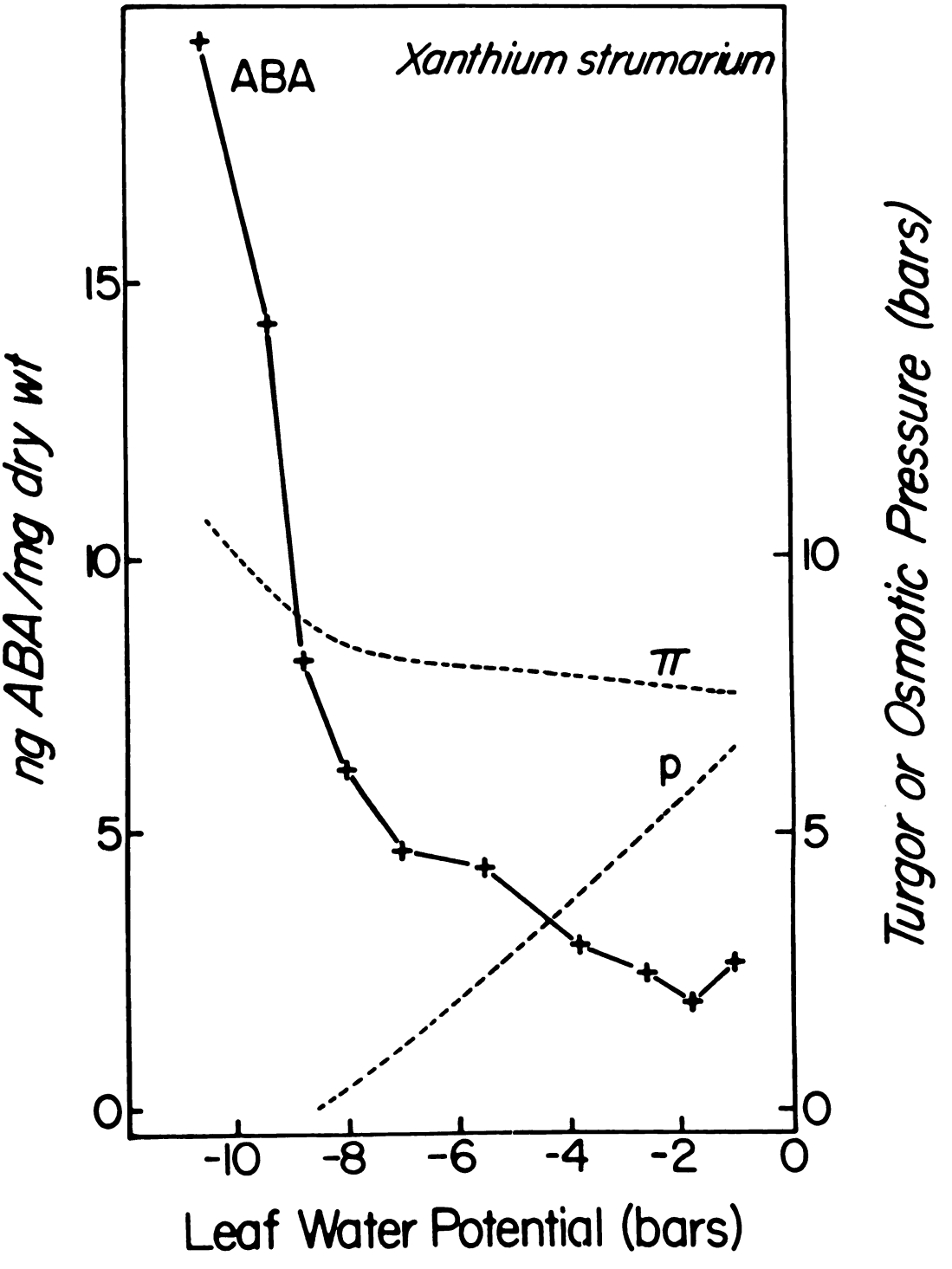

ABA
Xanthium strumarium
ng ABA/mg dry wt
Turgor or Osmotic Pressure (bars)
π
p
15
10
5
0
10
5
0
-10
-8
-6
-4
-2
0
Leaf Water Potential (bars)

Figure 2-5

Figure 2-6. The relationship between turgor and leaf water potential, and the effect of ψ_{leaf} on abscisic acid content in two leaves of Phaseolus vulgaris. The leaves were detached from plants cultivated in either a growth chamber or a greenhouse. (Turgor: ·····, ABA: ▲,△.)

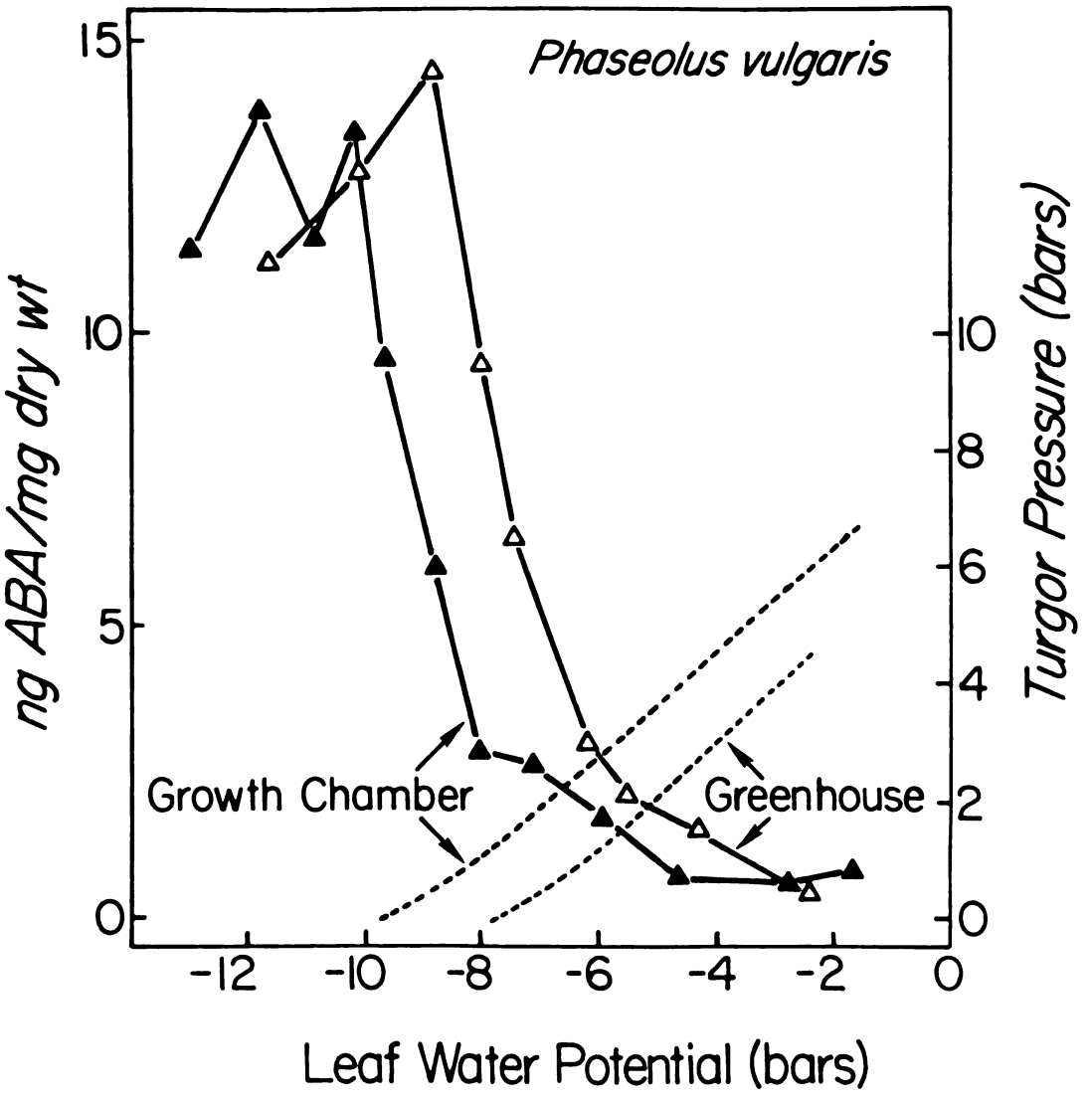

Phaseolus vulgaris
ng ABA/mg dry wt
Turgor Pressure (bars)
Growth Chamber
Greenhouse
Leaf Water Potential (bars)
15
10
5
0
-12
-10
-8
-6
-4
-2
0
10
8
6
4
2
0

Figure 2-6

equivalent displacement between the curves of increasing ABA content. P. vulgaris samples with a turgor of 1 bar developed levels of ABA which were four to eight times the level of ABA in fully turgid samples. Below p = 1 bar, ABA content increased sharply to as much as forty times the level found in unstressed samples. As in Figure 2-5, both examples in Fig. 2-6 show that the steepest slope in the relation between ABA content and ψ_{leaf} occurred at zero turgor. Some of the curves of ABA content versus ψ_{leaf} in Figures 2-4, 2-5, and 2-6 indicate a tendency for the response to level off within several bar of the point of zero turgor. Saturation of the response is particularly obvious in Figure 2-6.

The dependence of ABA production on loss of turgor is summarized in Figure 2-7. ABA content progressively increased as turgor approached zero. Accumulation of ABA in the example from G. hirsutum plants which were well-supplied with water was exceptionally abrupt; all of the increase in ABA above unstressed levels took place below p = 0.5 bar. For the other leaves, more than 80% of the increase in ABA above unstressed levels took place at less than 1 bar turgor.

2.3.2. The effect of elevated N_2 pressure on accumulation of ABA by wilted leaves. Elevated atmospheric pressure increases ψ by the amount of the added pressure. That allows the possibility of having a wilted leaf (p = 0) with a high ψ (ψ_{leaf} = 0). This experiment was designed to

Figure 2-7. Abscisic acid content as a function of turgor in single, detached leaves of Xanthium strumarium, Phaseolus vulgaris, and Gossypium hirsutum. ABA content is plotted as the percent of the maximum ABA content accumulated by the same leaves at any ψ_{leaf} tested. The relationship between ABA content and ψ_{leaf} for these leaves is shown in Figures 2-4, 2-5, and 2-6.

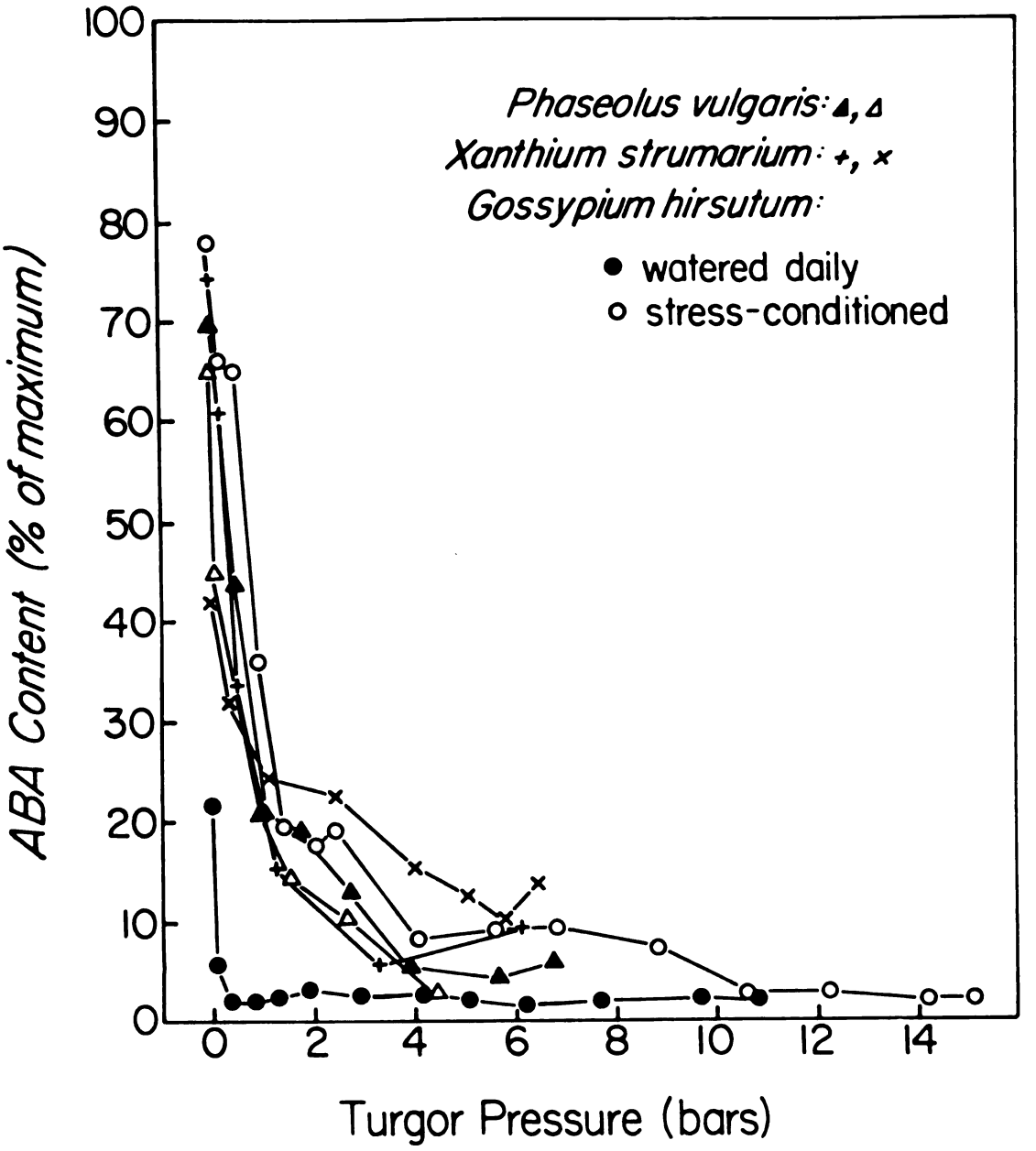

Phaseolus vulgaris: ▲, △
Xanthium strumarium: +, ×
Gossypium hirsutum:
● watered daily
○ stress-conditioned
ABA Content (% of maximum)
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
Turgor Pressure (bars)

Figure 2-7

test whether a high ψ_{leaf} in a wilted leaf could prevent the normal water-stress induced accumulation of ABA.

Mature leaves of Xanthium strumarium were used. After a sample was cut from a turgid leaf, the remainder of the leaf was allowed to wilt. The wilted part was divided into two samples, one of which was put in a pressure chamber. All three samples were wrapped in foil to prevent water loss during a subsequent 4-h incubation. Nitrogen was added to the pressure chamber until the pressure in the chamber balanced the wilted leaf π raising ψ_{leaf} to zero.

The results are presented in Table 2-1. The turgid samples contained the amount of ABA that is typical of unstressed Xanthium leaves (e.g. Figure 2-3). The wilted samples which were maintained at elevated N_2 pressure consistently tripled their content of ABA in the 4-h period, but the wilted samples that experienced normal atmospheric pressure increased their content of ABA 7 to 10-fold.

The role of ψ_{leaf} per se in affecting accumulation of ABA was not clarified by these results. If the leaf samples had been brought to $\psi_{leaf} = 0$ by immediate rehydration rather than by applied pressure, one would expect that they would contain no more ABA than unstressed leaves. (This was the case in other experiments.) Pressure-induced high ψ_{leaf}, on the other hand, did not completely prevent accumulation of ABA. It is unlikely that lack of O_2 was responsible for attenuating accumulation of ABA by samples in the pressure chamber. During the 4-h period,

Table 2-1. The effect of elevated N_2 pressure on accumulation of ABA by wilted leaves.

The ψ_{leaf} of the turgid samples ranged between -1 and -3 bar. Wilting occurs in Xanthium leaves near ψ_{leaf} = -10 bar; water loss was allowed to continue until the wilted samples reached ψ_{leaf} = -11 bar. Eleven bar N_2 pressure was added to the pressure chamber. All samples were incubated in darkness at constant weight for 4 h, and were frozen and lyophilized for subsequent extraction of ABA.

Leaf Number	Turgid Sample	Wilted Sample, Bench Incubation	Wilted Sample, Pressure Chamber Incubation
	ng ABA mg^{-1} DW		
1	1.9	13.3	5.2
2	1.8	13.9	5.6
3	0.8	8.1	2.4
	ABA Relative to Unstressed Level		
1	1.0	7.2	2.8
2	1.0	7.9	3.2
3	1.0	10.4	3.0

respiration by the tissue would have consumed less than 2% of the available O_2. The effect of the increased concentration of dissolved N_2 in and around the compressed cells is not known. It is thought that the most likely explanation, of how pressure could reduce the accumulation of ABA by wilted leaves, would be the possibility of applied pressure mimicking turgor in the cells. (See Section 2.4.3.)

2.4. DISCUSSION

2.4.1. Leaf ψ and π influence production of ABA through their effect on turgor. ABA content in water-stressed leaves increased at different water potentials for different leaves (Figures 2-4, 2-5, and 2-6), but in each case the capacity for accumulation of ABA rose sharply as turgor approached zero (Figure 2-7). These results are interpreted to mean that ψ_{leaf} *per se* does not control ABA content. It is unlikely that bulk leaf π directly influences production of ABA either for the following reasons: (1) Increases in ABA content were not uniquely related to π just as they were not uniquely related to ψ_{leaf}. (2) In most leaves the entire change in ABA content occurred over a range of π of about 2-4 bar, for instance from 7 to 9 bar in *P. vulgaris* or from 14 to 18 bar in *G. hirsutum* (*c.f.* Figure 2-5). Changes in π of this magnitude can normally occur in leaves during the course of a day and *may* be attributed to solute accumulation rather than dehydration, as Acevedo *et al.* (1979) reported for leaves of maize and sorghum. Even larger diurnal changes in π were observed in cotton leaves (Cutler *et al.* 1977) when some dehydration accompanied solute accumulation during the day, but even so the stomata did not close, suggesting ABA levels did not rise in this case. The changes in bulk leaf π which were observed in the present study were almost certainly not responsible for the 10 to 40-fold accumulation of ABA that

occurred. The 2-4 bar change in π represents a 30% increase in solute concentration; a change of that size, for any component of π, is unlikely to trigger accumulation of ABA. (3) It is probably concidental that a nearly linear relationship appears if ABA content for an individual leaf is plotted versus π. The linearity results from the fact that with decreasing ψ_{leaf} only small changes in leaf π and ABA occur so long as turgor is positive, but that these changes increase after turgor has been lost (e.g. Figure 2-5). It is not possible to assign significance to the linearity because the relation holds only for a narrow (2-4 bar) range of π. (4) Not all, but at least half, of the leaves showed saturation of the response within the range of water potentials tested (see Figures 2-4 and 2-6); increases in solute concentration above about 30% were less effective or not at all effective in promoting accumulation of ABA. Thus, accumulation of ABA in water-stressed leaves is most probably a turgor-dependent process rather than a ψ_{leaf}- or π-dependent one.

2.4.2. The question of a "threshold" ψ_{leaf} for accumulation of ABA. Single mature leaves were used in these experiments in the hope of getting a clearer answer as to whether ABA production in response to water stress exhibits a threshold phenomenon or not. In one case--that of G. hirsutum well-supplied with water--the response curve versus ψ_{leaf} in Figure 2-4 showed an abrupt change of slope coincident with the point of zero turgor. In the rest of the examples in Figure 2-4, 2-5, and 2-6, the curves were more sigmoid in shape, just as Wright (1977) found for

detached wheat leaves, showing no clear threshold. Although Zabadal's (1974) data produced the appearance of a threshold ψ_{leaf} for the accumulation of the ABA, the data of this study and that of Wright, in fact, showed much steeper responses. The data of Zabadal and Wright have been replotted in Figure 2-8 for comparison. Soil and plant ψ were falling throughout Zabadal's experiment. Presumably he would have obtained results more like those of this study and Wright's study if time had been allowed at each ψ_{leaf} for full expression of the effect on accumulation of ABA.

2.4.3. How is accumulation of ABA related to turgor? The results, when plotted against turgor (Figure 2-7), showed a lack of response at high turgor and a progressive increase in accumulation of ABA by the leaf samples as they approached zero turgor. This pattern of response may reflect the nature of turgor sensing by the individual cells of the leaf. The curves are of the same shape as that found for the turgor-dependence of K^+ influx into cells of Valonia utricularis (Zimmermann and Steudle 1977).

According to the electrochemical model of turgor sensing by plant cell membranes (Coster et al. 1977, Coster and Zimmermann 1976, Zimmermann 1978), turgor is sensed by membrane compression. Both mechanical forces (pressure) and the electric field in a membrane can affect membrane thickness. These authors have presented evidence that the elastic, compressive modulus for plant cell membranes is

Figure 2-8. The effect of ψ_{leaf} on ABA levels; a comparison of data redrawn from Wright (1977) and Zabadal (1974). ABA in excised wheat leaves: ■ - ■, right-hand scale, (Wright 1977). ABA in leaves of Ambrosia artemisiifolia: ● - - ●, left-hand scale, and Ambrosia trifida: ▲ - ▲, left-hand scale, during a period of progressive dehydration of intact plants (Zabadal 1974).

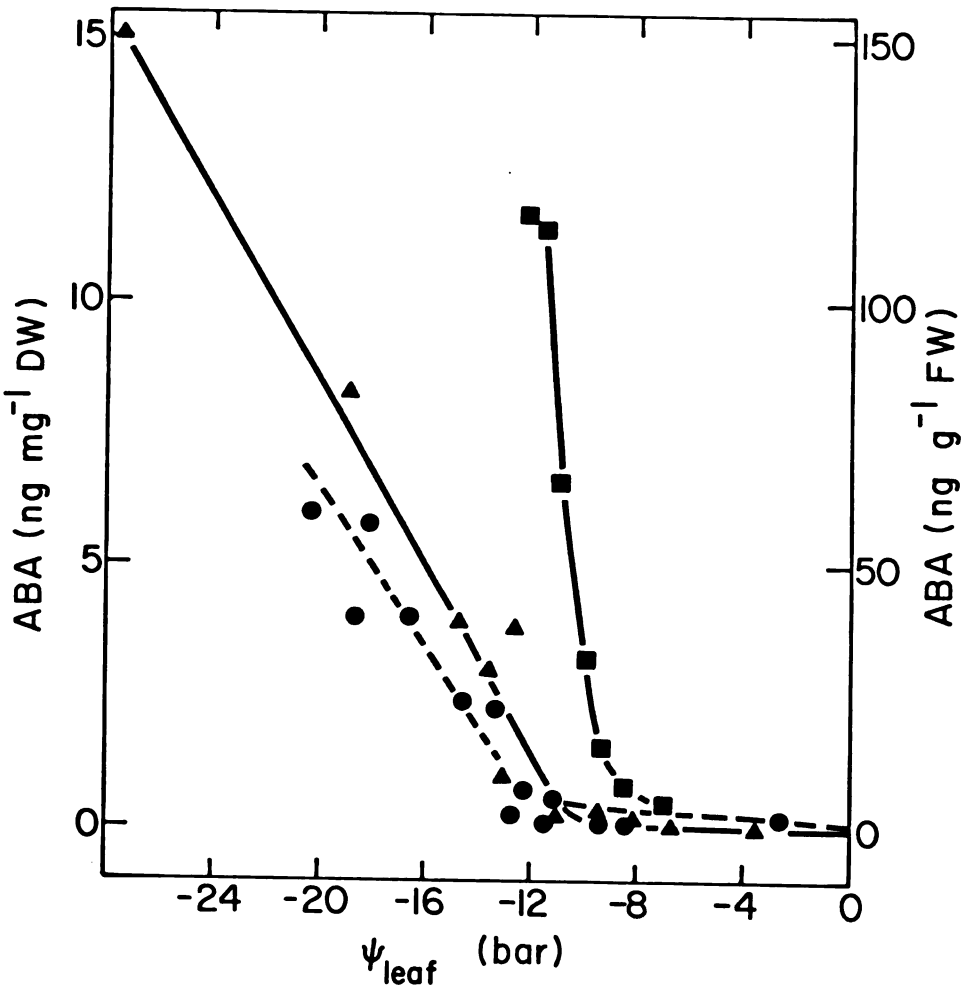

15
10
5
0
150
100
50
0
ABA (ng mg^{-1} DW)
ABA (ng g^{-1} FW)
-24
-20
-16
-12
-8
-4
0
ψ_{leaf} (bar)

Figure 2-8

sufficiently low that significant changes in membrane thickness may be expected within the normal range of cell turgor. Thickness is also affected by stretching in the plane of the membrane due to changes in cell or organelle volume. All membranes in a cell are subject to changes in thickness due to compression or stretching, not just the plasmalemma. The cell wall is thought to control membrane stretching if the plasma membrane is mechanically closely coupled to the cell wall. It is not clear what changes might occur in electrical or mechanical stresses in the cell membranes as plant cells are dehydrated beyond the wilting point.

A model of turgor sensing by an effect on membrane thickness can account for the results that were obtained by putting wilted leaves under N_2 pressure (Table 2-1). Pressure, whether from turgor or the atmosphere, will compress cell membranes; in addition, turgor, but not atmospheric pressure, will result in membrane stretching. The fact that atmospheric pressure was less able than turgor to prevent accumulation of ABA would indicate, according to this model, that change in membrane thickness due to stretching (from longitudinal stress) dominated over change due to transverse stress.

The triggering of ABA synthesis in individual leaf cells may be more closely coupled to zero turgor than is shown by the response curves derived from leaf sections (Figure 2-7). A close coupling between zero turgor and the stimulation

of accumulation of ABA may indicate that the stimulation occurs when the plasmalemma no longer presses against the cell wall. In this case, turgor sensing would reside in the change in wall/membrane interaction rather than in the change in membrane thickness. Part of the accumulation of ABA in leaf samples having apparently positive turgor was certainly due to leaves not being homogeneous populations of cells. Inverse balancing pressures plotted versus tissue volume (or fresh weight) will not look like a straight line unless nearly all of the cells have lost turgor; only then will "pressure-volume curves" indicate zero turgor has been reached (Cheung et al. 1976). Thus, any variation in π among cells of a leaf would spread elimination of turgor in some cells to higher water potentials than that corresponding to zero turgor according to the pressure-volume curve. Variation in the volumetric modulus of elasticity (ε), which affects the relative change in volume that occurs as turgor is lost, should do the same. According to this view, we could expect that the more uniform the cells of a leaf are, the more abrupt should be the onset of accumulation of ABA with respect to loss of turgor.

2.4.4. How reliable are the determinations of turgor? Irrespective of how gradual or abrupt the onset of accumulation of ABA was, in every leaf that was analyzed for ABA content as a function of ψ_{leaf}, π, and p, determined from measurements with the pressure bomb, we found that

the accumulation of ABA was most sensitive to changes in ψ_{leaf} at turgor less than 1 bar. When coincident determinations of water status were made by the dew-point method, ψ_{leaf} values were obtained which agreed fairly well with determinations of balancing pressures made with the pressure bomb. However, π (and hence p) values were always several bars lower than those indicated by the "pressure-volume curve," displacing the point of zero turgor to higher ψ_{leaf} values and also resulting in some negative values for p (see also Figure 2-2). While the measurement of π on frozen-thawed tissue or expressed sap seems adequate for relative measurements (as in Figure 2-2), apparent underestimation of π (Boyer and Potter 1973, Tyree 1976) produces ambiguous negative values for p, and makes this method questionable for determining the point of zero turgor. If psychrometric (or dew-point) measurements of ψ_{leaf} are plotted as ψ_{leaf}^{-1} versus water content, p can be determined from the resulting graph just as it is from "pressure-volume curves" (Talbot et al. 1975). (See Figure 3-5.) There was agreement between determining the point of zero turgor by this method and by the pressure-bomb method.

2.4.5. Implications for stomata of the observed relation between accumulation of ABA and leaf turgor. The requirement for low turgor in order for leaves to accumulate ABA has consequences concerning stomatal response to water stress: (1) The guard cells themselves, if they possess the ability to make ABA, are probably the last cells in the

leaf to be stimulated to do so. In general, guard cells have by far the highest osmotic pressures of any cell type in the leaf even when the stomata are closed (see review by Raschke 1979); hence, the guard cells should be among the last cells in the leaf to reach zero turgor during water stress. It is thus unlikely that ABA production by the guard cells themselves initiates stomatal closure. Rather, if ABA does mediate stomatal closure during water stress, it most likely comes to the guard cells as a result of a rapid transfer of ABA from those leaf cells that are first to reach zero turgor.

(2) An insensitivity of stomata to changes in ψ_{leaf} in the region of high leaf turgor has been observed by many investigators (summarized in Turner 1974). This insensitivity may reflect lack of stimulation of the system producing ABA. However, as the results in Figure 2-3 demonstrate, wilted leaves can require more than one hour before any increase in whole-leaf ABA can be seen, but stomata generally close within 10-20 min after a leaf loses turgor (e.g. Beardsell and Cohen 1975). (See also Chapter 3.) As Beardsell and Cohen pointed out, in order for ABA to trigger stomatal closure during water stress, changes in ABA concentration in the vicinity of the guard cells must happen much faster than changes in whole-leaf ABA. Accumulation of ABA near the guard cells will be accelerated if a high rate of transpiration sweeps (pre-existing or newly-produced) ABA from the mesophyll to the epidermis. The possibility that

some cells in a leaf reach zero turgor before the majority provides candidates for a source of ABA that could reach the stomata by the time bulk-leaf turgor has been lost.[2]

2.4.6. Implications of the results with stress-conditioned G. hirsutum. The known ability of G. hirsutum plants to increase π in response to reduced water supply (Brown et al. 1976, Cutler and Rains 1978) and thereby decrease the ψ_{leaf} at zero turgor was used successfully to alter the relation between ABA content and ψ_{leaf}. Compared to the response of leaves from plants which were watered daily, increases in ABA content in leaves from stress-conditioned plants were displaced to lower ψ's (Figure 2-4). Jones and Turner (1978) and Fereres et al. (1978) documented osmotic adjustment to dry environments in sorghum. One would expect that the critical range of ψ_{leaf} from -8 to -10 bar for sorghum below which ABA levels increased (Beardsell and Cohen 1975) would be shifted to lower ψ's by prior stress conditioning. Further, the demonstration that stress conditioning of plants can displace accumulation of ABA to

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[2]Stomatal closure does not always correlate with zero bulk-leaf turgor. Time of year and canopy position appear to affect the turgor at which stomata closed in detached Sitka spruce shoots (Beadle et al. 1978). Radin and Parker (1979a and b) found during soil-drying experiments with cotton plants that under normal environmental conditions stomata closed as the leaves wilted, but at air temperature of 40-42°C plants wilted before the stomata closed, and under conditions of nitrogen deficiency the stomata closed before leaf turgor was lost. These results are not surprising since environmental and physiological conditions are known to affect stomatal response to closing stimuli, e.g. ABA (Raschke 1979), and growing conditions also affect the relation between bulk-leaf turgor and accumulation of ABA (see Section 2.4.6).

lower ψ's provides a likely explanation of why stress conditioning can also displace drought-induced stomatal closure to lower ψ's (e.g. Jordan and Ritchie 1971, McCree 1974).

Field-grown plants often resemble stress-conditioned plants in the respect that turgor-dependent processes such as growth by cell expansion and stomatal opening are extended to lower ψ's in these plants than in laboratory plants that do not normally experience water stress (e.g. Jordan and Ritchie 1971, Cutler et al. 1977). Very small diurnal fluctuations in ABA content occurred in field-grown cotton regardless of whether the crop was irrigated or not (McMichael and Hanny 1977). One possible explanation of those results is that the unirrigated ("stressed") field cotton failed to accumulate ABA during the day when ψ_{leaf} fell to -30 bar because prior adaptation to drought allowed positive turgor to be maintained.

In addition to drought adaptation, conditions such as leaf or plant age or canopy position, by altering cell elasticity, volume, or solute content, affect what ψ_{leaf} and π are when $p = 0$. Judging from the results of this investigation, one would expect leaves of different age or canopy position to accumulate ABA at different ψ's, according to the ψ_{leaf} at which turgor becomes zero. Obviously one cannot generalize for a given species or variety on what will be the critical range of ψ_{leaf} for the accumulation of ABA. The point of zero turgor may even

change during the course of a slow soil-drying experiment as a result of drought adaptation. Turgor cannot be predicted; it has to be determined.

Not only did stress conditioning of G. hirsutum displace accumulation of ABA to lower ψ's, but it also resulted in a more gradual increase in ABA content with declining p. This loss of sharpness in the onset of accumulation of ABA was probably the result of an increased variance in solute content and wall elasticity of the cells capable of producting ABA. Whether a gradual increase in ABA production offers an advantage to plants over an abrupt one in the toleration of drought remains for investigation.

2.4.7. "Incipient wilting" and the accumulation of ABA. According to Figure 2-7, a ψ_{leaf} which is several bar above the ψ_{leaf} which is corresponding to zero turgor can induce some accumulation of ABA. These results are in agreement with reports of Most (1971) and Wright (1972) according to which some accumulation of ABA occurred under conditions of mild water stress where wilting symptoms were not seen. ABA content at p = 1 bar averaged approximately 4 times the level found in fully turgid samples (see Figure 2-7) when sufficient time was provided for development of a new steady-state level of ABA. Figure 2-7 shows that a doubling of ABA content may occur below p = 4 bar with as little as a 1 bar decrease in turgor. Therefore, any investigation into the chain of events between infliction of a stress and production of ABA should include a determination of whether a small change in

turgor was involved (see Section 1.3).

Chapter 3

Measurements of ABA in epidermis of *Commelina communis* during stomatal closure caused by water stress

3.1. INTRODUCTION

This chapter concerns the possible involvement of ABA in stomatal closure during water stress. The evidence which implicates ABA as a factor in the limitation of water loss from leaves during water stress was presented in the General Introduction. The hypothesis has been based partly upon a correlation between stress-induced stomatal closure and accumulation of ABA (Hiron and Wright 1973, Loveys and Kriedemann 1973, Walton *et al.* 1977), or a correspondence between the ψ's at which stomata close and ABA accumulates (Beardsell and Cohen 1975). Chapter 2 (Pierce and Raschke 1980) provided further indirect evidence: namely, accumulation of ABA correlates with ψ's which bring a leaf close to zero turgor, and the same was found for stomatal closure (Turner 1974).

Considerable variation has been found in the time required by wilted leaves to produce a significant increase in the amount of ABA which they contain. Hiron and Wright (1973) found a 50% increase in 10 min in the level of ABA in primary leaves of bean seedlings that were being exposed to a stream of warm air. Walton *et al.* (1977) found no increase in the level of ABA in primary leaves of bean seedlings by 21 min but a 75% increase by 35 min after the ψ of the root medium was lowered to -5 bar. In detached wheat leaves that had been reduced in fresh weight by 6%, Wright (1969) found doubling of the ABA content

within 40 min. Loveys and Kriedemann (1973) detected a 75% increase in the level of ABA in grape leaves within 15 min after they had been detached from the vine. In contrast, more than 2 h elapsed before detached maize leaves significantly increased their level of ABA (Beardsell and Cohen 1975). Detached Xanthium leaves that were decreased by 10% of fresh weight showed about a 50% increase in the level of ABA in 30 min and about a doubling in 1 h (Zeevaart 1980). Time courses (Figure 2-3) for mature, detached leaves of Xanthium, bean and cotton all indicated a lag period of about 1 h after wilting before accumulation of ABA was apparent.

Sometimes stomata have been observed to close before any increase in ABA was detected in the leaves, as when Walton et al. (1977) reduced the ψ of solution around the roots of bean plants of -5 bar with PEG-6000, or when Henson (1981b) severed pearl millet shoots from their roots, or when Beardsell and Cohen (1975) detached maize leaves from their stalks. Beardsell and Cohen (1975) had found that when water was withheld from intact maize and sorghum plants, the stomata closed and ABA levels were elevated at the same ψ_{leaf}, but during a soil-drying treatment of maize plants, Davies et al. (1980) found partial stomatal closure well before the level of ABA had increased.

Although one can find instances in which accumulation of ABA in a stressed leaf has occurred fast enough to account for stomatal closure, one cannot make any such

generalization. Clearly, if ABA is responsible for the closure of stomata during water stress, stomata must be sensitive to a concentration of ABA in the environment of the guard cells which can be accommodated by an insignificant change in the level of ABA in the whole leaf. When (±)-ABA was added to the transpiration stream of bean and rose leaves, stomatal closure was complete when the leaves had taken up an amount of ABA that was approximately equal to their endogenous level (Kriedemann et al. 1972). As not all of the ABA absorbed would be in the vicinity of the guard cells, the amount of ABA required by the epidermis in order for stomata to close could be a small amount relative to the total leaf content of ABA. Raschke (1975a) found that a dose of ABA as small as 1-2% of the endogenous level was enough to trigger a stomatal response in Xanthium leaves.

It appears likely that the redistribution of a small fraction of a leaf's ABA to a pool in contact with the guard cells would be sufficient to initiate stomatal closure during water stress, as was proposed by Raschke (1975b). If ABA is to account for stomatal closure during water stress, one would expect increases in epidermal content of ABA to correlate better with stomatal closure than what has been found for the total leaf content of ABA. The experiments presented here were designed to test that prediction. Commelina communis leaves were used because epidermis can be removed easily with little contamination by mesophyll cells,

and because reports have been published on the sensitivity of the stomata to exogenous ABA (Raschke et al. 1975, Weyers and Hillman 1979). These reports were used to estimate the changes one could expect in epidermal ABA during stomatal closure, for comparison with the changes that were actually found. A preliminary report of this work was presented at the 1979 meeting of the International Plant Growth Substances Association (Pierce and Raschke 1979). Measurements of ABA levels in Vicia faba epidermis have been made (Loveys 1977), and several other reports of ABA levels in Commelina epidermis have recently appeared (Singh et al. 1979, Dörffling et al. 1980).

Accumulation of K^+ by guard cells is reduced and, consequently, stomatal opening is suppressed in the presence of exogenous ABA (Mansfield and Jones 1971, Horton and Moran 1972). Conversely, loss of K^+ from the guard cells occurs when exogenous ABA induces stomatal closure (Ehret and Boyer 1979). Hsiao (1973a) and Ehret and Boyer (1979) reported, for leaves of Vicia faba and Helianthus annuus respectively, that water-stress-induced stomatal closure was paralleled by loss of K^+ from the guard cells. Earlier work (Iljin 1914, Steinberger 1922, both cited in Stålfelt 1955) had indicated that stomatal closure in wilting leaves was associated with loss of solute from the guard cells. In the present study, epidermal strips were stained for potassium in order to know the phase of stomatal closure during which loss of K^+ from the guard cells occurred and,

hence, during which one might expect increases in epidermal ABA to have occurred.

3.2. MATERIALS AND METHODS

3.2.1. Plants. Plants of Commelina communis L. were cultivated in a growth chamber, which had a 14.5-h photoperiod at 85 W m^{-2} of light from General Electric (Cleveland, O., USA) cool-white fluorescent lamps. Day temperature was 22°C; night temperature was 20°; relative humidity was 85%. The three youngest, fully expanded leaves from non-flowering branches were used for the experiments when the plants were 5-6 weeks old. These leaves had the characteristics listed in Table 3-1.

Table 3-1. Characteristics of Commelina communis leaves.

Dry weights were determined on lyophilized tissue.

	Abaxial epidermis	Mesophyll + Adaxial epidermis	Intact leaf section
mg DW cm^{-2}	0.25	3.1	3.4
mg DW mg^{-1} FW	0.06	0.15	0.14
stomata mm^{-2}	60		

3.2.2. Preparation of leaf tissue wilted in air. Leaves were cut from the plants in the morning, wiped with a damp tissue, and divided in half by cutting out the midrib. Leaf-halves were floated, abaxial epidermis upwards, in

dishes of distilled water. The dishes were placed on moistened paper toweling under inverted glass trays in a lighted chamber (85 W m^{-2} from General Electric mercury-vapor lamps, H400RDX33-1, with 5 cm water as filter). Humidified air passed continuously over the sample dishes at 30 l h^{-1}. The temperature was 23°C. After 3 h the leaf sections were removed from their water supply, blotted, and replaced in the chamber in empty dishes. At selected times thereafter, intact leaf sections were photographed for later determination of stomatal aperture (20 apertures were measured per section), and the lower epidermis was stripped from the sections. The epidermis was stained for investigation of the K^+ content of the guard cells, or the epidermis and the remainder of the leaf were frozen for separate determinations of ABA concentration. Virtually all guard cells and stomatal accessory cells were intact on the epidermal peels. Approximately 70% of the ordinary epidermal cells remained intact on epidermis stripped from turgid leaves; for wilted leaves the figure was 60%. Intact cells were determined by their ability to exclude Evan's blue (Gaff and Okong'O-Ogola 1971). Contamination, by adhering mesophyll cells, of the epidermal samples, from which any obvious areas of green were cut away, amounted to 0.05% on a dry weight basis, whether the leaf was turgid or wilted.

3.2.3. Preparation of leaf tissue wilted in solution. Leaf-halves were floated on water in the light as described

in the previous paragraph. After 2 h the leaf-halves were trimmed to uniform size (4.2 cm^2), and the abaxial epidermis was removed. Epidermis (10 pieces per treatment) was rinsed in basal medium (with 2.5 g PVP-40 l^{-1}, drained, and transferred to 15 ml of incubation medium. Mesophyll with upper epidermis attached (2 pieces per treatment) was rinsed, blotted and transferred to 20 ml of incubation medium. Samples were incubated in the light chamber for 4 h in stoppered glass tubes through which humidified air bubbled at 3 l h^{-1}. Incubation solutions were: basal medium (π = 1.5 bar), basal medium made 0.5M in mannitol (π = 13.6 bar), and basal medium made 0.75M in mannitol (π = 20.9 bar), (1 bar = 0.1 MPa). Basal medium consisted of 10 mM HEPS buffer (pH 6.5) plus 25 mM KCl. After incubation, tissue was drained, frozen and lyophilized in preparation for determination of ABA content. In addition, samples of epidermis and mesophyll were frozen without the incubation. Abscisic acid was also extracted from the incubation solutions.

3.2.4. Analysis of ABA. Tissue samples were extracted and analyzed for ABA content as described in Chapter 2. Analysis of the incubation media began with extraction with ethyl acetate at low pH. Tritiated (±)-ABA (250 Bq = 0.15 ng) was added to samples before they were extracted, in order to assess the recovery of ABA at the end of the purification procedure. In no case did the exogenous ABA represent more than 25% of the total ABA in a sample. Results

were corrected for losses during purification and for the amount of (±)-[^{3}H]ABA that had been added. Recovery of ABA after purification from epidermal samples averaged 48%.

Purified extracts were analyzed for ABA by GLC-EC of the methylated material, as described in Chapter 2. Some samples were also analyzed by GLC-EC of the methylated material following isomerization, and by bioassay. Isomerization to an approximately 50:50 mixture of ABA and the 2-*trans*-ABA isomer (Mousseron-Canet *et al.* 1966, cited in Milborrow 1970) was achieved by a 2-day exposure of the methylated material in acetone to light from a fluorescent lamp. A standard mixture of the isomers was a gift from J.A.D. Zeevaart. For bioassay, samples (hydrolzyed to give the free acid) were diluted with 10 mM MES buffer, pH 4.2, to concentrations of 10^{-8} or 10^{-9} M ABA, from estimates based on the GLC-EC data. The bioassay was based on the rate of closure of stomata in *Commelina communis* epidermal strips. Epidermal strips ($\sim$15 mm^2) with wide open stomata were placed on 50 µl drops of test solution in little wells drilled in plexiglas. The strips were incubated in the light chamber in compartments flushed with humidified, CO_2-free air. Strips were removed from the chamber and examined for stomatal aperture at various times. Fifteen apertures were averaged for each data point. The ability of tissue extracts to cause stomatal closure was compared with that of authentic ABA in solutions of known concentration.

3.2.5. Estimation of guard cell potassium content. Formation of potassium cobaltinitrite was used to identify K (Macallum 1905). Staining was done as described by Raschke and Fellows (1971). Stained epidermal strips were photographed. Guard cell pairs were scored for their degree of formation of the cobaltous sulfide precipitate as the percent of their area which appeared black in the photographs. Twenty stomata were scored for each data point.

3.2.6. Measurement of leaf water potential. The dew-point method was used to measure ψ_{leaf} as described in Chapter 2, except that samples were prepared from leaf-halves by cutting them into 12-mm segments.

3.3. RESULTS

3.3.1. Abscisic acid in Commelina epidermal tissue. In the first analysis of ABA in epidermal strips, the identification of ABA by GLC-EC of its methyl ester in a purified extract was supplemented by two other diagnostic tests for ABA. A second GLC-EC analysis of samples, after light-induced isomerization, showed a reduced peak at the proper retention time for the naturally-occurring 2-cis isomer and the appearance of a new peak at the proper retention time for 2-trans-ABA. A bioassay for ABA, based on closure of stomata in epidermal strips of Commelina communis, was also used. The ability of purified extracts to cause stomatal closure was compared with that of solutions of known concentration of authentic ABA. The biological activity of extracts agreed very well with their content of ABA as estimated from GLC-EC analysis. It was clear that epidermal tissue of Commelina leaves (from 10 leaves in this case) contained measurable amounts of ABA.

In another preliminary experiment the ABA content of epidermis from wilted Commelina leaves was compared with that from turgid leaves (Figure 3-1). Three hours of incubation in the wilted state, as compared to the turgid state, produced a 4-fold increase in the ABA content of Commelina leaves. Abaxial epidermis taken from the wilted leaves contained two to three times more ABA than did epidermis taken from the turgid leaves.

Figure 3-1. Increases in the level of abscisic acid in Commelina communis abaxial epidermis and the remainder of the leaf ("mesophyll") due to the wilting of intact leaves. Detached leaves were reduced in fresh weight by 10% and incubated in plastic bags. Other leaves were kept turgid and similarly incubated. After 3 h, abaxial epidermis was stripped from each leaf, adhering mesophyll cells were counted. Each sample consisted of epidermis (or the rest of the leaf) from 20 leaf-halves.

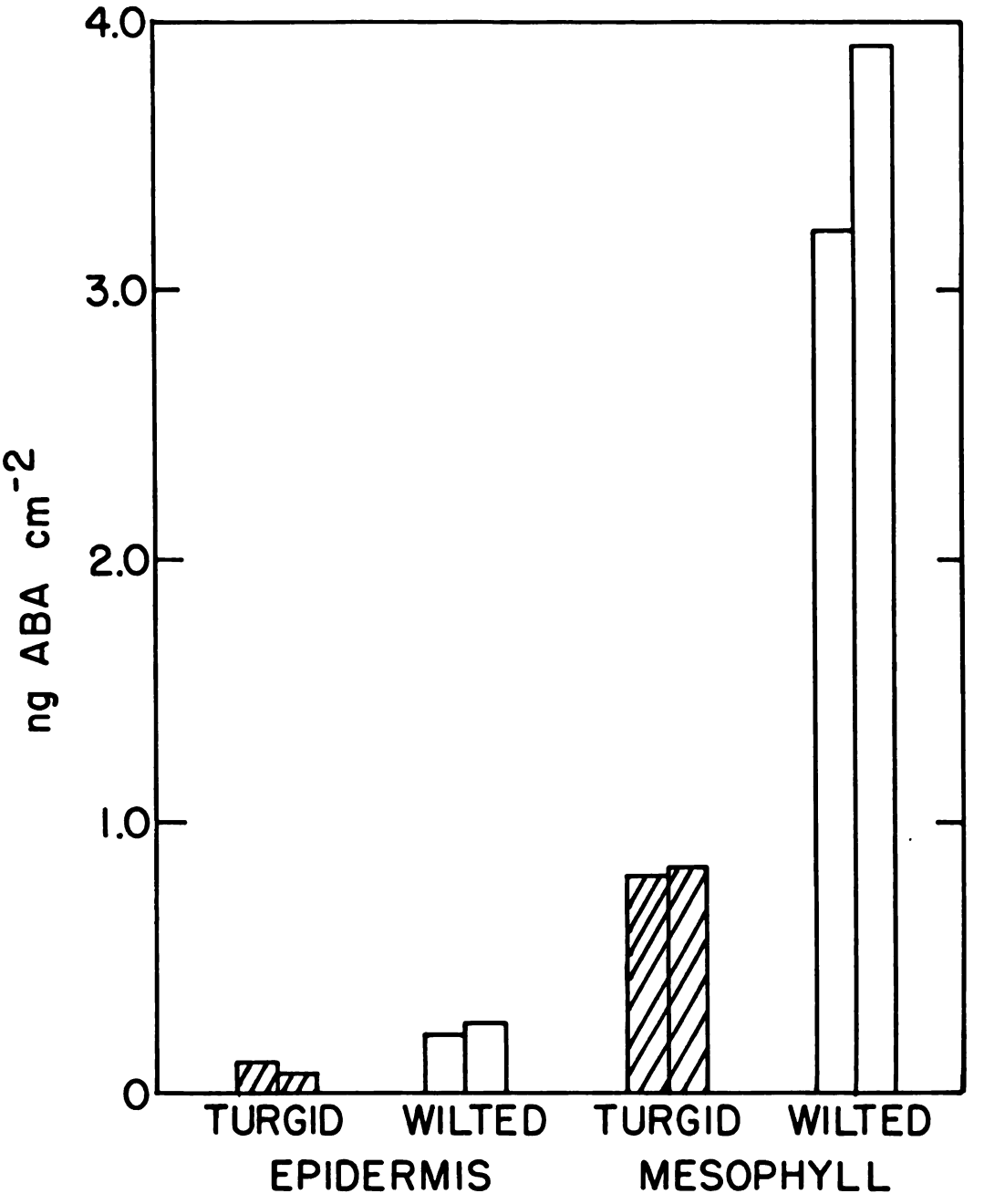
4.0
3.0
2.0
1.0
0
ng ABA cm-2
TURGID
WILTED
TURGID
WILTED
EPIDERMIS
MESOPHYLL

Figure 3-1

3.3.2. Decline in ψ_{leaf}, accumulation of ABA, and stomatal closure. A comparison was made of the time course of accumulation of ABA in the abaxial epidermis and in the rest of the leaf during dehydration of the intact leaf (Figure 3-2). For 30 min after leaf sections were removed from their water supply the level of ABA in the epidermis and in the mesophyll remained unchanged. Thereafter, an increase in ABA content was more obvious in the epidermis than in the mesophyll. By 1 h, epidermal ABA had almost doubled whereas mesophyll ABA was within 11% of the original level.

The series of GLC-EC traces for the epidermal samples in this experiment is shown in Figure 3-3. This set was chosen for illustration because the samples were of similar size and recovery of ABA after purification was 50-60% in all samples, so that the accumulation of ABA with time can be seen directly in a comparison of the chromatograms.

Further experiments focused on the initial 1.5 h after interruption of the water supply to the leaves. Figure 3-4 shows a time course of the decrease in water potential in the leaf sections. Leaf turgor reached zero 23 min, on average, after the leaf sections were removed from water. The ψ_{leaf} at which turgor reached zero was determened from a graph of $-\psi_{leaf}^{-1}$ versus percent of original fresh weight (Figure 3-5).

Time courses of the effect of water stress on stomatal aperture and the level of ABA in epidermal tissue are

Figure 3-2. Accumulation of ABA in the lower epidermis and the rest of the leaf ("mesophyll") of Commelina communis during dehydration of intact leaf sections. Leaf sections were removed from their water supply at time = 0 h. Each measurement of ABA was on epidermis or mesophyll from 8 leaf-halves.

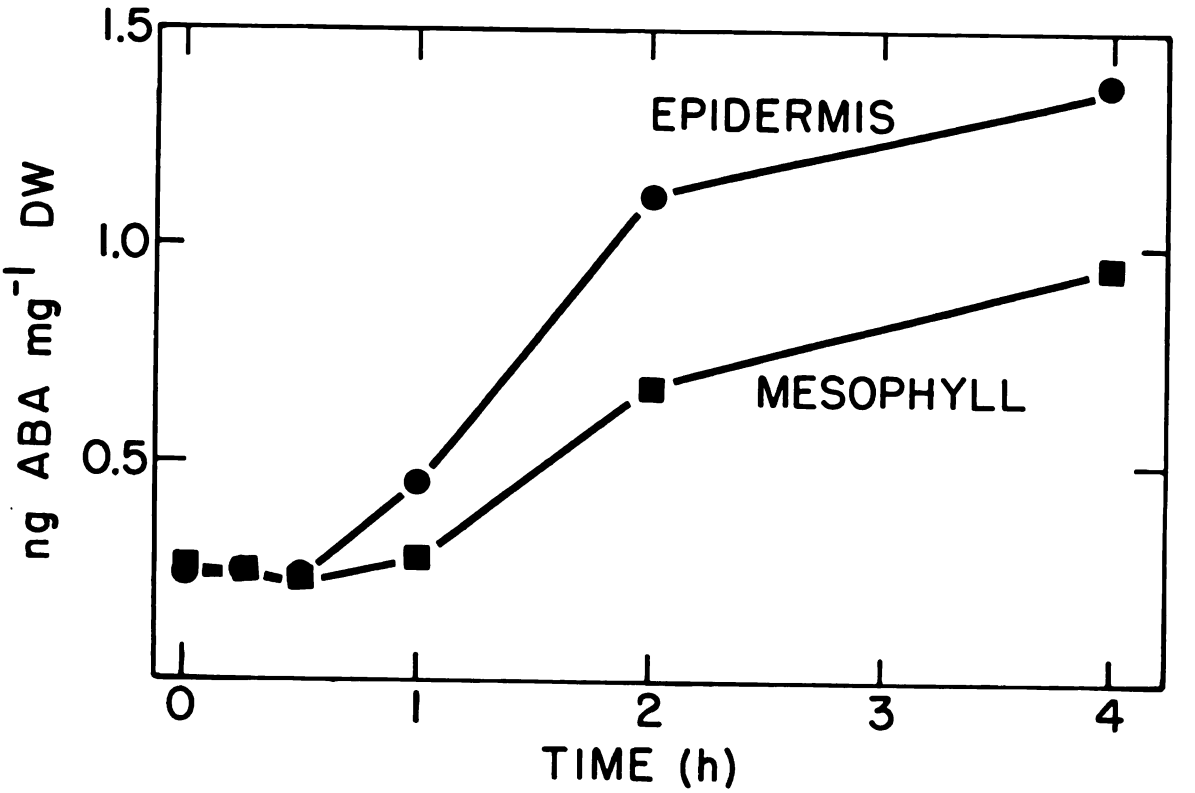

Figure 3-2

Figure 3-3. A series of GLC-EC chromatograms indicating the presence of ABA in purified extracts from Commelina communis epidermis. These GLC-EC traces correspond to the epidermal samples in Figure 3-2. The times, after removal of leaf sections from water, at which epidermis was stripped from the leaves and frozen, are indicated above the chromatograms. The retention time of authentic Me-ABA is indicatied by the arrow. Each injection was 2 µl and represented 1/50 of the total sample.

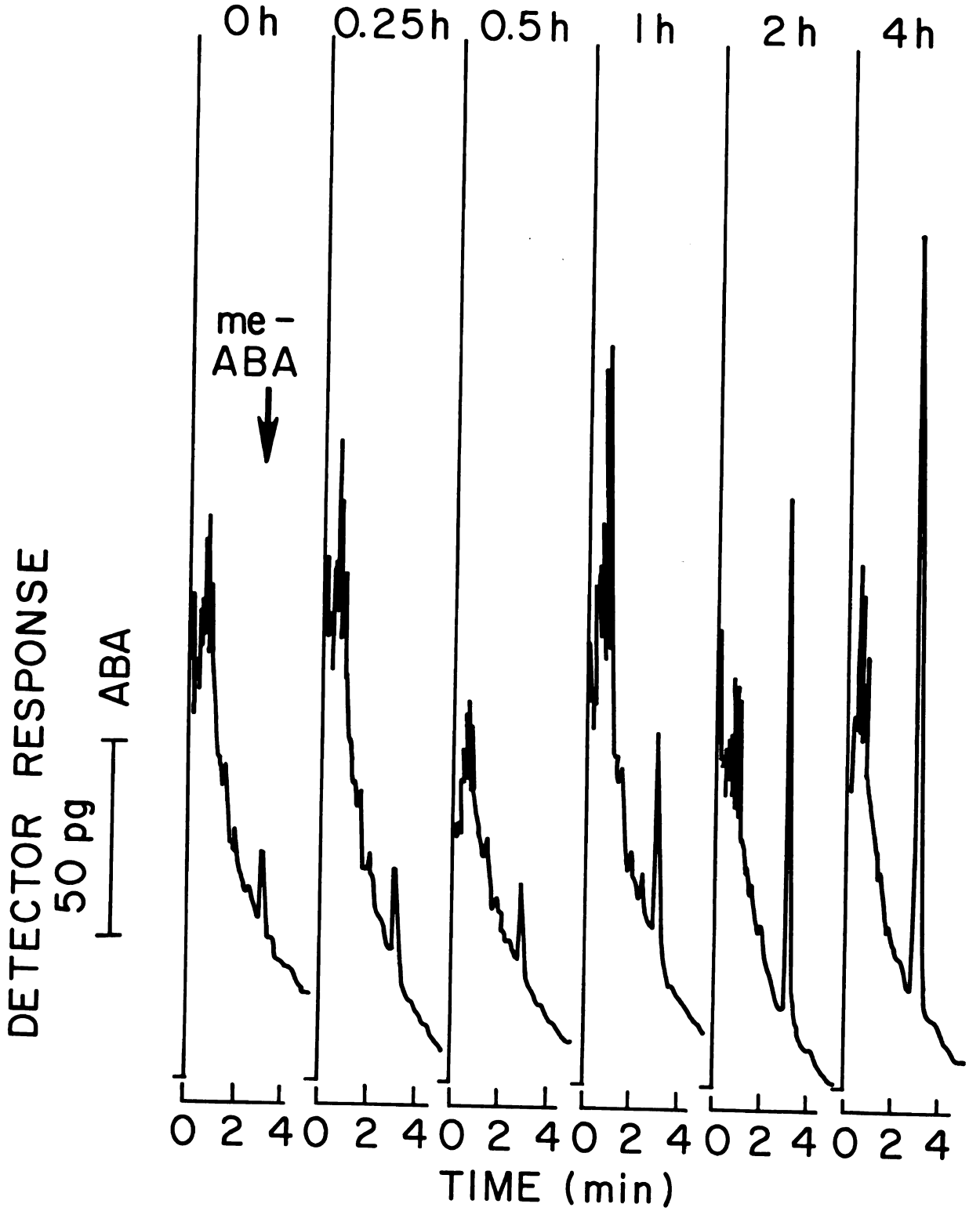
0h
0.25h
0.5h
1h
2h
4h
me-
ABA
DETECTOR RESPONSE
50 pg
ABA
0 2 4 0 2 4 0 2 4 0 2 4 0 2 4 0 2 4
TIME (min)

Figure 3-3

Figure 3-4. The decline in ψ_{leaf} with time after removing sections of Commelina communis leaves from water. Each data point represents a measurement on a single leaf section; points having the same symbol represent sections from the same leaf.

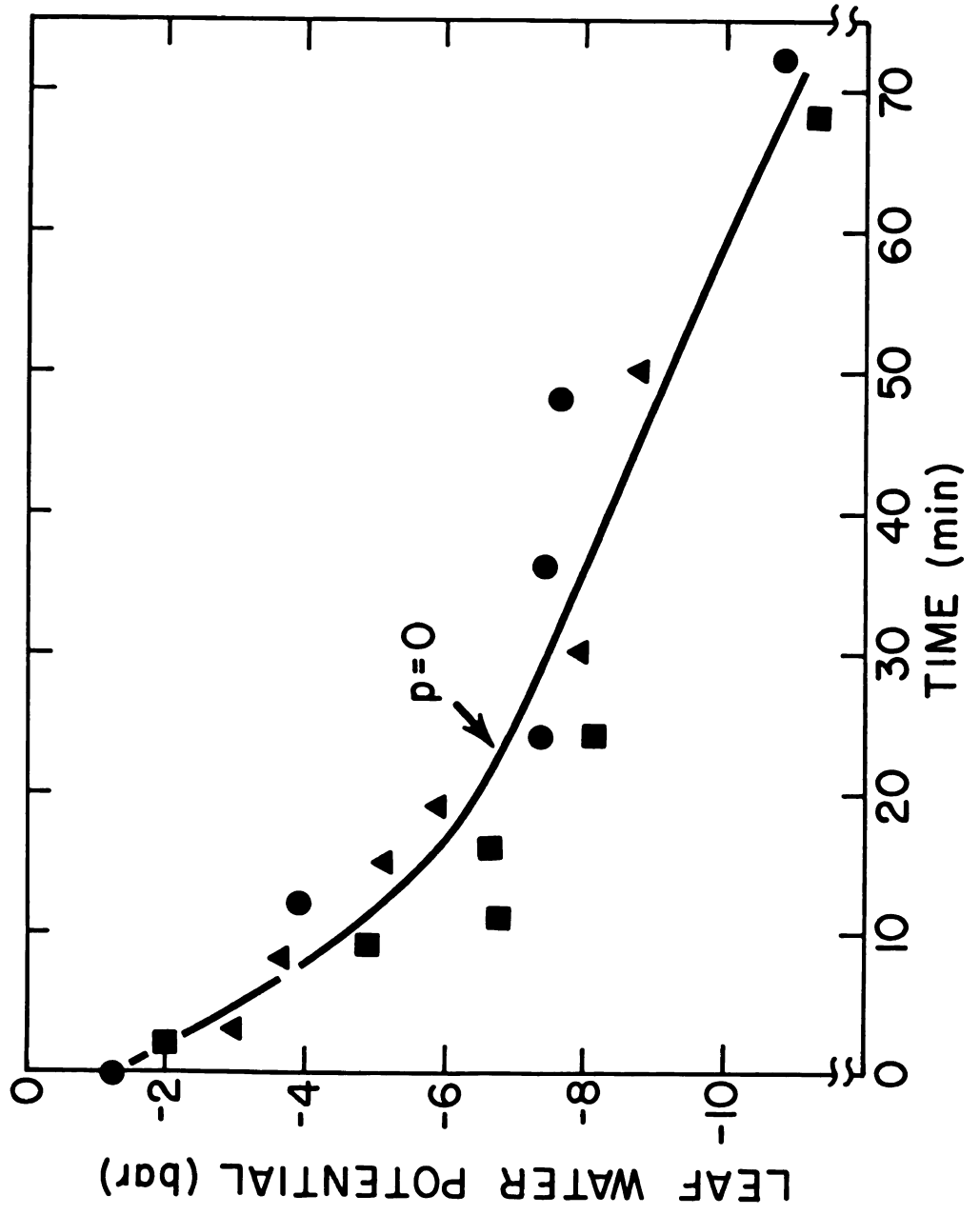
LEAF WATER POTENTIAL (bar)
0
-2
-4
-6
-8
-10
p=0
0
10
20
30
40
50
60
70
TIME (min)

Figure 3-4

Figure 3-5. The relation between $-\psi_{leaf}^{-1}$ and % of original fresh weight for Commelina communis leaves. Leaf sections were blotted and weighed immediately after being removed from water; they were weighed again just before ψ_{leaf} was determined. Leaf water potentials were determined by the dew-point method. The data for Figure 3-5 were obtained from the same samples which were used for Figure 3-4.

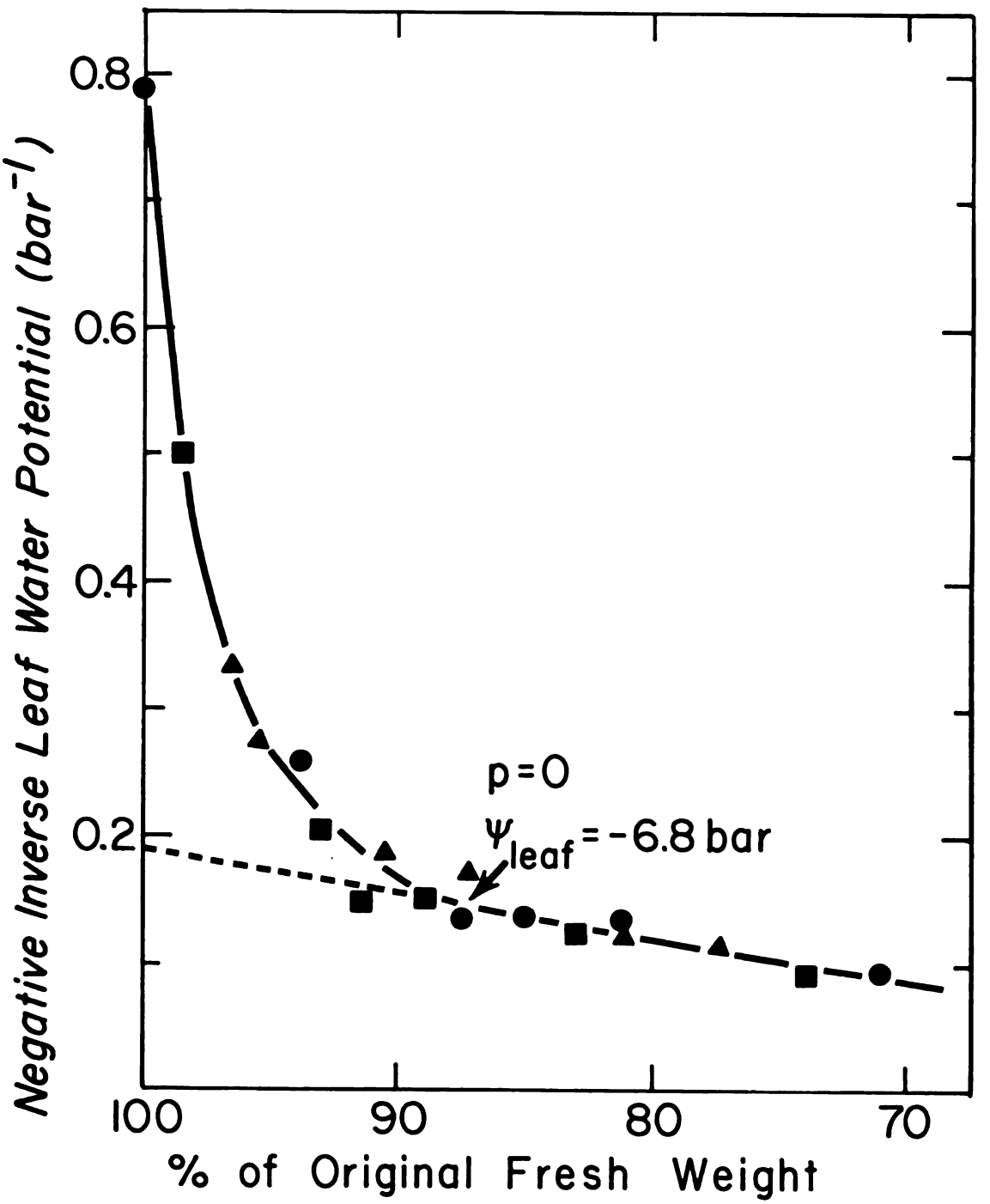
Negative Inverse Leaf Water Potential (bar⁻¹)
0.8
0.6
0.4
0.2
p=0
ψleaf = -6.8 bar
100
90
80
70
% of Original Fresh Weight

Figure 3-5

compared in Figure 3-6. No more than 1 min elapsed between the photographic recording of stomatal apertures and separation of the epidermis from the mesophyll. As shown in Figure 3-6, a gradual increase in epidermal ABA paralleled the period of stomatal closure. The initiation of stomatal closure coincided with the initiation of accumulation of ABA in the epidermis. They both occurred after the measurements at 24 min were made. Stomatal closure was complete by the time that the level of ABA in the epidermis had increased by 50%. As in the previous determinations of ABA, accumulation of ABA was obvious sooner in the epidermis than in the mesophyll; no significant change occurred in the level of ABA in the mesophyll within 84 min. The level of ABA in the mesophyll was in this case 0.2 ng mg^{-1} dry weight.

Transient stomatal opening, which occurs (as a result of reduction in epidermal pressure on the guard cells) upon interruption of the water supply to leaves (Raschke 1975b), preceded the stomatal closing, as shown in Figure 3-6. The opening response lasted at least 24 min; in some leaves this response was very strong. There was 10-20 min range in the duration of the transient opening response, and that resulted in large standard deviations for the stomatal apertures measured at 36,48, and 60 min after removing the leaves from water, as indicated in the legend to Figure 3-6. In some leaf sections, stomatal closure was complete by 48 min, and in some others, closure was not

Figure 3-6. Stomatal closure and accumulation of abscisic acid in the lower epidermis of Commelina communis during dehydration of intact leaf sections. Leaf sections were removed from their water supply at time = 0 min. Four of the 8 leaf-halves per sample were photographed for determination of stomatal apertures. Stomatal aperture means and standard deviations: 0 min, 11.8 ± 1.5 μm; 12 min, 12.1 ± 2.2 μm; 24 min, 13.0 ± 2.3 μm; 36 min, 8.5 ± 5.7 μm; 48 min, 5.2 ± 3.9 μm; 60 min, 3.5 ± 6.0 μm; 72 min and 84 min, all stomata were colsed. Stomatal aperture: ■, ABA: ●.

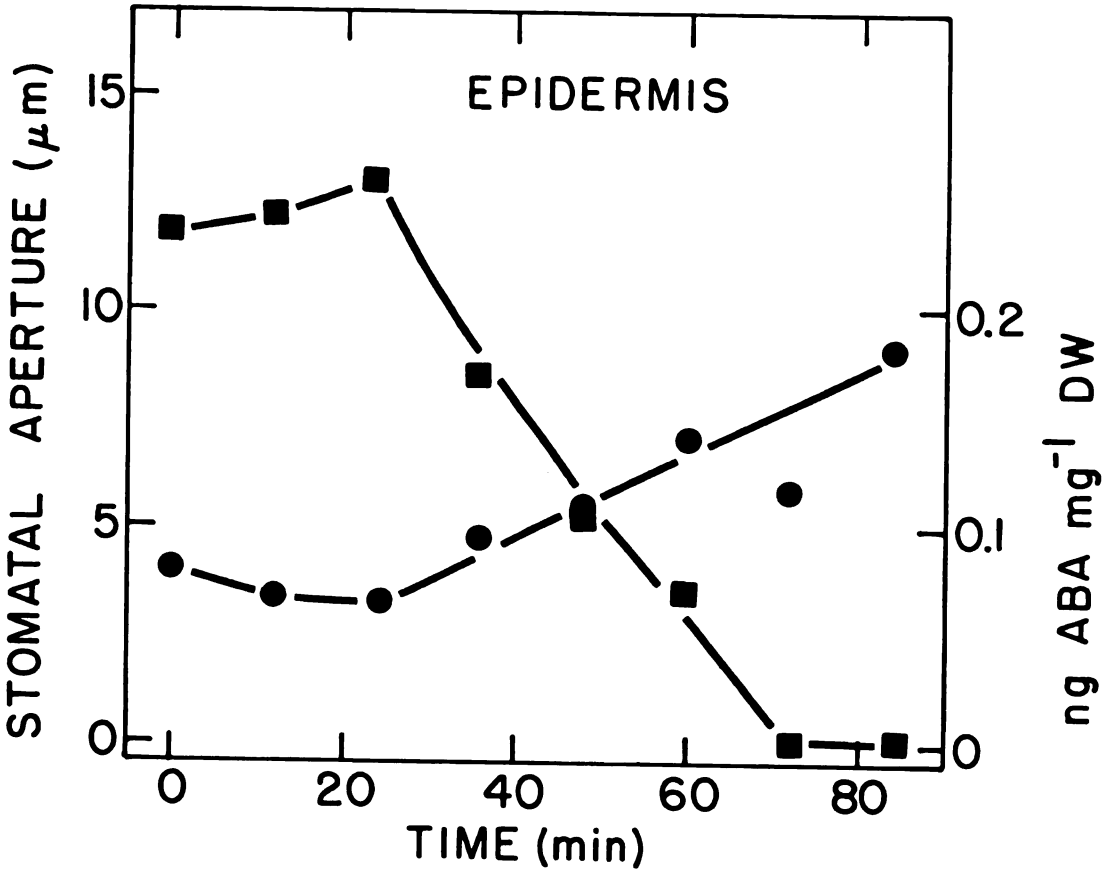
EPIDERMIS
STOMATAL APERTURE (μm)
15
10
5
0
0
20
40
60
80
TIME (min)
0.2
0.1
0
ng ABA mg⁻¹ DW

Figure 3-6

complete until 72 min.

The 10-20 min range in onset of stomatal closure prompted the next experiment, in which leaf samples for anaylsis of ABA content were combined on the basis of similar stomatal aperture rather than time after removal of the leaves from water. After waiting for the transient stomatal opening to occur, leaves were photographed and stripped of abaxial epidermis, one after another, for the next 45 min. Lyophilized epidermal pieces were combined for extraction, after apertures had been determined. In this set of samples, the level of ABA in mesophyll tissue, from leaves having wide open stomata, was 0.1 ng mg^{-1} dry weight.

The results from the epidermal samples are plotted in Figure 3-7. They agreed well with the results in Figure 3-6. Stomatal closure below the initial aperture was accompanied by a gradual increase in the level of ABA in the epidermis. Closure was almost complete when the concentration of ABA had increased by 60%.

3.3.3. Loss of potassium from guard cells and stomatal closure. Was the stomatal closure that was observed in the previous experiments accompanied by loss of K^+ from the guard cells? Stomatal aperture and relative guard cell K^+ content were compared for individual leaf sections. Within 2 min of photographing a leaf section for determining stomatal aperture, epidermal strips were stained for potassium. Figure 3-8 shows a close correspondence between stomatal closure and loss of K^+ from the guard cells.

Figure 3-7. Stomatal aperture versus level of ABA in abaxial epidermis during dehydration of intact leaf sections of Commelina communis. As indicated by the arrow, the average stomatal aperture prior to dehydration was 13.9 µm. Samples were collected beginning at the peak of the stomatal opening movement which preceded the period of stomatal closure. Each sample consisted of epidermis from five leaf-halves.

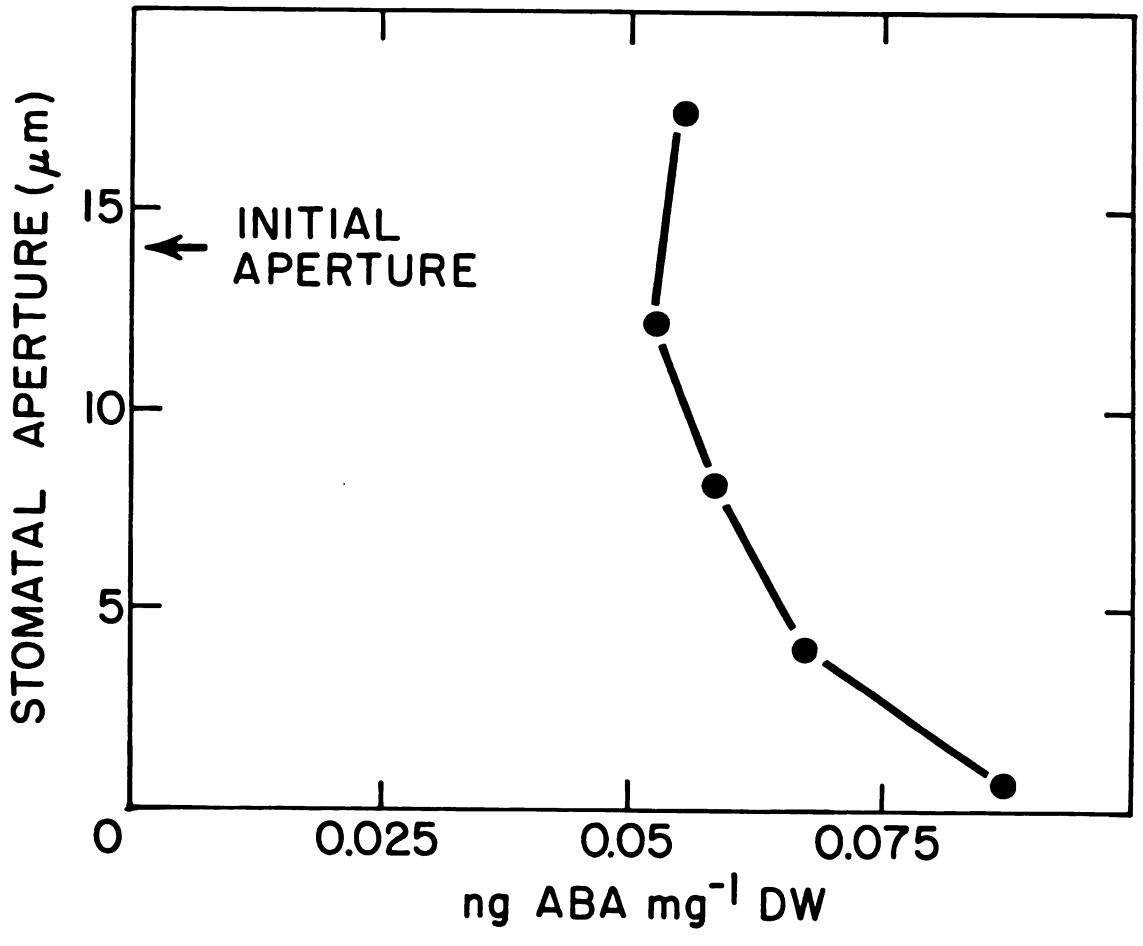
STOMATAL APERTURE (μm)
15
10
5
0
INITIAL
APERTURE
0.025
0.05
0.075
ng ABA mg⁻¹ DW

Figure 3-7

Figure 3-8. Correlation between stomatal aperture and relative potassium content of guard cells during stomatal response upon removal of Commelina leaf sections from their water supply. Relative potassium content was determined as the % of guard cell area which appeared black with precipitate. Each data point represents the average stomatal aperture and K^+ determination for an individual leaf-half.

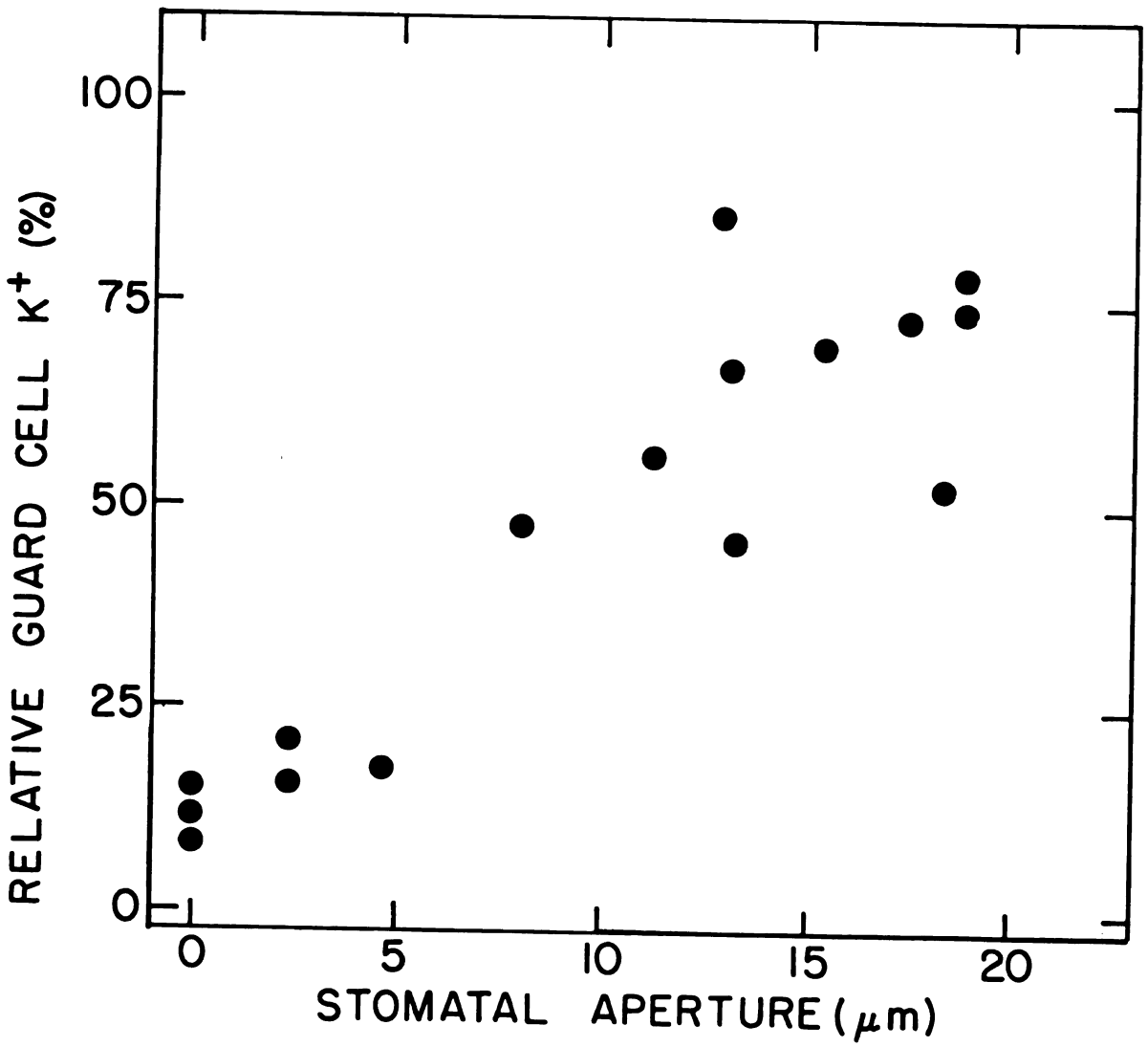
RELATIVE GUARD CELL K+ (%)
100
75
50
25
0
0
5
10
15
20
STOMATAL APERTURE (μm)

Figure 3-8

3.3.4. Accumulation of ABA in tissue which was wilted in solution. The observation of ABA levels increasing earlier after water stress in the epidermis than in the mesophyll could mean production of ABA by the epidermis or a water-stress-induced redistribution of the leaf's ABA in favor of the epidermis. Mesophyll and epidermis were examined for their ability to independently accumulate ABA during water stress, in solutions of mannitol (Figures 3-9 and 3-10). The tissue and the solutions were analyzed separately for ABA content. Both mesophyll and epidermis lost ABA to the incubation media. All solutions contained approximately as much ABA as the leaf tissue that they had incubating in them.

Conditions were found for incubation in which the leaf minus the lower epidermis, in solution, accumulated ABA as well as, or even better than, the wilted, intact leaf in air did. Compare Figure 3-9 with Figure 3-2. The incubation solution with π = 20.9 bar was more than three times as effective as that with π = 13.6 bar in eliciting accumulation of ABA in mesophyll tissue.

Epidermis in solutions of mannitol, under the same conditions as those provided for rest of the leaf, failed to produce any significant increase in ABA. The mannitol treatment of the isolated epidermis caused stomatal closure and cell breakage as well as cell plasmolysis, as indicated in Table 3-2.

Figure 3-9. Accumulation of ABA by mesophyll tissue of Commelina during osmotic stress. Basal medium: KCl + 10 mM HEPES buffer, pH 6.5, π = 1.5 bar. Basal medium + 0.5 M mannitol: π = 13.6 bar. Basal medium + 0.75 M mannitol: π = 20.9 bar. Hatched area: tissue ABA. Clear area: ABA lost to the incubation solution per mg dry weight of incubation tissue.

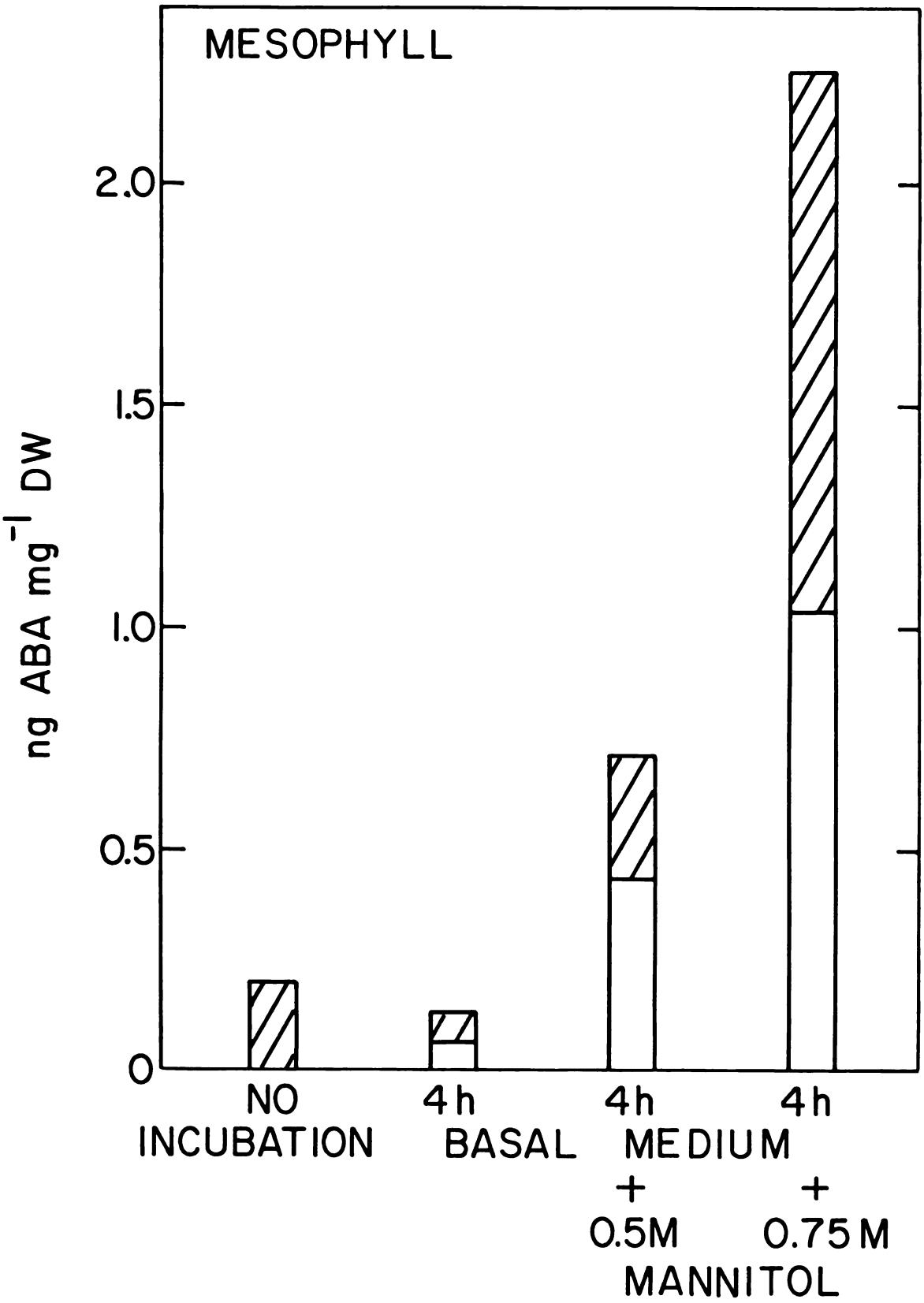
MESOPHYLL
2.0
1.5
1.0
0.5
0
ng ABA mg⁻¹ DW
NO INCUBATION
4h BASAL
4h MEDIUM + 0.5M MANNITOL
4h + 0.75M MANNITOL

Figure 3-9

Figure 3-10. The effect of osmotic stress on the level of ABA in Commelina leaf epidermal tissue. Incubation solutions as described in Figure 3-9. Hatched area: ABA in abaxial epidermis. Clear area: ABA lost to the incubation solution per mg dry weight of incubating epidermis.

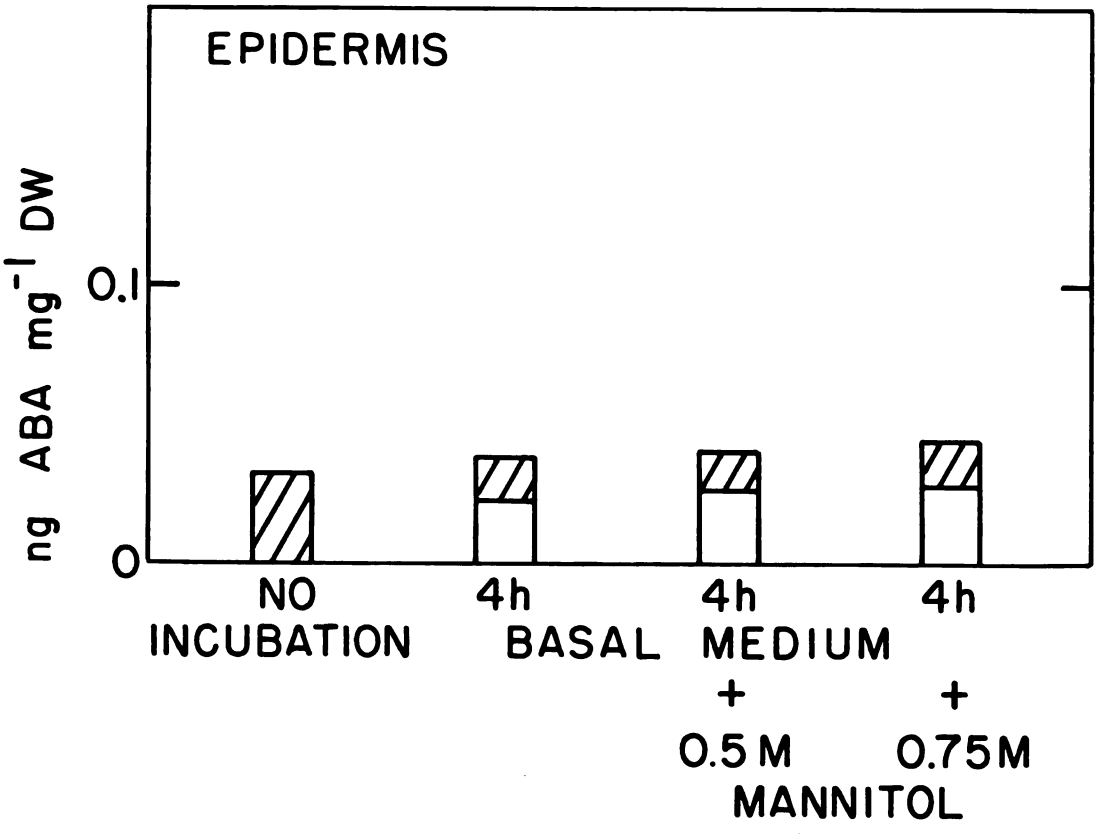
EPIDERMIS
ng ABA mg^{-1} DW
0.1
0
NO INCUBATION
4h BASAL
4h MEDIUM + 0.5 M MANNITOL
4h + 0.75M

Figure 3-10

Table 3-2. Observations during incubation of epidermal strips of Commelina leaves in osmoticum

Treatment	Cell breakage	Cell plasmolysis	Stomatal aperture
Initial Conditions	No Guard Cells or Subsidiary Cells broken. <25% Ordinary Epidermal Cells broken.	None	12 - 15 μm
Basal Medium 4 h	No Guard Cells or Subsidiary Cells broken. <40% Ordinary Epidermal Cells broken.	None	1 - 2 μm (Stomata remained wide open for at least 1 h).
0.5 M Mannitol + Basal Medium 4 h	No Guard Cells or Inner Subsidiary Cells broken. ∿25% Outer Subsidiary Cells broken. >80% Oridinary Epidermal Cells broken.	All cells plasmolyzed.	0 μm (Closure in progress by 40 min).
0.75 M Mannitol + Basal Medium 4 h	(Same as for 0.5 M treatment.)	All cells plasmolyzed.	0 μm (Closure in progress by 25 min).

3.4. DISCUSSION

3.4.1. The level of ABA prior to stress-induced accumulation. The level of ABA in epidermal tissue from unstressed leaves of Commelina was reported by Singh et al. (1979) and by Dörffling et al. (1980) to be, on the average, between 0.1 and 0.2 ng mg^{-1} dry weight. The results presented here agree with that estimate. Vicia faba epidermis from unstressed leaves appears to contain much more ABA than that, 0.8 nm mg^{-1} dry weight (Loveys 1977). A comparison of the same reports indicates that Vicia mesophyll also contains a higher basal level of ABA than Commelina mesophyll does.

It is puzzling that the absolute level of ABA in epidermis, prior to accumulation as a result of experimentally-imposed water stress, was found to vary considerably from one group of plants to another: from 0.05 (Figure 3-7) to 0.25 ng mg^{-1} dry weight (Figure 3-2). To some extent the variation of ABA level in the epidermis paralleled a variation in ABA level in the mesophyll. We already know from experiments with Xanthium strumarium (Raschke et al. 1976) that the basal level of ABA in a leaf, as a whole, can vary several-fold without an apparent effect on the stomata. Now it seems that, possibly, ABA can be somewhere even within the epidermis without affecting the stomata. In some groups of leaves (Figures 3-6 and 3-7) the stomata were closed at a level of ABA which, in another

group of leaves (Figure 3-2) was well below the initial value. Understanding the compartmentation of ABA within the epidermis may prove to be critical for an evaluation of the role which ABA plays in regulating stomatal behavior.

3.4.2. Changes in ABA content and stomatal aperture related to decline in ψ_{leaf}. An increase in ABA in the epidermis was apparent by 36 min after removing the leaf-halves from water (Figure 3-6), which was within 13 min of when leaf turgor fell to zero (Figure 3-4). If ABA content from Figure 3-6 were plotted against ψ_{leaf} from Figure 3-4, the ψ_{leaf} at which leaf turgor equalled zero would appear as a threshold for increasing ABA content in the epidermis. It is not possible, however, to conclude what the exact relation was between leaf turgor and epidermal ABA content; since ψ_{leaf} was falling throughout the experiment, any ψ_{leaf} above the apparent threshold could have initiated the increase in ABA level.

A comparison of Figure 3-4 and 3-6 indicated, as one might expect, that the peak of transient stomatal opening occurred as turgor in the leaf sections reached zero.

3.4.3. Stomatal closure and accumulation of ABA by epidermis. Increases in ABA in response to water stress were evident sooner in the abaxial epidermis than in the remainder of the leaf. The level of ABA in the epidermis gradually increased as the stomata closed. These observations support the idea that the stomatal closure could have been caused by ABA. On the other hand, the

changes in the amount of ABA in the epidermis were small, less than a doubling by the time the stomata were closed. Loss of potassium from the guard cells occurred in parallel with decreases in stomatal aperture (Figure 3-8). Had the accumulation of ABA by the epidermis begun by the time stomata had started to close? The accumulation of ABA certainly did not start before stomatal closure; the two processes became apparent at about the same time (Figure 3-6).

The data in Figures 3-6 and 3-7 indicate that Commelina stomata closed to 50% of the original aperture with about a 10-20% increase in the level of ABA in the epidermis, and complete closure was associated with a 50-100% increase in level of ABA. How does that result compare to the amount of exogenous ABA that is required to close stomata in Commelina abaxial epidermis?[1] Raschke (1975a) showed that the amount of ABA, which detached leaves take up in the transpiration stream by the time stomatal closure is initiated, depends on the concentration in which the exogenous ABA is supplied. The amount of ABA increased with a power of about 0.67 of the concentration of ABA in the transpiration stream. The concentration in which endogenous ABA appears in the epidermis when leaves wilt is not known. A reasonable estimate may be that concentration of exogenous

[1]The comparison will be affected by any variation in stomatal sensitivity to ABA. Stomatal sensitivity to ABA is affected by factors such as potassium (Cummins 1971), CO_2 supply (Raschke 1975a), or previous exposure to water stress (Davies 1978).

ABA which causes stomatal closure with a time course similar to the one which was observed in this study. According to Figure 3-6, significant stomatal closure occurred within about 30-40 min after the leaves were removed from their supply of water. In the experiments of Weyers and Hillman (1979), when (±)-[2-^{14}C]ABA was fed via the transpiration stream to detached Commelina leaves, a concentration between 1 and 5 · 10^{-6}M was required to obtain significant stomatal closure in 40 min. These authors used the ^{14}C activity to estimate how much ABA the leaves took up in 40 min and what percent of that was partitioned to the epidermis. Using the dry weight/area ratio in Table 3-1, the data of Weyers and Hillman (from their Table 2) were converted to give the estimate that significant stomatal closure would occur in 40 min by the addition of less than 0.12 ng (+)-ABA,[2] but probably more than 0.03 ng (+)-ABA mg^{-1} dry weight of epidermis. Whether this estimate amounts to a significant increase in epidermal ABA depends, of course, on how much ABA was in the epidermis to begin with, and, as mentioned, that level was variable. Using an average from all sources (this work, Singh et al. 1979, Dörffling et al. 1980) of 0.1 ng mg^{-1} dry weight, epidermal ABA would be expected to increase by 30-120% as stomatal closure occurred. This projection and the results that were actually obtained (Figures 3-6 and 3-7) are not widely

[2]The estimate by Weyers and Hillman was divided by 2 and expressed as (+)-ABA because Cummins and Sondheimer (1973) demonstrated that the (-)-enantiomer has little or no effect on stomata.

disparate. It is still conceivable that ABA plays a major role in closing stomata during water stress.

Would a doubling of the level of ABA in the abaxial epidermis represent a significant increase in the level of ABA in the whole leaf, for instance an addition of 0.1 ng mg^{-1} dry weight of epidermis (= 25 pg cm^{-2})? One cm^2 of leaf would contain (at 0.235 ng mg^{-1} dry weight) 800 pg ABA. Thus, 25 pg would represent 3% of the total ABA in a cm^2 section of an unstressed Commelina leaf. Such a small change could hardly be detected.

Another estimate can be made, which, like the others, highlights the smallness of the active pool of ABA and, in doing so, makes a major role of ABA in regulating stomata seem credible. This is an estimate of how much the concentration of ABA might change around the guard cells if the level of ABA in the epidermis, as a whole, doubled. The ABA in the epidermis of an unstressed leaf may be rather uniformly distributed; since the basal level of ABA can vary by several-fold, presumably a large portion is not in contact with the guard cells. If additional ABA arrives from the mesophyll during stress, there is reason to suspect preferential deposition of that ABA in the vicinity of the guard cells. Certainly ABA which enters the apoplast and moves with the transpiration stream ends up mostly near the guard cells (Weyers and Hillman 1979; Raschke 1979). The implication is clear, then, that what may be a modest increase in terms of the whole epidermis, may really be a

very large increase in the concentration of ABA near the guard cells. Commelina epidermis weighs roughly 4 mg fresh weight cm^{-2}, and thus has a volume of about 4 µl cm^{-2}. At 6000 stomata cm^{-2} and roughly 20 pl per guard cell pair (including cell walls) (Raschke 1979), the guard cells make up about 0.12 µl of the 4 µl cm^{-2} of epidermis, or 3% of the volume. If 30% of the additional ABA were to go to the guard cells, during a doubling of the level of ABA in the whole epidermis, then the concentration of ABA at the stomata would have increased 10-fold.

In conclusion, the small and gradual increase in ABA in the epidermis, which paralleled the decrease in stomatal aperture in leaves of Commelina (Figures 3-6 and 3-7), is sufficient for us to still maintain the hypothesis that ABA is a major factor in closing stomata during water stress. The preceding calculations point out the difficulty we will have in proving whether or not the hypothesis is correct. The difficulty which is imposed by a requirement for only a very small active pool of ABA is compounded by the knowledge that stomata are sensitive to environmental and physiological parameters other than ABA that also pertain to plant water economy. As pointed out by Raschke (1979), biological control systems appear to depend on signals from more than one source.

Efforts to evaluate the role of ABA would be greatly helped by the discovery of an inhibitor of endogenous production of ABA. Meanwhile, the following experiment might yield interesting results. We know that guard cells, but not ordinary epidermal cells, can retain their viability after

epidermal strips have been sonicated (Ogawa et al. 1978). We also know that compounds can be transferred from the mesophyll to the epidermis even if the epidermis has been removed and then replaced (Dittrich and Raschke 1977). If sonication removes the large background pool of ABA in the epidermis, then it should be easier to see a subsequent stress-induced accumulation of ABA after replacing the epidermis on the mesophyll and subsequently wilting the leaf sections.

3.4.4. Accumulation of ABA by tissue in solution. One of the purposes of this set of experiments was to check whether conditions, which induced the rest of the leaf to accumulate ABA, would also induce its accumulation in isolated epidermis. The results were negative (Figure 3-10). Loveys (1977) and Dörffling et al. (1980) also did not detect any increase in ABA in stressed, isolated epidermis. The negative result was expected, since production of ABA by the epidermis would diminish the effectiveness of such a signal of water-stress coming from the mesophyll. The absence of accumulation of ABA by isolated epidermis certainly does not prove, however, that the mesophyll provides ABA to the epidermis during water stress. Several interpretations are possible, especially since mannitol treatment caused stomatal closure and epidermal cell breakage (Table 3-2).

The main purpose of the experiments with solutions of mannitol was to develop a system in which mesophyll tissue,

in solution, would produce ABA just as well as dehydrated leaves do. This purpose was achieved. Incubation of mesophyll tissue (leaf minus lower epidermis) in basal medium plus 0.75M mannitol induced accumulation of ABA in 4 h to a greater degree than was observed in dehydrated leaves in 4 h (Figure 3-9 vs. Figure 3-2). Loveys (1977) reported higher levels of ABA in mesophyll tissue that was infiltrated with a 0.88M solution of mannitol rather than buffer, but the total ABA in that tissue plus incubation medium was, for unknown reasons, at a lower level than the content of ABA which Loveys reported for unstressed leaves. Recently Mawson et al. (1981) found enhanced production of ABA by leaf slices of Phaseolus vulgaris that were incubated in medium which contained 0.77M sorbitol. Others have found that addition of mannitol to suspension cultures caused enhanced production of ABA by grape pericarp tissue (Loveys et al. 1975) and tobacco cells (Wong and Sussex 1980).

The results in Figure 3-9 are preliminary to extending the study that was presented in Chapter 2. There it was reported that leaves were slightly stimulated to accumulate ABA if turgor fell to within a few bars of being zero. The stimulation progressively increased as turgor approached zero. There was evidence of saturation of the response as ψ_{leaf} was further reduced. The consequences of reducing turgor by osmotic stress should provide further insight on the relationship between loss of turgor and accumulation of ABA. The results in Figure 3-9 are, in fact, curious in

this respect. Commelina communis leaves are wilted at ψ_{leaf} = -6.8 bar (Figure 3-5), and yet there was a large difference in the accumulation of ABA between Commelina tissue incubated in solution at ψ = -13.6 bar versus -20.9 bar, even though both sets of samples were expected to have been without turgor. The phenomenon requires investigation; the possibility exists that the tissue at ψ = -13.6 bar regained turgor part way through the incubation by taking up solute from the medium.[3] That suggestion is supported by the following observation. As the leaf-halves wilted upon being immersed in either solution of mannitol, they curled with the long axis along the circumference of the incubation tube, whereas the leaf-halves in buffer were turgid and hung vertically in the tube. By the end of the incubation period, the sections at ψ = -20.9 bar were still curled, but the sections at ψ = -13.6 bar had straightened.

An in vitro system of osmotic stress greatly increases the options for studying the induction of accumulation of ABA. Some examples follow.

1) An in vitro system will allow direct measurements

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[3]Adding the equivalent of 0.15 M KCl to the cell sap would have re-established turgor in the tissue that was incubated at ψ = -13.6 bar. If that had happened in 2 h, the required rate of K^+ uptake would have been 75 μmole g^{-1} fresh weight h^{-1}. This rate is half that calculated for Zea mays guard cells in response to light (Raschke and Fellows 1971) and 3.6 times the rate reported for barley roots in 25 mM KCl (Epstein et al. 1963). The required rate of K^+ uptake would be reduced by the extent to which mannitol was taken up. It may be worth noting that K^+ influx into cells of Valonia utricularis is stimulated by loss of turgor (Zimmermann and Steudle 1977).

of cell turgor with a pressure probe (Zimmermann and Steudle 1974). 2) Assuming that changes in turgor are monitored; it should be possible to decrease ψ_{leaf} beyond the wilting point in order to find out if accumulation of ABA saturates once all cells in a sample have lost turgor. Attempts to decrease ψ_{leaf} much beyond the wilting point, by using a pressure bomb to force water out of a leaf, were thwarted by breakage of cells. 3) Populations of cells can be uniformly exposed to osmotic stress so that one may be able to distinguish whether the progressive induction of accumulation of ABA that was observed in leaves was due to a progressive induction within the individual cells or to differences in the water relations among the cells in a leaf. 4) Turgor-dependent processes may depend on the exact position of the plasmalemma relative to the cell wall. Can protoplasts accumulate ABA? How does accumulation of ABA compare in response to osmotica which affect cells in different ways physically? Polyethylene glycol 6000 causes protoplast and cell wall to shrivel; mannitol causes cells to plasmolyze; ethylene glycol causes only a temporary loss of turgor since it penetrates cells rapidly. Ethylene glycol has been used to distinguish turgor-dependent processes since it affects ψ_{leaf} and π without affecting p (Greenway *et al.* 1972). 5) An *in vitro* system would facilitate testing the effect of potentially inhibitory or stimulatory compounds and testing hypotheses for turgor-transduction such as membrane compression (Coster *et al.*

1977).

Chapter 4

Synthesis and metabolism of abscisic acid in detached leaves of *Phaseolus vulgaris* L. after loss and recovery of turgor

4.1. INTRODUCTION

As demonstrated by the results presented in Chapters 2 and 3, loss of turgor causes the amount of ABA in a leaf to increase many-fold. Upon rehydration of a leaf, its ABA content returns to a level typical of leaves of unstressed plants (for review, see Wright 1978). The level of ABA will depend on the relative rates of synthesis and metabolic removal of this hormone. Knowledge of the factors that affect accumulation and disappearance of ABA will contribute to an understanding of how plants cope with water stress and recover from it.

Harrison and Walton (1975) and Zeevaart and Milborrow (1976) showed that, in leaves of Phaseolus vulgaris, ABA is metabolized either by hydroxylation then rearrangement to phaseic acid (PA) followed by oxidation of PA to dihydrophaseic acid (DPA), or by formation of alkaline-hydrolyzable conjugated ABA. Of these pathways, the former appears to be the predominant one.

Harrison and Walton (1975) fed (+)-[2-^{14}C]-ABA to leaves of P. vulgaris and found that metabolism of ABA continues during water stress. This chapter examines how the pool sizes of the important metabolites of ABA change with time during and after water stress, in order to compare the rate of synthesis with the rate of metabolic removal of ABA and to estimate how much each of the two pathways for the metabolism of ABA contributes to the reduction of

the ABA level in leaves during recovery from water stress. Recently, Zeevaart (1980) presented data on this subject obtained with detached leaves of Xanthium strumarium. Among his results was the finding that much more ABA is converted to PA than is being conjugated, both in wilted and rehydrated leaves of Xanthium.

Chapter 2 (i.e. Pierce and Raschke 1980) provided evidence that turgor, rather than ψ_{leaf} or π, is the critical parameter of leaf water status that affects accumulation of ABA. This chapter reports on the influence of turgor on the metabolism of ABA. Effects of changes in turgor were examined both with increasing severity of water stress and with increasing relief from water stress. This work is presented in Pierce and Raschke (1981).

4.2. MATERIALS AND METHODS

4.2.1. Plants. Plants of Phaseolus vulgaris L. cv. Mecosta (red kidney bean) were cultivated as described in Section 2.2.1. The terminal leaflets of fully developed leaves were used for the experiments when the plants were 4.5-6 weeks old. For simplicity's sake, "leaf" is used throughout instead of "leaflet".

4.2.2. Experimental procedure. On the evening before an experiment was performed, a leaf was excised under water. The leaf was left to hydrate overnight by standing it in a beaker of water in a darkened cabinet (relative humidity close to 100% at 25°C). After hydration, the ψ_{leaf} was always $\geq$ -2 bar (1 bar = 0.1 MPa). The fresh weight of the leaf which corresponded to a ψ_{leaf} of -2 bar was taken as the leaf's original fresh weight, and will hereafter be referred to as such.

The measurements of ψ_{leaf} were made with a pressure chamber as described in Section 2.2.3. In some experiments measurements of ψ_{leaf} were made periodically on the same leaf as it became more and more dehydrated. Then, in order to determine turgor and π, inverse balancing pressures ($= -\psi_{leaf}^{-1}$) were plotted versus decreasing leaf fresh weight, as in Figure 4-1.

In some experiments measurements of ψ_{leaf} were also made by the dew-point method, as also described in Section 2.2.3.

Figure 4-1. Decrease in ψ_{leaf} with loss in fresh weight for a leaf of Phaseolus vulgaris. (Inverse balancing pressure = $-\psi_{leaf}^{-1}$; $1/\pi$ = inverse bulk osmotic pressure.)

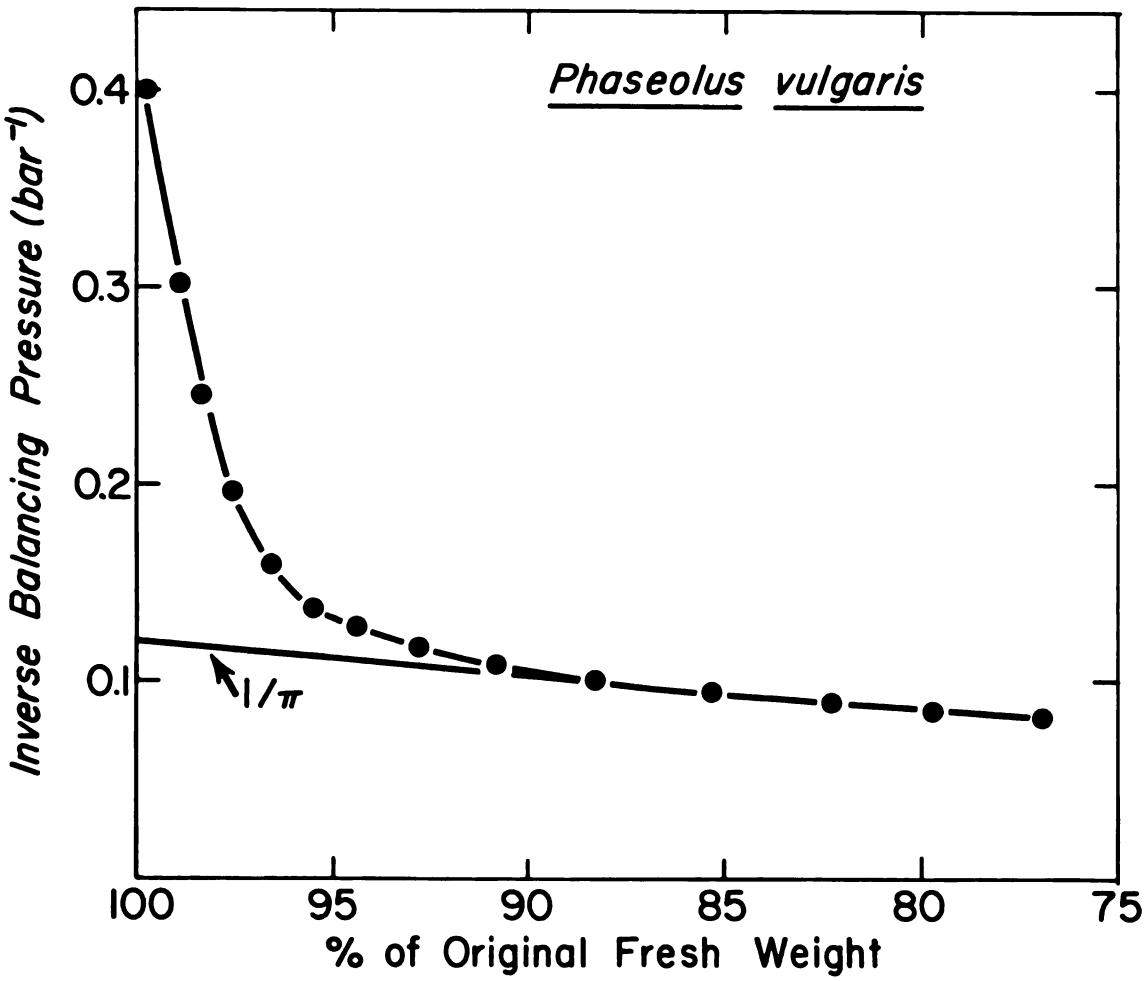
Phaseolus vulgaris
Inverse Balancing Pressure (bar⁻¹)
0.4
0.3
0.2
0.1
1/π
100
95
90
85
80
75
% of Original Fresh Weight

Figure 4-1

The experiments were of three general types: (1) Accumulation of ABA and its metabolites in leaves of P. vulgaris was studied as a function of leaf water deficit. This type of experiment was performed as described in Sections 2.2.4, 2.2.5, and 2.2.6.

(2) ABA synthesis and metabolism were studied as a function of time during and upon recovery from water stress. Individual, detached leaves were removed from their water supply and allowed to transpire until they reached 82% of their original fresh weight, the degree of water deficit which produced the greatest accumulation of ABA in the first type of experiment. They were then wrapped in foil and maintained at constant weight for up to about 8 h from the time the leaf lost turgor. Part of the leaf was removed from foil after usually 5 h, and fully rehydrated by floating on water in a covered dish for up to 0.5 h. This part of the leaf was kept further in the rehydrated condition. Sections of wilted and rehydrated parts of the leaf were frozen at various times for subsequent analysis of the levels of ABA and its metabolites.

(3) Metabolism of ABA was studied as a function of degree of rehydration. Curves for inverse balancing pressure versus fresh weight were determined for leaves until they reached 82% of their original fresh weight. These curves were used to determine by what percentage the fresh weight of a wilted leaf section would have to be increased in order to re-establish a desired degree of turgor. After allowing

a wilted leaf to accumulate ABA for a period of about 5 h, leaf sections were cut. These sections were frozen immediately, left wilted for 3 h longer, or else rehydrated to varying degrees and kept for a further 3 h in the rehydrated state. Leaf sections were rehydrated by floating them on water in a covered dish until they reached the desired, pre-determined weight.

4.2.3. Analysis for ABA and its metabolites. Each lyophilized sample was homogenized at room temperature in 15 ml methanol containing 1% (v/v) glacial acetic acid and 10 mg l^{-1} of antioxidant 2,6-di-tert-butyl-4-methylphenol (Milborrow and Mallaby 1975). The methanol extract was separated from the debris by vacuum filtration, and the debris was re-extracted by shaking overnight in another 15 ml of methanol. The second methanol fraction was combined with the first. Tritiated (±)-ABA, [^{3}H]PA, [^{3}H]DPA, and conjugated [^{3}H]ABA (250 Bq each) were added to the methanol extract for monitoring recovery. Hereafter, (±)-[^{3}H]ABA is referred to simply as [^{3}H]ABA. Five ml H_2O were added to the methanol extract, and the methanol was evaporated under reduced pressure. Material insoluble in H_2O was removed by filtration through a Millipore (Bedford, Mass., USA) AP prefilter.

The filtered aqueous solution was loaded on a SEP-PAK C_{18} cartridge (Waters Associates, Milford, Mass., USA) and followed by a wash with 5 ml 1% aqueous acetic acid. This method of extraction and preliminary purification was highly

efficient. A single extraction of the tissue with methanol and subsequent purification of the treated extract on a SEP-PAK cartridge recovered more than 95% of the radioactivity added through the application of a known amount of ^{3}[H]ABA to leaf sections just prior to freezing. Ethanol and 1% acetic acid were combined in a ratio 40:60 (v/v), and ABA, PA, DPA, and conjugated ABA were all removed from the cartridge with 7 ml of this solvent mixture. A sample was evaporated to a smaller volume under reduced pressure, filtered through a 0.45-µm Millipore filter, and evaporated to dryness under a stream of N_2.

Further purification was achieved with a high-performance liquid chromatography (HPLC) system (Model SP 8000; Spectra-Physics, Santa Clara, Cal., USA) using a procedure modified from the one developed by Zeevaart (1980). The two solvents used for the first HPLC column were re-distilled 95% ethanol (A) and 1% (v/v) aqueous acetic acid (B). A sample was dissolved in 0.5 ml of 10% A: 90% B and injected on a 0.5-ml sample loop connected to a guard column filled with ODS pellicular packing (Whatman, Clifton, N.J., USA) followed by an analytical column packed with 10 µm Spherisorb ODS (Spectra-Physics). The sample was eluted with a linear gradient of 10 - 30% A in B in 1 h following 10 min at initial conditions. Solvent flow rate was 1 ml min^{-1} at $\simeq$ 7 MPa (1000 pounds per square inch, psi).

The collection times for ABA, PA, DPA, and conjugated ABA were based on retention times of standards, which were

detected in the HPLC eluant by absorption of light at 254 nm. Abscisic acid (racemic mixture) was purchased from Calbiochem, La Jolla, Cal., USA. Phaseic acid and DPA were a gift from T.D. Sharkey (see Sharkey and Raschke 1980). The conjugated ABA (β-D-glucopyranoside ester of (±)-ABA was a gift from J.A.D. Zeevaart (see Zeevaart 1980). The retention time of alkaline-hydrolyzable conjugated ABA in extracts of P. vulgaris leaves was originally determined from HPLC trials in which fractions throughout the gradient were subjected to mild alkaline hydrolysis and re-run, collecting at the retention time for ABA. Subsequently it was found that the retention time originally determined for alkaline-hydrolyzable conjugated ABA of P. vulgaris corresponded to the retention time of synthetic glucosyl ester of (±)-ABA and to the retention time of a major radioactive metabolite when [^{3}H]ABA was supplied to leaves of P. vulgaris.

Conjugated ABA which was collected from the reverse-phase HPLC column was hydrolyzed at pH 13 (adjusted with 5 N NaOH) at 60°C for 30 min (Milborrow and Mallaby 1975). After hydrolysis, the pH was adjusted to 2.5 with phosphoric acid, and ABA was extracted from the solution by partitioning three times with an equal volume of ethyl acetate. The ABA in ethyl acetate was further purified using the same procedure as for the free-ABA fraction collected from the reverse-phase HPLC column.

The fractions containing ABA, PA, or DPA were taken to dryness under a stream of N_2 and methylated with diazomethane

in ethyl acetate. The methylated fractions were filtered through a 0.5-µm Millipore FHLP filter and taken to dryness. A sample was dissolved in 0.5 ml of a mixture of ethyl acetate and hexane and injected on a 0.5-ml sample loop connected to a guard column filled with PAC pellicular packing (Whatman) followed by a µBondapak-NH_2 analytical column (Waters Associates). A sample was injected and eluted with solvent of constant composition. A mixture of 40% ethyl acetate in hexane was used for ABA; 50% ethyl acetate in hexane was used for PA; 70% ethyl acetate in hexane was used for DPA. Solvent flow rate was 1 ml min^{-1} at $\simeq$ 2 MPa (300 psi). Retention times were determined with standards.

The extracts of the individual leaf sections contained amounts of ABA and its metabolites too small to be analyzed quantitatively by absorption of light at 254 nm. Quantitative analysis was therefore performed by GLC-EC as described in Section 2.2.2. The concentrations of standards for calibration were determined by ultraviolet spectrophotometry in methanol using published extinction coefficients (Milborrow and Robinson 1973, Harrison and Walton 1975).

Samples were transferred to scintillation vials and taken to dryness. Overall recovery was estimated by determining the radioactivity in samples using a Packard (Downer's Grove, Ill., USA) model 3255 Tri-Carb Liquid Scintillation Spectrometer. Recovery of ABA from the

original methanol extract was generally between 60 and 70%; recovery of the metabolites of ABA was generally between 40 and 60%. The [^{3}H]ABA, specific activity $8.3 \cdot 10^{14}$ Bq mol^{-1}, was purchased from Amersham Corp., Arlington Heights, Ill., USA, prepared according to Walton et al. (1977). The [^{3}H]PA, [^{3}H]DPA, and conjugated [^{3}H]ABA were extracted and purified from P. vulgaris leaves which had been fed [^{3}H]ABA through the transpiration stream and incubated for 24 h (see Harrison and Walton 1975). The specific activities of the radioactive metabolites varied from one preparation to another. In no case did the amount of radioactive metabolite, which was added to a sample for monitoring recovery, equal more than 10% of the endogenous metabolite, and in most cases it was less than 1%. When warranted, the results were corrected for the quantity of radioactive material that was added to the samples. All results were corrected for degree of recovery and expressed as pmol mg^{-1} dry weight.

4.3. RESULTS

4.3.1. Accumulation of ABA and its metabolites as a function of leaf water deficit. Leaf samples were prepared according to the first type of experiment outlined above in Section 4.2.2: As a leaf lost known amounts of water its ψ_{leaf} was monitored with a pressure bomb and sections were cut and wrapped for incubation. A sample graph of the water relations parameters is shown in Figure 4-1. After about 8 h of incubation, a new steady-state level of ABA for each ψ_{leaf} had been reached in the samples (see Figure 2-3). The result of the experiment, shown in Figure 4-2, illustrates again what was found before for ABA (Figure 2-6): Eighty percent or more of the maximum increase in ABA content, that was developed above the unstressed level, occurred at a turgor of less than 1 bar (= 0.1 MPa). Contents of PA, conjugated ABA, and free ABA were nearly the same in samples having a high water potential (>-5 bar). Unstressed leaf sections contained about three times as much DPA as ABA. The DPA content was not measurably affected by decreasing water potential (<-5 bar) within the time of the experiment ($\leq$ 10 h). Samples with a low ψ_{leaf} contained twice as much conjugated ABA as samples with a high ψ_{leaf}. In contrast, with loss of leaf turgor, PA content increased in parallel with increasing ABA content to more than 10 times the basal level. The sum of the contents of ABA and PA more accurately reflects how much ABA was synthesized during the incubation

Figure 4-2. The relationship between turgor (·····) and ψ_{leaf} and the effect of ψ_{leaf} on the content of ABA, PA, DPA, and conjugated ABA in samples from a single, detached leaf of Phaseolus vulgaris.

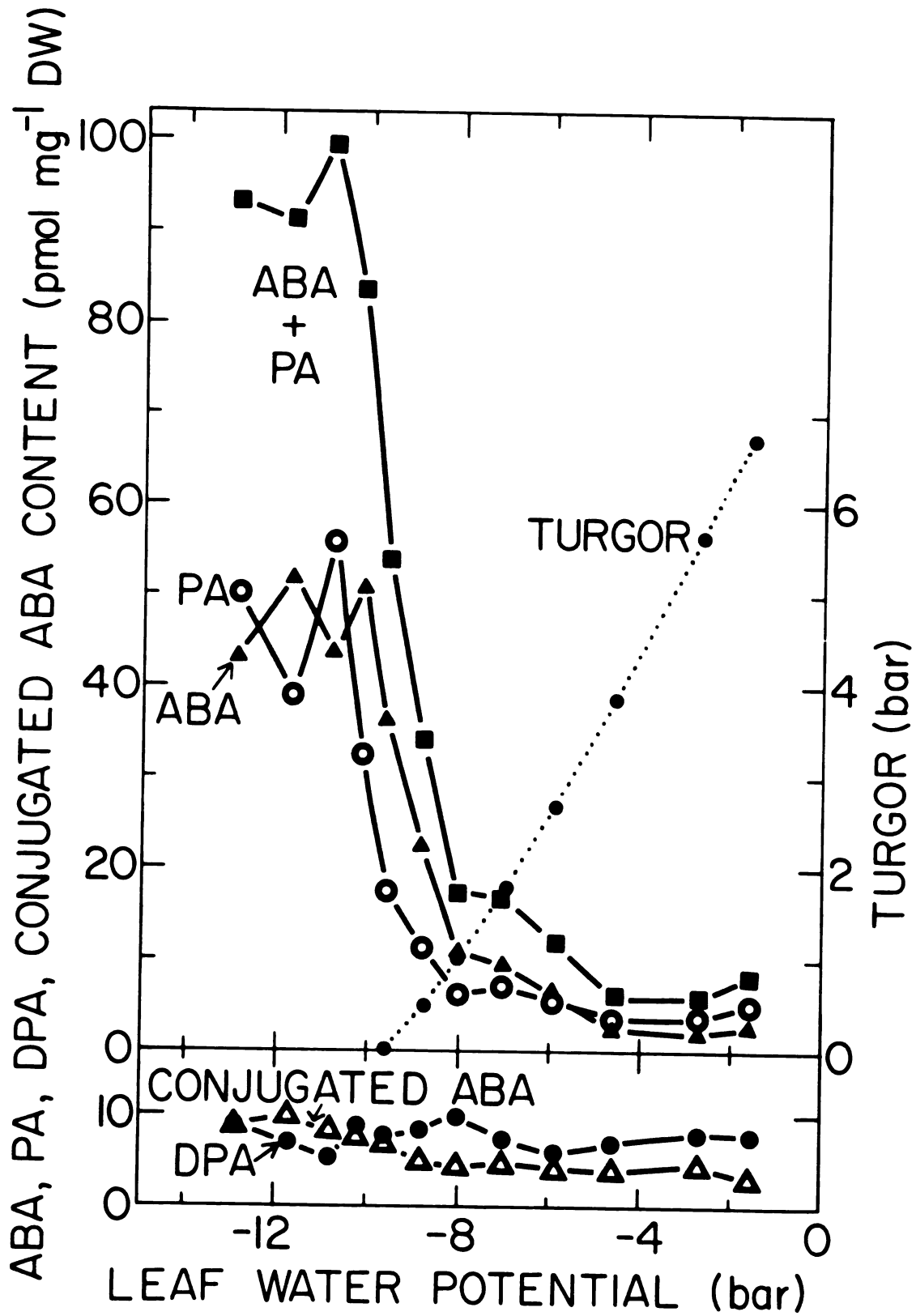
ABA, PA, DPA, CONJUGATED ABA CONTENT (pmol mg⁻¹ DW)
100
80
60
40
20
0
ABA
+
PA
PA
ABA
TURGOR
6
4
2
0
TURGOR (bar)
CONJUGATED ABA
10
0
DPA
-12
-8
-4
0
LEAF WATER POTENTIAL (bar)

Figure 4-2

period as a result of water deficit than does the ABA content alone. A similar experiment produced essentially the same results, although the extent of the accumulation of PA in the wilted samples was somewhat less than in the experiment shown in Figure 4-2. The continuation of metabolism of ABA, especially to PA, during water stress was certainly a factor in limiting the levels of ABA in stressed bean leaves.

4.3.2. Identification of metabolites of ABA in bean leaves during recovery from water stress. In detached leaves, changes in the pool sizes of an endogenous compound and its metabolites can be used to calculate rates of metabolism. In order to obtain a good estimate of the rate of synthesis of ABA, the pool sizes of ABA and of all of its important metabolites must be determined. Although the identity of the metabolites of ABA is known for fresh and for wilted leaves of P. vulgaris (Harrison and Walton 1975, Zeevaart and Milborrow 1976), it was necessary to determine the pattern of metabolism also for leaves recovering from water stress, when the rate of disappearance of ABA is especially high. Leaves were prepared according to the second type of experiment outlined in Section 4.2.2. In this particular experiment, leaf sections were rehydrated in water containing [^{3}H]ABA. The tissue was analyzed for radioactive compounds 3 h later. Other samples were prepared by applying a known amount of [^{3}H]ABA directly to rehydrated leaf sections and allowing no time for

metabolism. Radioactive material was extracted from the tissue and transferred to SEP-PAK C_{18} cartridges (Section 4.2.3). Three fractions were prepared from the cartridges: (1) the 1% acetic-acid solution from which the sample was loaded onto the cartridge; (2) the fraction eluted from the cartridge with 40% ethanol in 1% acetic acid; and (3) the fraction eluted with 95% ethanol. Whether a leaf sample was given time to metabolize the [^{3}H]ABA or not, most of the radio-activity recovered from the SEP-PAK cartridge was contained in 40% ethanol fraction, an average of 95% in 8 samples that were rehydrated in the presence of [^{3}H]ABA.

The radioactive compounds in the 40% ethanol fraction were separated by HPLC on a reverse-phase analytical column. The distribution of radioactivity in the HPLC elution profile is shown for several samples in Figure 4-3. When [^{3}H]ABA was added to samples just before freezing them, most of the radioactivity co-chromatographed with ABA (Figure 4-3b), an average of 83% of the total radioactivity, in two trials. The remaining radioactivity appeared in several small peaks as well as in a low level of radioactivity spread over the whole profile; presumably this residual radioactivity resulted from non-metabolic breakdown of the [^{3}H]ABA.

When 3 h were allowed for the metabolism of [^{3}H]ABA (Figure 4-3a,c), major peaks of radioactivity appeared which co-chromatographed with PA, conjugated ABA and residual free ABA. Very little, if any, radioactivity above background was associated with DPA. If DPA had been labeled to a

Figure 4-3a-c. HPLC elution profiles of radioactivity from leaf sections of Phaseolus vulgaris. The retention times of standards are indicated by arrows. For (b), rehydrated leaf tissue was frozen immediately after a drop of [^{3}H]ABA solution was added to it. Profiles (a) and (c) show two examples of leaf tissue which had been wilted for 5 h, was rehydrated in water containing [^{3}H]ABA and allowed to metabolize the [^{3}H]ABA for 3 h. During rehydration the amount of ABA absorbed by the tissue was 1-2 pmol mg^{-1} DW. Profiles (a) and (b) were obtained by HPLC conditions as described in Materials and Methods; (c) is from samples which were eluted with a slightly steeper gradient of solvent composition.

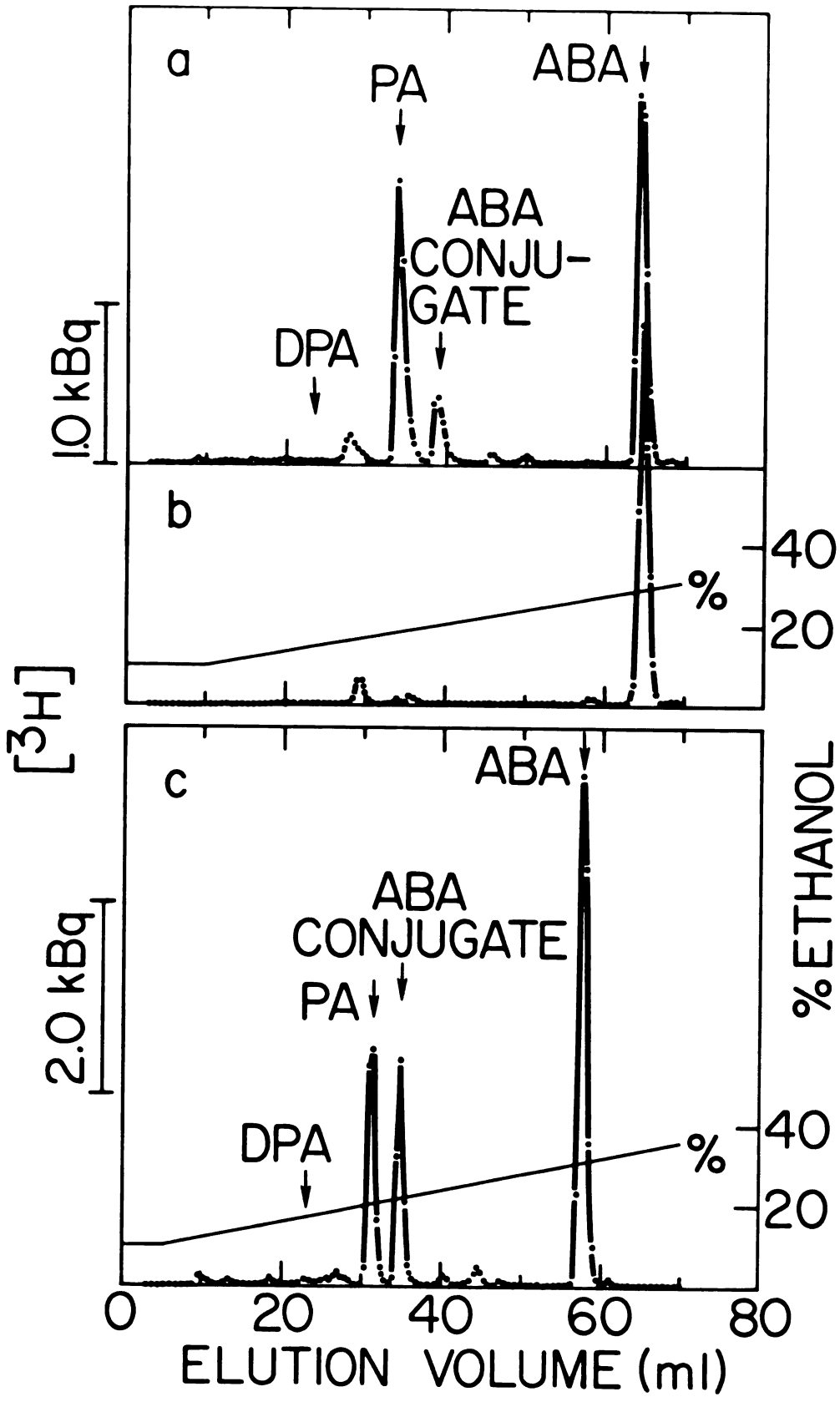
a
PA
ABA
ABA CONJU-GATE
DPA
10 kBq
b
40
%
20
[3H]
c
ABA
ABA CONJUGATE
PA
2.0 kBq
DPA
%ETHANOL
40
%
20
0
20
40
60
80
ELUTION VOLUME (ml)

Figure 4-3a-c

measurable degree, we would see it in the HPLC elution profile because the fractions which eluted at the retention time for DPA were collected and concentrated, and actually found to contain DPA. The conversion of PA to DPA appears to be a relatively slow process. Harrison and Walton (1975) reported that, in wilted bean leaves, at 4 h after feeding [^{14}C]ABA, DPA contained one-sixth as much radioactivity as did PA.

The peak of radioactivity which in all profiles eluted several minutes prior to the PA peak was probably an artefact of the extraction procedure. It was not caused by an alkaline-labile conjugate of ABA, of PA, or of DPA since its retention time was not changed after the extract was subjected to conditions for mild alkaline hydrolysis.

The radioactivity which co-chromatographed with PA on the reverse-phase HPLC column also co-chromatographed with the methyl ester of PA, after methylation and subsequent chromatography on a μBondapak-NH_2 analytical HPLC column. When analyzed by GLC-EC, the methylated radioactive compound produced a single peak with the retention time of PA methyl ester.

The peak of radioactivity which co-chromatographed with synthetic glucosyl ester of (±)-ABA (designated "ABA conjugate" in Figure 4-3) was indeed caused by a conjugate of ABA: after mild alkaline hydrolysis of this radioactive material, a second HPLC elution profile showed one major peak of radioactivity at the retention time for ABA.

When (±)-ABA is fed to tissue, conjugated ABA contains an excess of the unnatural (-)-enantiomer (Milborrow 1970). Thus, in the profiles of radioactive products in Figure 4-3 the extent to which conjugated ABA would be produced from endogenous ABA in bean leaves during 3 h after rehydration is overestimated.

In the four trials conducted, the sum of the radioactivities which appeared in the eluates corresponding to DPA, PA, conjugated ABA and free ABA accounted for an average of 86% of the total radioactivity in the HPLC elution profile. The remaining radioactivity can reasonably be attributed to non-metabolic breakdown of the [^{3}H]ABA or of its metabolites, based on the results presented above from leaf samples which were not given any time to metabolize the radioactive ABA. Changes in the endogenous pool sizes of PA, DPA, and alkaline-hydrolyzable conjugated ABA account for the bulk of metabolism of ABA in bean leaves during the first few hours of recovery from water stress, PA being by far the principal metabolite during this time.

4.3.3. Accumulation of ABA and its metabolites as a function of time during and upon recovery from water stress. Samples were prepared according to the second type of experiment described in Section 4.2.2. A bean leaf which was dehydrated to 82% of its original fresh weight had a ψ_{leaf} close to -11 bar (Figure 4-1). In this material, a ψ_{leaf} of -11 bar was sufficiently low to elicit maximum accumulation of ABA (Figure 4-2). The time course of

accumulation of ABA in the wilted leaf and the dissappearance of ABA after rehydration is plotted in Figure 4-4. Two and one-half h after turgor had been lost, ABA had increased to seven times its original value, and by 7.5 h the ABA content was leveling off at 16 times its original value. Rehydration required about 20 min, and by 40 min later the level of ABA had started to decline. After 4.5 h of recovery, 84% of the ABA which had accumulated in 5 h of stress had been metabolized.

Figure 4-5 shows the time course of accumulation of PA and DPA in the same samples which were used for the determinations of ABA that are shown in Figure 4-4. The level of DPA increased, but only slightly, during the course of the experiment. This is in agreement with the results presented in Figures 4-2 and 4-3. Phaseic acid accumulated at a constant rate in water-stressed tissue after a lag period of less than 2.5 h. After 9.5 h, the wilted tissue had a level of PA which was 10 times the pre-stress level. While rehydration caused the level of ABA to fall rapidly, it caused a surge in the rate of synthesis of PA to as much as four times the rate that was found in the wilted tissue. In the face of a declining pool size of ABA, the conversion of ABA to PA was enhanced. Since DPA accumulates as an end product in bean leaves (Zeevaart and Milborrow 1976), and since the levels of DPA were higher rather than lower in the rehydrated tissue as compared to the wilted tissue, rehydration did not inhibit conversion of

Figure 4-4. Changes with time in content of ABA in sections of a single, detached leaf of Phaseolus vulgaris during water stress (——) and recovery (----).

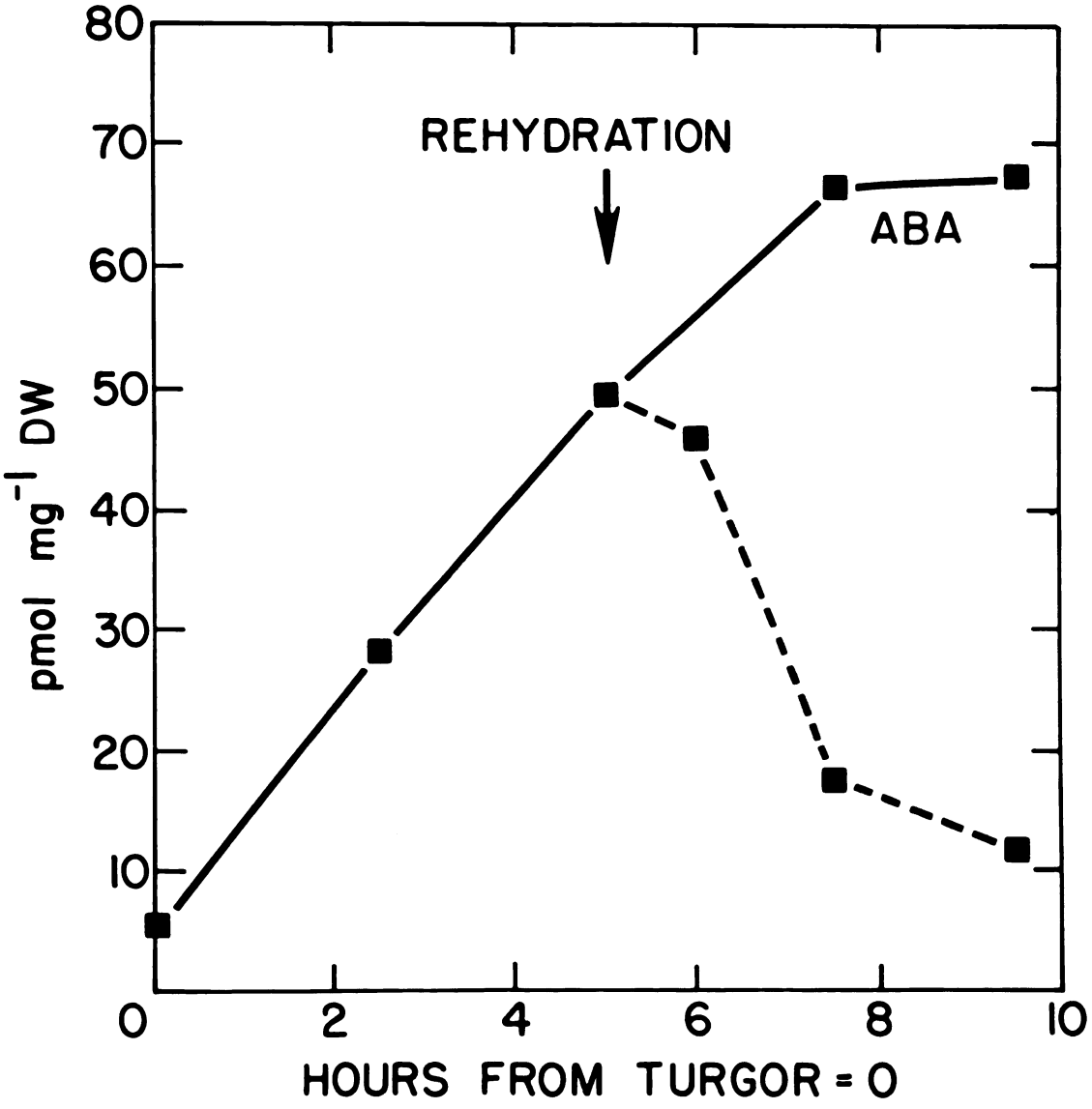
80
70
60
50
40
30
20
10
0
2
4
6
8
10
REHYDRATION
ABA
pmol mg⁻¹ DW
HOURS FROM TURGOR = 0

Figure 4-4

Figure 4-5. Changes with time in content of PA (▲) and DPA (●) in the same leaf sections that were analyzed for ABA content (Figure 4-4) during water stress (____) and recovery (----).

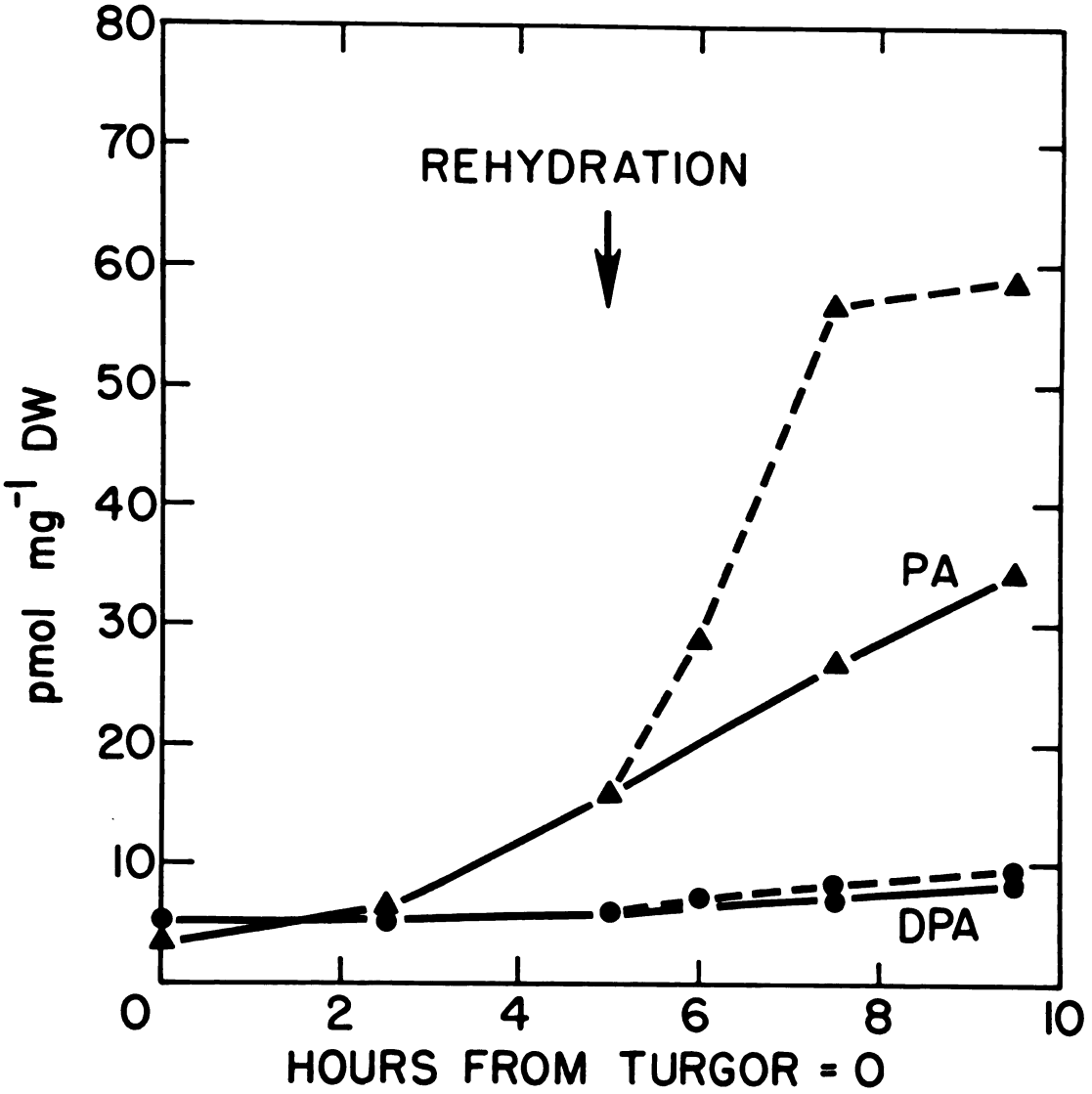
80
70
60
50
40
30
20
10
0
2
4
6
8
10
REHYDRATION
PA
DPA
pmol mg⁻¹ DW
HOURS FROM TURGOR = 0

Figure 4-5

PA to DPA.

During stress and during recovery the formation of conjugated ABA occurred to a much smaller extent than did the formation of PA, and rehydration did not accelerate the conjugation of ABA. Unstressed bean leaves were found to contain about 3.8 pmol of conjugated ABA mg^{-1} dry weight. In the time-course experiment illustrated in Figures 4-4 and 4-5, the content of conjugated ABA increased to 11 pmol mg^{-1} dry weight in the sample which was kept wilted for 9.5 h and to 5 pmol mg^{-1} dry weight in the sample which had been wilted but then was allowed to rehydrate for 4.5 h. In another experiment, the corresponding wilted sample also contained more than twice as much conjugated ABA as the rehydrated sample did.

Rates of synthesis and metabolism of ABA were estimated from the data of the time-course experiments. This was possible because detached leaves were used; translocation of ABA or its metabolites from the leaf did not contribute to the changes that were observed. The rate of synthesis of ABA equals the rate of change in the content of ABA plus the rates of change in the contents of its metabolites. The rate of metabolism of ABA equals the sum of the rates of change in the levels of just the metabolites of ABA. The maximum rate of synthesis of ABA averaged 15 pmol mg^{-1} dry weight h^{-1} in 5 experiments, with a range from 6 to 23 pmol mg^{-1} dry weight h^{-1}. The calculations for the results shown in Figures 4-4 and 4-5 are presented in Figure 4-6. After wilting occurred,

Figure 4-6a,b. Effect of wilting (——) and rehydration (----) on the average rate of synthesis (▲) and metabolism (●) of ABA during intervals of time after loss of turgor in samples from a single, detached leaf of Phaseolus vulgaris. Panel (a): continued wilting. Panel (b): rehydration after 5 h. The data points are placed at the midpoints of the intervals.

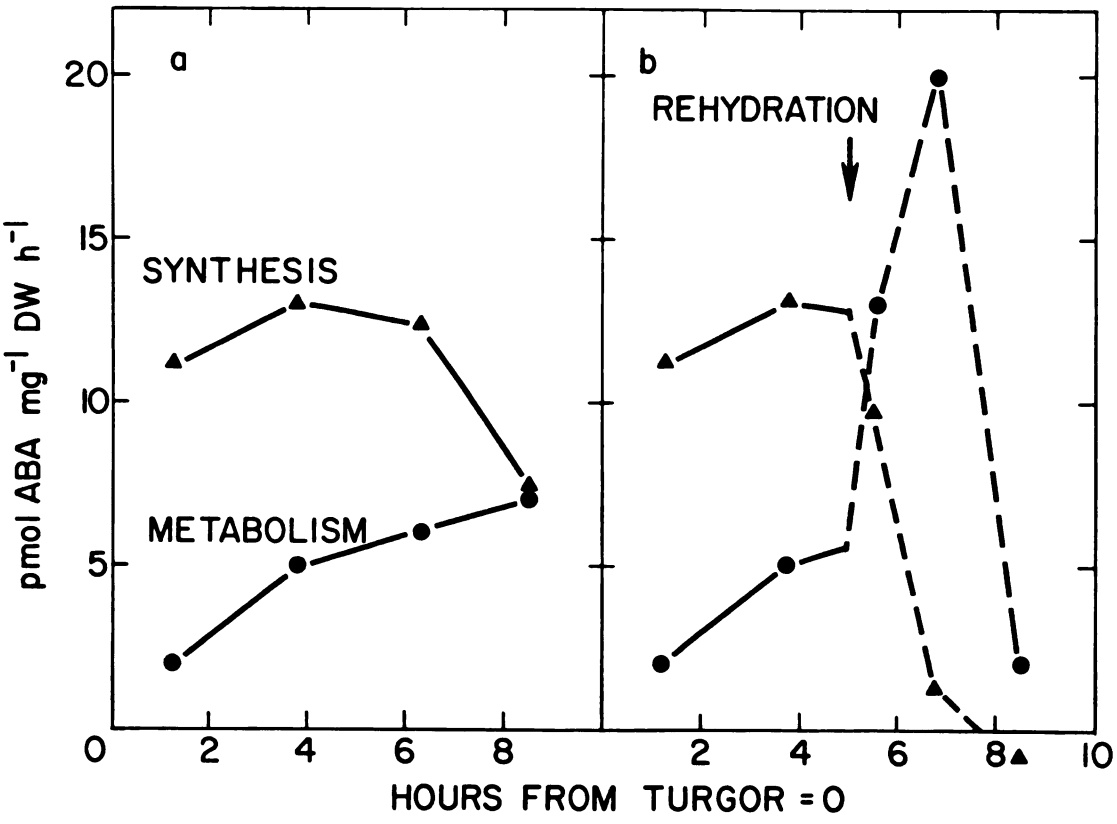
a
b
REHYDRATION
SYNTHESIS
METABOLISM
pmol ABA mg⁻¹ DW h⁻¹
20
15
10
5
0
2
4
6
8
2
4
6
8
10
HOURS FROM TURGOR = 0

Figure 4-6a,b

Figure 4-6a,b. Effect of wilting (——) and rehydration (----) on the average rate of synthesis (▲) and metabolism (●) of ABA during intervals of time after loss of turgor in samples from a single, detached leaf of Phaseolus vulgaris. Panel (a): continued wilting. Panel (b): rehydration after 5 h. The data points are placed at the midpoints of the intervals.

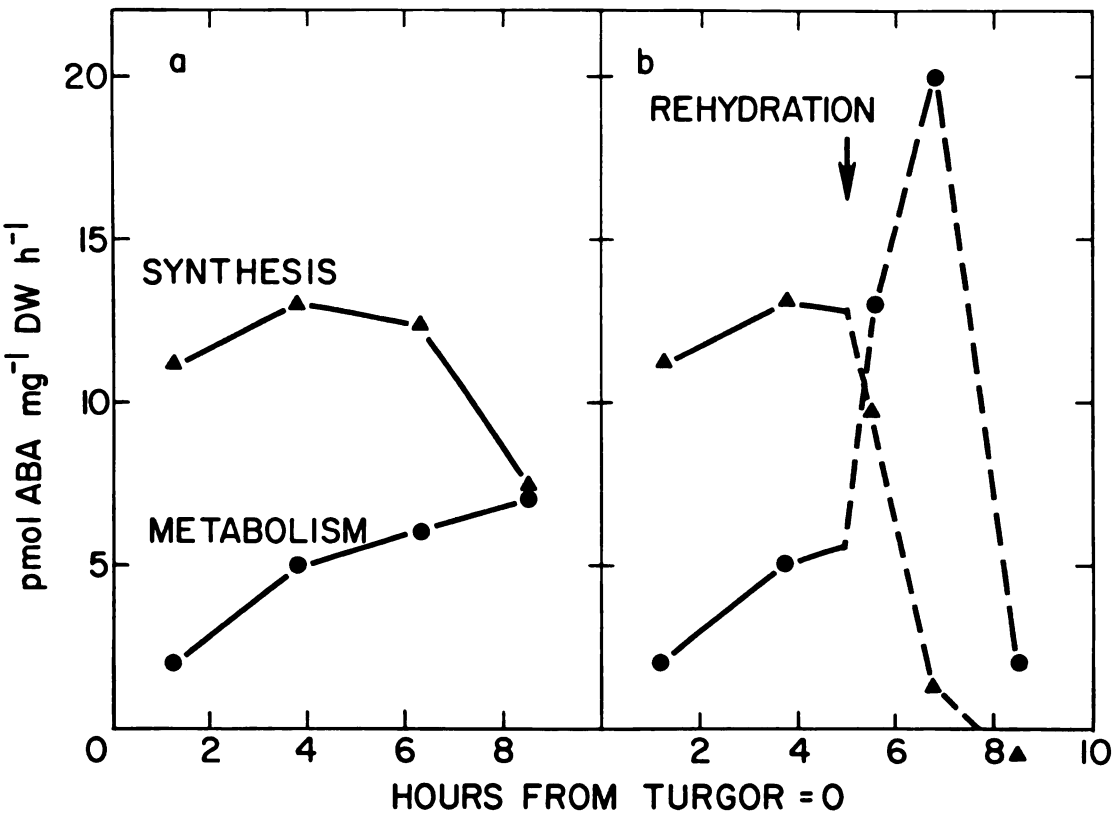
a
b
REHYDRATION
SYNTHESIS
METABOLISM
pmol ABA mg⁻¹ DW h⁻¹
0
5
10
15
20
2
4
6
8
10
HOURS FROM TURGOR = 0

Figure 4-6a,b

the rate of synthesis of ABA jumped to a maximum that averaged 13 pmol mg^{-1} dry weight h^{-1} between 2.5 and 5 h. Thereafter, the rate of synthesis of ABA subsided to an average of 7 pmol mg^{-1} dry weight h^{-1}. In wilted tissue the rate of metabolism of ABA climbed steadily until it matched the rate of synthesis between 7.5 and 9.5 h.

Upon rehydration of the leaf tissue, the synthesis of ABA did not cease abruptly. The pool size of the metabolites of ABA increased by 13 pmol mg^{-1} dry weight, and the level of ABA fell by 3 pmol mg^{-1} dry weight during the first hour after rehydration, which meant that ABA was being synthesized at an average rate of 10 pmol mg^{-1} dry weight h^{-1}, down from the maximum rate found just prior to rehydration. Synthesis of ABA fell rapidly after that to zero within about 3 h after rehydration.

If the loss of stimulation of ABA synthesis had been the only effect of rehydration, ABA would have disappeared at a rate of 6 or 7 pmol mg^{-1} dry weight h^{-1}, which was the average rate of metabolism of ABA in wilted tissue. However, after 1 h of recovery, ABA disappeared at a rate of more than 19 pmol mg^{-1} dry weight h^{-1}, which was possible only if rehydration actually enhanced the metabolic removal of ABA. Later, as synthesis of ABA slowed down and the conversion to PA proceeded rapidly, the supply of ABA was depleted and turnover of ABA slackened in the last period of recovery.

The rate of accumulation of PA during the period of rehydration was compared with the rate in tissue that was left wilted. Results of five experiments are shown in Table 4-1. Individual leaves varied considerably in their response. In three experiments, rehydration caused increased production of PA; in two experiments no large change occurred (Table 4-1, left column). In spite of the declining size of the precursor pool, accumulation of PA in rehydrated bean leaves was equal to or greater than it was in wilted tissue. An acceleration of the conversion of ABA to PA becomes strikingly apparent if the rates of PA formation are related to the sizes of the ABA pools and coefficients of conversion are obtained (Table 4-1, right column). The same data also indicate that the relative rates of ABA conversion to PA in rehydrated tissue were similar in all experiments (between 0.4 and 0.5 pmol ABA converted $pmol^{-1}$ ABA present h^{-1}). What appears to have varied from one experiment to the next is the rate of conversion of ABA to PA in the wilted tissue. The cause of this variation is not known at present.

4.3.4. Conversion of ABA to PA as a function of degree of rehydration. In leaf samples differing in degree of water deficit, synthesis of ABA was stimulated by loss of turgor (Figure 4-2 and results in Chapter 2). Is regulation of the metabolism of ABA to PA also related to turgor? The following experiments were designed to test whether enhanced

Table 4-1. Effect of wilting and rehydration on the average rate of accumulation of PA in samples from individual, detached leaves of Phaseolus vulgaris.

Leaves were wilted for approx. 5 h, after which part of each leaf was rehydrated. The change in level of PA that occurred during the subsequent approx. 3 h was determined for both the rehydrated tissue and tissue which was kept wilted. Coefficient of conversion: pmol ABA converted to PA h^{-1} $pmol^{-1}$ ABA present.

Expt.	Treatment	Average rate of change in PA during period following approx. 5 h of wilting	
		pmol PA mg^{-1} DW h^{-1}	coefficient of conversion (h^{-1})
1	Continued wilting	1.6	0.05
	5.5 h wilted + 2.5 h rehydrated	7.8	0.51
2	Continued wilting	4.3	0.07
	5 h wilted + 2.5 h rehydrated	16.3	0.49
3	Continued wilting	9.3	0.13
	5.75 h wilted + 3.25 h rehydrated	20.9	0.42
4	Continued wilting	10.4	0.29
	5 h wilted + 3 h rehydrated	9.7	0.36
5	Continued wilting	9.4	0.27
	5 h wilted + 3 h rehydrated	10.6	0.42

conversion of ABA to PA correlates with re-establishment of turgor. The experimental procedure was of the third type described in Section 4.2.2. In general, leaves were wilted and left to accumulate ABA for 5 h. Then leaf sections were rehydrated to varying degrees and 3 h later examined for their production of PA.

A graph of $-\psi_{leaf}^{-1}$ versus percent of original fresh weight (e.g. Figure 4-1), which was determined during dehydration of a leaf, was used as a calibration curve for calculating turgor when sections from the same leaf were subsequently rehydrated to different percentages of their original fresh weights. It was important to determine whether the original relation between ψ_{leaf} and fresh weight could be used to calculate the turgor of the rehydrated samples. Figure 4-7 shows the results of one kind of test. A bean leaf was dehydrated, yielding one set of ψ_{leaf} and fresh weight values. When the same leaf was rehydrated and then dehydrated a second time the second set of ψ_{leaf} and fresh weight values yielded a curve which closely matched the original relationship. The two curves fell within about 0.25 bar of each other.

In another kind of test (Figure 4-8) a relation between ψ_{leaf} and fresh weight was determined using a pressure bomb, then the leaf was cut into sections, and these were rehydrated to varying degrees. The ψ_{leaf} values of the rehydrated samples were determined by the dew-point method. Once again the agreement was good between the two sets of

Figure 4-7. A comparison of duplicate determinations on the same leaf of the relationship between ψ_{leaf} and fresh weight. After the first set of measurements, the leaf was floated on water until it had rehydrated.

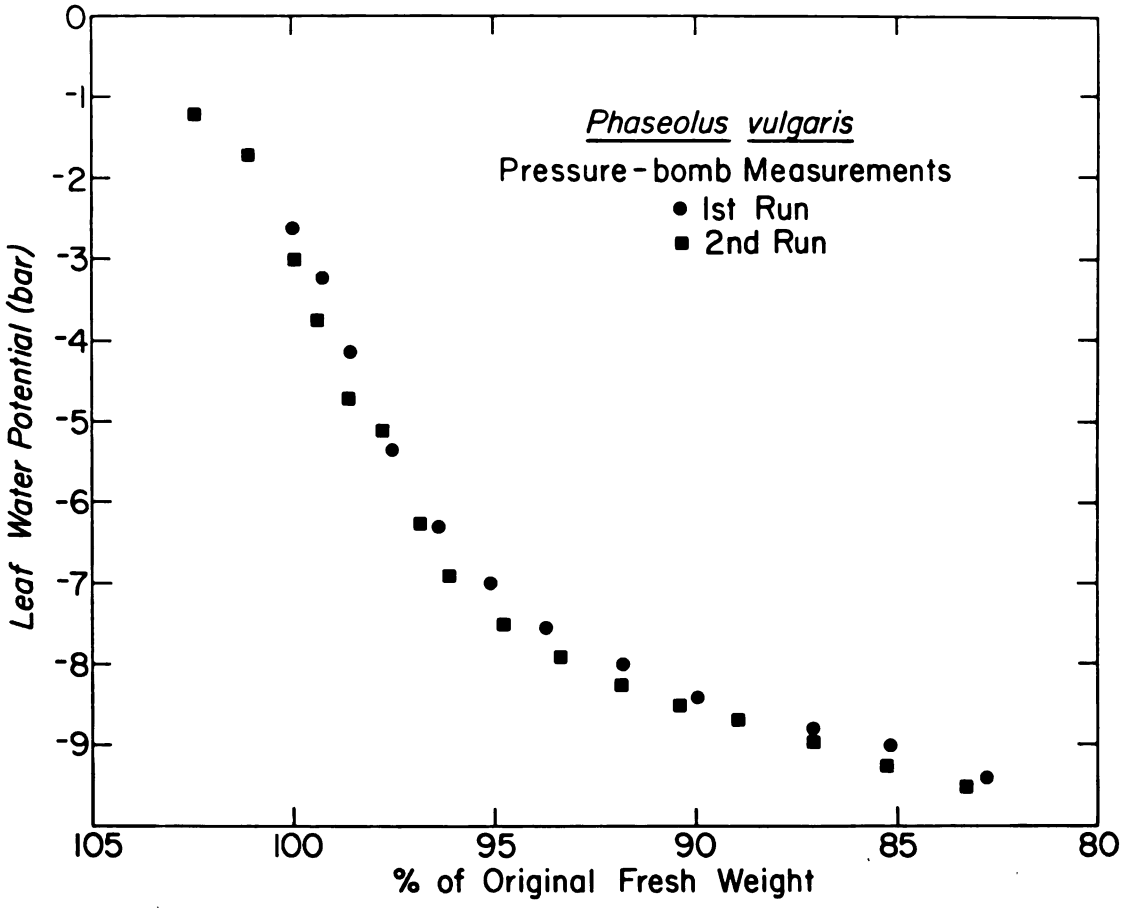
Phaseolus vulgaris
Pressure-bomb Measurements
1st Run
2nd Run
Leaf Water Potential (bar)
0
-1
-2
-3
-4
-5
-6
-7
-8
-9
105
100
95
90
85
80
% of Original Fresh Weight

Figure 4-7

Figure 4-8. Measurements of ψ_{leaf}, which were determined with a pressure bomb, versus % of the leaf's original fresh weight compared to measurements of ψ_{leaf} on sections of the same leaf, which were made by the dew-point method, versus the % to which those sections were rehydrated.

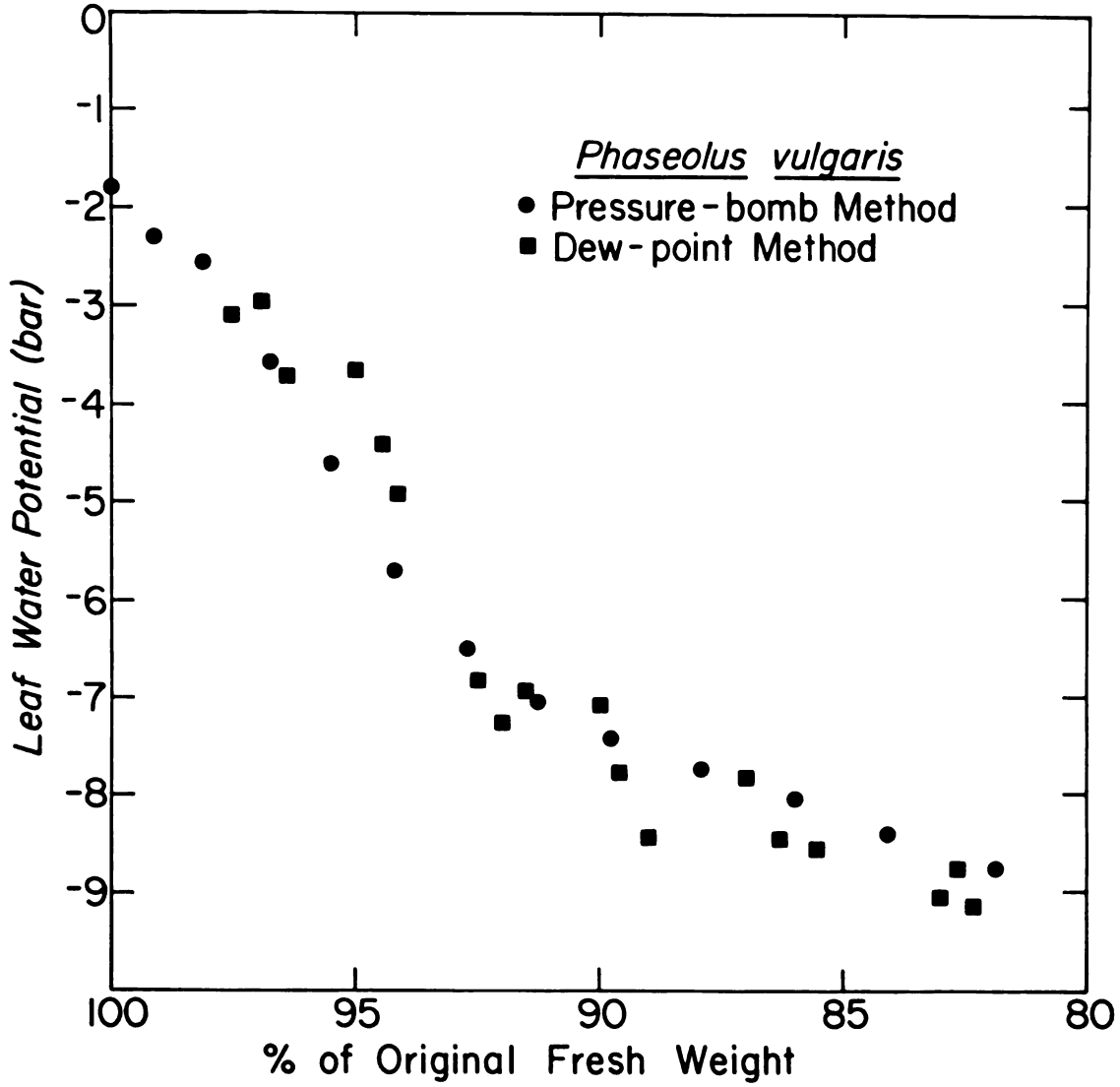
Phaseolus vulgaris
Pressure-bomb Method
Dew-point Method
Leaf Water Potential (bar)
0
-1
-2
-3
-4
-5
-6
-7
-8
-9
100
95
90
85
80
% of Original Fresh Weight

Figure 4-8

measurements. Figure 4-8 shows that ψ_{leaf} values of individual leaf pieces, as determined by the dew-point method, may deviate by as much as 1 bar from ψ_{leaf} values as originally determined by the pressure-bomb method for the entire leaf. Calculations from the original relation between ψ_{leaf} and fresh weight were reasonable estimates of ψ_{leaf} and turgor for the rehydrated samples.

Bean leaf samples were prepared for analysis of the content of ABA and its metabolites. Tissue was frozen to assess the contents after 5 h of wilting. A number of leaf sections were rehydrated and incubated for another 3 h, after which they were also frozen. The results of this experiment are shown in Figure 4-9. The changes in ABA, PA, or conjugated ABA from the levels after 5 h of wilting, that occurred during the 3 h recovery period, were plotted versus the ψ_{leaf} which a sample had during the recovery period. The relation between ψ_{leaf} and turgor for these samples is also indicated. The sample having the lowest ψ_{leaf} (-10.9 bar) and no turgor had not been rehydrated at all. It can be compared to the samples in the time course experiments (Figures 4-4 and 4-5) which were kept wilted throughout the experiment. The sample having the highest ψ_{leaf} (-2.3 bar) and the highest turgor (5.6 bar) had been rehydrated to 100% of its original fresh weight and can be compared to the samples which were rehydrated in the time course experiments.

Rehydration triggered the disappearance of ABA and

Figure 4-9. The effect of degree of rehydration on the change in content of ABA, PA, and conjugated ABA in sections of a single leaf of Phaseolus vulgaris during a 3 h period of recovery from water stress. Degree of rehydration is indicated by (i) water potential and (ii) turgor which the leaf sections had during the period of recovery.

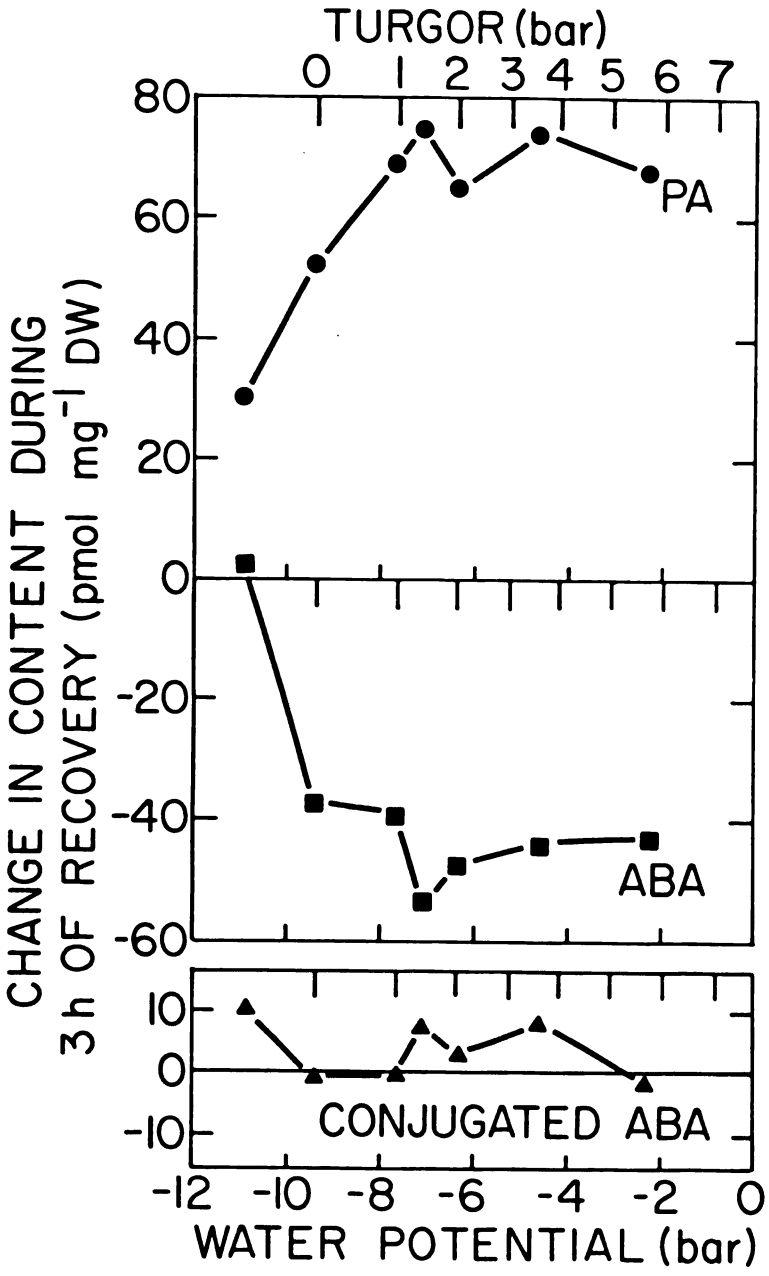
TURGOR (bar)
0 1 2 3 4 5 6 7
80
60
40
20
0
-20
-40
-60
PA
ABA
10
0
-10
CONJUGATED ABA
-12 -10 -8 -6 -4 -2 0
WATER POTENTIAL (bar)
CHANGE IN CONTENT DURING 3 h OF RECOVERY (pmol mg-1 DW)

Figure 4-9

increased the production of PA (Figure 4-9). Samples having turgor accumulated more than twice as much PA during the recovery period than did the sample having the lowest ψ_{leaf}. In all samples, the magnitude of the decrease in ABA was less than the rise in PA, which indicates that synthesis of ABA must have continued during at least part of the recovery period. These results are consistent with those presented in Figures 4-4 and 4-5.

Increased synthesis of PA and disappearance of ABA was not a linear function of the degree of rehydration. Re-establishment of the slightest turgor was sufficient to elicit maximum enhancement of the conversion of ABA to PA. We cannot tell whether the response anticipated re-establishment of turgor in the leaf or not. The position of zero turgor on the ψ_{leaf} scale in Figure 4-9 could be incorrect by about ± 1 bar (refer to Figure 4-8). Re-establishment of turgor did not increase the production of conjugated ABA (Figure 4-9).

4.4. DISCUSSION

4.4.1. Synthesis and metabolism of ABA after loss and gain of turgor. The results of these experiments demonstrate that leaf turgor affects both the metabolic system which catalyzes the synthesis of ABA, and that which catalyzes the conversion of ABA to PA. The systems respond to changes in turgor in opposite ways. Synthesis of ABA is more rapid in wilted than in turgid leaves; metabolism of ABA to PA is more rapid in leaves that have regained turgor than in wilted leaves. The results are a net accumulation of ABA in wilted tissue, and a decline of the ABA content to a low level in tissue to which turgor has returned.

4.4.2. Stimulation of ABA synthesis after loss of turgor. The conclusion that turgor loss causes an increase in the rate of synthesis of ABA is based on the elimination of other possible causes of ABA accumulation, namely (1) inhibiton of the metabolism of ABA to PA, (2) release of ABA from a conjugated form, or (3) inhibition of the formation of ABA conjugates. With respect to the first possibility, Harrison and Walton (1975) were the first to show that metabolism of ABA continued in leaves under water stress. The extents to which [^{14}C]ABA disappeared and labeled products appeared in the tissue were the same in both stressed and unstressed leaves. The content of PA and DPA actually built up in wilted bean leaves. As shown by Harrison and Walton (1975) and indicated by Figures 4-2 and

4-5, inhibition of the metabolism of ABA through the PA - DPA pathway was not responsible for the accumulation of ABA that had occurred. Hydrolysis of ABA conjugates could not have been the source of free ABA either. As demonstrated by Figure 4-2, bean leaves which had not experienced water stress did not contain sufficient alkaline-labile conjugated ABA to yield the amounts of ABA which appeared in the wilted tissue (see Zeevaart 1980 and review by Wright 1978). Furthermore, Milborrow and Noddle (1970) obtained more conversion of labeled mevalonate to ABA in wilted than in turgid wheat leaves. Turning to the third possibility, we see from Figure 4-2 that the level of conjugated ABA was slightly higher in wilted than in turgid leaves. Inhibition of the metabolism of ABA to conjugated ABA cannot have been the cause of the observed accumulation of ABA. As the pathways via PA and alkaline-labile conjugated ABA account for the bulk of the metabolism of ABA in bean leaves (e.g. Figure 4-3), it follows that the principal cause of the accumulation of ABA in water-stressed bean leaves was the stimulation of the synthesis of ABA.

Simultaneous with the accumulation of ABA in wilted leaves occurred an accumulation of PA and, to a much smaller degree, of conjugated ABA (Figures 4-2 and 4-5). Since ABA and PA (and DPA) increased in parallel fashion with respect to changes in ψ_{leaf} (Figure 4-2), and with respect to time (Figures 4-4 and 4-5), the accumulation of the metabolites of ABA in wilted tissue can be explained as an effect of mass

action of ABA.

With confidence that determinations of changes in the levels of PA, DPA, and alkaline-labile conjugated ABA would account for the metabolism of ABA during a short-term wilt-recovery cycle in bean leaves (see Figure 4-3 and Harrison and Walton 1975), rates of synthesis and metabolism of ABA were calculated. Seven h after turgor was lost, accumulation of ABA leveled off, then production and metabolic removal of ABA balanced each other. At this time, the rate of synthesis or metabolism was 7 pmol mg^{-1} dry weight h^{-1} (Figure 4-6). This is about two times the rate Harrison and Walton (1975) determined for a steady-state level of ABA in wilted primary leaves of two-week old bean plants (at 15% dry weight, 0.15 µg ABA g^{-1} fresh weight h^{-1} correspond to 3.8 pmol mg^{-1} dry weight h^{-1}). Harrison and Walton felt that twice the rate they calculated was closer to being accurate. Wilted Xanthium leaves appear to possess a capacity to synthesize and metabolize ABA at rates very similar to those of wilted bean leaves. The data of Zeevaart (1980, Figures 3 and 4, time interval 5-12 h) indicate a rate of 8 pmol mg^{-1} dry weight h^{-1} for the steady state.

4.4.3. Stimulation of ABA metabolism after recovery of turgor. After the return of turgor to the tissue, conversion of ABA to PA accelerated (Figure 4-5, 4-9, Table 4-1). Since this stimulation of the metabolism of ABA occurred at a time of declining ABA content (Figures 4-4 and 4-9), recovery of turgor must have caused an increase in

the amount or the activity of the enzyme(s) involved in the conversion of ABA to PA; or, possibly, recovery of turgor increased the accessibility of critical substrates to the enzyme(s). A stimulation of the conversion of ABA to PA after rehydration was also discovered by Zeevaart (1980) in leaves of Xanthium strumarium. Previously, it was possible to consider that the concentration of ABA falls when plants are re-watered because of diminished capacity for synthesis in combination with, as Milborrow (1979) wrote, "a constant, large degradative capacity for ABA...". The evidence indicates that the capacity of bean leaves to remove ABA by metabolism is not constant but increases when turgor returns to a wilted leaf (Table 4-1, Figure 4-9). This acceleration becomes particularly evident when the rate of ABA conversion is expressed relative to the amount of ABA present in the tissue (yielding a coefficient of conversion; second column in Table 4-1). In rehydrated tissue, almost one half of the amount of ABA present was converted to PA per h (0.44 ± 0.06 h^{-1}, as compared to 0.16 ± 0.11 h^{-1} in wilted tissue). An increase in the rate of conversion appears also in Zeevaart's data on Xanthium leaves. Calculating from Figures 3 and 4 of Zeevaart, during periods when PA was increasing linearly with time, the coefficient of conversion averaged 0.40 h^{-1} for leaves rehydrated after four hours of wilting (time interval: 0-4 h after rehydration), and it averaged 0.12 h^{-1} for wilted leaves (time interval: 4-12 h of wilting).

The stimulation of the metabolic removal of ABA after rehydration was restricted to the PA pathway. Conjugation of ABA continued at a low rate, if at all (Figure 4-9). An early report by Hiron and Wright (1973) indicated some conversion of ABA to conjugated ABA during recovery of bean seedlings from water stress. But neither this work with bean leaves, nor that of Dörffling et al. (1974), who tested pea seedlings, nor that of Zeevaart (1980), who analyzed Xanthium leaves, showed the formation of conjugated ABA to be of importance during short-term stress and recovery from it. Prolonged or repeated stress may be a different matter: leaves from cotton plants which had experienced 8 wilt-recovery cycles over the course of two weeks contained 20 to 30 times more conjugated ABA than did comparable samples from unstressed plants. (See also Hiron and Wright 1973, Zeevaart 1980.)

CONCLUSION

Abscisic acid has come to be considered a plant "stress hormone" (Jones 1978). In the biological control system by which plants regulate CO_2 and water vapor exchange, Raschke (1979) positioned ABA as a signal in a feedback loop controlling plant water status. The results of this thesis strengthen the candidacy of ABA as an important signal. Accumulation of ABA and disappearance of ABA from leaf tissue are tightly linked to loss and recovery of turgor (Chapters 2 and 4). Plants have the ability to adapt to stress conditions by adjustment of their solute content (Turner and Jones 1980). Linking the control of ABA level to turgor automatically takes into account the plant's success in making an osmotic adjustment, when ABA is produced as a signal of water stress.

Abscisic acid appears to play an important role in the way plants cope with water stress. In wilting Commelina leaves, an increase in ABA was obvious in the epidermis before it was obvious in the mesophyll, and the increase paralleled stomatal closure (Chapter 3). At least in this species, ABA is likely to be an important factor in causing stomatal closure during water stress. Stomatal closure in response to ABA is probably only one among several

ameliorative reactions. For instance, ion transport as well as water permeability of roots have been reported to be enhanced by ABA (Glinka 1980).

Changes in ABA are net changes from effects on both synthesis and metabolism of ABA (Chapter 4 and Zeevaart 1980). Conversion of ABA to PA continues when ABA is being synthesized in wilted tissue, and, coupled with slackening of ABA synthesis, leads to a steady state. Synthesis of ABA continues for about an hour after turgor has been regained and conversion of ABA to PA has been accelerated. Thereafter, synthesis of ABA declines.

Accumulation of ABA sets in before turgor of the bulk of the leaf has become zero; conversion of ABA to PA is accelerated at the slightest indication of a regain of turgor (Chapters 2 and 4). Anticipatory production of removal of ABA could be caused by deviations from bulk properties in the solute content or the cell-wall elasticity of individual cells in the tissue, but turgor sensing may also be a progressive response in individual cells (Zimmermann (1978).

Of the questions arising from this work, what the turgor sensor is and how it causes changes in the opposing mechanisms of synthesis and metabolic removal of ABA appear particularly important and challenging.

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