ON THE STOMATAL RESPONSE TO ABSCISIC ACID

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ABSTRACT

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Stomates of excised primary leaves of Hordeum vulgare started to close within a very short time after the introduction of abscisic acid (ABA) to the transpiration stream. Treatment with 10^{-5}M ABA initiated closure within 2.6 min. There was no response within 20 min following treatment with 10^{-8}M ABA. The lag period between the start of treatment and the onset of the response was a function of the inverse of the concentration of ABA applied. A relatively simple method involving continuous leaf temperature measurements has been described for showing changes in transpiration rates following treatment of leaves.

The closure response was specific for cis,trans-(+)-ABA. Other ABA analogs were relatively ineffective, in this short-time assay.

Removal of the supply of ABA resulted in a reversal of the closing response. This reversal could not be

explained solely on the basis of catabolism of ABA since no significant breakdown of ^{14}C-ABA occurred within the time in which reversal could be shown to have occurred. ABA treatment of isolated epidermal strips from Vicia faba leaves was effective in causing closure of stomates when the strips were floated on solutions containing low concentrations of potassium ions. The action on epidermal strips indicated that the ABA acted directly on the guard cells.

Studies of changes in the levels of intercellular $C0_2$ in leaves following treatment with inhibitors, showed that this may be a suitable method for screening for compounds with antitranspirant activity that do not inhibit the photosynthetic mechanism. Such studies coupled with results of experiments with flacca, a wilty mutant of tomato, demonstrated that ABA caused stomatal closure without disruption of the photosynthetic mechanism.

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By

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ABBREVIATIONS

ABA	Abscisic acid (unless specifically modified this means the racemic mixture of cis,trans-(RS)-abscisic acid).
TLC	Thin-layer chromatography
GLC	Gas-liquid chromatography
UV	Ultra-violet light
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethyl-urea
Ci	Curie
ORD	Optical rotatory dispersion
r_{H_2O}	Diffusion resistance to water vapor. This includes stomatal resistance and boundary layer resistance.
$[CO_2]_i$	The concentration of carbon dioxide inside the leaf (intercellular).

INTRODUCTION AND LITERATURE SURVEY

ABA and Stomatal Closure

Little and Eidt (1968) were the first to show that ABA treatment affected transpiration. Cuttings from Picea glauca (white spruce) placed in sealed vials containing aqueous ABA solutions lost less water after six days than similar cuttings kept only in water. Mittelheuser and van Steveninck (1969) repeated this experiment and monitored water loss from graminaceous leaves. The stomates of ABA-treated leaves opened only partially.

Excised wheat leaves showed a forty-fold increase in the concentration of ABA as shown by ORD determination and by bioassay following a 4-hour period of wilting when compared to similar leaves kept well supplied with water (Wright, 1969; and Wright and Hiron, 1969).

Radioactively labeled mevalonic acid was incorporated into (+)-ABA in excised barley leaves which had been left to wilt for several hours (Milborrow and Noddle, 1970). These data suggested that water stress could stimulate the biosynthesis of (+)-ABA in leaves, and that large increases in ABA concentration could be found within a short period after the application of the stress. No data

have yet been published to show the time course of the increase in ABA concentration.

When roots of Nicotiana tabacum plants were subjected to stress by increasing the osmotic strength of the solution surrounding the roots, leaves excised 4 or 48 hours after the start of such stress were shown to contain greater quantities of an ABA-like inhibitor than leaves from non-stressed plants (Mizrahi et al., 1970). The ABA content of such leaves remained high even after the leaves had apparently recovered full turgor. Gale et al. (1967) reported that the stomates of cotton plants under salinity stress were only partially open and that transpiration was considerably reduced even after the leaves regained full turgor.

Stålfelt (1929) investigated the changes in stomatal aperture following the initiation of water stress. Leaves of Vicia faba were exposed to water deficits. The first response was an opening of the stomates. Such rapid "hydropassive" opening, which has also been shown to occur in leaves of Zea mays following the application of negative pressure to the leaf's water supply (Raschke, 1970), is caused by the reduction in water potential of the xylem resulting in a reduction in turgor of the epidermal cells and a decrease in the pressure exerted by the epidermis on the guard cells of the stomates. Willis et al. (1963), reported similar data. They found that the stomatal

resistance started to decrease very rapidly when V. faba leaves were excised. The resistance continued to fall for at least 10 minutes after excision of the leaf. If this were the only response then the rate of water loss in stressed leaves would be accelerated by the action of the stomates. However, the transient opening of stomates as a response to water stress is followed by a second response. Starting ca. 13 min after exposure of V. faba leaves to a water deficit greater than 3% (Stålfelt, 1929) or after excision of turgid leaves (Willis et al., 1963) closure of stomates could be observed. Stålfelt called this "hydroactive closure" and assumed that an active closure mechanism was activated during periods of water deficit.

Evidence cited above indicates that one of the physiological roles of ABA may be to protect plants from drying out during periods of drought by mediating hydroactive stomatal closure.

The Effect of Other Factors on Stomatal Responses

Stomata are capable of responding to a host of stimuli including treatment with chemicals; therefore, caution must be exercised in evaluating and understanding the responses of stomata to exogenously applied agents such as ABA.

Increased CO_2 concentration in the leaf leads to stomatal closure, while reduction of the CO_2 level causes

stomatal opening (Heath, 1961; Linsbauer, 1916). Therefore, illumination causing photosynthetic CO_2 fixation leads to stomatal opening. There is possibly also a light-activated, CO_2-independent opening response since stomates on epidermal strips kept in CO_2-free air open more in the light than in the dark (Humble and Hsiao, 1969).

The humidity of the air around the stomates also affects the extent of opening (Lange et al., 1971).

Minshall (1960) suggested that any compound that affects photosynthesis also affects the stomatal apparatus by way of changes in intercellular CO_2 levels. He showed that excised leaves of Phaseolus vulgaris treated through the petiole with various inhibitors of the Hill reaction transpired less. Application of herbicides (Thorne and Minshall, 1964) and fungicides (Smith and Bucholtz, 1964) to whole plants and roots reduced transpiration. Many workers have investigated the effects of auxin on transpiration; in some cases, auxin solutions were supplied to the roots of potted plants of Tropaeolum majus (Ferri and Lex, 1948), or by spraying auxin solutions on whole bean plants (Brown, 1946), or by dipping excised leaves of kidney beans into auxin solutions (Bradbury and Ennis, 1952). Player (1950), Kasperik (1955), and Mansfield (1967) showed that monocotyledonous plants, which are resistant to the herbicidal effects of auxins, showed no decrease in

transpiration rates following auxin treatment which effectively closed stomata in susceptible dicotyledonous plants.

A toxin, fusicoccin, extracted from a fungus which is a pathogen of almond and peach trees, causes opening of stomata on excised leaves of P. vulgaris and N. tabacum (Turner and Graniti, 1969).

Treatment with various metabolic inhibitors results in a variety of stomatal responses. Treatment with 2,4-dinitrophenol and sodium azide, uncouplers of oxidative phosphorylation, inhibited opening of stomates (Mouravieff, 1953). Stålfelt (1957), on the other hand, showed that treatment with sodium azide at 10^{-2}M prevented hydroactive closure suggesting that active metabolism is involved in mediating that response in excised V. faba leaves. Treating leaves with inhibitors of glycolic acid oxidase (Zelitch, 1961) or with phenyl mercuric acetate (Mansfield, 1967), an inhibitor of noncyclic photophosphorylation (Nozaki et al., 1961), inhibited transpiration.

Cytokinins and Hydroactive Closure

Itai and Vaadia (1965) demonstrated a decrease in the concentration of cytokinin-like material in the root exudate collected from plants, the roots of which had been subjected to a water stress as compared to exudate from unstressed plants. Excised barley leaves treated with a cytokinin or gibberellic acid showed greater transpiration

rates than untreated leaves (Livne and Vaadia, 1965). This work was confirmed by Luke and Freeman (1967) using a 48-hour bioassay period. Meidner (1967) presented evidence that cytokinin-treated, excised mature primary barley leaves had increased rates of CO_2 uptake as well as greater transpiration rates when compared to untreated leaves. O'Leary and Tarquinio Prisco (1970) gave evidence that kinetin treatment increased the stomatal diffusion resistance (i.e., closed stomates) in salt-stressed bean plants. Pallas and Box (1970) provided evidence based on psychrometric data that kinetin-treated, excised leaves of barley show decreased turgor pressure potentials which may account for the opening of the stomata by an osmopassive response. The most rapid response of stomates to cytokinin treatment was shown by Meidner (1967) to occur 3 hours after treatment.

Except for the report by O'Leary and Tarquinio Prisco (1970) on cytokinin-induced stomatal closure, it seems that reduced cytokinin supply may partially account for hydroactive closure of stomates in a period of water stress; or, in other words, a continued cytokinin supply may be needed for optimum stomatal operation. If this is true and if the root is the site of synthesis of cytokinins (Kende, 1964; Humphries and Thorne, 1964) then excised leaves placed in water should show a rapid (starting within ca.13 minutes) decrease in transpiration rate unless they are treated with cytokinin. No such data have yet been

presented; the results of Livne and Vaadia (1967) definitely do not show such fast decreases of transpiration in excised water-supplied leaves.

The Objective of this Thesis

The evidence to date suggests that ABA may play a physiological role in the conservation of water in plants during periods of water stress. The objective of this thesis was to determine if this hypothesis is supported by experimental evidence concerning the characteristics of the stomatal response to ABA treatment.

MATERIALS AND METHODS

Plant Material

Barley seeds (Hordeum vulgare L., cv. Himalaya) were sown in trays of vermiculite. Twice a week they were irrigated with half-strength Hoagland's solution; once a week with distilled water. The conditions under which the plants were grown were as follows: 16 hr day length; 23°C (day), and 20°C (night) temperatures; 80% relative humidity; fluorescent light supplemented with incandescent light of ca. 3000 ft candles light intensity. Primary leaves were excised from 9-14 day old plants 6-8 hr after the beginning of the light period.

Tomato seedlings (Lycopersicum esculentum Mill., cv. Rheinlands Ruhm, wild-type and flacca, a wilty mutant) were grown in vermiculite from seeds supplied by Dr. C. M. Rick of the University of California at Davis. Growth conditions were as stated above. Flacca is a recessive point mutation produced by x-ray treatment located on chromosome number 7 as indicated by Dr. Rick (see Tal, 1966) from tests of allelism and linkage. The phenotype was described by Tal (1966), and Imber and Tal (1970).

Three weeks after germination the tomato seedlings were transferred to pots containing a 3:1 mixture of potting soil and sandy soil. They were irrigated daily with distilled water. In some cases the seedlings were transferred to pots containing a mixture of vermiculite and soil. These were then placed into aerated half-strength Hoagland's solution. Similarly-shaped leaves were chosen from the wild-type and flacca plants, excised under water and subjected to analysis.

Broad beans (Vicia faba L., cv. Improved Longpod) were grown from seeds in a 3:1 mixture of Bacto potting soil and sandy soil. The plants were irrigated daily with distilled water. Three to 4 weeks after germination, leaves were excised under water, and epidermal strips were taken from the abaxial leaf surface.

Abscisic Acid and Analogs

Cis,trans-(R,S)-ABA was obtained from Dr. J. van Overbeek of Texas A and M University and judged to be pure by thin-layer chromatography and by ultraviolet (UV) spectroscopic examination.

Trans,trans-(R,S)-ABA was kindly supplied by Dr. E. Sondheimer of Syracuse University. Gas liquid chromatography (GLC) showed that the sample contained 6% of cis,trans-(R,S)-ABA (Figure 6).

Cis,trans-dihydroionylideneacetic acid was prepared by Dr. E. Sondheimer. Solutions of known concentration were prepared simply by dissolving weighed amounts of this isomer in distilled water. The solutions were not tested for purity. It was selected because of its chemical similarity to ABA and its relative inactivity in other biological assays.

(+) and (-)-Cis,trans-ABA: the (+) and (-) optical isomers of cis,trans-ABA were supplied by Dr. E. Sondheimer. Concentrations of stock solutions of these optical isomers were determined by optical rotatory dispersion (ORD) and UV spectroscopy. The (+) and (-) enantiomers gave mirror-image ORD curves (Figure 7). Measurement of the concentrations by ORD and UV agreed very closely (Table 1) indicating very little, if any, contamination of one enantiomer with the other. Each sample contained contamination with trans,trans-ABA as indicated by GLC (7% in the (+) preparation, 4% in the (-) preparation). Both UV and GLC results indicated considerable contamination of the (+) preparation with unknown compounds. Sharp peaks at 264 and 268 nm marked the UV spectra of this preparation and three extra peaks were detected by GLC. The (-) preparation was free of such contaminants.

Since there is uncertainty reflected in the literature (see Cornforth et al., 1967; Burden and Taylor, 1970) as to the designation of absolute configuration of (+)-ABA

Table 1. Comparison of concentrations of (+) and (-) ABA preparations determined by ORD and UV.

PREPARATION	CONCENTRATION (M)	
	ORD	UV
(+) cis,trans-ABA	1.7×10^{-4}	1.7×10^{-4}
(-) cis,trans-ABA	1.6×10^{-4}	1.5×10^{-4}

(i.e., either R or S), the nomenclature is herein restricted to the optical properties of preparations of the two enantiomers.

Radioactively labeled ABA: ^{14}C-cis,trans-(R,S)-abscisic acid was received from two sources. The first lot was synthesized by Dr. O. Smith, University of California, Riverside and contained the label in the alpha carbon of the side chain. Its specific activity was 26 Ci mole^{-1}. Thin-layer chromatography showed it to contain less than 5% of the trans,trans-isomer and no other contaminant.

The second lot was custom-synthesized by Mallinkrodt/Nuclear, St. Louis, Mo. and contained the label in the carboxy carbon of the side chain. It was radio-chemically pure as judged by TLC. GLC showed it to contain 4% of the trans,trans-isomer. Its specific activity was 23 Ci mole^{-1}.

Extraction of Leaves

Following exposure to labeled ABA for varying periods of time, leaves were immersed into methanol which had been cooled with solid CO_2. The frozen leaf was finely chopped with scissors and left in methanol which was then allowed to warm to room temperature overnight. The suspension was filtered and the leaf pieces reextracted in methanol. This was repeated until the leaf extract became colorless.

The combined methanol filtrate was diluted with ten volumes of distilled water and evaporated under partial vacuum to remove the alcohol. The resulting aqueous solution of the total leaf extract was adjusted to pH .0 with a saturated solution of $NaHCO_3$ thrice partitioned against diethyl ether; the combined ether phases will be referred to as neutral fraction. The pH of the remaining aqueous phase was then adjusted to 3.0 with 0.1N H_2SO_4 and again partitioned three times against diethyl ether; this ether phase will be referred to as the acidic fraction of the extract. Following hydrolysis of the aqueous phase at 60° C and pH 11.0 for 20 min. partitioning again of diethyl ether was repeated at pH 3.0 yielding the hydrolized fraction. The ethereal phases from each of these treatments were then dried over Na_2SO_4 and evaporated to small volumes under partial vacuum. Aliquots from each of these partitioned phases as well as from the remaining aqueous phase were transferred into scintillation vials to which a dioxane-based scintillation fluid was added. The vials were examined for radioactivity using a Packard Model 3375 liquid scintillation spectrometer. Efficiency of counting was 85% as determined by external standardization. The acidic fraction of the extracts were then divided: one-third was chromatographed directly by TLC. One-third was co-chromatographed with a standard solution of ^{14}C-cis,trans-(R,S)-ABA. The final third was chromatographed after it had been methylated.

Alternatively, when a leaf had been exposed to low levels of radioactivity, the initial methanol extract was chromatographed directly by TLC.

Methylation of Samples

Methylation was carried out following the procedure of Schlenk and Gellerman (1960) as modified by Powell (1964). Since the procedure allows for complete methylation of ABA samples, the reaction products could be directly chromatographed (either by TLC or more generally by GLC).

Gas-Liquid Chromatography (GLC)

A Packard Model 7300 gas-liquid chromatograph was used throughout this investigation. This instrument was equipped with dual glass columns, flame-ionization detectors or electron-capture detectors. Gas chrome Q, 60-80 mesh, was used as the solid support, and 3% DC 200 was used as the liquid phase. Nitrogen was passed through the column at 40 ml min^{-1} at 40 p.s.i. The amplifier output (detector response) was recorded on a linear flat-bed recorder.

Retention times were uncorrected and used only for comparative purposes. Standard ethereal solutions of methylated ABA were injected into the column to determine standard retention times. The standard ABA solution was a 1:1 mixture of cis,trans and trans,trans-isomers of the (±) synthetic ABA supplied by the Reynolds Tobacco Company, Winston-Salem, N.C.

Quantitative estimates of the isomers were obtained by comparison of the relevant peak area with that of a known quantity of standard. The peak area was calculated as a function of the weight of the excised chart paper delineated by the peaks.

Thin-Layer Chromatography (TLC)

Thin-layer plates from Brinkman Instruments Inc., E.M. Division, Westbury, N.Y., precoated with a 0.25 mm layer of silica gel, were washed in ethanol and activated at 105°C for 10 min prior to use. The developing solutions were prepared from redistilled solvents.

Since most of the solutions to be chromatographed were crude extracts, the plates were first developed three times in a hexane-ethyl acetate, 1:1 (v/v) to move a large portion of the pigments to the front. Then the plates were developed in benzene-ethyl acetate-acetic acid, 50:5:2 (v/v). This general method for whole leaf extract chromatography was described by Milborrow (1970).

Optical Rotatory Dispersion (ORD)

Determination of concentration of solutions of the (+) and (-) enantiomers of ABA was carried out as described by Zeevaart (1971) using a Durrum-Jasco Spectropolarimeter model J-5 assuming equal absolute magnitudes of rotation of the optical isomers.

Ultraviolet Spectroscopy (UV)

Concentrations of (+), (-) and (±) preparations of cis,trans-ABA were determined after examination of UV spectra. All isomers of ABA were dissolved in ethanol containing 0.005N sulfuric acid and gave absorbance maxima at 261 nm. Solutions of cis,trans-(±)-ABA made up by weighing a crystalline sample supplied by Dr. J. van Overbeek showed molar extinction coefficient of 2.2×10^4 which is in agreement with the value published by Milborrow (1970).

Analysis of Gas Exchange

Small plexiglas chambers were designed and built for analysis of small leaves. The leaf blade under study was gently pressed between two silicone rubber gaskets. The two halves of the chamber were then pressed over the gaskets. The gaskets were wide enough to prevent leaf damage from excess pressure yet an airtight seal could be maintained around the leaf edges. The gaskets bordered an area of 2.0 cm^2 on each of the 2 surfaces of the leaf. Air flowed into each half of the chamber, over each surface of the leaf and was vented.

Fixed into one gasket were fine threads of copper and of constantan fashioned so that they lay along 4 cm of the abaxial leaf surface. The junction between them (one thermocouple junction) was touching this leaf surface. The reference thermocouple junction was kept in an ice

bath at 0°C. This arrangement allowed continuous monitoring of accurate leaf temperatures.

Once excised from the plant, the base of the leaf blade (or the cut surface of the petiole in the case of tomato leaves) was kept under water. When each leaf had been placed into its separate chamber, its base was immersed into a beaker containing 10 ml of distilled, deionized water. The beaker was shaded to keep it cool and to avoid the possibility of photo-conversion of added solutes. The leaves were vertically oriented and the light from an overhead, water-cooled Xenon arc lamp (Osram XBF 6000) was reflected off a mirror of mylar coated aluminum film through an infra-red filter and through one side of the plexiglas chamber onto the adaxial surface of the leaf. The four chambers used in each study were positioned, using a small silica photocell so that they each received equal irradiation (ca. 40 mW cm^{-2} of photosynthetically useable light). Fans were positioned around the chambers to prevent excessive heating.

Addition of solutes to the leaf was carried out as follows: each identical beaker under each leaf was filled to capacity (10 ml); 1 ml was drawn from each and 1 ml of experimental or control solution was then added to each. In this way precise concentrations of solutes could be administered to each leaf.

Each surface studied was aerated with a constant flow (usually 50 l hr^{-1}) of air, the dew point (usually

11.8± 0.05°C) and the CO_2 concentration (usually 300 ± 2 $\mu l\ l^{-1}$) of which were specific and constant. The composition of efflux gas from each leaf surface was independently compared to the composition of influx gas using 2 differential infra-red gas analysers (URAS-2 Hartmann and Braun, Frankfurt) in series. One of them monitored changes in water vapor concentration, the other measured CO_2 concentration differences. The air flowing from each chamber or leaf surface was sequentially diverted to the gas analysers by solenoid valves activated by a timing mechanism. The data were recorded on analog recorders as well as through a digital data acquisition system on magnetic tape for computation using appropriate programs. The gas analysis and data acquisition systems were designed and assembled by Dr. K. Raschke and will be fully described (Raschke, in preparation).

Computation of Diffusion Resistance to Water Vapor and Inter-cellular Carbon Dioxide Concentration

From the data recorded as described above, the diffusion resistance (including stomatal and boundary layer resistances) to water vapor (rH_2O) and the inter-cellular CO_2 concentration $[CO_2]_i$ as well as the transpiration rate and the CO_2 assimilation rate could be computed (Raschke, in preparation) using calculations similar to those described by Moss and Rawlins (1963).

Preparation of Epidermal Strips

Stomata on isolated epidermal strips were studied as described by Humble and Hsiao (1969). Epidermal strips were removed from the abaxial surface of bean leaves (Vicia faba L., cv. Improved Long Pod) and transferred to buffered solutions (0.5 mM tris-maleate pH 6.0) containing 0.2 meq liter^{-1} of Ca^{+2} and concentrations of KCl ranging from 0 to 100 meq liter^{-1}. The solutions on which the strips were floated were then either placed in darkness, or illuminated with 8.5 mWcm^{-2} visible light (filtered through water to remove infra-red) from 2 mercury vapor lamps. These solutions were continuously flushed in a stream of humidified, CO_2-free air.

Once the stomata were allowed to open for 2 hours in the light, the strips were transferred to solutions containing abscisic acid. After one hour the strips were removed from the solutions, blotted dry and temporarily fixed in immersion oil. The degree of opening of the stomata was examined under a microscope fitted with an ocular micrometer.

During preparation of the strips, the majority of epidermal cells were ruptured (Humble and Hsiao, 1969), but the guard cells remained intact. Intact epidermal cells could be distinguished by observing the strips under low magnification using small-angle incident light. Intact cells appeared convex and bulging.

RESULTS

Transpiration Measured as Weight Loss

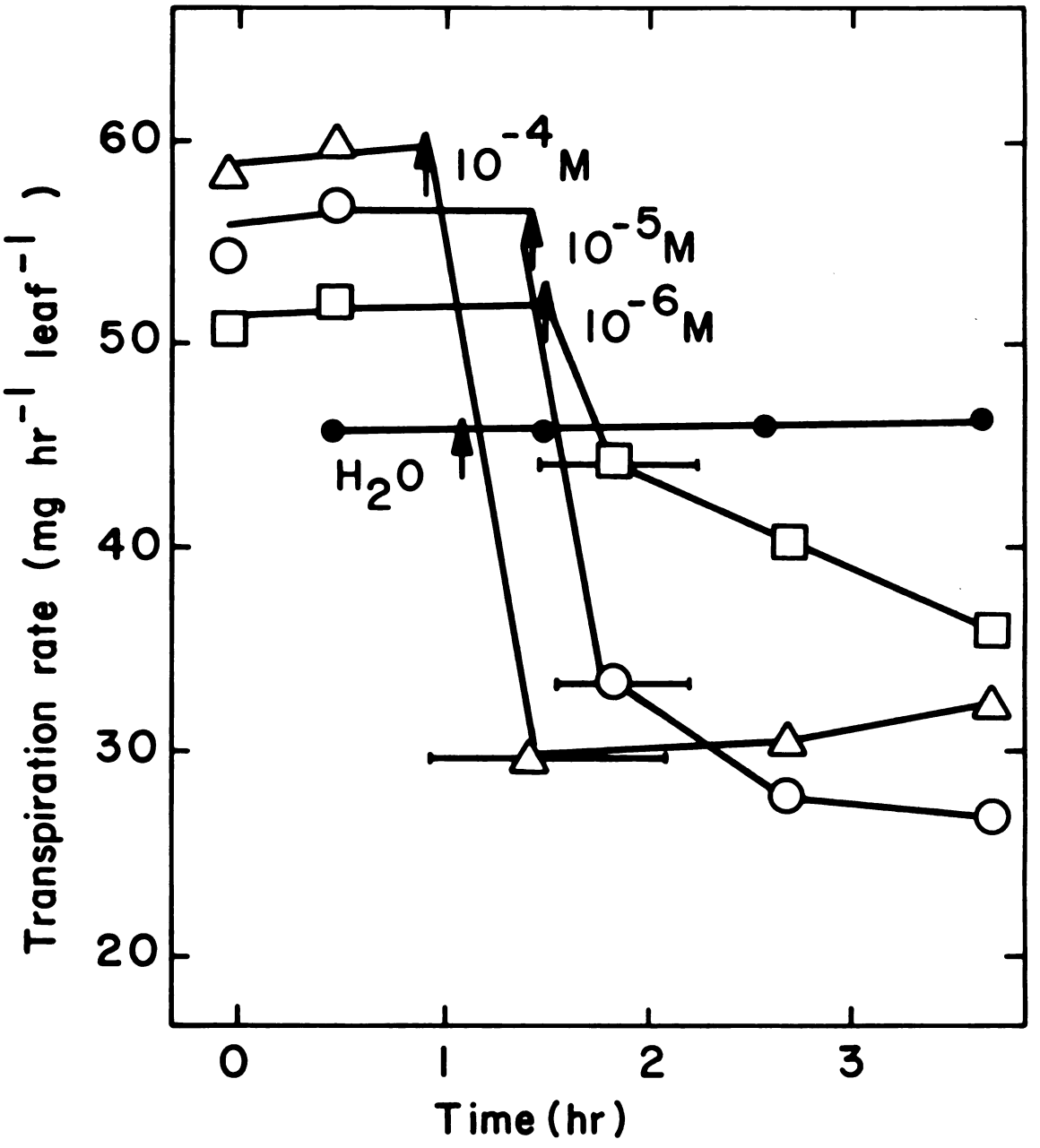
The bases of 3 primary barley leaves were immersed into a glass vial containing water. In order to prevent evaporation, the opening of the vial around the leaf bases was sealed with parafilm. Transpiration rates were determined as functions of weight loss from tared vials.

Figure 1 shows the results from one such experiment. Transpiration rates were measured before and after abscisic acid addition to the irrigating solution. The weighing intervals were 1 hour apart, and as can be seen from the results, decreases in transpiration rates occurred within the first hour after addition of abscisic acid. Final concentrations of ABA in the irrigating solutions of 10^{-4}, 10^{-5} and 10^{-6}M all caused decreases which became evident as soon as could be measured by this technique.

ABA-Induced Changes in Diffusion Resistance to Water Vapor

Using the gas analysis technique, the lag for abscisic acid action was determined. Values for the leaf diffusion resistance to water vapor (r_{H_2O}) were calculated

Figure 1.--Transpiration rate of excised barley leaves as a function of time before and after treatment with cis,trans-(RS)-abscisic acid at 10^{-4}, 10^{-5} and 10^{-6}M concentrations. Arrows indicate time of application of the inhibitor. The horizontal bars on the first readings after treatment indicate the interval over which weight loss was determined. Transpiration rates were determined as a function of the loss of weight from vials, each containing 3 leaves. Each point is the average of 3 determinations (i.e., 9 leaves). The experiment was carried out under fluorescent lights at 20°C. The leaves were placed 50 cm below a bank of 4 Sylvania Lifeline FR40W-235 fluorescent tubes.


60
50
40
30
20
0
1
2
3
Time (hr)
Transpiration rate (mg hr⁻¹ leaf⁻¹)
10⁻⁴M
10⁻⁵M
10⁻⁶M
H₂O

at 10 second intervals for a barley leaf before, during, and after addition of abscisic acid to the irrigating solution.

Figure 2 shows that within 7 minutes of changing the irrigating solution from pure water to a 10^{-7}M ABA solution, the r_{H_2O} had started to increase and continued to increase (indicating closure) for about 30 minutes, while no change in r_{H_2O} of the untreated leaf took place.

ABA-Induced Changes in Leaf Temperature

Subsequent examination of temperature changes in leaves led to an easier method for determining time course of the ABA response.

Figure 3 shows a typical leaf temperature versus time plot. When the light was turned on, the leaf temperature rose instantaneously. As the stomates began to open, however, transpiration started to cool the leaf. The leaf temperature stabilized when transpiration rate reached a constant level. In such experiments radiation was kept constant for each leaf (approximately 40 mW cm^{-2} from a xenon arc lamp). The air flow past each surface of the leaf was also constant at 50 l h^{-1} and the rate of heat loss from the chambers was kept constant by fans directing air around the outside of the chambers.

Very soon after ABA was added to the irrigating solution, the leaf temperature started to increase,

Figure 2.--Leaf diffusion resistance to water vapor as a function of time. At time zero the solutions irrigating the excised primary barley leaves were changed by adding either cis,trans-(RS)-abscisic acid (final concentration was 10^{-7}M) or water. The ABA-treated leaf showed an increase in resistance starting 5 to 6 minutes after treatment. In this experiment air flowed around the leaf over both leaf surfaces so the results indicate a mean resistance for both sides of the leaf. Total air flow around leaf was 50 l hr^{-1}. Leaf temperature ranged between 27° and 29°C. The dewpoint of the air was 11.8°C. The CO_2 concentration was 300 µl l^{-1}. Light source was a xenon arc lamp giving irradiance of ca. 40mWcm^{-2}.

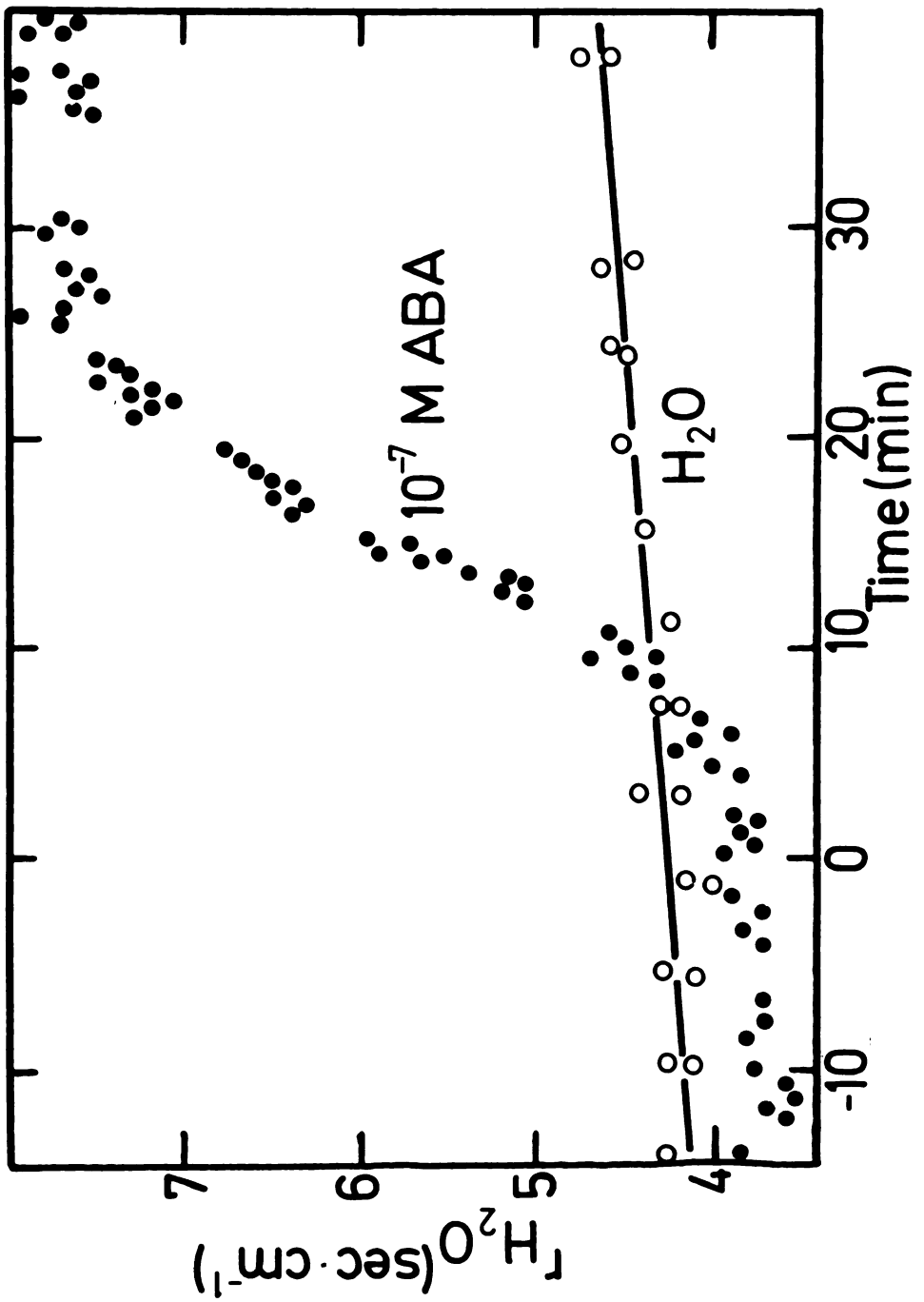
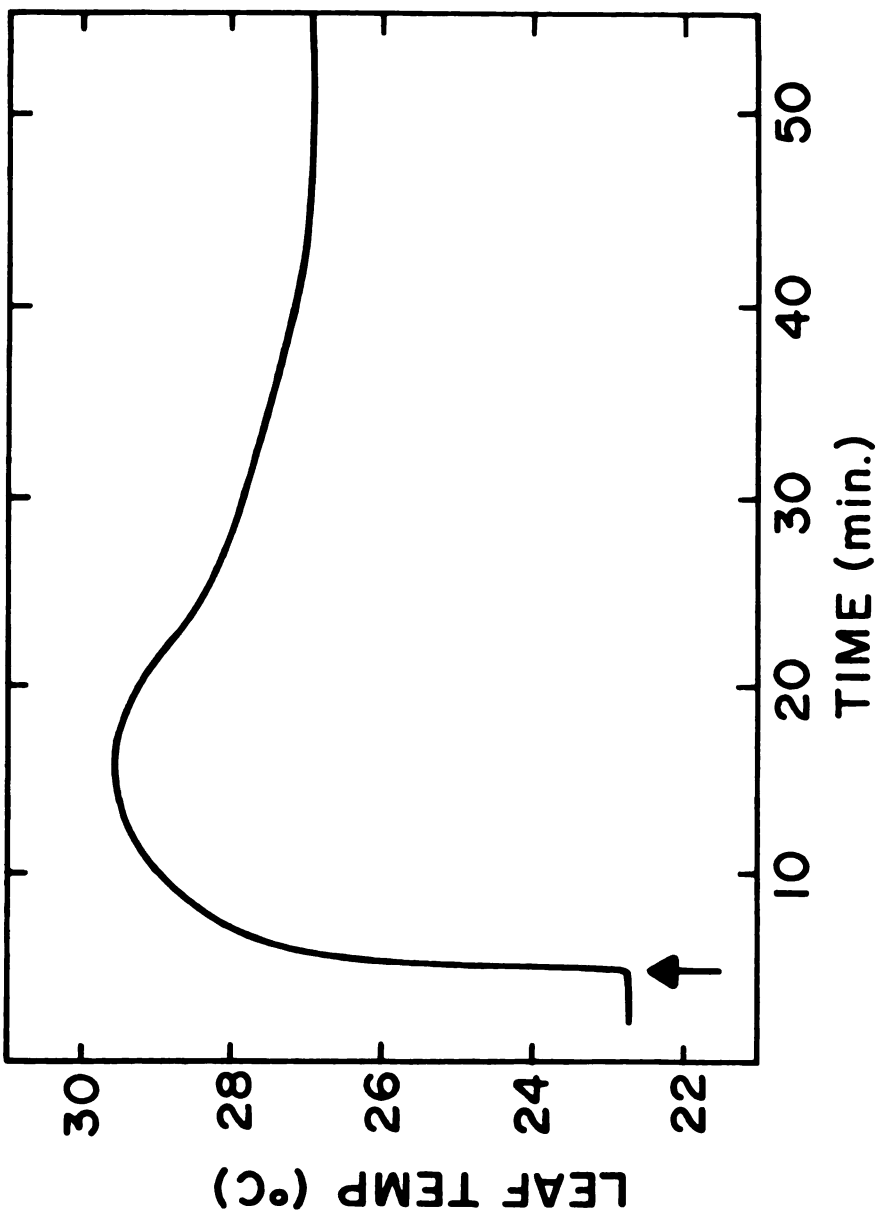

r_{H_2O}(sec·cm^{-1})
7
6
5
4
-10
0
10
20
30
Time(min)
10^{-7} M ABA
H_2O

Figure 3.--Leaf temperature changes with time upon illumination (at the time indicated by the arrow) with light from a xenon arc lamp filtered to exclude infra-red radiation and giving ca.40 mW cm^{-2} of photosynthetically useable light. Conditions were the same as indicated for Figure 2 except for the following: (a) air flowed over both leaf surfaces independently at a rate of 50 l hr^{-1}; (b) smaller chambers were used wherein the leaf area exposed was 0.5 x 4.0 cm or 2.0 cm^2 per side; (c) the thermocouple leads were in direct contact with the lower leaf surface above the entire 4 cm dimension.


LEAF TEMP (°C)
30
28
26
24
22
10
20
30
40
50
TIME (min.)

indicating that transpiration was reduced and that stomates had started to close. Figure 4 shows such increases above leaf temperature at equilibrium. The temperature change started as early as 2 minutes after adding ABA (10^{-5}M final concentration) to the irrigating solution. The lag before the start of the temperature rise appeared to be a function of the inverse of the concentration of the ABA applied.

Estimation of the Rate of Flow of ABA through Leaves

The rate of flow of ABA through the leaf was estimated in order to determine what proportion of the lag time was due to transport of the inhibitor to its site of action.

Flow rate was determined for secondary leaves of barley because their size allowed the positioning of several gas analysis chambers along the length of the leaf. Fully-grown leaves were used so that all the stomates along the leaf would be fully developed. The transpiration rates per area of leaf were determined at each of the 3 positions monitored along the leaf for comparison. The flow of water (and presumably the dissolved ABA) through the leaf should be a function of the transpiration rate. Table 2 shows the results of one such experiment. The flow rate determined between the 3 positions was approximately 10 cm min^{-1}. The time required for abscisic

Figure 4.--Leaf temperature changes upon treatment of primary barley leaves with 10^{-6}M or 10^{-5}M cis,trans-(RS)-abscisic acid at time zero. The ABA supply was removed at the time indicated by the arrow. In each case the circles represent temperature of the treated leaf; the squares indicate temperature of the control leaf. Treatment with 10^{-6}M ABA caused initial change within 5 minutes; 10^{-5}M ABA treatment within 3 minutes.

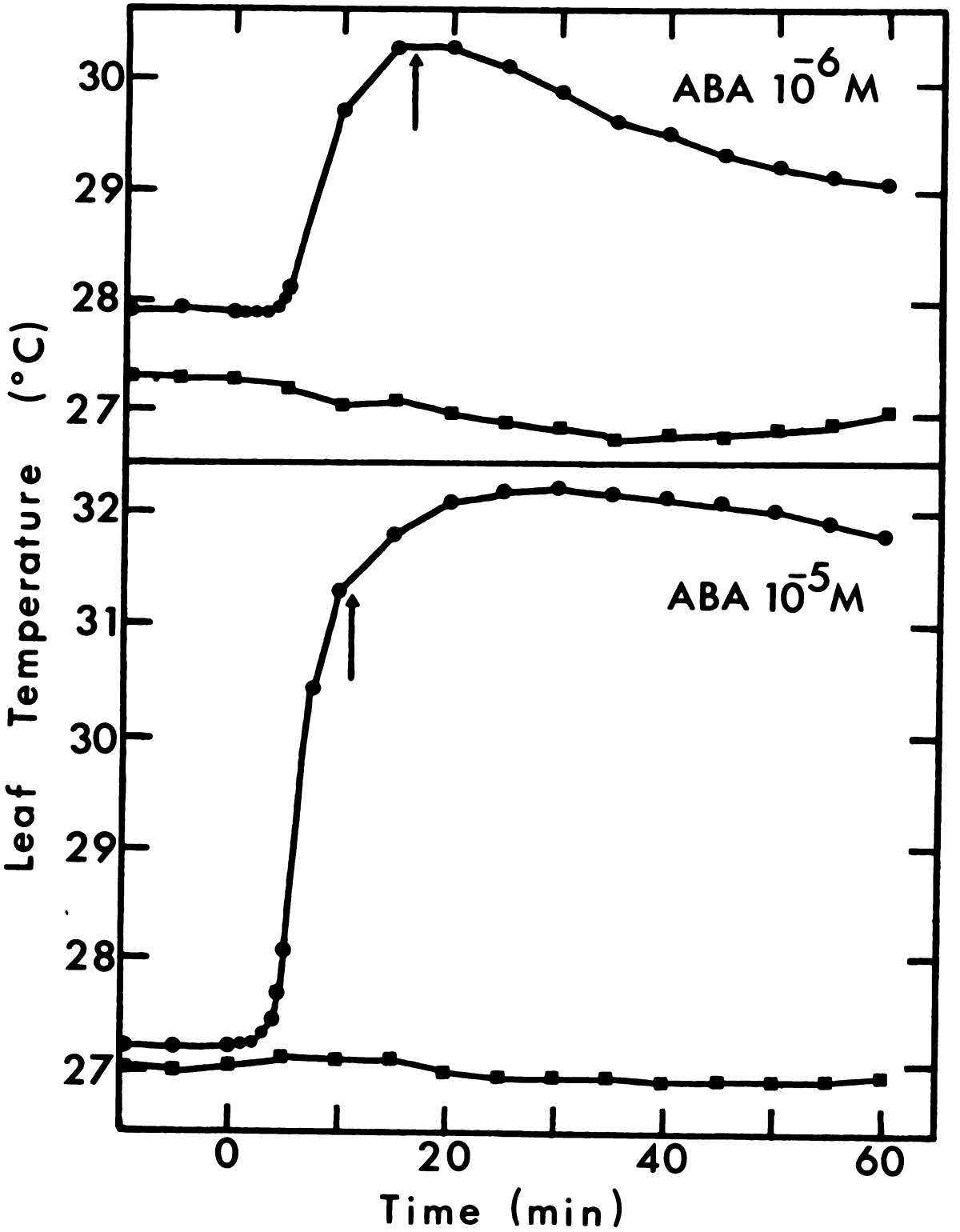

ABA 10^{-6} M
ABA 10^{-5} M
Leaf Temperature (°C)
30
29
28
27
32
31
30
29
28
27
0
20
40
60
Time (min)

Table 2. Estimation of ABA flow rate determined from the lags for leaf temperature changes in a secondary barley leaf as a function of distance from the base of the leaf where ABA at 10^{-5} was supplied.

DISTANCE FROM BASE (cm)	TOTAL LAG (min)	TRANSPIRATION RATE (g dm^{-2} hr^{-1})	FLOW[a] RATE (cm min^{-1})
18.5	4.5	1.5	
			10.4
23.7	5.0	1.5	
			10.3
34.0	6.0	1.3	

Total lag at 34 cm from source	6.0 min
Transport lag[b]	3.3 min
Net lag[c] (independent of transport)	2.7 min

[a]Flow rate was determined by dividing the difference in distances from the base of two monitored points by the difference in their total lags.

[b]Transport lag was determined by dividing the distance from the ABA source by the flow rate.

[c]Net lag is that time needed for leaf temperature change following the computed time of arrival of ABA to the center of the area of leaf that was monitored.

acid to reach the stomates covered by the 3 chambers (the transport lag) accounted for only a part of the total lag time. This means either that the estimate of flow rate is too high by a factor of 2 or that other considerations account for the net lag time.

If one considers the ABA that is added to the irrigating solution to move up through the leaf as a front from the cut surface then its rate of flow may be described according to Ohm's law as:

$$\text{flow rate} \propto \frac{\text{potential difference}}{\text{resistance}}$$

As the front moves away from the base the potential difference factor should decrease since there is a decrease in the total evaporating surface above the front. This evaporating surface is the major determining factor causing the flow of water in a leaf. The resistance to flow is assumed to increase only slightly as the front moves through more and more conducting tissue. Therefore the flow rate determined over an interval of 4 cm far from the water supply should be somewhat less than the flow rate determined over a similar interval closer to the water supply.

In these experiments it has been assumed that the flow rate is relatively constant along the entire leaf surface. This means that the flow rate has probably been somewhat underestimated.

Other factors which may contribute to the lag time are:

1. The time required for the ABA to diffuse along or through the evaporating surfaces to its site of action. If ABA does act on the guard cells directly, then ABA must diffuse along the evaporating surface (presumably that layer of liquid water covering the mesohyll cells) to the epidermis and the guard cells. There is no way to estimate this time but it conceivably contributes to the lag of the response.

2. A part of the lag must be due to the time required for ABA to cause closure once it has reached its site of action. For secondary leaves this time must be somewhat less than 2.7 minutes. The shortest lag for primary barley leaves was 2 minutes. In this case the distance traveled was less than 10 cm.

3. The measurement of leaf temperature was virtually instantaneous. There was no mechanical or instrument lag. The time required for a rise in leaf temperature once stomata start to close, however, is very difficult to estimate. It is conceivable that stomata start to close some seconds before the change in leaf temperature. However, the lag

times for change in transpiration rate and change in leaf temperature are virtually the same so this time must be only a few seconds.

Does ABA Flow in Both Directions?

To answer this question, secondary barley leaves were again fitted with three gas analysis chambers as before. This time the tips as well as the bases were cut and immersed under water. ABA (10^{-5}M final concentration) was added only to the solution bathing the tip. The pattern of response was different. Table 3 shows that the leaf portion farthest from the tip source did not respond to ABA (no change in leaf temperature or transpiration rate). Leaf portions closer to the ABA supply did respond.

These results indicate that ABA can move in either direction depending on the direction the water is moving. The leaf portions closer to the base draw their water from the solution bathing the base. The water flows in response to the water potential gradient established by evapotranspiration along the path of least resistance.

Specificity of the Response to Abscisic Acid

Analogs of abscisic acid which show varying degrees of biological activity in other assay systems (Sondheimer and Walton, 1970) were tested to determine whether their relative activities in the closing response paralleled their other inhibitory activities.

Table 3. Lag for temperature change following ABA application to solution bathing cut tip of a secondary barley leaf at various distances from the point of application.

DISTANCE FROM TIP SOURCE[a] (cm)	DISTANCE FROM BASE (cm)	TOTAL LAG (min)
6	16	3.3
11	11	16
16	6	>46

[a] ABA 10^{-5}M applied only to tip of leaf.

Table 4 shows the lag time for leaf temperature change as a function of the concentration of synthetic abscisic acid analogs: the higher the concentration of inhibitor present, the shorter the lag. These data are compared with data published by Sondheimer and Walton (1970) which show the relative effectiveness of these compounds in inhibiting the growth of embryonic bean axes.

As seen in Figure 5 the analogs are very similar to abscisic acid in chemical structure. All have a carboxylic acid terminating a conjugated side chain which is attached to a six-membered ring. The analogs demonstrated marked differences in biological activity which are paralleled in at least 2 very different types of assay systems, namely inhibition of growth of embryonic bean axes and inhibition of transpiration.

GLC of the trans,trans-ABA (Figure 6) showed it to contain about 7% of the cis,trans-isomer. This contamination may account for the relatively high (1-10%) activity of this sample in both assay systems since the same trans,trans-ABA preparations were used in both cases.

In the very rapid leaf temperature assay, there was no activity of the other analog (dihydroionylidene-acetic acid).

Such preparations contain equal amounts of both enantiomers, i.e., the (+) and the (-) optical isomers of the molecule. Only the (+) enantiomer occurs naturally in

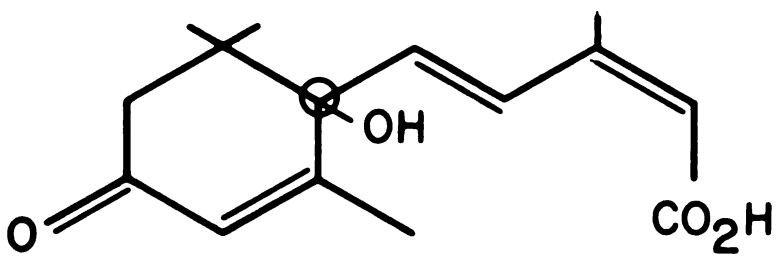
Table 4. Estimations of relative activities of the analogs of ABA compared to ABA in leaf temperature assay and embryonic axis assay.

TREATMENT	LAG (min)	% RELATIVE ACTIVITY IN	
		BARLEY LEAVES[a]	EMBRYONIC AXES[b]
cis,trans-(RS)-ABA		100	100
10^{-7}M	7		
10^{-6}M	4		
10^{-5}M	2.5		
trans,trans-(RS)-ABA		1-10	6
10^{-7}M	>35		
10^{-6}M	10		
10^{-5}M	6		
cis,trans-dihydro-ionylidenacicetic acid		<1	2
10^{-7}M	>35		
10^{-6}M	>35		

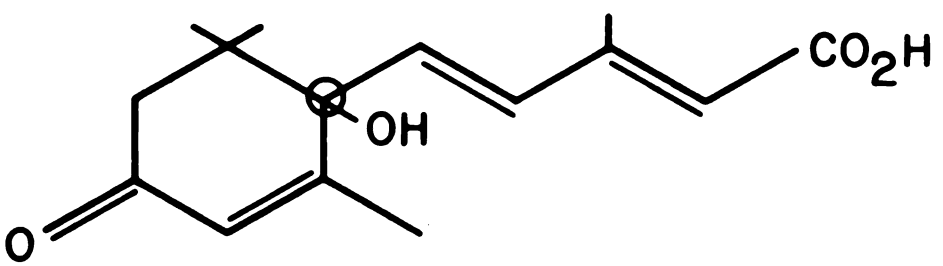
[a]Based on ability to change transpiration rates.

[b]From data of Sondheimer and Walton (1970) based on ability to inhibit elongation of bean embryonic axes.

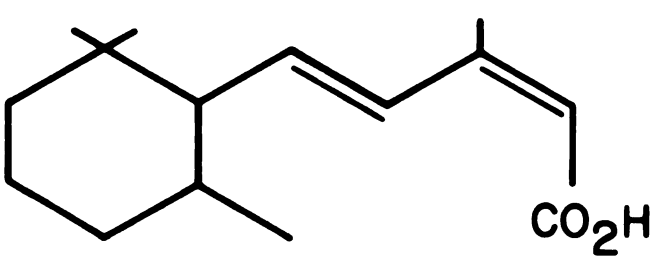
Figure 5.--Chemical structures of the ABA analogs used. These were synthetic preparations and, therefore, contained equal amounts of the R and S enantiomers (i.e., they were racemic mixtures). The circled atom is the assymmetric carbon. The (+) and (-) optical isomers of cis,trans-abscisic acid (not racemic mixtures) were also tested for activity.



cis, trans -(RS)- abscisic acid

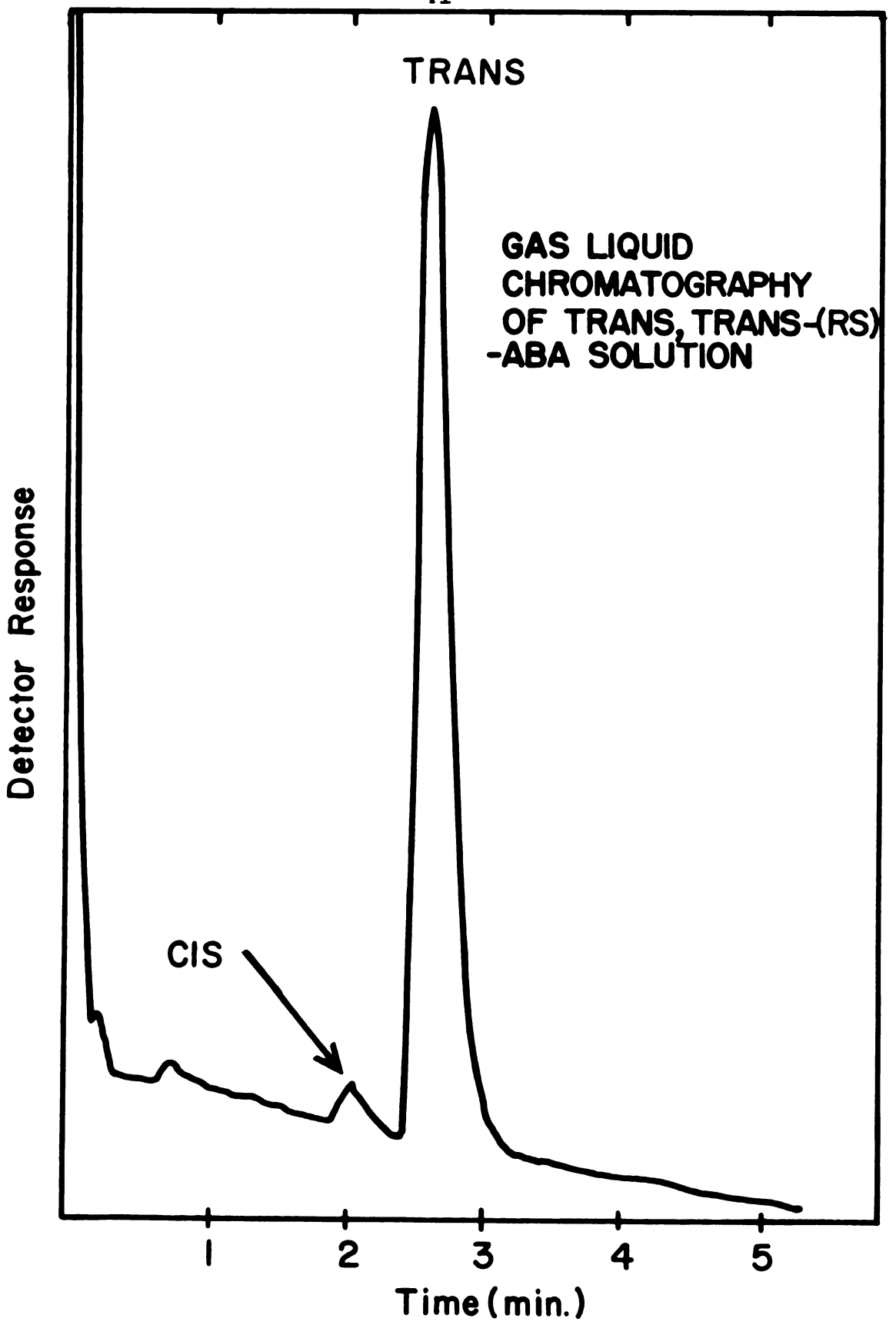


trans, trans -(RS)- abscisic acid



cis, trans-dihydroionylideneacetic acid

Figure 6.--Gas-liquid chromatography of a sample of the trans,trans-(RS)-abscisic acid following methylation. This chromatogram shows the high overall purity of the sample and the major contamination by a peak (accounting for 6% of the total) the retention time of which corresponds to that of the cis,trans-isomer.


TRANS
GAS LIQUID
CHROMATOGRAPHY
OF TRANS, TRANS-(RS)
-ABA SOLUTION
CIS
Detector Response
1
2
3
4
5
Time (min.)

plants (Milborrow, 1969). Indeed the optical activity of the (+) isomer is used to determine (+) abscisic acid concentrations from natural sources by ORD. Figure 7 shows ORD curves for the two optical isomers. Based on ORD and UV data (see Materials and Methods), stock solutions of known concentrations of the two isomers were prepared. Since the concentrations determined by the two methods agreed very closely (Table 1), we concluded that the (-) preparation contained little, if any, (+) ABA.

Table 5 shows the results of experiments to determine the relative activity of the two optical isomers. There was some activity of the (-) preparation at 0.8×10^{-5}M but virtually none at 10^{-6}M while the activity of the (+) preparation was comparable to or slightly greater than the activity of the racemic mixture. From such results we conclude that the (+) isomer, which is the naturally occurring one, is considerably more active than the (-) isomer preparation. Analysis of the two isomers by GLC indicated that both preparations were more than 77% pure (Figure 8).

Reversal of the ABA Response

The persistence of the ABA effect on stomata closure was investigated in the following manner. The ABA supply was removed by repeatedly diluting and draining the irrigating solution. During the draining and diluting

Figure 7.--Optical rotatory dispersion of the (+) and the (-) optical isomers of cis,trans-abscisic acid dissolved in 0.005N sulfuric acid in ethanol. The (+) enantiomer shows a positive rotation peak at 289 nm, zero rotation at 269 nm and a negative rotation peak at 246 nm. The curve for the (-) enantiomer shows a mirror image except that the point of zero rotation is slightly shifted to a lower wave length. There is no reasonable explanation for this small shift although it could indicate slight contamination of this enantiomer by an optically-active species containing a UV-sensitive chromophore.

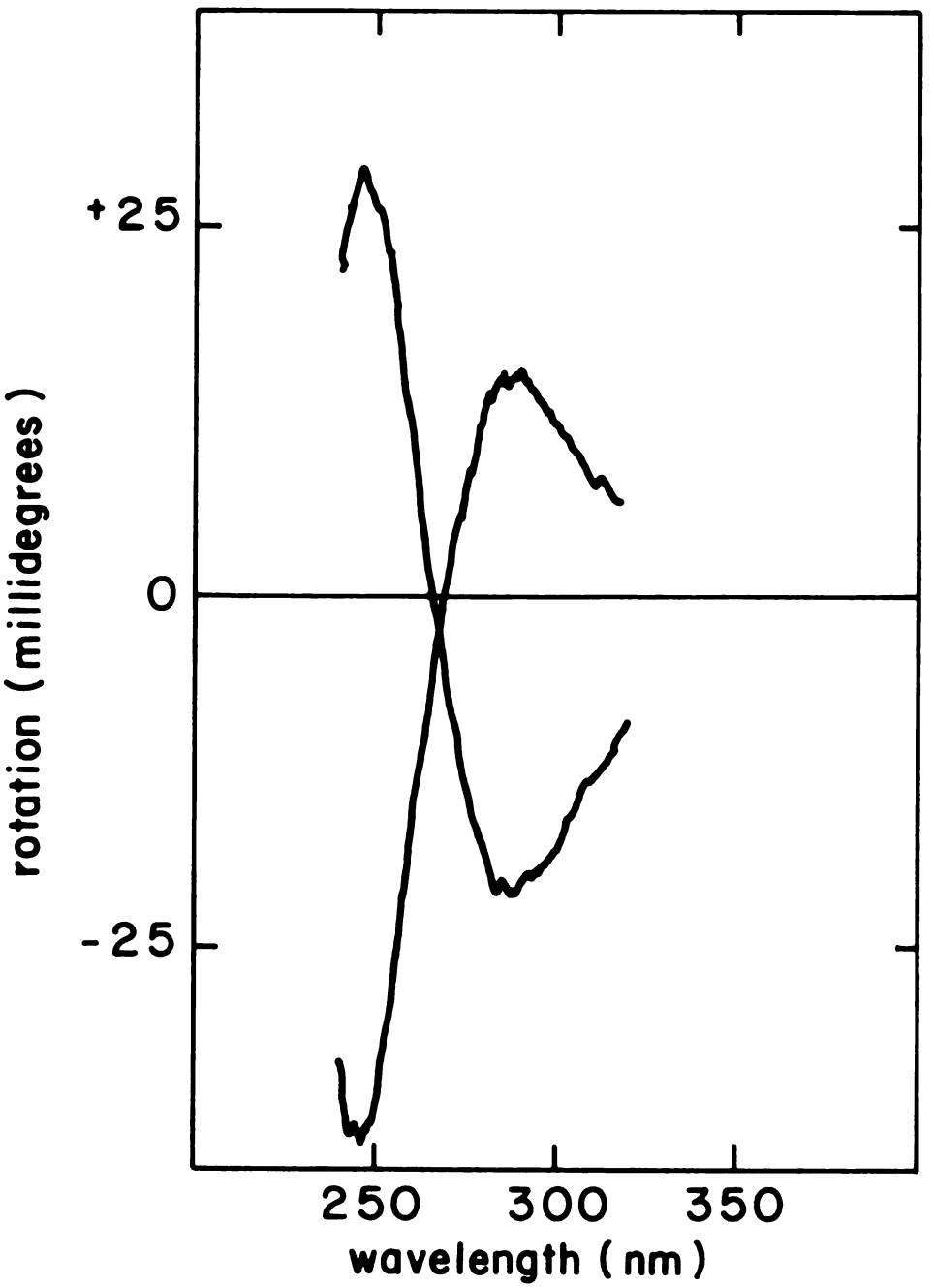

+25
0
-25
rotation (millidegrees)
250
300
350
wavelength (nm)

Table 5. The lag times for leaf temperature change following treatment of barley leaves with (+) and (-) ABA preparations.

ABA CONCENTRATION APPLIED (M)	TOTAL LAG (min)	LEAF TEMPERATURE		INITIAL TRANSPIRATION (g dm^{-2} hr^{-1})
		INITIAL (°C)	FINAL[a] (°C)	
1.0×10^{-6}[b]				
(±)	10	24.2	25.2	1.35
(±)	10	24.5	25.4	1.53
(+)	7	24.5	25.5	1.68
(-)	>30	24.8	24.6	1.53
0.8×10^{-5}[b]				
(+)	<7	-	26.7	1.35
(-)	12	25.8	26.0	1.35
(+)	5.5	25.4	26.9	1.28
(-)	12	25.8	26.0	1.35
1.6×10^{-6}[c]				
(+)	5	27.4	28.4	1.20
(-)	>20	27.9	27.9	1.35
(±)[d]	7	27.9	28.6	1.35

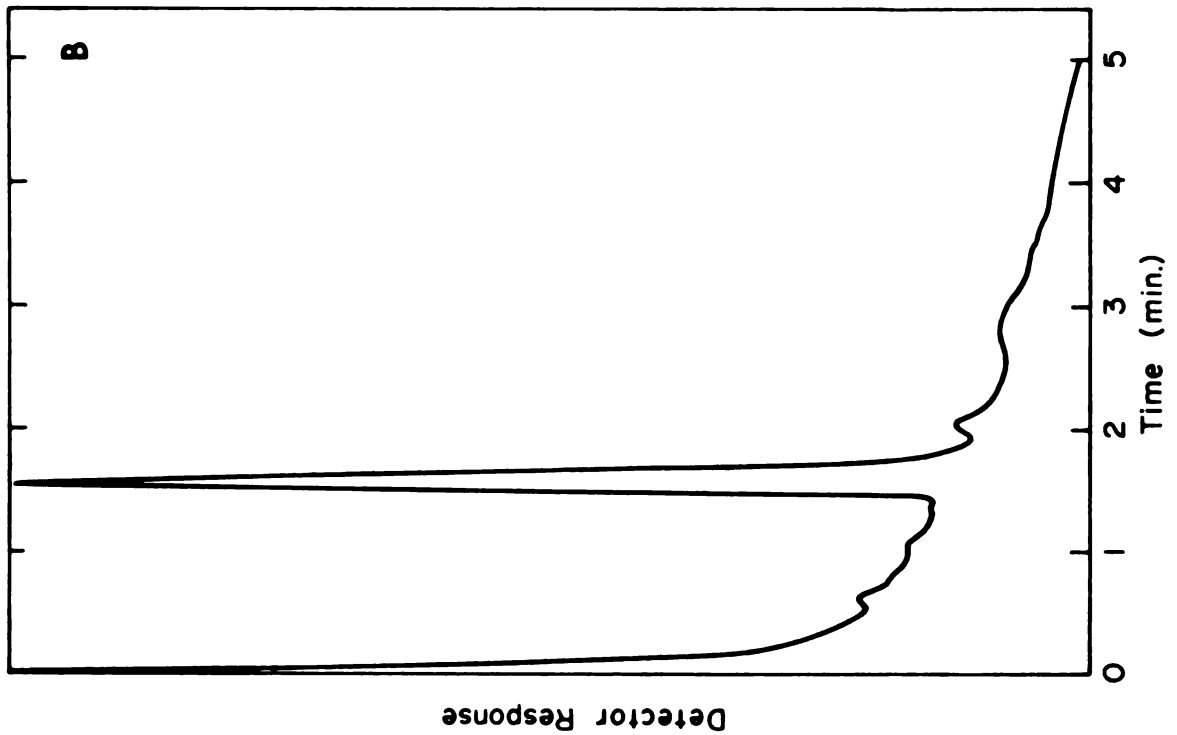
[a]Leaf temperature 30 min after treatment started.

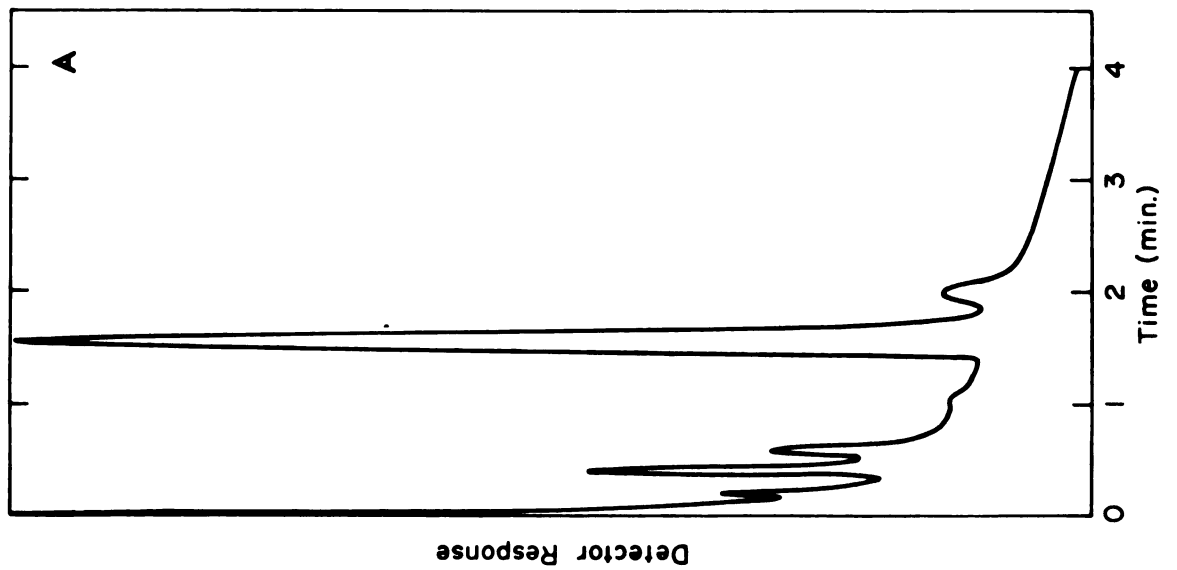
[b]Experiment carried out in CO_2-free air.

[c]Experiment carried out in air containing 300µl l^{-1} CO_2.

[d](±) applied to leaf which failed to respond to (-) preparation.

Figures 8A and 8B.--GLC of preparations of the (+) and (-) optical isomers of ABA, showing the degree of purity of the samples; 8A: (+) enantiomer; 8B: (-) enantiomer.


B
Detector Response
0
1
2
3
4
5
Time (min.)


A
Detector Response
0
1
2
3
4
Time (min.)

procedure, the base of the leaf was kept submerged so that the transpiration stream was not broken. Removal of the ABA supply caused a reversal of the ABA effect, i.e., the transpiration rates started to increase again (Figure 9). Those leaves to which ABA supply had been maintained showed continued decline of transpiration rates.

Such reversion of the response can most simply be explained in the following 2 ways. Active ABA must be removed from its site of action either by being sequestered into a cellular compartment which is different from its site of action, or else by being chemically altered to an inactive form. To determine which of the two possibilities is most likely, efforts were directed at following the metabolism of the applied ABA.

Short-Term Metabolism of ABA in Primary Barley Leaves

Primary barley leaves were fitted into gas analysis chambers where leaf temperature, CO_2 exchange and H_2O exchange could be monitored. After the leaves were illuminated for 2 hr, radioactively labeled ABA was added to the irrigating solution. After a labeling period of 15 min, when the stomates closed as a response to ABA treatment (Figure 10), one leaf was removed and immediately extracted. At the same time the ABA supply to the other leaf was removed as described above. Thirty-two minutes after removal of the ABA supply when reversal of the ABA

Figure 9.--Changes of transpiration rates upon the addition and removal of cis,trans-(RS)-abscisic acid. The solution irrigating the blades of excised primary barley leaves were treated at time zero with either 10^{-7}M ABA (closed circles) or distilled water (open circles) each containing 0.002% ethanol. At the time indicated by the arrows the ABA was removed from the irrigating solution by repeated dilution and partial drainage. The open triangles indicate the transpiration rate of the leaf upon removal of the ABA supply. The other treated leaf remained in the ABA solution. The transpiration rate showed an increase ca. 10 minutes after removal of the ABA supply. Conditions were identical to those described for Figure 2.

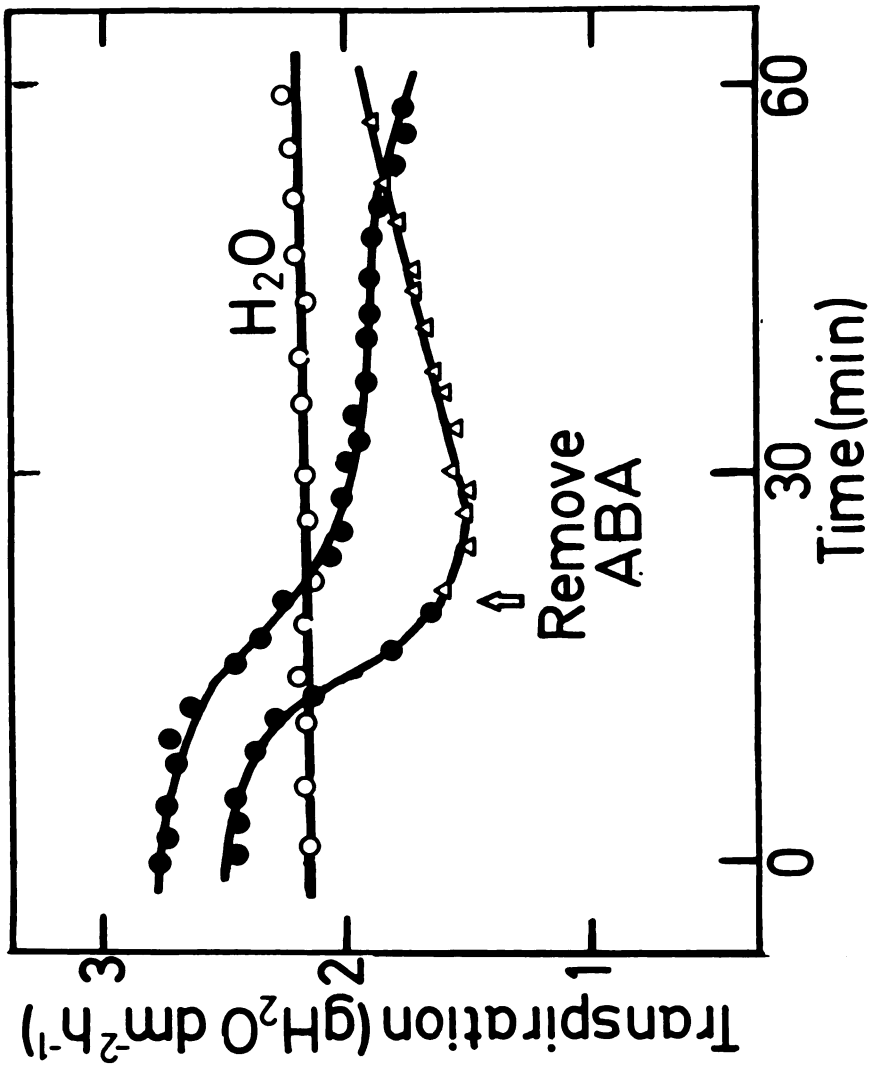
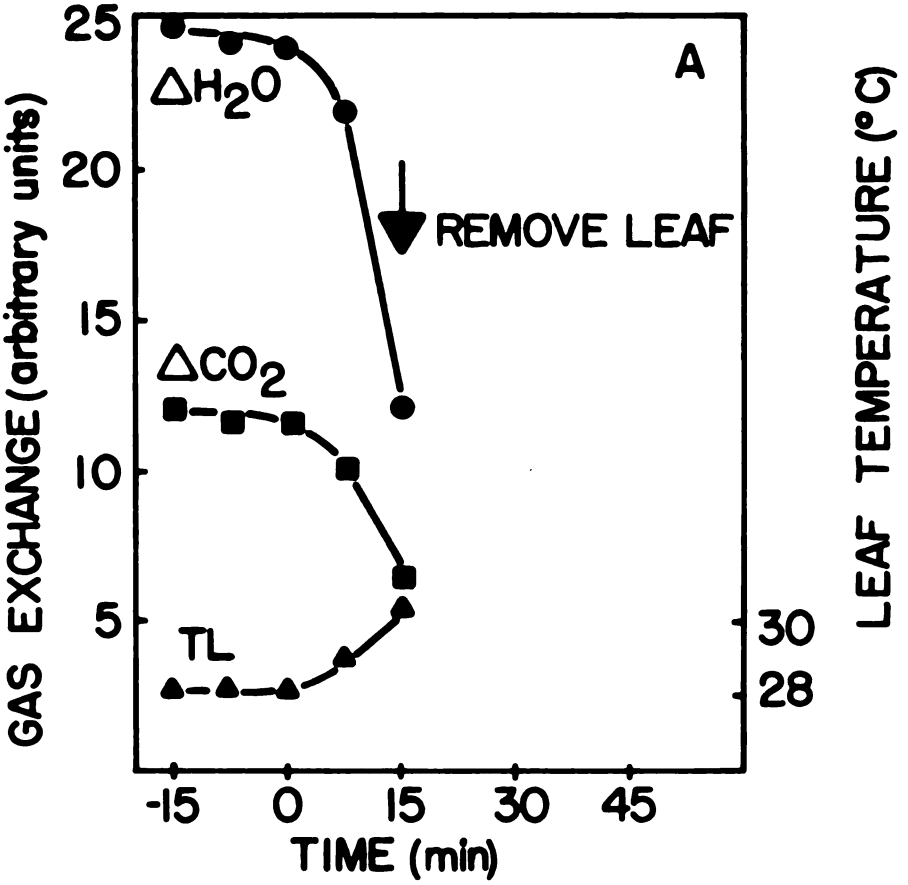
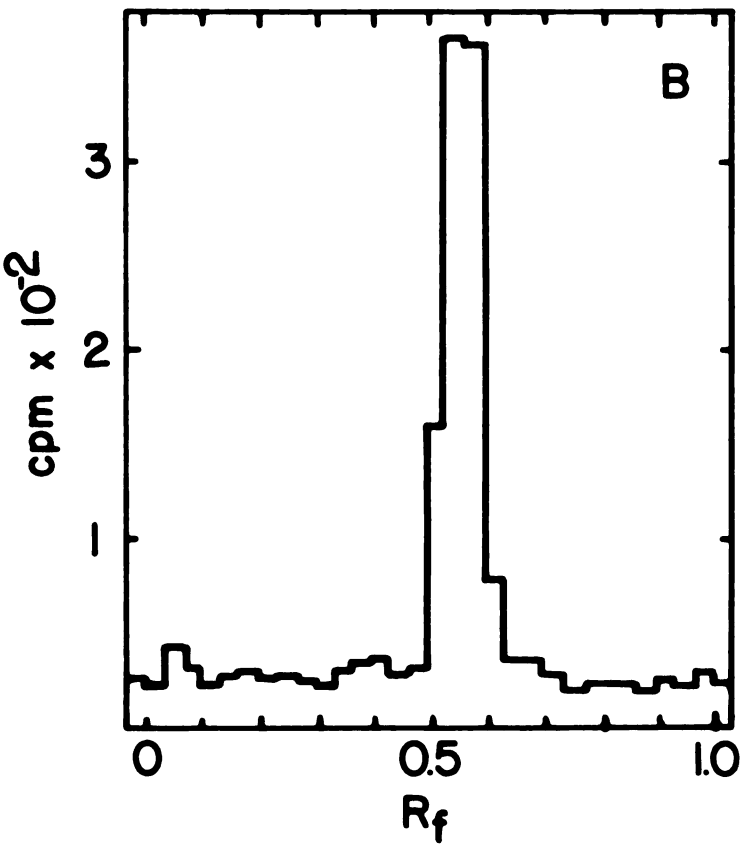

Transpiration (gH2O dm-2h-1)
3
2
1
0
30
60
Time(min)
H2O
Remove
ABA

Figure 10A.--Changes in water loss, net CO_2 uptake and leaf temperature with time for a primary barley leaf supplied with ^{14}C-α carbon labeled 10^{-6}M cis,trans-(RS)-ABA at time zero. At the time indicated by the arrow the leaf was removed and extracted. ΔH 0 is the difference in water vapor concentrations in the air flow into and out of the chamber. ΔCO_2 is the difference in CO_2 concentration between these two air flows. The units for the two are arbitrary and not related. TL is the leaf temperature. Conditions identical to those described for Figure 3.

Figure 10B.--Thin-layer chromatogram of the total extract from the leaf described in Figure 10A.


A
ΔH2O
ΔCO2
TL
REMOVE LEAF
GAS EXCHANGE (arbitrary units)
LEAF TEMPERATURE (°C)
25
20
15
10
5
30
28
-15
0
15
30
45
TIME (min)


B
cpm x 10-2
3
2
1
0
0.5
1.0
Rf

effect was evident (Figure 11A), this leaf was removed and immediately extracted. The total leaf extract from each set of leaves was then plated directly onto TLC plates as described above. This chromatographic system was described by Milborrow (1970) for the separation of the glucosyl ester of ABA, "metabolite A" and "metabolite C" (a phaseic acid precursor) from ABA.

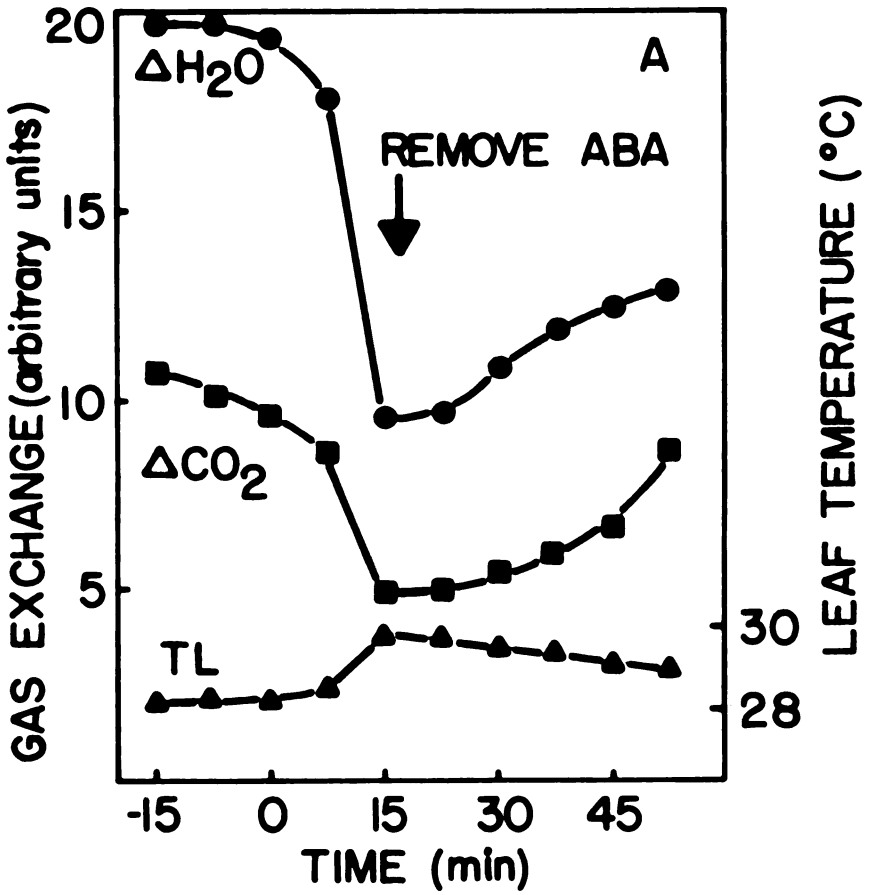
The TLC results indicate no significant metabolism of ^{14}C-ABA during a 15-minute exposure to ABA (Figure 10B); during a subsequent 32-minute period without external supply of ^{14}C-ABA in which there is demonstrable reversion of the physiological response, there is a slight metabolic conversion of ^{14}C-ABA amounting to less than 10% of the total radioactivity (Figure 11B).

Long-Term Metabolism of ABA

Longer term exposures of leaves to radioactively-labeled ABA resulted in some metabolism of ABA. In these experiments leaves were labeled during a 25 min exposure to 10^{-5}M ^{14}C-ABA. They were extracted and partitioned either immediately ("zero time extract") or after an additional 2 hours in the light (light extract) or darkness (dark extract). Ninety-eight % of the radioactivity from the zero time extract stayed in the acidic phase (Table 6). It is this phase into which abscisic acid partitions. Figure 12 shows that all of the radioactivity from this

Figure 11A.--Partial reversion of the rates of water loss, net CO_2 uptake and leaf temperature upon the removal of ABA supply. Conditions identical to those described in Figure 10A, except that the leaf was not extracted until 32 min after the ABA supply was removed.

Figure 11B.--Thin-layer chromatogram of the total extract from the leaf described in Figure 11A.


20
15
10
5
ΔH2O
A
REMOVE ABA
ΔCO2
TL
30
28
GAS EXCHANGE (arbitrary units)
LEAF TEMPERATURE (°C)
-15
0
15
30
45
TIME (min)

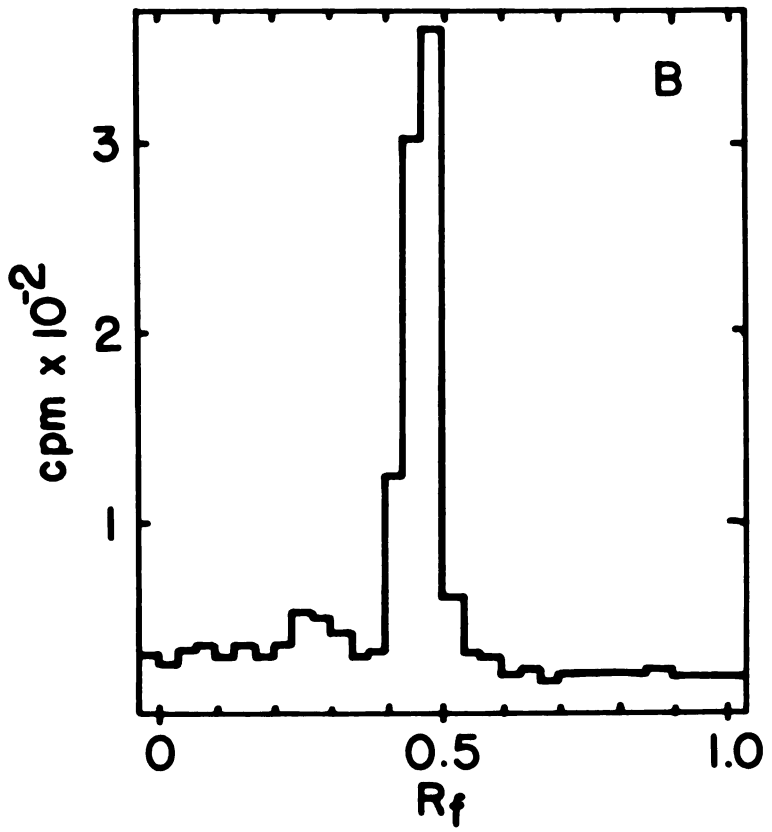

B
3
2
1
cpm x 10-2
0
0.5
1.0
Rf

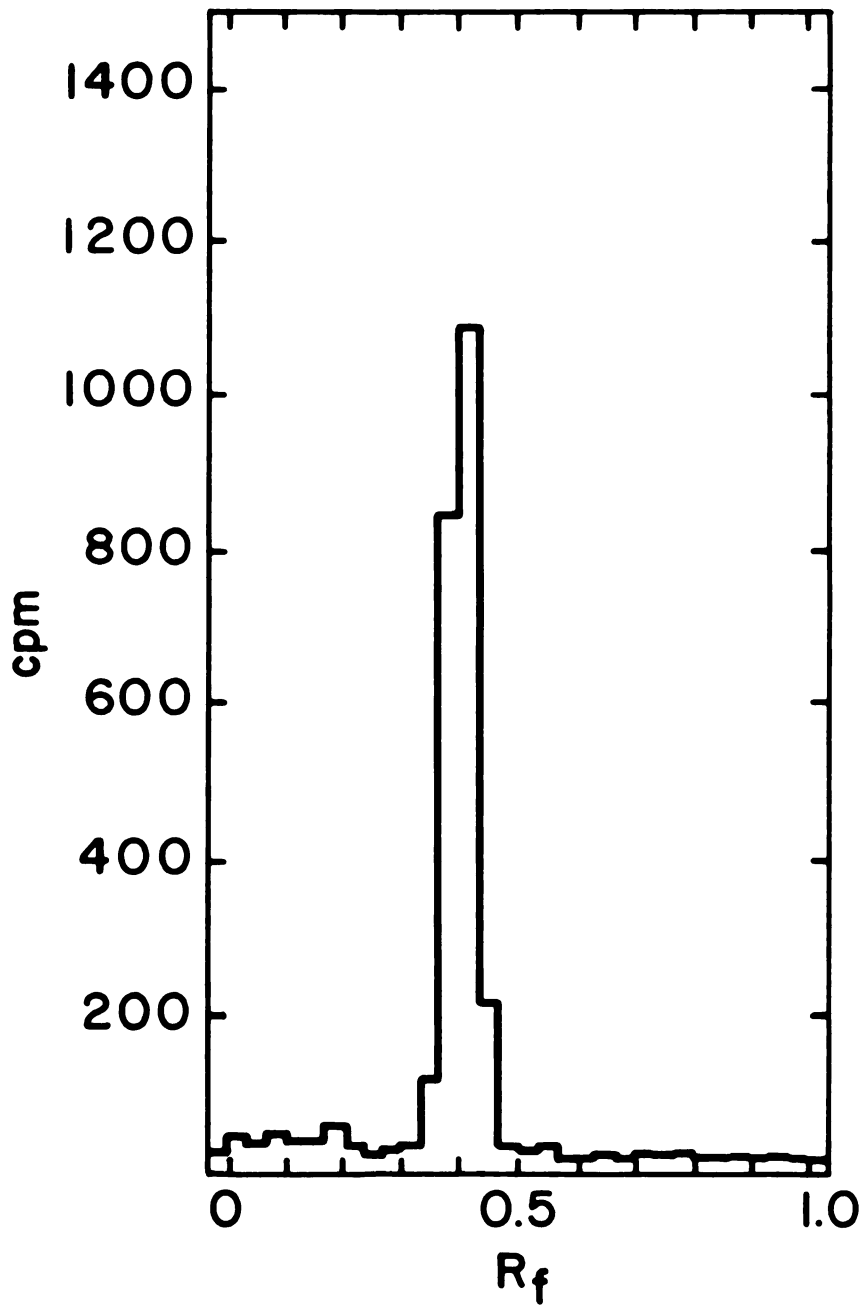
Table 6. Partitioning of radioactivity from leaves fed ^{14}C-ABA.

FRACTION	LIGHT		DARK		ZERO	
	cpm x 10^{-3}	%	cpm x 10^{-3}	%	cpm x 10^{-3}	%
Insoluble residue	0	0	0.1	0.3	0	0
Neutral	0	0	0	0	0	0
Acidic	13.4	88	23.2	87	14.6	98
Hydrolysate[a]	1.0	7	2.6	9.7	0.3	2
Aqueous[b]	0.8	5	0.8	3.0	0	0
Total	15.2		26.7		14.9	

[a]Hydrolysate is that radioactivity which partitioned into ether at pH3.0 after hydrolysis.

[b]Aqueous is that radioactivity remaining in the aqueous phase following all the partitioning.

Figure 12.--Thin-layer chromatogram of an aliquot from the acidic phase extract of primary barley leaves at time zero. The leaves in this experiment were illuminated for 1 hr prior to the addition of ^{14}C-ABA (10^{-5}M final concentration) to the water irrigating the leaves and were extracted 25 minutes after the addition of the radioactive ABA.


1400
1200
1000
800
600
400
200
cpm
0
0.5
1.0
R_f

phase chromatographed like standard ABA. Less radioactivity of the light and dark extractions partitioned into the acidic phase.

Figure 13 shows the chromatogram of the acidic phase from the "light extract." Much of the radioactivity did not move with the major peak. Co-chromatography (Figure 14) showed that the major peak moved with standard cis,trans-(RS)-ABA. Figure 15 shows the chromatogram of the acidic phase from the "dark extract." This is virtually identical with Figure 13 and indicates that the breakdown of ABA occurred at the same rate in either light or darkness. Figure 16 shows that this chromatographic system was capable of separating the ^{14}C-cis,trans-(RS)-ABA from ^{14}C-trans,trans-(RS)-ABA which arose after UV treatment of the former.

The results of the study of the metabolism of ^{14}C-ABA indicate that even after 2 hr, the majority of the radioactivity remains as cis,trans-ABA and no discernible isomerization to trans,trans-ABA occurred in either the light or darkness.

The reversion of the closing response did not take place as readily in leaves that had been treated with high concentrations of ABA. As shown in Figure 4, removal of the ABA supply after treatment with 10^{-5}M ABA did not result in noticeable reversion. It must be concluded therefore that barley leaves have a limited

Figure 13.--Thin-layer chromatogram of an aliquot from the acidic phase extraction from "light" leaves. Such leaves were extracted 2 hr after the 25 min labeling period and had been exposed to continuous light for the entire pre-treatment, labeling and metabolizing periods.

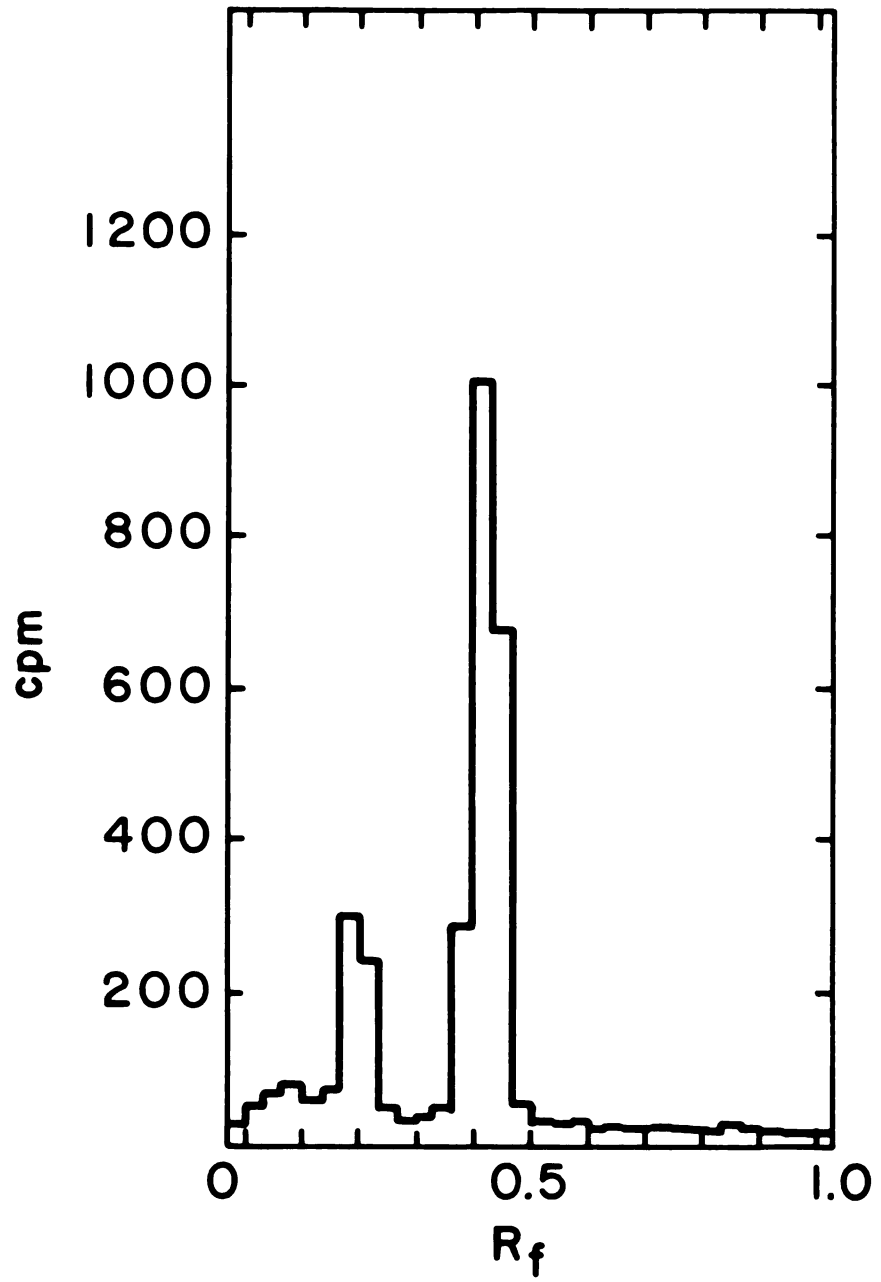

1200
1000
800
600
400
200
cpm
0
0.5
1.0
R_f

Figure 14.--Thin-layer chromatogram of an aliquot from the acidic phase extraction from "light" leaves which was co-chromatographed with an aliquot of a standard solution of radio-labeled ^{14}C-ABA.

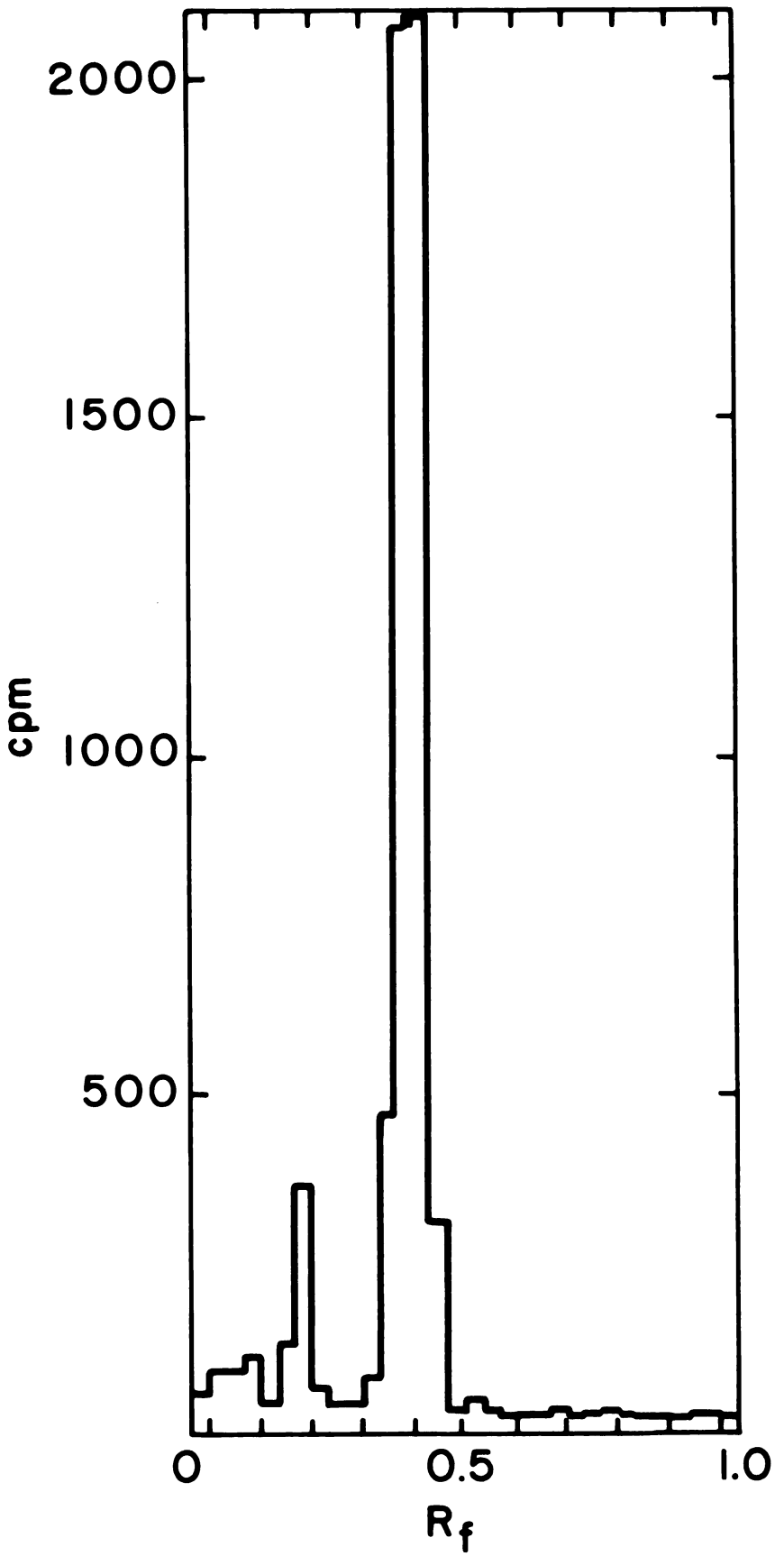

2000
1500
1000
500
cpm
0
0.5
1.0
R_f

Figure 15.--Thin-layer chromatography of an aliquot from the acidic phase extract from "dark" leaves. Such leaves were identical to the so-called "light" leaves except that they were kept in the dark during the two-hour period following the 25 min labeling period and prior to extraction.

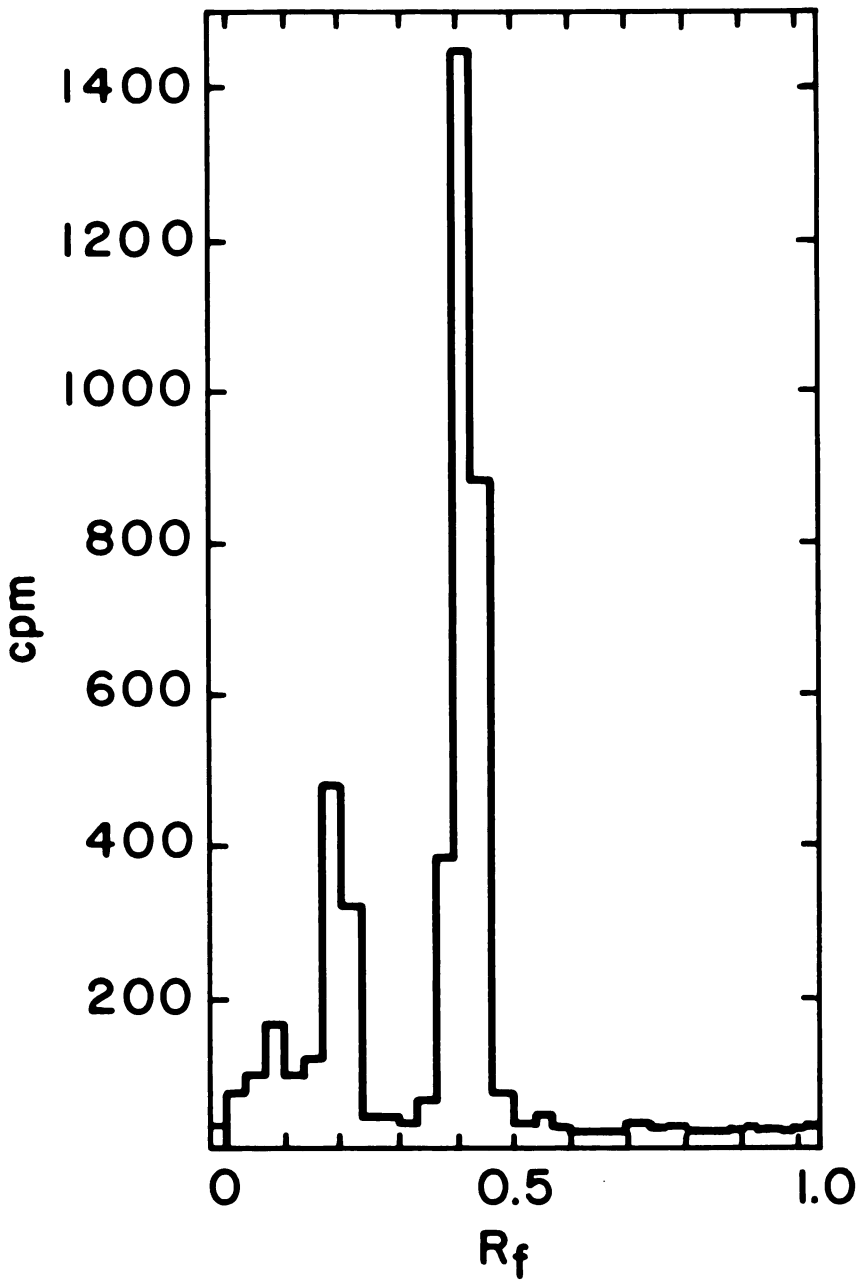

1400
1200
1000
800
600
400
200
cpm
0
0.5
1.0
Rf

Figure 16.--Thin-layer chromatogram of a methanolic solution of ^{14}C-ABA which had been irradiated for 1 minute with UV light. Light source: Mineralight UVS·11 lamp (Ultra-violet Products, Inc., San Gabriel, California) at a distance of 1 cm. The chromatogram was developed sequentially first in the solvent system described in Figure 17, then in the system described in Figure 18. After only 1 min of UV light, a new peak (the R_f value of which corresponds to trans,trans-ABA) emerges ahead of the standard larger peak of cis,trans-ABA. This demonstrates that the solvent system used is capable of separating these two isomers.

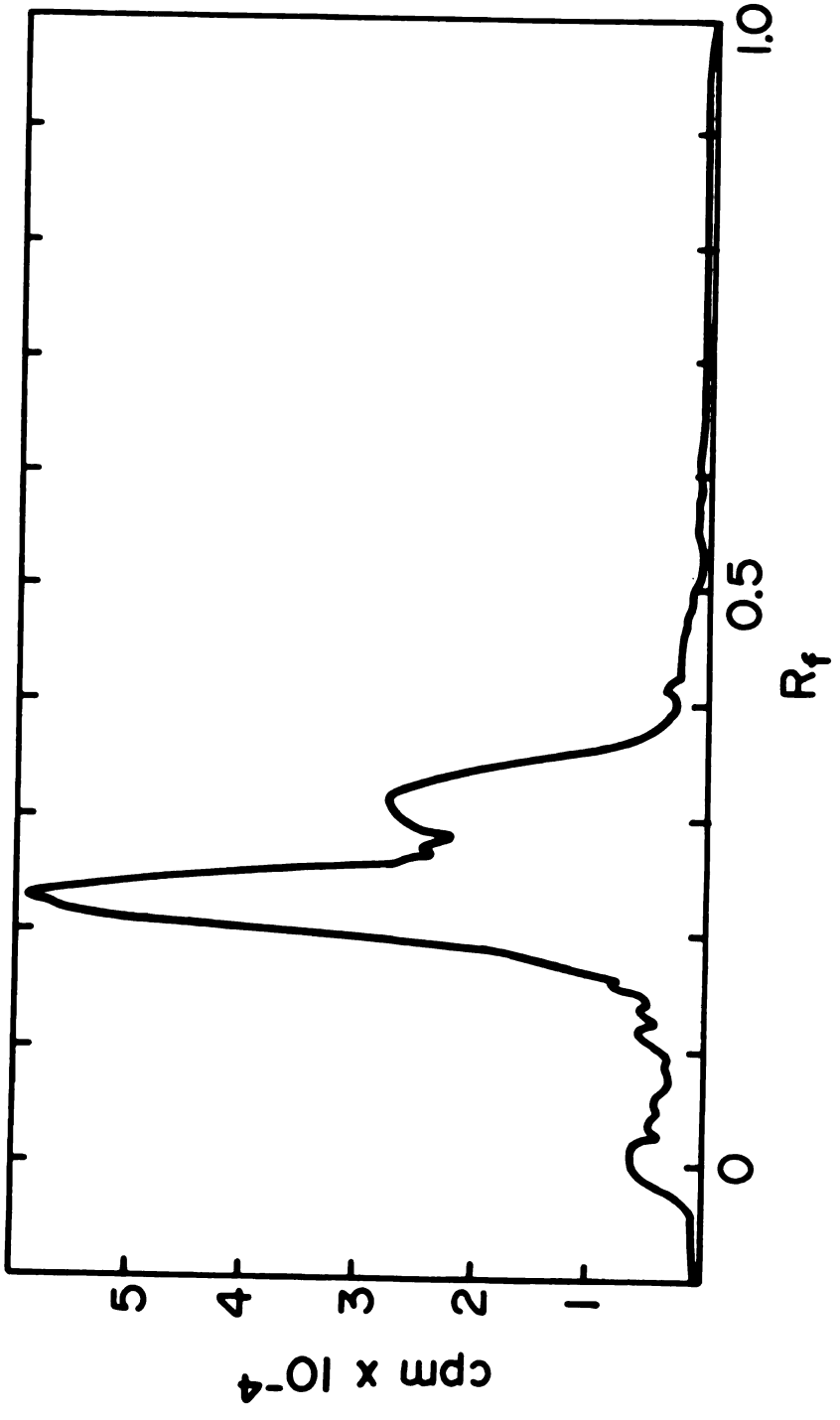

cpm x 10⁻⁴
1
2
3
4
5
0
0.5
1.0
R_f

Figure 17.--Thin-layer chromatogram of a stock methanolic solution of ^{14}C-ABA developed in the initial solvent system used to clear the plates of chlorophyll and other pigments. Solvent system used was hexane-ethyl acetate, 1:1 v/v.

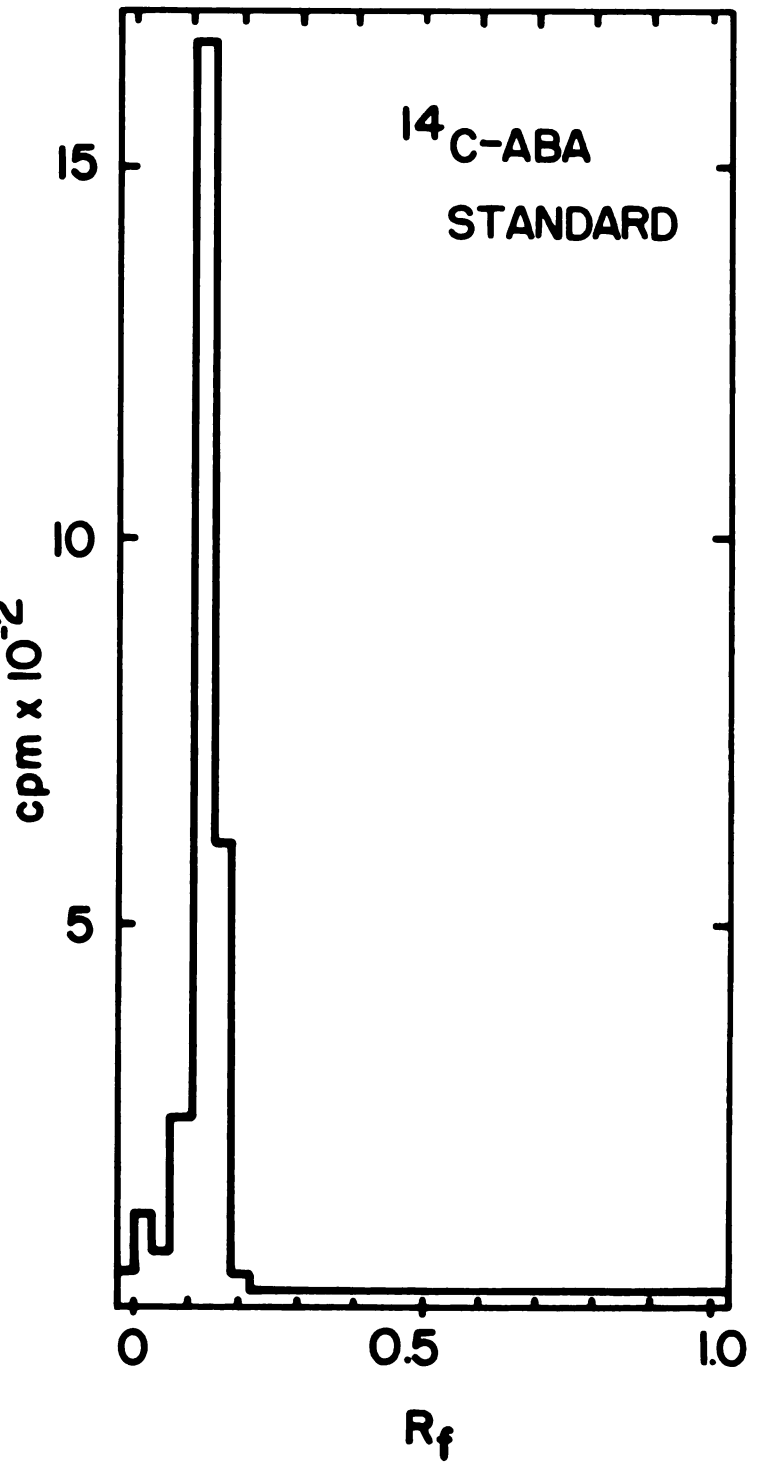
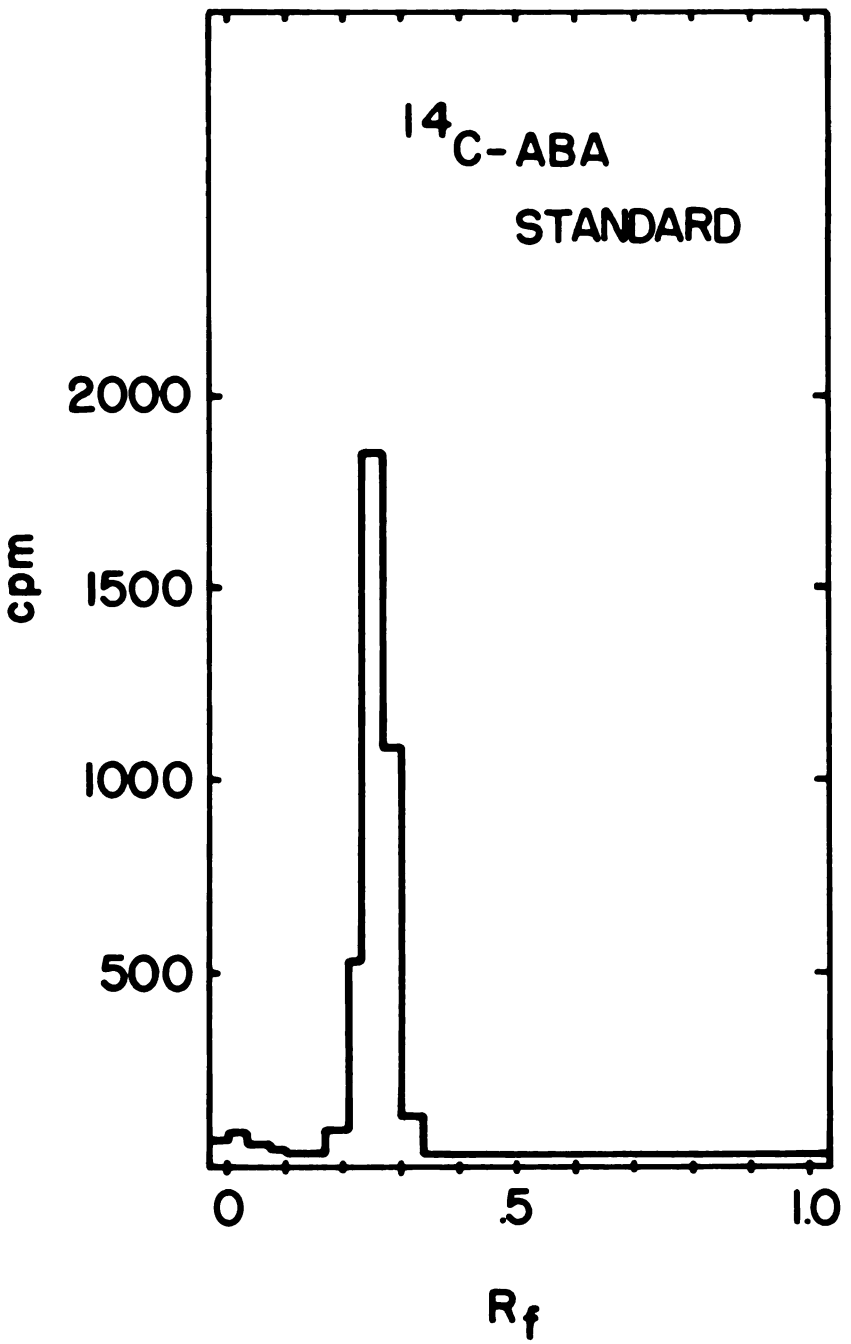

^{14}C-ABA
STANDARD
15
10
5
cpm x 10^{-2}
0
0.5
1.0
R_f

Figure 18.--Thin-layer chromatogram of a methanolic solution of ^{14}C-ABA developed in solvent system: benzene:ethyl acetate acetic acid, 50:5:2 v/v.


^{14}C-ABA
STANDARD
2000
1500
1000
500
cpm
0
.5
1.0
R_f

capacity to sequester ABA into an inactive compartment. This is the simplest hypothesis which can account for the reversion of the short-term ABA response upon removal of the ABA supply. The observed low rate of ABA metabolism cannot entirely be responsible for the reopening of the stomates.

Does ABA Affect Photosynthesis Directly?

Figure 19 shows the change in computed $[CO_2]_i$ and r_{H_2O} after cis,trans-(R,S)-ABA treatment. $[CO_2]_i$ decreased as the stomata closed. This indicates that the photosynthetic CO_2 fixation mechanism remained functional after the stomata started to close. That is, an effective sink for CO_2 remained. On the other hand when DCMU, an inhibitor of photosynthesis, was added to the irrigating solution (Figure 20) the stomata also closed with a comparably short lag. In this case, however, the $[CO_2]_i$ increased during closure. Since it is known that increased external CO_2 concentrations will cause stomatal closure, (Linsbauer, 1916) and since DCMU does not cause closure of stomata on isolated epidermal strips of V. faba leaves, in CO_2-free air (Humble and Hsiao, 1970), one may conclude that DCMU probably causes closure in the whole leaf through changes in internal CO_2 concentration.

Since ABA treatment at 10^{-7}M did not cause $[CO_2]_i$ to rise (it actually fell) one may conclude that ABA probably does not effectively inhibit photosynthetic CO_2

Figure 19.--Changes in r_{H_2O} and $[CO_2]_i$ (in ppm or $\mu l\ l^{-1}$) following addition at time zero of water (control leaf) or of ABA (10^{-7}M final concentration) to the distilled water irrigating primary barley leaves.

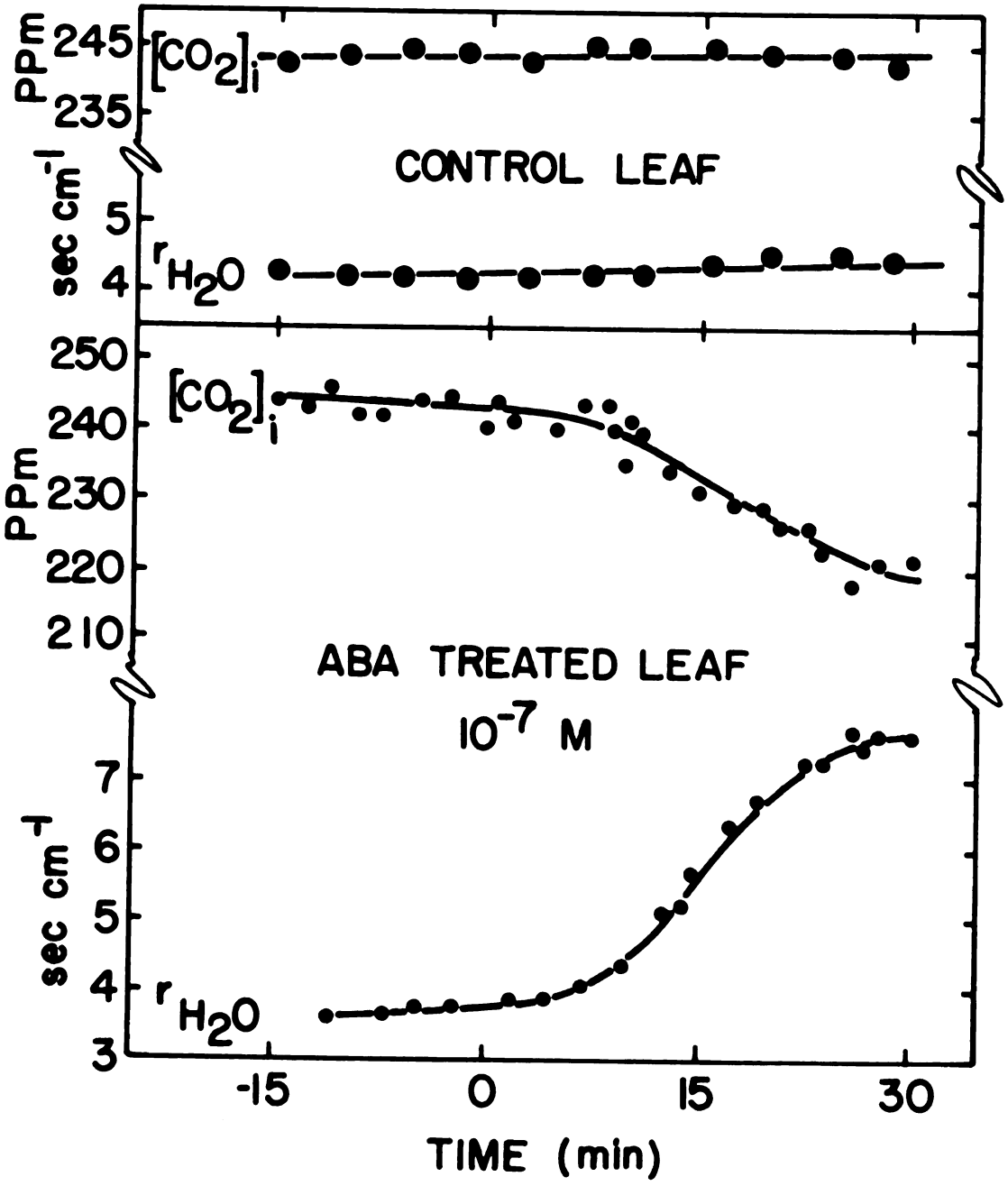
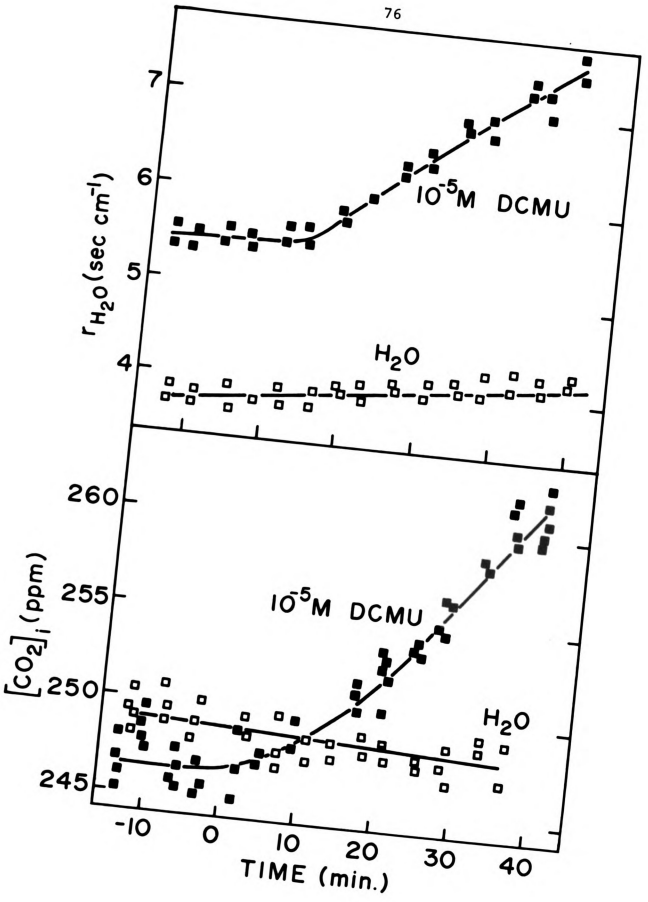

PPm
245
235
$[CO_2]_i$
CONTROL LEAF
sec cm^{-1}
5
4
r_{H_2O}
250
240
230
220
210
$[CO_2]_i$
PPm
ABA TREATED LEAF
10^{-7} M
7
6
5
4
3
sec cm^{-1}
r_{H_2O}
-15
0
15
30
TIME (min)

Figure 20.--Changes in r_{H_2O} and $[CO_2]_i$ (in ppm or $\mu l\ l^{-1}$) following addition at time zero of DCMU (10^{-5}M final concentration) to the distilled water irrigating primary barley leaves. Both the DCMU and the so-called H_2O additions contained equal concentrations of the solvent used to dissolve the DCMU.


r_{H_2O} (sec cm^{-1})
7
6
5
4
10^{-5}M DCMU
H_2O
$[CO_2]_i$ (ppm)
260
255
250
245
10^{-5}M DCMU
H_2O
-10
0
10
20
30
40
TIME (min.)

fixation (at least in mesophyll cells) and therefore ABA most probably acts more directly on the stomatal mechanism. Since ABA does cause closure it is only by limiting the supply of CO_2 to the photosynthetic tissues that it effectively decreases CO_2 fixation.

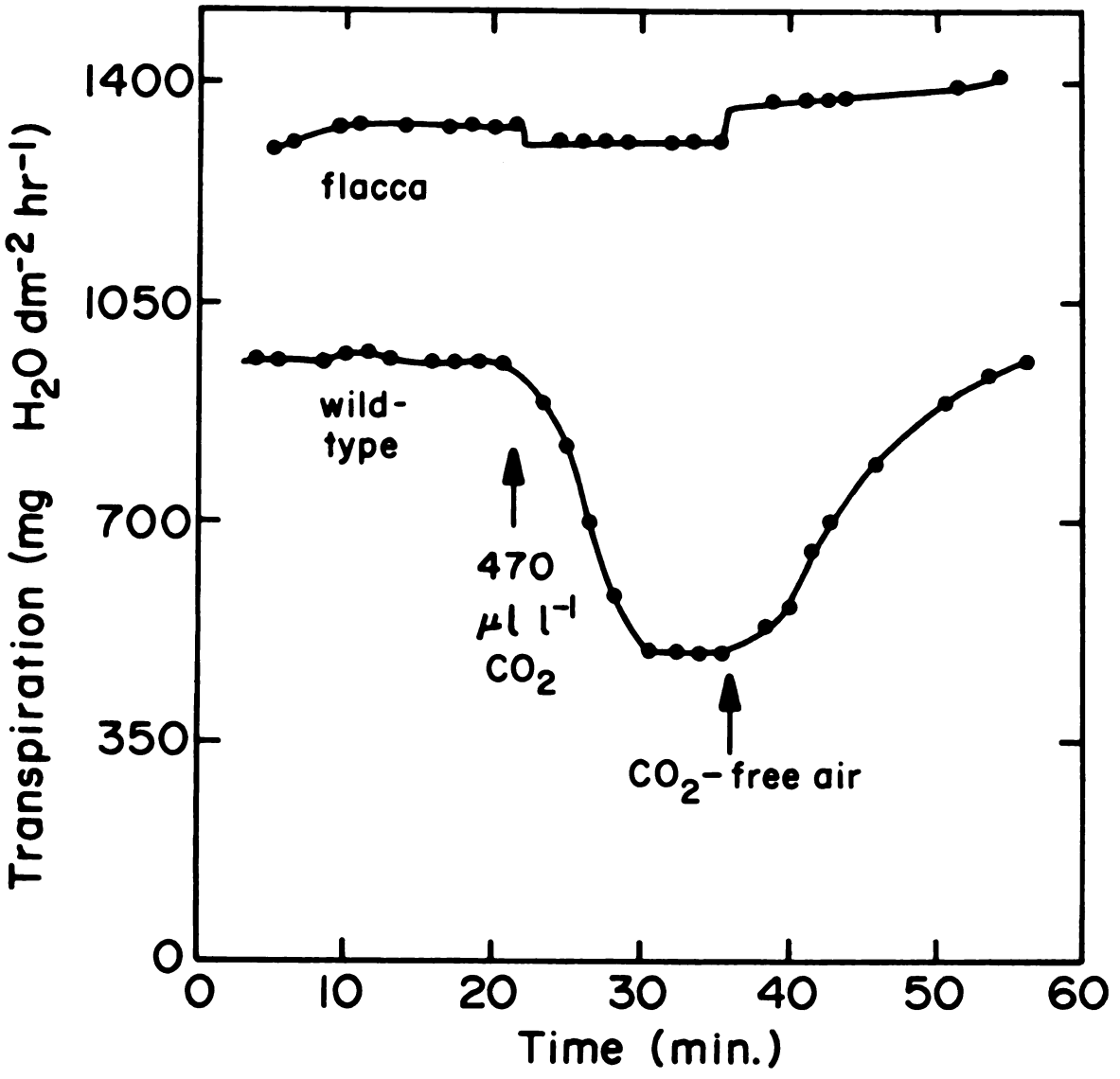
It is interesting to note (Table 5) that ABA treatment caused decreased transpiration with comparable effectiveness in leaves exposed to CO_2-free air as in leaves exposed to air containing 300 $\mu l\ l^{-1}$ CO_2.

Experiments with a Wilty Mutant of Tomato

In an effort to further demonstrate that the ABA-induced closure is independent of changes in CO_2 concentration, several experiments were carried out using *flacca*, a wilty mutant of tomato. The stomates of *flacca* do not respond to many stimuli which cause either closure or opening in the wild-type. Neither darkness, wilting, guard cell plasmolysis, nor treatment with phenyl mercuric acetate cause closure of *flacca* stomates (Tal, 1966). On the other hand, ABA is reported (Imber and Tal, 1970) to cause stomatal closure in excised *flacca* leaves.

Figure 21 shows the changes in transpiration rates when the CO_2 concentration of the air flowing over the leaves was first increased from 270 to 470 $\mu l\ l^{-1}$ and then decreased to 0 $\mu l\ l^{-1}$. Only the wild-type leaves responded. The very slight and abrupt changes in the apparent

Figure 21.--Changes in calculated transpiration rates in excised flacca and wild-type leaves of L. esculentum cv. Rheinlands Ruhm following changes in concentration of CO_2 in the air flowing over the leaves. At 21 min, (first arrow) the CO_2 concentration was increased from 270 $\mu l\ l^{-1}$ to 470 $\mu l\ l^{-1}$. At 36 min, (second arrow) the CO_2 supply was stopped and CO_2-free air flowed over the leaves. Both leaves were of nearly identical size and shape taken from analogous positions on 80-day-old plants. No correction was made for air flow rate changes resulting from changing rate of injection of CO_2 into the CO_2-free air supply.

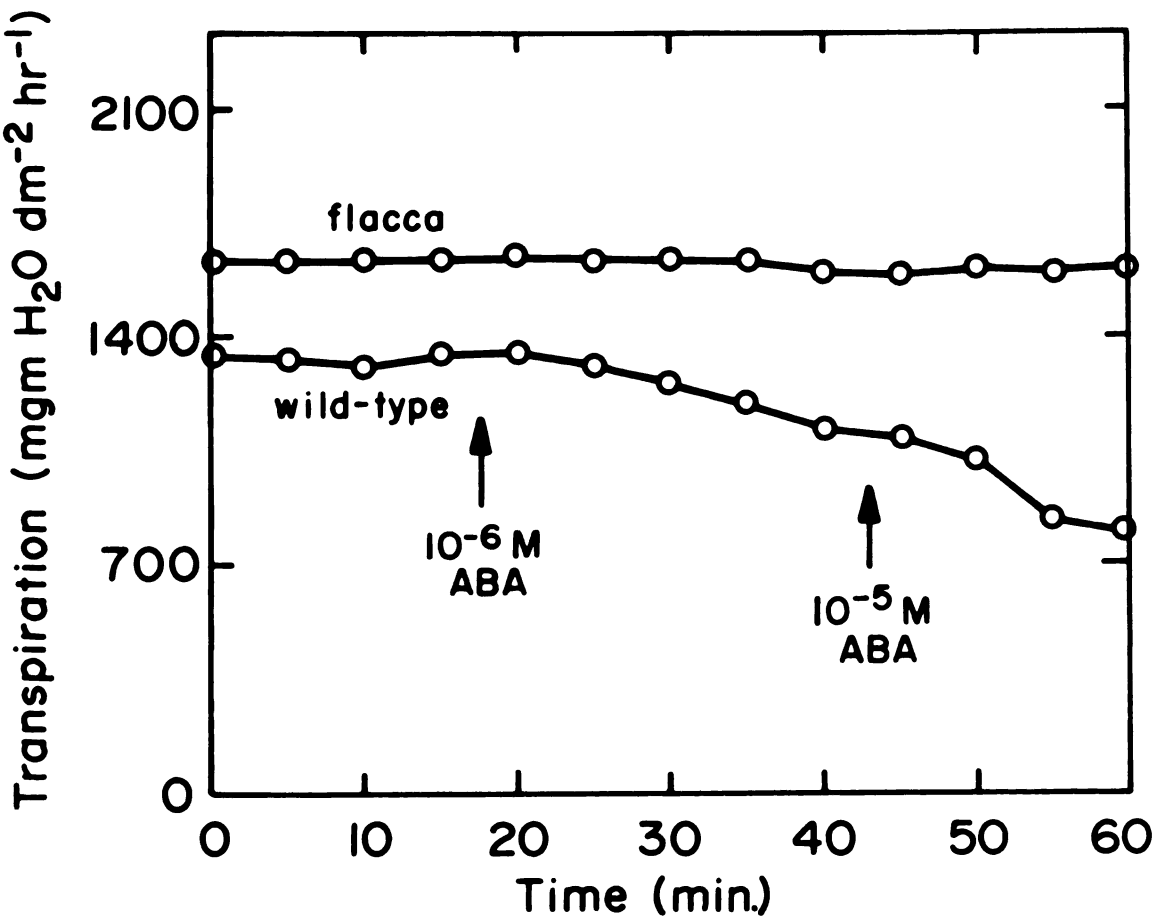

1400
1050
700
350
0
Transpiration (mg H2O dm-2 hr-1)
flacca
wild-
type
470
µl l-1
CO2
CO2-free air
0
10
20
30
40
50
60
Time (min.)

transpiration rate of the flacca leaves arose as an artifact due to the changes in rate of air flow past the leaves when CO_2 containing air was added to or withdrawn from the system. These slight changes in air flow were not taken into account when the transpiration rates were calculated. The lack of response to CO_2 of the flacca leaves is understandable since flacca stomates respond to neither light nor darkness (Tal, 1966).

Having established that CO_2 did not affect stomata of flacca, I then tried to demonstrate a response to ABA treatment. As shown in Figure 22, only the wild-type stomates responded to ABA treatment. The flacca leaves showed no change in transpiration within 40 minutes of treatment with either 10^{-6} or 10^{-5}M ABA.

The reported (Imber and Tal, 1970) closure of stomates in excised flacca leaves was observed only in darkened leaves whereas in experiments reported here both flacca and wild-type leaves were illuminated equally. Imber and Tal do not report on experiments to show stomatal closure in flacca leaves in the light, nor on ABA responses that occur earlier than 24 hours after start of the treatment. It seems that the reported closure of flacca leaves following long-term ABA treatment is a phenomenon quite different from the rapid responses observed with the wild-type leaves.

Figure 22.--Changes in calculated transpiration rates with time for leaves excised from flacca or wild-type tomato, following addition of ABA to the distilled water irrigating the leaves. At 18 min ABA was injected into irrigating water to give a final concentration of 10^{-6}M. At 43 min the ABA concentration was raised to 10^{-5}M. The air supply flowing over each leaf contained 295 μl^{-1} CO_2. Comparable-sized leaves were excised from analogous positions on 80-day-old plants. Transpiration rates were calculated for lower leaf surface only.


Transpiration (mgm H_2O dm^{-2} hr^{-1})
2100
1400
700
0
flacca
wild-type
10^{-6} M ABA
10^{-5} M ABA
0
10
20
30
40
50
60
Time (min.)

These experiments suggest that flacca leaves would be an excellent system to study rapid physiological responses in leaves which are uncoupled from known stomatal controls. Since the guard cells do not respond to plasmolysis (Tal, 1966), flacca may be a morphological mutant with relatively inflexible guard cell walls.

One other very interesting observation was made during these investigations of flacca. ABA treatment at 10^{-6}M or 10^{-5}M did not cause any change in the rate of CO_2 uptake in excised flacca leaves. If the mutation just renders the stomates non-functional, this is quite good proof that ABA does not inhibit photosynthetic CO_2 fixation, and that ABA normally causes stomatal closure by acting directly upon the guard cells.

ABA Action on Stomates in Isolated Epidermal Strips

The response of stomates in epidermal strips from V. faba leaves was investigated in an effort to demonstrate more directly an effect of ABA on the stomatal system. Strips were isolated from the leaves as described in the methods section; they were floated under illumination on buffered solutions containing $CaCl_2$, various concentrations of KCl and were aerated with humidified CO_2-free air. After two hours, when the stomates had opened, some strips were transferred to solutions identical to those on which they had been floated before but containing 10^{-6}M ABA.

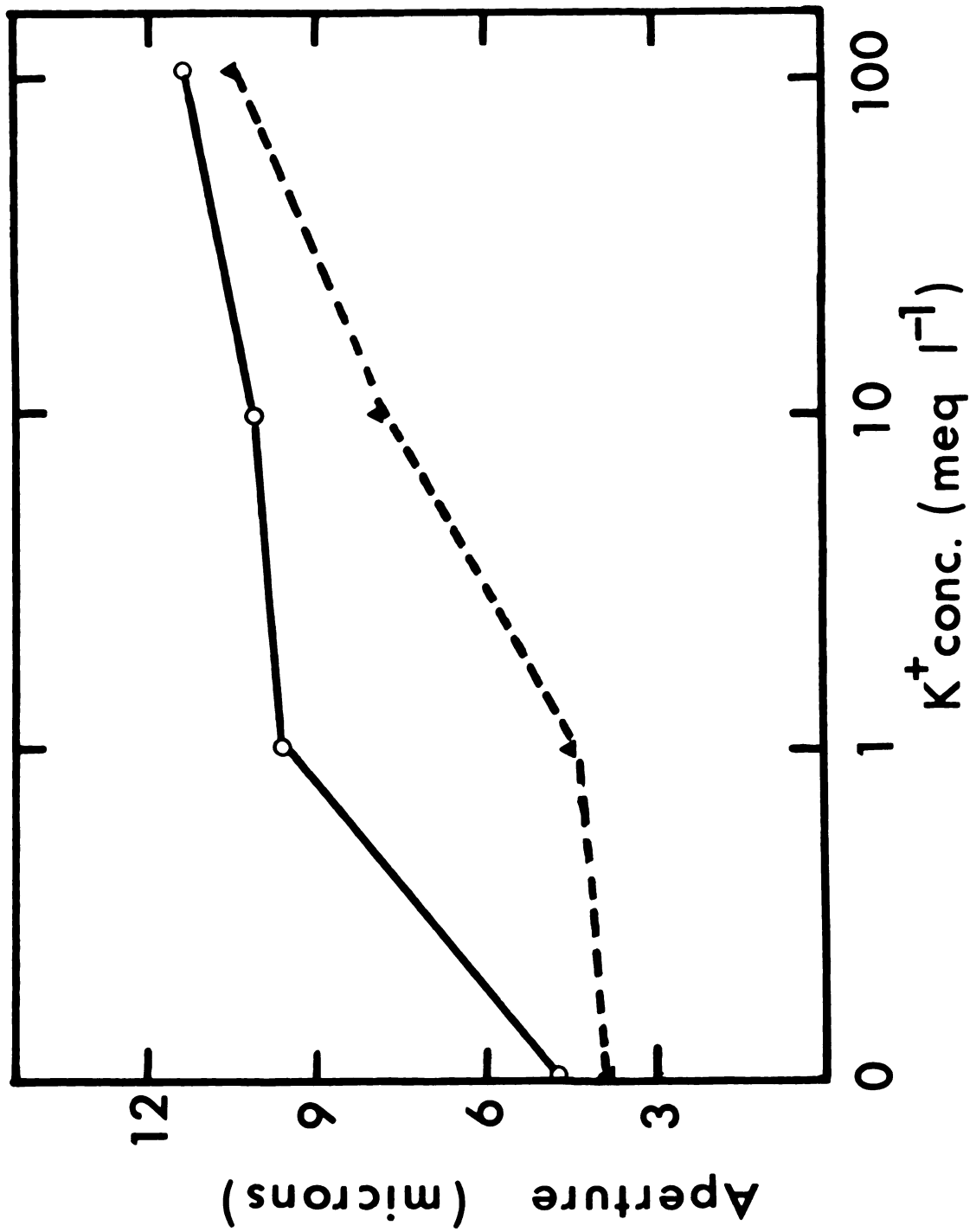
Figure 23 shows the stomatal aperture on such strips after the strips had floated for one hour on ABA solutions.

Apparently stomates that opened over solutions of high K^+ concentration did not close as a result of ABA treatment. Those that opened over solutions of low K^+ concentration (1-10 meq $liter^{-1}$) did close in response to ABA.

Since the strips were prepared in such a manner that at least 80% of the non-stomatal epidermal cells were ruptured, it is reasonable to conclude that ABA causes closure by a direct action on the guard cells and not on the epidermal cells. V. faba leaves were chosen as a source for epidermal strips because they are easily prepared and the system is well described (Humble and Hsiao, 1970). Attempts were made at treating epidermal strips from corn and barley in the same manner. However, the stomates from such strips did not stay open in either ABA-treated or control strips for long enough periods to show any differences in aperture due to ABA treatment.

The V. faba stomatal system is quite different from that of H. vulgare. The former does not have the well defined subsidiary cells of the latter, but does have larger and slower-moving guard cells which are apparently more resistant to the destruction that occurs when the strips are peeled from the leaf. With these points in mind, one cannot immediately transfer conclusions arising

Figure 23.--Stomatal aperture on epidermal strips after they were floated for 3 hours in the light on solutions containing various concentrations of KCl. The solid line indicates aperture on strips floated on solutions without ABA. The broken line indicates aperture of stomates on strips which were exposed to 10^{-6}M ABA during the last hour of the light period.


Aperture (microns)
12
9
6
3
0
1
10
100
K+ conc. (meq l-1)

from epidermal strip studies in V. faba to responses found in intact excised leaves of barley. Nonetheless the analogy is presented.

Stomates on strips floated on solutions of high K^+ concentration open even in the dark (Humble and Hsiao, 1970). Light facilitates opening predominantly at lower K^+ concentrations.

It seems that ABA may reduce the aperture to the same degree that light increases aperture. If this proves to be correct then ABA may be inhibiting the same process which causes opening when activated by light.

DISCUSSION AND CONCLUSIONS

The results have shown that ABA applied to barley leaves causes very rapidly stomatal closure, and that the rapidity of the response is a function of the inverse of the concentration of ABA applied. This is the most rapid response to ABA that is known. Mittelheuser and van Steveninck (1971) have recently shown closure to occur within 10 minutes of application of 3.8×10^{-6}M ABA to barley leaves. Jones and Mansfield (1970) reported closure within 30 minutes after treatment of excised tobacco leaves with 10^{-4}M ABA. Warner and Leopold (1971) have recently shown that treatment of individual pea plants with 10^{-5}M ABA resulted in a decrease in the growth rate which started 5.1 min after treatment.

Loveys *et al.* (1971) have reported results regarding the rapidity and specificity of the ABA-induced stomatal closure in leaves from several species. They also determined that the ABA content needed for closure was of the same order as the endogenous levels.

Such rapid responses to ABA treatment indicate that ABA may be involved in the inhibition of discrete

enzyme systems which are involved in maintaining stomatal aperture. Gene repression by ABA seems to be unlikely since the lag for the ABA-induced response is shorter than 2 min (after transport lag is deducted), and since the typical time for assembly of a complete protein molecule in higher organisms is estimated to be 5 min (Goodwin, 1963; also see discussion by Evans and Ray, 1969).

The lag time might be shortened even more. Since there is a concentration dependence of the lag time, a diffusion process may be involved in the involvement of ABA to its site of action. Possibly this diffusion step could be reduced if leaves were immersed in a solution and the stomates continuously examined microscopically after the addition of ABA to the solution.

Since the response is so rapid, ABA is probably not acting by a general enhancement of senescence such as enhanced degradation of cellular constituents.

There is probably no irreversible damage done to the stomatal system by the ABA treatment since removing the supply of ABA results in a reopening of the stomates. If ABA is the agent responsible for hydroactive closure, this is very significant since leaves do recover from hydroactive closure with time.

The reversal of the closing response upon removal of the ABA supply cannot readily be explained on the basis of metabolism of ABA. In leaves where reversal of

the response was observed after withdrawal of ^{14}C-ABA from the medium, at least 90% of the radioactivity was still associated with unchanged ABA. Some other mechanism, such as compartmentation of ABA must then account for the reversal.

The closure may be reversed by an adaptation of the guard cells to the presence of low concentrations of ABA. The opening may also reflect an action of ABA on the other epidermal cells. If ABA treatment also caused a loss of turgor of the epidermal cells the stomates might partially open by a hydro-passive response. This is not likely, however, since continuing the supply of ABA to the transpiration stream did not result in reopening.

The long-term closure caused by water stress probably reflects a continued production of ABA by the leaf. It is not known when ABA synthesis ceases after a water stress has been relieved.

After 2 hr and 25 min exposure to ABA there was significant metabolism of ABA as indicated by partitioning followed by TLC. Thirteen percent of the radioactivity did not partition into the acidic fraction. Most of this radioactivity was found in the hydrolysate fraction indicating possible esterification of ABA. Of the radioactivity which partitioned into the acidic phase 75% co-chromatographed with ABA. The remainder moved to a Rf characteristic of the hydroxylated ABA (a precursor of

phaseic acid as described by Milborrow, 1970). As judged by partitioning and TLC, approximately 66% of the radioactivity was still present in the form of cis,trans-(RS)-ABA. Esterification of an aliquot of the acidic phase followed by TLC again showed the majority of the radioactivity to move as cis,trans-(RS)-ABA methyl ester. This is quite good evidence that no further metabolism had occurred. No attempt was made to differentiate between the amounts of the (+) and (-)-isomers metabolized. Milborrow (1970) showed that in tomato the (-)-isomer is metabolized more rapidly than the (+)-isomer.

The demonstrable metabolism of ABA within 2.5 hr after its application, suggests that ABA may not be useful as an antitranspirant. It may be metabolized too quickly. However, the use of repeated applications of ABA or of analogs of ABA which show activity but are not as easily catabolized may prove practical.

This work has also shown that the stomatal apparatus responds specifically to cis,trans-(+)-ABA. The response to trans,trans-(RS)-ABA is significantly lower and may even be entirely accounted for by contamination of the preparation with the cis,trans-isomer. This latter point could be demonstrated better if samples with fewer impurities were available. However, it is very difficult to separate the two isomers with greater efficiency except by preparative GLC. It is also possible that some

isomerization occurs in the leaf after treatment with trans,trans-ABA to yield cis,trans-ABA. However, this appears less probable for two reasons:

1. ABA absorbs very little light energy at wave lengths above 300 nm (see UV spectral curve published by Milborrow, 1970). The absorption peak is at 240-260 nm, depending on the degree to which ABA is dissociated. In the experiments described here, the leaves were always shielded with glass filters which blocked the transmission of short wave-length irradiation.

2. In experiments using radioactive cis,trans-(RS)-ABA there was no detectable isomerization after exposing the leaves for 2 hr and 25 min to the labeled preparation either in the light or in darkness, whereas such isomerization was detected after exposing methanolic solutions of the labeled hormone to UV light for 1 minute (Figure 18).

The optical isomer of the naturally occurring cis,trans-(+)-ABA showed very little activity in closing stomates. Only at 0.8×10^{-5}M did it cause a slight change in leaf temperature which started 12 min after treatment. It had no activity at 10^{-6}M. This result was surprising since Milborrow (1968) claimed that both optical isomers were biologically active. It is not clear from

Milborrow's report, however, in which assay system and at which concentration the activity of the cis,trans-(-)-ABA was tested. Sondheimer et al. (1971) claim that the activity of the (-)-isomer was either equal to or less than that of the naturally occurring (+)-form in inhibiting the germination of barley seeds and the growth of roots and shoots. These assays involved 56 hr treatments of the seeds with the (+) and (-)-ABA preparations. It appears then that the short-time inhibition of transpiration is very specific for the (+)-isomer and that the (-)-isomer is much less effective in initiating this fast response. The (-)-isomer possibly acts only during long periods of incubation. The relatively low effectiveness of the ABA analogs in causing stomatal closure indicates that the response is very specific and not caused by a trivial mechanism such as a lowering of the pH of the solution in the transpiration stream. The high degree of specificity of the response to cis,trans-(+)-ABA also means that the response is very likely of physiological significance and not a pharmacological phenomenon. This is especially significant since it is the cis,trans-(+)-ABA which occurs naturally in plants and which is synthesized in leaves in response to water stress.

There must be highly specific receptor sites, probably in (or on) the guard cells which recognize ABA and are involved in translation of the ABA stimulus to give the observed response.

The results also indicate two novel methods for differentiating between direct effects of "anti-transpirant" preparations either on the stomatal apparatus or on the photosynthetic mechanism. The first method involves the determination of changes in the $[CO_2]_i$ after treatment of excised leaves. ABA treatment caused decreased levels of $[CO_2]_i$ with stomatal closure, whereas DCMU treatment caused an increase in $[CO_2]_i$ while the stomates closed. This has been interpreted as meaning that ABA treatment caused the stomates to close without affecting the CO_2-fixing mechanism of the leaf, while DCMU treatment caused closure because it inhibited photosynthetic CO_2-fixation and thereby allowed $[CO_2]_i$ to increase sufficiently to effect closure.

The second method involves the use of the wilty mutant flacca, the stomates of which do not respond to increased CO_2 levels nor to ABA during short-time experiments. By monitoring the CO_2 assimilation rate in the mutant after treatment with a potential "anti-transpirant" preparation, it is possible to monitor effects on the ability of the leaf to assimilate CO_2 in the absence of stomatal movement. ABA treatment did not change the

assimilation rate at all in this mutant. It would be interesting to investigate the effect of photosynthetic inhibitors on flacca. It would also be important to understand why ABA does not cause stomatal closure in flacca in short-term experiments, yet apparently does during extended exposure to ABA (Imber and Tal, 1971).

Mittelheuser and van Steveninck (1970) monitored r_{H_2O} and photosynthetic CO_2 fixation rates in leaves as a function of time following application of ABA. CO_2 fixation was measured by supplying the leaves with air containing $^{14}CO_2$ and determining the amount of radioactivity assimilated. They concluded that closure of stomates in response to ABA treatment preceded a decrease in the rate of CO_2 fixation. These results support the ones described in this thesis insofar as they show that ABA treatment causes closure without inhibiting the photosynthetic mechanism. However, it is very difficult to understand how the results were obtained since closing of the stomates restricts the supply of CO_2 to the photosynthetic mechanism. This should, therefore, cause decreases in the net rate of CO_2 uptake. The interpretation of these results is made difficult by the fact that the determinations of stomatal aperture and CO_2 fixation were carried out on different sets of leaves. Even in the leaves which were not supplied with ABA, the rate of CO_2 fixation varied considerably. It is concluded that the

gas analysis method coupled with $[CO_2]_i$ determinations yields more conclusive evidence concerning the lack of a direct effect of ABA treatment on the photosynthetic apparatus.

The results obtained after ABA treatment of isolated epidermal strips also suggest that ABA acts directly on the guard cells. Horton (1971) and Tucker and Mansfield (1971) have reported similar results showing that ABA inhibits stomatal opening in epidermal strips. Horton (1971) also showed that stomates of ABA-treated strips would open when the strips were transferred to solutions which did not contain ABA. Therefore, the response in strips is also reversible. Since the epidermal cells on such strips are disrupted, it can be concluded that the site of ABA action is localized at the guard cells. Humble and Hsiao (1970) have shown that light activates the influx of potassium into guard cells as the stomates open. Humble and Raschke (1971) demonstrated that the guard cells of open stomates contain a twenty-fold higher content of potassium than guard cells of closed stomates. They also postulated that potassium is the predominant cation involved in the maintenance of turgor pressure of open stomates. Since the ABA response is so fast, it is reasonable to postulate that ABA may be involved in the regulation of potassium transport in guard cells. ABA could cause closure by one of three mechanisms:

1. Inhibiting a potassium pump.
2. Rendering the guard cell membrane(s) more leaky to potassium (or ions in general).
3. Interfering with anion production in the guard cell.

The movement of leaflets in Albizzia have many features in common with stomatal closure. Such movements result from the differential turgor changes in dorsal and ventral pulvinule cells which are attributable to potassium ion fluxes. ABA treatment promotes the closure of excised pulvinules (Satter and Galston, 1971).

ABA treatment (Glinka and Rheinhold, 1971) apparently increases the permeability of carrot root cells to water. This is the only reported direct effect of ABA on membranes. It is hard to see how increased water permeability of guard cell membranes would cause closure, unless this observation reflects a general increase in permeability of the membrane which allowed increased efflux of potassium ions as well as water from the guard cell.

The evidence on the response to ABA treatment suggests that ABA may be involved in the hydroactive closure. It remains to be determined if ABA synthesis occurs rapidly enough to account for this closure in the stressed leaf.

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