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Effects of Phaseic Acid and Dihydrophaseic Acid on Stomata and the Photosynthetic Apparatus[1]

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ABSTRACT

Plant extracts containing phaseic acid (PA), as well as solutions of purified PA and dihydrophaseic acid (DPA) were applied to leaves, isolated mesophyll cells, and isolated epidermal strips. In *Commelina communis*, stomatal closure began 4 minutes after the addition of either 20 micromolar (±)-abscisic acid or 10 micromolar PA. Stomata closed less rapidly after treatment with 10 micromolar PA than after treatment with 10 micromolar (±)-abscisic acid in *Amaranthus powelli*, *Hordeum vulgare*, *Xanthium strumarium*, and *Zea mays* and did not respond at all to PA in *Vicia faba*. DPA (10 micromolar) did not cause stomatal closure in any species.

Plant extracts containing PA reduced photosynthesis, as reported by Kriedemann *et al.* (Aust J Plant Physiol 2: 553-567, 1975). Subsequent experiments with PA purified by crystallization and with residues of solvents employed in the extraction of PA proved that it was not PA that impaired photosynthetic O_2 evolution or CO_2 uptake but unidentified contaminants of the allegedly pure solvents.

Many plants accumulate abscisic acid in response to water stress (8, 10). ABA is metabolized to PA[3] and further to DPA (7), both of which also accumulate in plants during and after water stress (7, 11). Water stress also causes stomatal closure and a decline in the rate of photosynthesis (3, 10, 11). Reduction of photosynthesis can be the consequence of either a reduced supply of CO_2 caused by stomatal closure or the result of reduced photosynthetic capacity of the mesophyll. Exogenous ABA can cause stomatal closure (5, 9, 15), and it is likely that the stomatal closure observed in response to water stress is caused by endogenous ABA. Similarly, Kriedemann *et al.* (11) proposed that endogenous PA causes the reduction of the photosynthetic capacity of plants after periods of water stress; they had found that plant extracts containing PA inhibited photosynthesis and that, following water stress or fruit removal, reduced photosynthetic capacity was correlated with increased levels of PA (11, 12).

We investigated whether stomata responded to PA and DPA, and whether PA could indeed reduce the photosynthetic capacity of plants, as Kriedemann *et al.* (11) proposed. We compared effects of PA with those of ABA on leaves, leaf sections, isolated epidermal strips, and mechanically isolated mesophyll cells. Effects of DPA were studied only on leaves and isolated epidermal strips.

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[3] Abbreviations: PA: phaseic acid; DPA: dihydrophaseic acid; Pipes: piperazine-*N*,*N*'-bis(2-ethanesulfonic acid).

MATERIALS AND METHODS

Plants. *Xanthium strumarium* L. was grown in liter-size plastic pots and *Spinacia oleracea* L. cv. Savoy hybrid 612 in small plastic cups in a gravel soil mixture in a greenhouse. The natural photoperiod was extended to 20 h by supplementary illumination with Sylvania Gro-lux fluorescent tubes of 0.3 w m^{-2} intensity. Temperature maxima were between 23 and 29 C and RH between 70 and 80%. *Amaranthus powelli* S. Wats., *Zea mays* L. cv. Michigan 500, and *Arachis hypogaea* L. were grown in a growth chamber with 27 C day and 20 C night temperature. RH was approximately 70% and peak irradiance was 160 w m^{-2} from General Electric lamps H 400 DX 33-1 (mercury vapor) and LU 400 (high temperature discharge sodium vapor). Day length was 20 h. *Hordeum vulgare* L. cv. Himalaya, *Vicia faba* L. cv. Improved Long Pod, *Brassica oleracea* L., *Brassica campestris* L., and *Commelina communis* L. were grown in a growth chamber with 14-h days, 85% RH, and temperatures of 22/20 C, day/night. Light intensity was 85 w m^{-2} from General Electric cool-white fluorescent tubes.

Gas Analysis. Gas exchange of leaves was monitored by measuring humidification and CO_2 depletion of air passing over 2.44-cm^2 leaf lamina. The flow rate was 50 liters h^{-1} each over the adaxial and abaxial surfaces. Changes in the molar fluxes of H_2O and CO_2 were measured with differential IR gas analyzers (URAS 2, Hartmann and Braun, Frankfurt a.M., Federal Republic of Germany). The temperature of the leaf chamber was kept at 23 C, and the dew point of the air was kept constant at 18 C by passage through a glass condenser. The temperature of the leaf was monitored with a fine copper-constantan thermocouple (part no. SCPSS-020E from Omega Engineering, Inc. Stamford, Conn.) pressed along the abaxial leaf surface for 1 cm or more (to minimize thermal conductivity errors).

We measured three parameters of stomatal closing in response to the various compounds as described by Raschke (16). These parameters are explained in Table I.

The CO_2 concentration in the intercellular spaces of the leaf was calculated using the equation:

$$c_i = c_a - 1.6\ A/g$$

where c_i is the CO_2 concentration in the intercellular space, c_a is the CO_2 concentration in the air passing over the leaf, A is the rate of CO_2 assimilation, g is the conductance for water vapor, and 1.6 is the ratio of the diffusivities of water vapor and CO_2 in air. Relating the rate of CO_2 assimilation and stomatal conductance to c_i gave an indication of whether a reduction in CO_2 assimilation was caused by stomatal closure or by a reduction of the photosynthetic capacity of the mesophyll.

Measurement of Stomatal Aperture in Detached Epidermis. The first and second fully expanded leaves from 3- to 4-week-old *V. faba* and *C. communis* plants were cut into approximately 1-cm^2 pieces and floated abaxial side up on deionized H_2O. Light (85 w m^{-2}, as measured by an Eppley pyranometer with a Corning No. 4600 IR absorbing filter) was provided by two General

Electric mercury vapor lamps (H 400 RDX 33-1) shining through a 5-cm water filter, and humidified CO_2-free air was passed over the strips. After at least 3 h, epidermal strips were peeled from the leaf sections and placed on a piece of Plexiglas to cover a 2-mm-diameter well in the Plexiglas. The epidermal surface that is normally in contact with the mesophyll faced the well. The well was continuously flushed with O_2-saturated 10 mM K-citrate (pH 6.2) at a rate of 0.5 ml min^{-1}. ABA, PA, or DPA was added to the buffer to give 10 μM of the naturally occurring (+)-enantiomer; (±)-ABA was used at 20 μM whereas PA and DPA were presumed to be exclusively the (+)-enantiomer and were therefore supplied at 10 μM. Stomatal apertures before and after addition of the compounds were measured through a microscope to which a television camera was attached.

Isolated Mesophyll Cells of *X. strumarium*. We used a method of rapid mechanical isolation of mesophyll cells originally developed by B. G. Drake while he was at this laboratory. Cells prepared from *Xanthium* plants grown in a greenhouse photosynthesize at a rate one-half to two-thirds of that determined in intact leaves (when rates are determined on the basis of Chl content). For isolation of cells, the midribs of four fully expanded leaves were removed and the remaining leaf parts cut into 2-cm^2 pieces. The leaf pieces were ground in a Waring Blendor for 40–60 s at medium speed in 60 ml of 0.1 M Hepes (pH 7) with 200 mg PVP-40. The slurry was filtered through 16 layers of cheesecloth to catch veins and large aggregations of cells. The suspension was then centrifuged at low speed for 1 min in a clinical centrifuge. The supernatant, which contained chloroplasts and cellular debris, was discarded. The pellet contained primarily whole cells; it was twice resuspended and recentrifuged. For assays at pH 7, 0.1 M Hepes was used for resuspension. For assays at pH 5.6, each resuspension contained more Mes (pH 5.6) and less Hepes (pH 7) (total molarity always equal to 0.1) than the previous medium until the final medium was 0.1 M Mes (pH 5.6). It was necessary to use a grinding medium with a pH of 7 since cells isolated at any other pH are photosynthetically inactive (B. G. Drake, personal communication). One per cent CO_2 in air was bubbled through the final suspension of cells. This suspension was used within 6 h. The photosynthetic activity of the cells generally rose during the first 0.5 h after isolation, then remained constant for the rest of the day.

To determine Chl content, cells were extracted twice with methanol. The methanol was evaporated; the Chl was dissolved in 80% aqueous acetone, and its concentration was determined by the method of Arnon (1).

Measurement of O_2 Evolution. O_2 evolution of isolated cells and leaf sections was followed with a Clark-type O_2 electrode (Yellow Springs Instrument Company, Yellow Springs, Ohio). The electrode was used in a 3-ml cylindrical Plexiglas cuvette which had a Pyrex window to admit light. The light path (depth of cylinder) was 1 cm. The cuvette was illuminated by a tungsten lamp behind a Corning No. 4600 IR-absorbing filter. The light intensity at the window of the cuvette was 450 w m^{-2}. The cuvette was stirred by a 1-cm magnetic stir bar. The temperature was not controlled, but by inserting a thermocouple into the cuvette, we verified that the temperature was constant over the time of an individual assay. The electrode was calibrated daily with air-saturated water and dithionite.

Sources of Chemicals. ABA was obtained from Calbiochem and was used without recrystallization. PA is not available commercially. The method used to extract PA is described below. Since some of the results presented in this paper deal with solvent effects, it is important to list the origins of the major solvents used: ethyl acetate, labeled "distilled in glass," was obtained from Burdick and Jackson, lot nos. 9638 and AA708. Diethyl ether was obtained from Mallinckrodt, lot BVR, and Fisher Scientific, lot no. 764424.

Preparation of PA. PA was extracted from *P. vulgaris* plants that had been fed commercial (±)-ABA, and from mature *P. vulgaris* beans in a manner similar to that of Zeevaart and Milborrow (18). Plants were cut off at the ground level and the stems were put into a solution of ABA (between 1 and 10 mM). The plants took up all of the solution within 3 h and were then placed into distilled H_2O for 3 days. They were then ground in methanol, to which 0.5 M phosphate buffer (pH 8.2) was added. The methanol was evaporated *in vacuo* at 35 C. The buffer was extracted once with ethyl acetate, then the pH was lowered to 2.5 with HCl, and the buffer was extracted three times with ethyl acetate. After evaporating the ethyl acetate, the extract was chromatographed on thin layer (0.3-mm) silica gel plates. Two solvent systems were used: toluene-ethyl acetate-acetic acid (50:30:4, v/v/v), and 1-butanol-1-propanol-ammonium hydroxide (58%) (20:60:30, v/v/v). The plates were developed at least twice in the acid solvent system. The amount of extracted PA was determined spectrophotometrically using the extinction coefficient of 16,900 at 263 nm (7). Five per cent of the applied ABA was recovered as PA. The PA extract was used directly in some of the early assays; it is identified in other parts of this paper as the "plant extract containing PA."

DPA and additional PA were extracted from 7 kg ground navy beans (*P. vulgaris*). A methanolic slurry of part of the meal was poured into a glass cylinder (120 cm long, 4.7-cm diameter), and was extracted with 3 liters of methanol. This was repeated until all of the bean meal was extracted. Phosphate buffer (0.2 M, pH 8.2) was added to the methanol; the methanol was then evaporated at 35 C *in vacuo*. The residual buffer was washed with petroleum ether and acidified with HCl to pH 2 to 3. An extraction with four changes of ethyl acetate followed. PA and DPA were then back-extracted with phosphate buffer of pH 8.2. The pH was again lowered to 2 to 3 and then the solution was loaded onto charcoal columns (three columns of 3 g charcoal and 6 g Celite each). PA and DPA were eluted from these columns with 150 ml 55% acetone. After the acetone was evaporated, the pH was adjusted to 2 to 3, and the residue was extracted three times with ethyl acetate. After concentration by evaporation, the extract was placed on 2-mm silica gel plates. The plates were developed in toluene-ethyl acetate-acetic acid (50:30:4, v/v/v) after which the DPA was pure enough to be crystallized. The PA was rechromatographed in hexanes-isopropyl alcohol-acetic acid, (80:20:4, v/v/v). Authentic PA and DPA for co-chromatography were supplied by J. A. D. Zeevaart, MSU-DOE Plant Research Laboratory; PA and DPA were crystallized from ethyl acetate-heptane solutions. The liquor from the DPA crystallization was treated with CrO_3 in pyridine, then added to the PA crystallization solution to increase the yield of PA. The identity of the crystallized PA and DPA was confirmed by direct probe MS with Varian CH 5 (double focusing) and Hewlett-Packard 5985 instruments.

RESULTS

Effects of PA and DPA in Detached Leaves on Stomatal Conductance for Water Vapor. Table I shows data obtained in early experiments with PA given to us by J. A. D. Zeevaart. Both PA and ABA were applied at 10 mM. The PA used in these experiments was presumed to be the naturally occurring (+)-enantiomer; the ABA used was composed of both the (+) and (−)-enantiomers. Therefore, the concentration of naturally occurring (+)-ABA was one-half that of the PA. The magnitude of stomatal responses to PA covered a wide range. In *C. communis*, the relative slope of closing in response to PA was nearly as steep as that in response to ABA. In *Z. mays*, the relative slope was less than 1% of that caused by ABA. Table II contains data obtained with purified PA and DPA the identity of which had been confirmed by MS. The wide range of stomatal sensitivity to PA was again observed. Stomata of *C. communis* responded to PA, stomata of the other two species responded only slightly; DPA

Table I. *Three Parameters of Stomatal Closing in Response to (±)-ABA and PA*

Values were calculated from the time course of the total conductance for water vapor (upper plus lower epidermis) of one leaf per treatment. The CO_2 concentration in the air was 320 $\mu l\ l^{-1}$, light intensity was 300 w m^{-2}.

Species	Compound 10 μM	Delay[a]	Relative Slope[b]	Relative Final Conductance[c]
		min	*10^{-3} min*	%
Amaranthus powelli	ABA	6	65	47
	PA	18	10	61
Commelina communis	ABA	5	102	11
	PA	4	95	37
Hordeum vulgare	ABA	6	172	15
	PA	39	5	80
Zea mays	ABA	4	1890	48
	PA	15	12	91

[a] The time between application of the compound and a reduction of conductance by 5% of the conductance at the time of application of the compound.

[b] The steepest decline of conductance divided by the conductance at the time of application of the compound.

[c] One hundred times the conductance measured 60 min after application of the compound divided by the conductance at the time of application.

Table II. *Three Parameters of Stomatal Closing in Response to 20 μM (±)-ABA, 10 μM PA, and DPA*

PA and DPA were purified by crystallization. Values were calculated from the time course of the total conductance of one leaf. Explanation of the parameters is given in Table I. The CO_2 concentration was 320 $\mu l\ l^{-1}$, light intensity was 270 w m^{-2} for *Xanthium* and 140 w m^{-2} for *Commelina* and *Vicia*.

Species	Compound 20 μM	Delay	Relative Slope	Relative Final Conductance
		min	*10^{-3} min^{-1}*	%
Commelina communis	ABA	4	37	20
	PA	4	31	79
	DPA	9	1.2	84
	Control	16	1.7	83
Vicia faba	ABA	10	27	43
	PA	>60	0	102
	DPA	>60	0	100
	Control	stomata oscillated		
Xanthium strumarium	ABA	12	46	29
	PA	46	4	83
	DPA	>60	0	97
	Control	>60	0	99

had no effect on stomata in any of the three species tested.

Effect of PA and DPA on Stomata in Isolated Epidermis. Xanthoxin, an analog of ABA, caused a reduction in gas exchange when fed to a leaf via the petiole, but did not cause stomatal closure when it was applied to an isolated epidermis (17). We felt that it was necessary to test whether stomata required the presence of mesophyll to be able to respond to PA as was the case with xanthoxin. Fortunately, *C. communis*, the species most responsive to PA, and *V. faba*, the species least responsive to PA, possess epidermes that can be peeled easily from the mesophyll. Stomata of *C. communis* became narrower after contact with solutions of ABA and PA, while stomata of *V. faba* responded only to ABA (Fig. 1). DPA did not elicit stomatal responses in isolated epidermal samples of either species.

Effect of Plant Extract Containing PA on Photosynthesis. Rates of photosynthetic O_2 evolution of leaf sections from eight species were determined with an O_2 electrode (Table III). ABA did not inhibit photosynthesis, except possibly in spinach, but the plant extract containing PA suppressed photosynthetic O_2 evolution completely or almost completely in all species tested.

Further experiments were conducted with isolated mesophyll cells of *X. strumarium*. Photosynthetic O_2 evolution from these cells was not inhibited by the extracts as long as the suspension medium was buffered at pH 7. However, the extracts were effective in stopping O_2 evolution when the pH of the cell suspension was adjusted to 5.6. All further tests were therefore made at this pH.

The Mehler reaction was employed to determine whether photosynthetic electron transport or the carbon metabolism of the cells was inhibited. When KCN is present to block carboxylation of RuBP as well as any enzyme-catalyzed splitting of H_2O_2, methyl viologen added to the cell suspension will catalyze the transfer of electrons from the high energy acceptor of PSI to molecular O_2, ultimately producing H_2O_2. Since two electrons (derived from 1 molecule of H_2O) are used to make 1 molecule of H_2O_2, there is a net uptake of one-half O_2 per pair of electrons transported instead of the normal evolution of one-half O_2 per pair. This light-dependent uptake of O_2 is called the Mehler reaction. Table IV shows that a plant extract containing PA did not reduce the rate of the Mehler reaction in isolated mesophyll cells. Kriedemann *et al.* (11) reported that 1.8 μM PA reduced the photosynthetic electron transport rate in spinach chloroplasts to 40% of the control rate. When we performed a similar experiment (using an extract containing PA at 100 μM) we found the photosynthetic electron transport rate of spinach chloroplasts reduced to only 70% of the control rate.

When the same extract was fed to leaves of *X. strumarium* through the transpiration stream, the rate of CO_2 assimilation was reduced from 23 to 18 mg CO_2 dm^{-2} h^{-1} within 1 h when the concentration of PA was 0.1 mM. This reduction of assimilation is much smaller than that reported by Kriedemann *et al.* (11) brought about by 75 μM PA in grape leaves, where the rate of CO_2 assimilation was reduced to less than one-half of the original rate.

Effects of Purified PA and DPA on Photosynthesis. When the experiments described so far were repeated with PA purified by crystallization, neither photosynthetic O_2 evolution from mesophyll cells nor photosynthetic CO_2 uptake by detached leaves showed signs of inhibition. After addition of pure PA to the cell suspension the O_2 trace had the same slope as in the control treatment (Fig. 2), but O_2 evolution stopped within 3 min if the same concentration of PA was established in the medium by the addition of the plant extract containing PA. A concentration as high as 0.3 mM of pure PA did not reduce photosynthetic O_2 production.

The absence of direct inhibitory effects of ABA, PA, and DPA on the photosynthetic apparatus in whole leaves is demonstrated by Table V. Although treatment with ABA lowered rates of CO_2 uptake, the simultaneous occurrence of reduced levels of intercellular CO_2 concentration indicates that reduced CO_2 supply (an effect of stomatal closure) and not an impairment of the photosynthetic apparatus caused the reduction. Dubbe *et al.* (6) determined that the relationship between CO_2 assimilation and intercellular CO_2 concentration was unaffected by the presence of ABA in leaves of *X. strumarium* and other species.

Effect of Solvent Residues on Photosynthesis. We suspected that the inhibitor of photosynthesis was not a plant product but a contaminant of the materials used in the extraction and separation procedure for PA. Although we used only redistilled solvents, they were evaporated *in vacuo* during the extraction of PA and not at

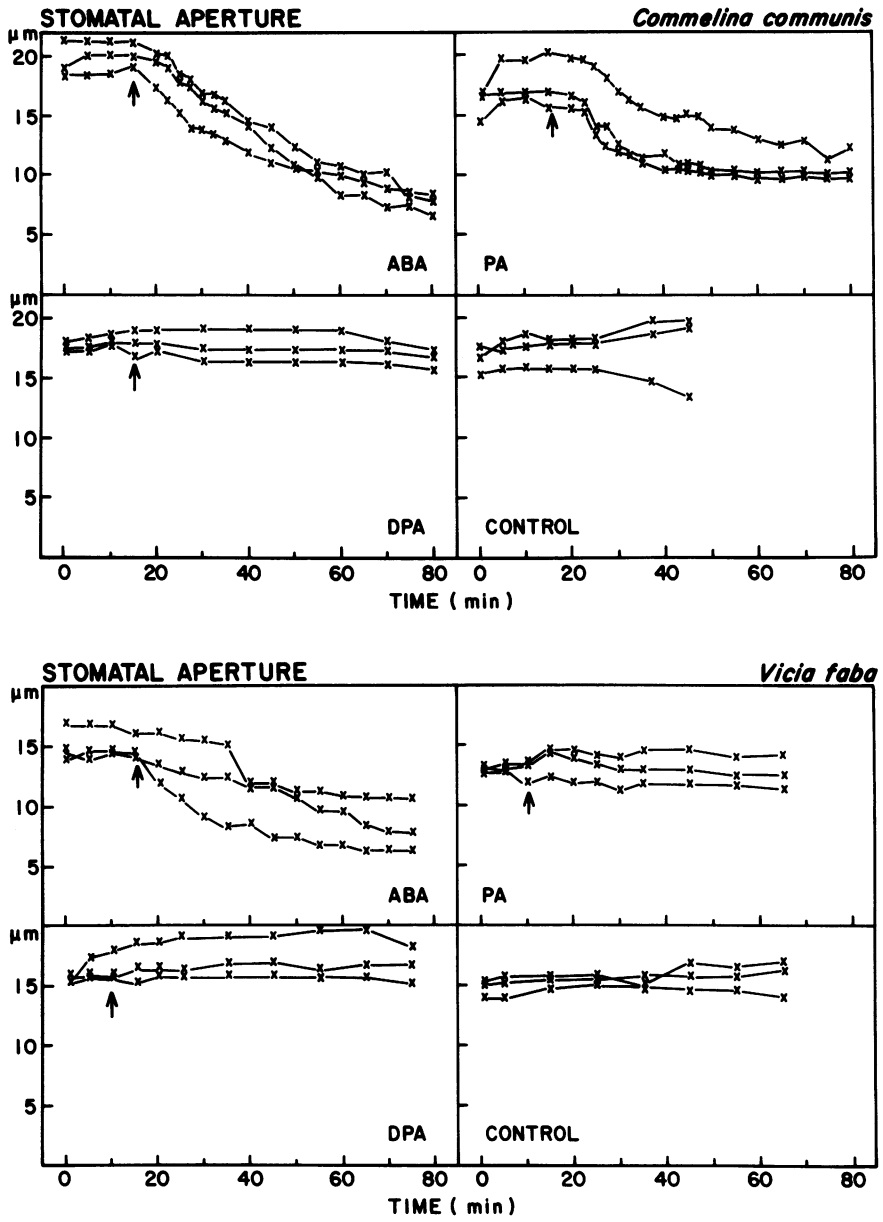

FIG. 1. Time courses of stomatal responses to ABA, PA, and DPA in epidermal strips of *C. communis* and *V. faba*, bathed at a rate of 0.5 ml min^{-1} with 10 mM K-citrate (pH 6.2). Each line represents behavior of one stoma. Control time courses were measured on each day, but only the time courses of the 1st day of each series of experiments are presented. (±)-ABA was 20 μM (to give 10 μM (+)-ABA), PA and DPA (both purified by crystallization) were 10 μM. Arrow indicates time at which the substances came into contact with the epidermal strip.

ambient pressure. Compounds may have remained in the residue which under normal pressure (and elevated temperature) would have been part of the distillate. One hundred ml each of six solvents commonly used for extraction and purification of PA were evaporated *in vacuo*. Each residue was added to a suspension of mesophyll cells of *X. strumarium* and assayed at two pH values for effects on photosynthesis. At pH 5.6, only the methanol residue was found to be free from substances inhibiting photosynthesis; at pH 7, residues from acetone and hexanes were inhibitory (Table VI). Since plant extracts containing PA reduced photosynthesis at pH 5.6 and not at pH 7, acetone or the hexanes could not have been sources of the inhibitor in the extract; but chloroform, ether, and ethyl acetate were possible candidates. Inasmuch as we had used large amounts of ethyl acetate in our extraction procedure we tested the residue of this solvent further. Besides being inhibitory at pH 5.6 and not at pH 7, the residue of ethyl acetate hardly reduced photosynthetic electron transport (from water to benzyl quinone, in experiments analogous to the one described in Table IV). Commercial ethyl acetate "Distilled in Glass" and ethyl acetate freshly distilled in our laboratory contained a substance (or substances) inhibiting photosynthetic O_2 evolution from isolated mesophyll cells (Fig. 3) but not affecting photosynthetic uptake of CO_2 by detached leaves (Fig. 4). The same observations were made with our extract containing PA. The observations made by Kriedemann *et al.* (11) were different. In addition to determining that their extract containing PA inhibited photosynthetic electron transport they found that their extract inhibited CO_2 assimilation by leaves. We suspected that the discrepancy between their and our observations was caused by the fact that Kriedemann *et al.* (13) used ether for the same purpose we used ethyl acetate. The residue of ether does inhibit uptake of CO_2 by detached leaves while the residue of ethyl acetate does not (Fig. 4).

Phthalates as Inhibitors of Photosynthesis. Martin *et al.* (14)

Table III. *Rates of O_2 Evolution from 10 4-mm² Leaf Sections in the presence of ABA or a Plant Extract Containing PA*

The compounds were added to 2 ml 50 mM Pipes (pH 5.6), in which the leaf sections were floating, followed by vacuum infiltration of the tissue. After incubation of the leaf sections for 1 h or longer they were transferred to the O_2-electrode cuvette which contained O_2-free distilled H_2O. Photosynthesis was initiated by adding bicarbonate (final concentration 8.3 mM).

Species	O_2 Evolution		
	Control	(±)-ABA 50 μM	PA
	nmol min⁻¹		
Arachis hypogaea	20	21	6
Vicia faba	15	13	0
Brassica campestris	22	23	0
Brassica oleracea	26	28	0
Spinacia oleracea	38	23	0
Amaranthus powelli	15	13	0
Xanthium strumarium	32	33	2
Commelina communis	14	14	0

Table IV. *Effect of Plant Extract Containing PA on the Mehler Reaction of Isolated Mesophyll Cells of Xanthium strumarium*

	O_2 evolution
	μmol O_2 mg Chl⁻¹ h⁻¹
Sequence 1	
Cells bubbled with 1% CO_2 in air	32
↓	
KCN added (to give 3 mM)	0
↓	
MV added (to give 0.1 mM)	−15
↓	
DCMU added (to give 15 μM)	0
Sequence 2	
Cells bubbled with 1% CO_2 in air	31
↓	
KCN added (to give 3 mM)	0
↓	
MV added (to give 30 μM)	−11
↓	
PA added (to give 30 μM)	−9
Sequence 3	
Cells bubbled with 1% CO_2 in air	36
↓	
PA added (to give 50 μM)	0
↓	
KCN added (to give 3 mM)	0
↓	
MV added (to give 30 μM)	−10

identified some of the compounds that often contaminate solvents and TLC plates. Among them were esters of phthalic acid. We tested a series of them for their ability to inhibit photosynthesis. At 50 μM, dibutyl phthalate and dimethyl phthalate inhibited photosynthetic O_2 evolution of isolated mesophyll cells completely. However, the inhibition of photosynthesis caused by dibutyl phthalate was not pH-dependent, whereas the inhibition of photosynthesis caused by the solvent residues depended on pH. When added to the transpiration stream of detached leaves, dibutyl phthalate did not reduce the photosynthetic capacity. Although the inhibitory effect of solvent residues on O_2 evolution could have been caused by phthalates we did not find inhibitory amounts of phthalate esters in the ethyl acetate residue.

DISCUSSION

Effects on Stomata. PA can cause stomatal closure but its effectiveness in closing stomata varies from species to species. The effect does not require the presence of mesophyll tissue, as in the case of stomatal closure in response to xanthoxin (17); it occurs in isolated epidermis (Fig. 1). Possibly, the ABA receptors in guard cells can also bind PA, although with varying affinity, depending

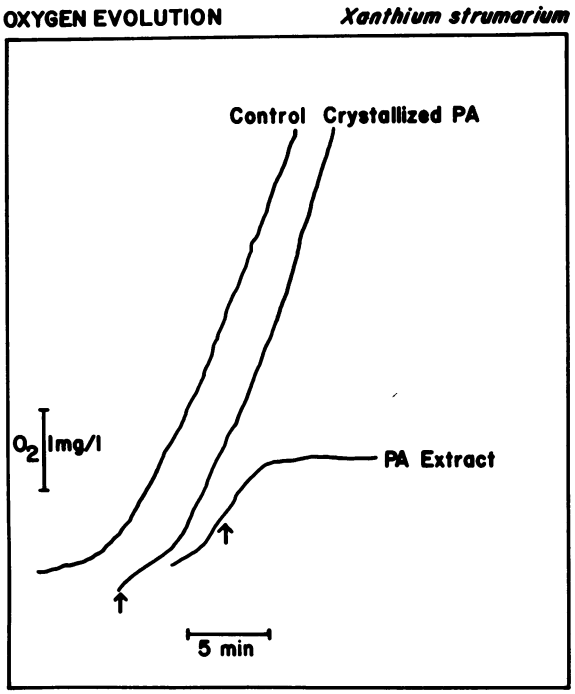

FIG. 2. O_2 evolution from mesophyll cells in the presence of plant extract containing PA and of a solution of PA purified by crystallization. Final concentration was in each case 50 μM. The suspension buffer was 0.1 M Mes (pH 5.6); control rate of O_2 evolution was 34 μmol O_2 mg Chl^{-1} h^{-1}. Arrows indicate time of addition of PA.

Table V. *Rates of CO_2 Assimilation and Intercellular CO_2 Concentrations of Leaves after Applications of ABA, PA, and DPA through the Transpiration Stream (Purified PA and DPA Were Used)*

Final values were measured 60 min after addition of (±)-ABA, PA, or DPA. Stomatal conductance values for this experiment are given in Table II.

Species	Compound 10 μM	Assimilation		Intercellular CO_2	
		Initial	Final	Initial	Final
		μmol m^{-1} s		*μl l^{-1}*	
Commelina communis	ABA	9.8	5.2	231	90
	PA	11.4	11.1	243	213
	DPA	10.7	11.2	250	223
	Control[a]	17.7	17.2	254	245
Vicia faba	ABA	8.6	6.9	229	102
	PA	9.6	9.3	245	246
	DPA	8.0	9.7	239	228
	Control[a]	14.9	15.6	242	253
Xanthium strumarium	ABA	19.8	14.7	260	165
	PA	22.1	20.1	263	246
	DPA	20.1	19.5	258	250
	Control[a]	24.1	23.4	257	252

[a] The rates of CO_2 assimilation of the controls are spuriously high because of a leak in the leaf chamber used for the control leaves.

on plant species. The magnitude of stomatal responses to PA may indicate relatively high affinity in *C. communis* and low or no affinity in *X. strumarium* and *V. faba* (Fig. 1 and Table II). Alternatively, the ABA receptor could have equal affinity for PA in all species, but some species metabolize PA much more quickly than others. The degree of stomatal closure in response to PA will depend on the amount of PA remaining in the tissue.

DPA did not cause stomatal closure in any of the three species tested. The keto function at the 4'-position of ABA and PA appears to be essential for causing a stomatal response. Xanthoxin has an hydroxyl group at the 4'-position and does cause stomatal closure. However, xanthoxin becomes effective on stomata only after passage through petiole and mesophyll of detached leaves where it is probably converted into ABA (17).

Beardsell and Cohen (2) reported that after temporary water stress, stomata of *Z. mays* were not able to reopen immediately after the ABA content of the leaves had returned to the prestress level. Therefore, doubts arise whether stomatal behavior after periods of stress is linked to the amount of ABA in the tissue. The role of ABA as a messenger of stress could be seen as one of initiating stomatal closure while the maintenance of closure could be ascribed to PA which accumulates as a result of a conversion of ABA to PA. Obviously, this hypothesis needs to be tested.

Effects on Photosynthetic Apparatus. In short term experiments, PA and DPA did not reduce the photosynthetic capacity of leaves. It is unlikely that these substances are involved in the impairment of the photosynthetic mechanism frequently seen to follow water stress.

Effects of Solvents Residues. Evidence was presented for the presence of contaminants in allegedly pure solvents that inhibit photosynthetic O_2 evolution in isolated mesophyll cells and CO_2

Table VI. *O_2 Evolution by Isolated Xanthium strumarium Mesophyll Cells in the Presence of Solvent Residues*

After evaporating 100 ml of each solvent *in vacuo* at 35 C or less, the residue was taken up into the cell suspension buffer and added to the cells. *p*-Benzylquinone was added to give a final concentration of 1 mM. Azide (0.1 mM final concentration) was present with the quinone. The control rates were 48, 107, and 234 μmol O_2 mg Chl^{-1} h^{-1} for cells at pH 5.6, pH 7.0, and at pH 5.6 with *p*-benzylquinone, respectively. About 100 μg Chl were used per assay.

Solvent	Rate of O_2 Evolution		
	pH 5.6	pH 7.0	pH 5.6 + *p*-benzylquinone
	% of control		
Acetone	0	21	68
Chloroform	15	104	
Ether	38	108	8
Ethyl acetate	0	101	73
Hexanes	−2	14	107
Methanol	93		

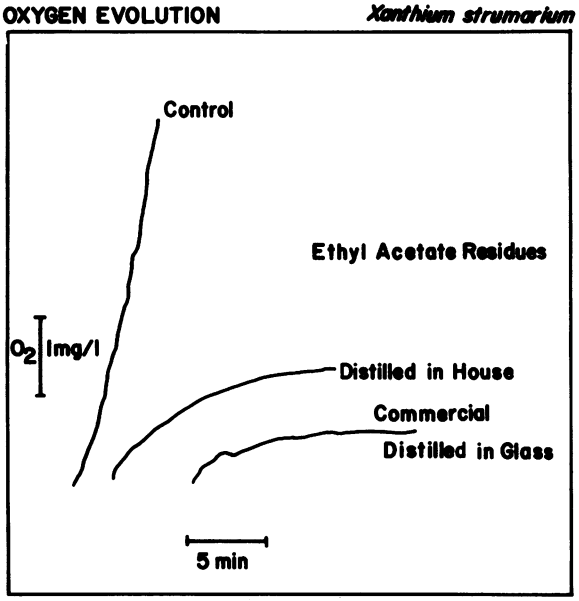

FIG. 3. O_2 evolution from mesophyll cells in the presence of the residues of 100 ml commercial "Distilled in Glass" ethyl acetate and ethyl acetate freshly distilled in house from bulk ethyl acetate. Suspension buffer was 0.1 M Mes (pH 5.6). Control rate of O_2 evolution was 83 μmol O_2 mg Chl^{-1} h^{-1}.

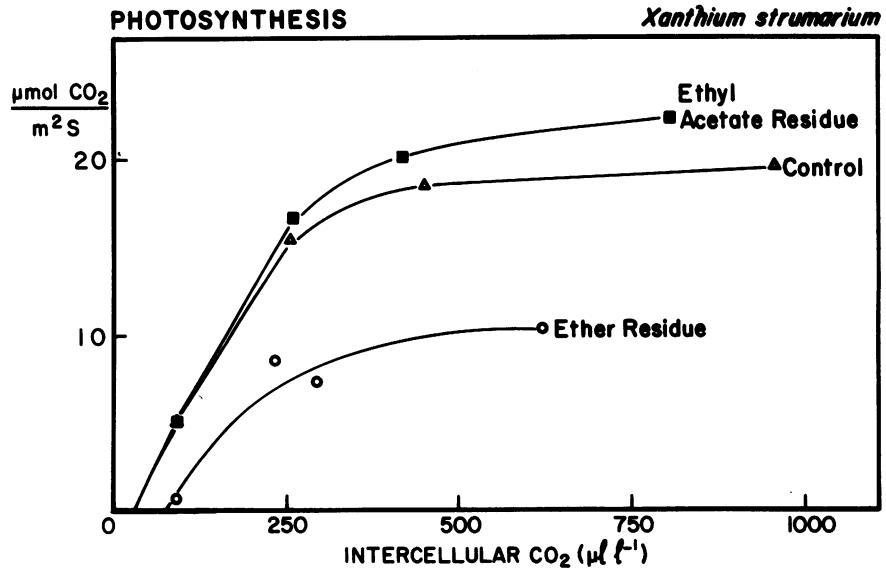

FIG. 4. CO_2 assimilation related to intercellular CO_2 concentration in leaves of *X. strumarium* that had been fed the residues of 500 ml each of ethyl acetate or diethyl ether obtained by evaporation of the solvents *in vacuo*. Each residue was taken up in 4.5 ml water into which the cut end of the petiole was placed.

uptake by detached leaves (Figs. 3 and 4 and Table VI). These contaminants were responsible for the apparent inhibitory activity of plant extracts containing PA. Residues from different solvents required different conditions for inhibitory activity; the inhibitory agent was therefore not the same in all solvents.

A substance contained in commercial ethyl acetate has caused a spurious effect in at least one other bioassay. Briggs (4) observed that a residue of ethyl acetate stimulated the release of reducing sugars from imbibed embryoless barley seeds, and thus mimicked an effect of gibberellins.

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LITERATURE CITED

1. Arnon DI 1949 Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol 24: 1–15
2. Beardsell MF, D Cohen 1975 Relationships between leaf water status, abscisic acid levels, and stomatal resistance in maize and sorghum. Plant Physiol 56: 207–212
3. Boyer JS 1976 Photosynthesis at low water potentials. Phil Trans R Soc Lond 273: 501–512
4. Briggs DE 1966 Residues from organic solvents showing gibberellin-like biological activity. Nature 210: 419–421
5. Cummins WR, H Kende, K Raschke 1971 Specificity and reversibility of the rapid stomatal response to abscisic acid. Planta 99: 347–351
6. Dubbe DR, GD Farquhar, K Raschke 1978 Effect of abscisic acid on the gain of the feedback loop involving carbon dioxide and stomata. Plant Physiol 62: 413–417
7. Harrison MA, DC Walton 1975 Abscisic acid metabolism in water-stressed bean leaves. Plant Physiol 56: 250–254
8. Hiron RWP, STC Wright 1973 The role of endogenous abscisic acid in the response of plants to stress. J Exp Bot 24: 769–781
9. Horton RF 1971 Stomatal opening: the role of abscisic acid. Can J Bot 49: 583–585
10. Hsiao TC 1973 Plant responses to water stress. Annu Rev Plant Physiol 24: 519–570
11. Kriedemann PE, BR Loveys, WJS Downton 1975 Internal control of stomatal physiology and photosynthesis. II. Photosynthetic responses to phaseic acid. Aust J Plant Physiol 2: 553–567
12. Kriedemann PE, BR Loveys, JV Possingham, M Satoh 1976 Sink effects on stomatal physiology and photosynthesis. *In* IF Wardlaw, JB Passioura, eds., Transfer and Transport Processes in Plants. Academic Press, New York, pp 401–415
13. Loveys BR, PE Kriedemann 1974 Internal control of stomatal physiology and photosynthesis. I. Stomatal regulation and associated changes in endogenous levels of abscisic and phaseic acids. Aust J Plant Physiol 1: 407–415
14. Martin GC, FG Dennis, P Gaskin, J MacMillan 1975 Contaminants present in materials commonly used to purify plant extracts for hormone analysis. HortScience 10: 598–599
15. Mittelheuser CJ, RFM Van Steveninck 1971 Rapid action of abscisic acid on photosynthesis and stomatal resistance. Planta 97: 83–86
16. Raschke K 1975 Simultaneous requirement of carbon dioxide and abscisic acid for stomatal closing in *Xanthium strumarium* L. Planta 125: 243–259
17. Raschke K, RD Firn, M Pierce 1975 Stomatal closure in response to xanthoxin and abscisic acid. Planta 125: 149–160
18. Zeevaart JAD, BV Milborrow 1976 Metabolism of abscisic acid and the occurrence of *epi*-dihydrophaseic acid in *Phaseolus vulgaris*. Phytochemistry 15: 493–500