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Nonstomatal Inhibition of Net CO_2 Uptake by (±) Abscisic Acid in *Pharbitis nil*

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ABSTRACT

(±) Abscisic acid (ABA) injected into petioles of attached transpiring leaves of *Pharbitis nil* Chois. cv violet reduced the photosynthetic capacity of the mesophyll of these leaves as well as the stomatal conductance to CO_2 diffusion. Greater than 75% of the injected ABA was recovered as ABA, suggesting that ABA rather than some metabolite thereof was the active compound. The nonstomatal effect of ABA increased from 30% reduction in photosynthesis at 0.25 micromolar ABA in the leaf blade to 90% reduction at 18 micromolar. Despite the effect of ABA on the nonstomatal component of leaf net CO_2 uptake, it was calculated that a substantial part of the reduction in leaf net CO_2 uptake (50–80%) could be accounted for by the effect of ABA on stomatal conductance.

It is well established that (±) ABA causes a decline in net photosynthetic CO_2 assimilation when supplied to green leaves. Because ABA has no effect on isolated cells (10, 16) or chloroplasts (9), it has been assumed that ABA reduces CO_2 assimilation only by its well known effect on stomatal movements (12). Some gas exchange studies have indeed indicated that stomatal closure is solely responsible for the decline of leaf net CO_2 uptake induced by ABA; Cummins *et al.* (5) showed that when ABA was added to the irrigation water of detached barley leaves the intercellular CO_2 partial pressure fell as photosynthesis declined, whereas when DCMU was added the intercellular CO_2 partial pressure increased. Dubbe *et al.* (6) showed that the relationship between CO_2 assimilation and intercellular CO_2 concentration was not changed by exogenous ABA.

In contrast, in longer term experiments, exogenous ABA has been shown to directly affect the photosynthetic apparatus. An inhibition of CO_2 uptake by ABA has been reported in *Lemna minor* and *Lemna gibba* which are devoid of functional stomata (1, 17). An ABA-induced decrease of RubisCo[1] activity, linked to a decrease in net photosynthesis, has also been reported in *Pennisetum* (14, 15). Inasmuch as ABA is synthesized as a result of various stress factors (2, 11), the question of whether this hormone causes a decrease of net photosynthesis through stomatal closure and/or via direct effects on photosynthesis reactions is important. We examine further this question by analyzing the variation of internal CO_2 partial pressure in intact attached leaves when supplied with physiological concentration of ABA during periods of active photosynthesis.

MATERIALS AND METHODS

Plants. *Pharbitis nil* Chois. cv violet plants were grown in vermiculite in the Gif phytotron. Growth conditions were: temperature, 27°C; RH, 70%; photoperiod, 16 h; quantum fluence rate (QFR), 250 μmol quanta m^{-2} s^{-1}. The experiments were performed 2 weeks after emergence, on the third unfolded leaf of the plant.

Gas Analysis. Gas exchange of the leaf was measured in an open system. Net CO_2 uptake was measured with an IR gas analyzer (COSMA Rubis 3000), the transpiration rate with a hygrodynamic sensor (American Instrument Company, type TH-3). Air of known CO_2, N_2 and O_2 was supplied from cylinders and circulated over hot water (80°C) and in a temperature controlled-water bath to establish the desired dew point temperature. Leaf temperature was monitored with a thin copper-constantan thermocouple pressed on the abaxial leaf surface. The CO_2 concentration in the intercellular spaces of the leaf (C_i) was calculated using the equation:

$$C_i = C_e - 1.6\,A/g$$

in which C_e is the CO_2 concentration in the chamber, A the rate of net CO_2 uptake, g the stomatal conductance to water vapor, and 1.6 the ratio of the diffusivities of water vapor and CO_2 in the air. The values of internal CO_2 partial pressure (P_i) were calculated from that of (C_i) by multiplying by the ambient pressure. Illumination was provided by two 450-w halogen lamps (Mazda MADF 450). Leaves were allowed to equilibrate 3 or 4 h in the assimilation chamber under the experimental conditions before the beginning of the measurements. During measurements QFR was 500 μmol quanta m^{-2} s^{-1}, and the leaf temperature 23 ± 1°C.

ABA Treatment. One hundred μl of a solution of [^{3}H]ABA (containing 1% or less of ethanol) was injected into the petiole using a gauge needle. The specific radioactivity of the ABA solution used was in the range of 2 to 20 μCi/μmol. Unlabeled ABA was added to obtain different concentrations.

As soon as the photosynthetic rate stabilized following treatment, the leaf was removed from the assimilation chamber and frozen in liquid N. It was either combusted to count the total activity in the leaf or used for further analysis to check whether or not the injected ABA was metabolized.

Measurement of the Radioactivity. The leaf was freeze dried. For total determination of the radioactivity after feeding by [^{3}H]-ABA, the leaf was fragmented into pieces of less than 300 mg (fresh weight) and burned in an Oxydiser (Oxymat Intertechnique); the radioactivity was counted in a liquid scintillation spectrometer (Beckman, type LS 7500).

Extraction of ABA and Analysis of its Metabolism. For the analysis of the metabolism of labeled ABA inside the leaf tissue, the experiment was as follows: three leaves which received [^{3}H] ABA were frozen as indicated above and extracted in 25 ml of methanol (with 1% acetic acid). The anti-oxidant 2,6-di-tert-butyl-4-methylphenol (BHT) was added to the solvent. After shaking overnight at +5°C in darkness the residue was removed by centrifugation and resuspended in 25 ml of the same solvent

[1] Abbreviations: RubisCo, ribulose bisphosphate carboxylase oxygenase; PA, phaseic acid; DPA, dihydrophaseic acid.

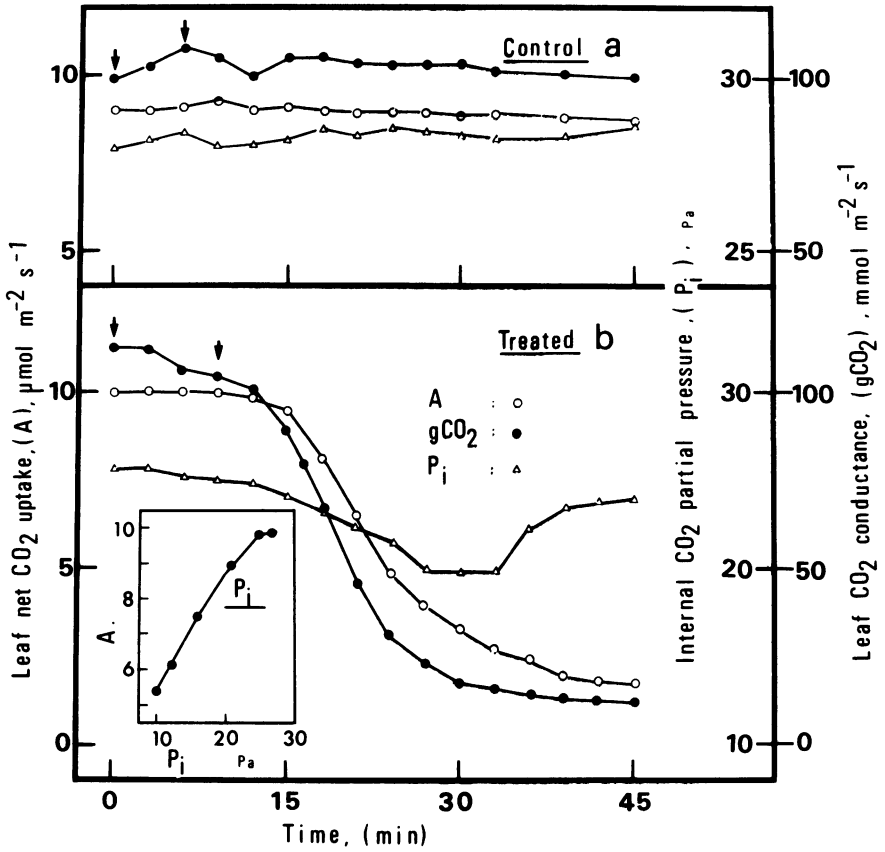

FIG. 1. Time course of leaf net CO_2 uptake (A: ○), leaf CO_2 conductance (g_{CO_2}: ●) and internal CO_2 partial pressure (P_i: △). a, 100 µl of 1% ethanol in water was slowly injected into the petiole of an attached leaf. The two arrows indicate the duration of the injection. b, 100 µl of a 0.1 mM ABA solution (in 1% ethanol in water) was injected into the petiole of an attached leaf. The two arrows indicate the duration of the injection. The inset represents the relationship between A and P_i measured on the same leaf before the treatment. The range of variation of P_i after the injection of ABA is shown in the inset. The data are representative of eight independent experiments. The average value observed for total inhibition was 85% (SE = 6).

under constant agitation in the same condition for 1 h. A second centrifugation gave a second supernatant which was added to the first. This total methanolic extract received a quantity of H_2O to produce an 80% methanol solution. All the methanol was evaporated under vacuum at 30°C. The pH of the aqueous phase was adjusted to 7.5 to 8.0 with 1 N NaOH and extracted with 1 volume of diethyl oxyd ethylic ether. The aqueous phase pH was adjusted to 2.5 to 3.0 with 1 N H_2SO_4 and extracted twice with ethyl ether. The ethereal fraction, which contains the free ABA, was evaporated to dryness and redissolved with 200 µl of ethyl ether before chromatography.

To measure the amount of conjugated ABA, the pH of the aqueous phase was adjusted to 11.0 and kept at 60°C for 60 min. After adjusting the pH to 2.5 to 3.0 with 1 N H_2SO_4, the solution was extracted with ethyl ether. This fraction which contains ABA released by alkaline hydrolysis from bound ABA was then treated as above before chromatography. The chromatographic system (TLC) is that proposed by Zeevaart and Milborrow (18): toluene:ethyl acetate:acetic acid (50:30:4). It can separate ABA from its main forms of metabolites: phaseic acid (PA) and di-hydrophaseic acid (DPA). The ethereal fractions were applied to Merck pre-coated Silica gel F_{254} plates (200 × 200 × 0.25 mm); two developments occurred, separated by 24 h. ABA samples were chromatographed with the extracts. After chromatography, the surface of the plate corresponding to an extract was delimited into 10 equivalent zones and scraped off. The powder was put in a vial and, after adding scintillation mixture (ACS, Amersham), the radioactivity was measured in a spectrometer.

RESULTS

Inhibitory Effect of ABA on the Nonstomatal Component of Photosynthesis. Figure 1 shows the time course of net CO_2 uptake (A), conductance to CO_2 diffusion (g_{CO_2}), and internal CO_2 partial pressure (P_i), either after water containing 1% ethanol (Fig. 1a, control experiments) or ABA (Fig. 1b) was injected into the petiole of an attached transpiring *Pharbitis nil* leaf. A and g_{CO_2} remained constant after the injection of 1% ethanol in water (Fig. 1a) but decreased simultaneously after the injection of ABA (Fig. 2b). After 45 min, A was reduced to 17% of the initial value. P_i decreased from 25 to 20 Pa during the first 30 min after injection but then increased nearly to the initial value.

The response of A to P_i before the ABA injection is shown in the inset in Figure 1b. If ABA-induced stomatal closure alone was responsible for the decline in A, then P_i would have varied as described by the curve in the Figure 1b inset. As P_i did not dramatically decline, the data in Figure 1b show that, in addition to the stomatal closure, ABA caused a direct reduction in the capacity of the mesophyll for photosynthetic assimilation.

The inhibitory effect of ABA on the nonstomatal component of photosynthesis was calculated as $(A_c - A)/A_c$ (see Fig. 2a), where A is the measured CO_2 uptake rate and A_c is the CO_2 uptake rate that would have occurred at the calculated P_i if there had been no direct effect of ABA on mesophyll metabolism. At the end of the experiment shown in Figure 1, the value of this inhibition was 80% and that of the total inhibition (involving stomatal and nonstomatal components) was 82%. Similar results were obtained in seven replicate experiments. The average values

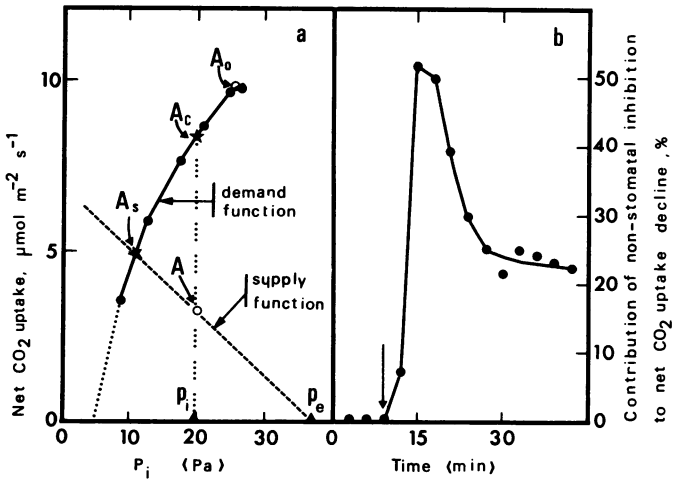

FIG. 2. Calculation (a) and time course of the contribution of nonstomatal inhibition to net CO_2 uptake decline of an attached leaf after an injection of 100 μl of 0.1 mM ABA solution into the petiole (b). a, Demand function is A. P_i response curve (——). The supply function of the leaf is the equation $A = g_{CO_2}(P_e - P_i)$ in which P_e is the CO_2 partial pressure inside the assimilation chamber. The supply function is drawn when A (the rate of net CO_2 uptake after the injection) = 3.2 μmol CO_2 m^{-2} s^{-1}, P_i = 20 Pa, and P_e = 37.4 Pa. Net CO_2 uptake of the leaf prior to the treatment is A_o. A_c is the leaf CO_2 uptake which would be observed at the measured P_i after ABA injection, if the treatment had no effect on mesophyll photosynthesis, A_s is the leaf net CO_2 uptake which would be observed at the measured leaf CO_2 conductance after ABA injection, if the treatment had no effect on mesophyll photosynthesis. The nonstomatal component of the decline in leaf net CO_2 uptake is calculated by the ratio $(A_s - A)/(A_o - A)$.

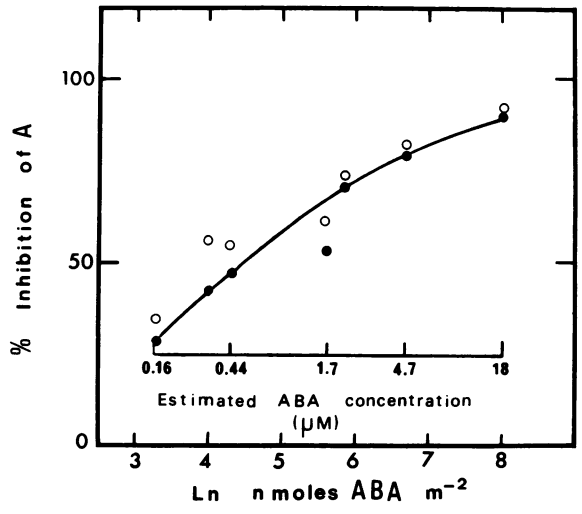

FIG. 3. Variation of the percentage inhibition of A as a function of the ABA content in the leaf. (O), Total inhibition, $(A_o - A)/A_o$; (●), inhibition of nonstomatal component, $(A_c - A)/A_c$ (see Fig. 2a). These ratios were calculated when A reached its lowest value. The inset scale indicates an estimated ABA concentration in the leaf, calculated assuming an homogeneous repartition of the injected ABA and that 78% of the injected ABA was not metabolized (see Fig. 6).

for the eight experiments were: for nonstomatal inhibition, 79% (SE = 9); for total inhibition, 85% (SE = 6). Leaf water potential increased from −0.10 MPa (SE = 0.04; n = 6) to −0.07 MPa (SE = 0.02; n = 6) by the ABA injection.

We calculated the contribution of the nonstomatal component to the decline of leaf net CO_2 uptake after injection of ABA. The

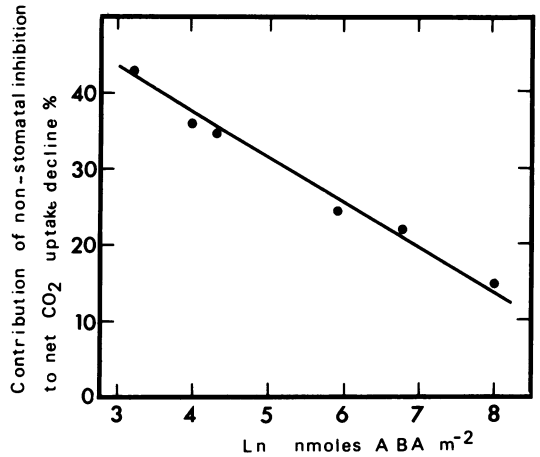

FIG. 4. Contribution of the nonstomatal effect of ABA to the total decline of net CO_2 uptake as a function of the ABA content in the leaf. This contribution was calculated with the ratio $(A_s - A)/(A_o - A)$ (see Fig. 2a) when A reached its lowest value after the treatment.

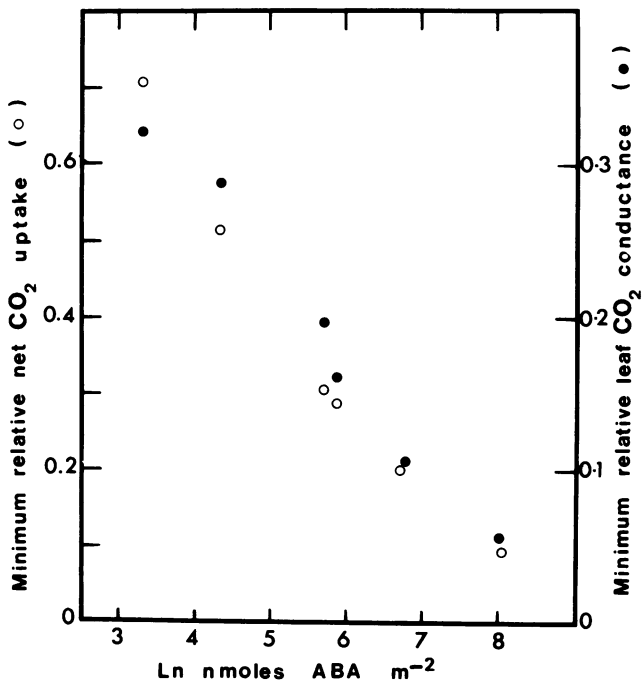

FIG. 5. Variation of minimal net CO_2 uptake (O) and minimal leaf CO_2 conductance (●) as a function of leaf ABA content. To eliminate a source of variation between the experiments, minimum leaf CO_2 conductance (g_{mCO_2}) and minimum net CO_2 uptake (A_m), observed after the ABA injection, were expressed, respectively, as the ratios g_{mCO_2}/g_{oCO_2} and A_m/A_c in which g_{oCO_2} is the leaf CO_2 conductance observed just before the treatment and A_c a value of A which has been already defined (see Fig. 2a).

method used and the results are shown in Figure 2. The A-P_i curve (Fig. 2a) represents the 'demand function' of the leaf before the injection, and the equation $A = g_{CO_2}(P_e - P_i)$ the 'supply function' (7). The supply function is represented when A = 3.2 μmol CO_2 m^{-2} s^{-1}, P_i = 20 Pa, and P_e = 37.4 Pa (observed values 30 min after the injection of ABA). The intersection of the demand and supply functions gives a theoretical value of $A = A_s$ which would be observed at the measured g_{CO_2} (the slope of the supply function) if no change in mesophyll photosynthesis had occurred during the treatment. The difference $A_s - A$ is a measure of the nonstomatal component of the decline in leaf net CO_2

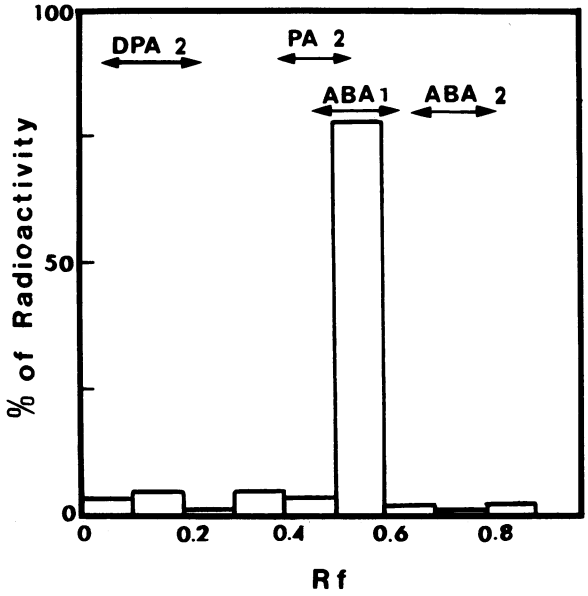

FIG. 6. Thin layer chromatogram of the total extract from three leaves which have been injected with 0.1 mM [^{3}H]ABA. ABA_1 refers to the position of the ABA in our system, whereas ABA_2, DPA_2, and PA_2 to that of ABA, DPA, and PA in the system described in Reference 18. Two similar experiments gave the same results.

uptake. The importance of this component in the total decline $A_o - A$, where A_o is the net CO_2 uptake at the beginning of the experiment, is calculated by the ratio $(A_s - A)/(A_o - A) = 26\%$. Thus, 30 min after the injection of ABA, 26% of the observed decline in leaf net CO_2 uptake is attributed to the nonstomatal effect of ABA.

Figure 2b shows the time course of the variations of $(A_s - A)/(A_o - A)$ after an injection of ABA. The nonstomatal component of the decline in leaf net photosynthesis increased rapidly after the injection (the arrow indicates the completion of the injection) to about 50%, and then decreases to reach a steady value of about 22%.

ABA Response Curve of the Nonstomatal Inhibition of CO_2 Uptake. The inhibition of the nonstomatal component of photosynthesis by ABA was calculated as explained above by the ratio $(A_c - A)/A_c$, considering the lowest value of A observed after the treatment. The nonstomatal effect of ABA increased with ABA content of the leaves (Fig. 3, ●). For each ABA content, the magnitude of total inhibition (○) was very similar to that of nonstomatal inhibition. The leaf water content was measured after the ABA injection on leaves similar to those used to obtain the results described in Figure 3. From that value and that obtained by scintillation counting, we estimated the concentration of ABA which had reached the leaf after the treatment, assuming that partition of ABA within the leaf was homogenous and that 78% of the total radioactivity was due to ABA (Fig. 6). The concentrations inside the leaf were within a physiological range (18–0.2 μM; see Fig. 3, inset scale). The contribution of the nonstomatal component to the total inhibition of leaf net CO_2 uptake (calculated as explained by Fig. 2a considering the lowest value of A after the treatment) increased when the ABA content of the leaf decreased (Fig. 4).

As both A and g were inhibited by ABA, we compared the effect of different concentrations of ABA on the minimum rate of CO_2 uptake and the minima leaf CO_2 conductance observed after the injection (Fig. 5). To eliminate a source of variation between experiments, the minimum leaf conductance g_{mCO_2} was expressed as the ratio g_{mCO_2}/g_{oCO_2}, in which g_{oCO_2} is the leaf CO_2 conductance observed just before the treatment. Similarly, the minimum rate of photosynthesis (A_m) was expressed as the ratio A_m/A_c in which A_c is the calculated rate of CO_2 uptake at the same internal CO_2 pressure from the A-P_i curve measured before the treatment. As shown by Figure 5, the sensitivity to ABA of photosynthetic carbon uptake was similar to that of leaf CO_2 conductance.

Metabolism of [^{3}H]ABA during the Inhibition. The main part of the radioactive ABA which reached the leaves was not metabolized, as 78% of the extracted radioactivity migrated exactly at the R_F of ABA measured in our experimental conditions (Fig. 6). Although this does not constitute an absolute proof that the high percentage of radioactivity is ABA, the exact concordance of the standard and of the radioactivity strongly suggests that this is the case. It is likely that the R_F of PA (phaseic acid) and DPA (dihydrophaseic acid), in our conditions, would be smaller, as the measured R_F of ABA is 0.50 to 0.55 and that mentioned by Zeevaart and Milborrow (18) is 0.64 to 0.75.

No radioactivity was found in the bound ABA fraction. Alkaline hydrolysis did not liberate measurable radioactivity. So, it is highly likely that the effects observed at the level of the photosynthesis activity are a consequence of the ABA molecule itself. Two similar experiments gave the same results.

CONCLUSION

In the present paper, we report a rapid inhibition of the nonstomatal components of net photosynthesis in attached leaves of *Pharbitis nil* by injection of a low concentration of ABA into the transpiration stream (Fig. 1). We obtained similar results after injection of ABA into leaves of two other species grown in the Phytotron (white mustard; french bean). As shown by the R_F measurements (Fig. 6), it is highly probable that most of the radioactive ABA (greater than 75%) which reached the leaves was not metabolized during the experiment. This latter observation is in close agreement with that of Cummins (4) using a similar technique. The concentration of the injected ABA inside the leaf was estimated assuming that its repartition was homogenous (Fig. 3, insets scale). It was within physiological range (0.5–18 μM). It is likely from the results of Heilmann *et al.* (8) and the calculation of Cowan *et al.* (3) that the concentration was the highest inside the chloroplast. Despite a large nonstomatal inhibition of photosynthesis caused by ABA (Figs. 1 and 3), the stomatal component of the decline in leaf net CO_2 uptake was quite substantial (Figs. 2b, 4).

Our results are in contrast with those of Dubbe *et al.* (6). These workers showed that the response curve of CO_2 uptake to increased internal CO_2 pressure, measured on detached leaves, was not changed when ABA was supplied through the petiole maintained in water. We are unable to explain the differences between our results and theirs. Direct action of ABA in photosynthesis has already been reported. However, in all cases, measurements were performed either on plants grown in the presence of ABA (14, 15) or on plants sprayed with ABA 12 h before the measurements (13). As discussed by Mawson *et al.* (10), such results can be understood since ABA has been shown to inhibit protein synthesis.

As far as we know there is only one report, with *Lemna gibba* (17) which lacks functional stomata, of a substantial direct inhibition of photosynthesis after 4 h of incubation in the presence of ABA. We report here a faster effect of ABA on net CO_2 uptake.

It is very puzzling that ABA does not decrease $^{14}CO_2$ fixation by *P. vulgaris* L. and *L. esculentum* L. isolated cells and isolated intact chloroplasts (9, 10, 16). Our own measurements on Spinach, *Xanthium* and *Pharbitis* isolated cells, as well as on intact spinach chloroplasts are in agreement with these results (Cornic, unpublished). However although it is true that isolated cells represent a system in which it is possible to study photosynthesis without the complication imposed by stomata it is also a system

in which the limiting reactions of photosynthesis may not be the same as in intact leaves.

In conclusion, the results which have been described here show that injection of a small amount of ABA into the transpiration stream of an intact attached leaf causes an inhibition of the nonstomatal component of photosynthesis. It is difficult at the moment to understand the mechanism of this inhibition as we have been so far unable to measure it either with isolated cells or isolated intact spinach chloroplasts. If we assume that ABA affects leaf net CO_2 uptake directly via an action on the chloroplast, our observations are interesting since ABA concentration inside the chloroplast could be quite high (3) even in unstressed plants and could be increased inside this organelle during a period of light, according to the pH gradient between the cytoplasm and the stroma (8). It would appear that ABA could inhibit photosynthesis either when it has been transformed in an 'active form' or when the physiological status of the plant reaches a 'critical state'. Nevertheless, whatever the mechanism of direct ABA action on net photosynthesis, the above results point to the possibility that part of the photosynthetic decline observed in plant submitted to water stress could be due to the increasing ABA concentrations experienced under such a condition.

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