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Compartmentation and Equilibration of Abscisic Acid in Isolated *Xanthium* Cells[1]

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ABSTRACT

The compartmentation of endogenous abscisic acid (ABA), applied (±)-[^{3}H]ABA, and (±)-*trans*-ABA was measured in isolated mesophyll cells of the Chicago strain of *Xanthium strumarium* L. The release of ABA to the medium in the presence or absence of DMSO was used to determine the equilibration of ABA in the cells. It was found that a greater percentage of the (±)-[^{3}H]ABA and the (±)-*trans*-ABA was released into the medium than of the endogenous ABA, indicating that applied ABA did not equilibrate with the endogenous material.

Therefore, in further investigations only the compartmentation of endogenous ABA was studied. Endogenous ABA was released from *Xanthium* cells according to the pH gradients among the various cellular compartments. Thus, darkness, high external pH, KNO_2, and drought-stress all increased the efflux of ABA from the cells. Efflux of ABA from the cells in the presence of 0.6 M mannitol occurred within 30 seconds, but only 8% of the endogenous material was released during the 20 minute treatment.

The distribution of ABA at the cellular level is determined by the pH gradients that are maintained within the cell (12, 13). In Hartung's (10, 12) model, the equilibrium of ABA among cellular compartments is determined by its protonation. ABA moves across membranes as the protonated species, whereas the dissociated ABA molecule does not penetrate membranes. Therefore, ABA is trapped within alkaline compartments of the cell. As much as 80% of the ABA is localized within the chloroplast, the compartment of the cell with the highest pH (10, 12, 14). Less than 10% of the endogenous ABA in the cell is located within the vacuole, the compartment of the cell with the lowest pH (4). The chloroplast could serve as a reservoir for ABA which would be redistributed within the cell in response to pH changes. Cowan *et al.* (6) predicted that decreasing the pH of the chloroplast would increase the availability of ABA to the free space, and thus increase the amount of ABA to reach the guard cell.

Many of the studies involving the uptake and release of ABA and the compartmentation of ABA within the cell have been done by following the movement of radiolabeled (±)-ABA within the cell (12, 13). The ABA molecule is a chiral molecule, the (+) form being the naturally occurring and active form (8). Studies employing radiolabeled (±)-ABA may not be accurate since the (−) enantiomorph may not behave similarly to the naturally occurring (+) enantiomorph.

Equilibration and efflux of endogenous ABA has not been previously studied. In this study, isolated intact *Xanthium* cells were used to investigate the efflux of endogenous ABA from the cell. Our first objective was to determine if radiolabeled (±)-ABA equilibrates with the endogenous ABA. Second, we examined the release of endogenous ABA to the free space using isolated *Xanthium* cells to determine if the movement of endogenous ABA was dictated by the pH gradients within the cell as predicted. In addition, we examined the effect of drought and osmotic stress on the distribution of endogenous ABA between the cell and the free space to determine if ABA is re-equilibrated within the cell during stress. This allowed us to test experimentally the predictions made by Cowan *et al.* (6). We found that exogenously applied (±)-ABA did not equilibrate with the ABA that was already present in the cell, suggesting that ABA movement within the cell involves a 'receptor-like' molecule. In addition, we determined that the movement of endogenous ABA was dependent upon the pH gradients within the cell by making measurements of compartmentation of endogenous ABA in isolated *Xanthium* cells. Stress to the plant resulted in redistribution of ABA within the cell with a small percentage of the total cellular ABA being available to the free space.

MATERIALS AND METHODS

Culture of Plant Material. *Xanthium strumarium* L., Chicago strain, was grown from seed in the greenhouse for 3 months as before (20). The most recently fully expanded leaves were harvested for these experiments.

Isolation of *Xanthium* Cells. Intact *Xanthium* cells were isolated using the methods of Sharkey and Raschke (18). Leaves were homogenized for 60 s in 0.1 M Hepes buffer (pH 7.0) and were washed by centrifugation three times. The pH of the medium was changed stepwise during the washes for subsequent incubations by combining 0.1 M Hepes buffer (pH 7.0) with 0.1 M Mes buffer (pH 5.0) until the desired pH was reached. The cells were divided into aliquots by volume for subsequent experiments.

Feeding (±)-[^{3}H]ABA to Cells. Cells were incubated in (±)-[^{3}H]ABA (16.4 Ci·mmol^{-1}) for a specified period of time. To ensure viability of the cells, they were incubated under a mercury vapor light (50 W/m^2) and air was bubbled through the medium (7). The cells were washed by centrifugation three times to remove any [^{3}H]ABA that remained on the cell walls. These cells were then used for export studies. Osmotic stress treatments were carrried out in 0.6 M mannitol for a specified period of time. Samples were collected at intervals by rapid centrifugation in a microfuge.

Cells were also fed (±)-*t*-ABA.[3] (±)-*t*-ABA was obtained by photoisomerization of (±)-ABA and was purified by HPLC.

DMSO Compartmental Analysis. Compartmental analysis

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[3] Abbreviation: (±)-*t*-ABA, (±)-*trans*-ABA.

was performed on isolated *Xanthium* cells using DMSO as described by Delmer (9). DMSO at different concentrations was supplied in the medium. After the appropriate incubation periods, the amount of ABA in the medium and in the cells was determined.

ABA Extraction and Analysis. After experimental treatments of the isolated cells, the amount of ABA and radiolabeled ABA was determined in the cell and medium samples. The supernatants were freeze dried. If the supernatant contained 0.6 M mannitol, the pH was adjusted to 2.5 with acetic acid, and the samples were passed through a Sep-Pak C_{18} (Waters Associates) to remove the mannitol. The Sep-Pak was washed with 1% acetic acid and the ABA was eluted with 50% ethanol in 1% acetic acid and the eluate was lyophilized (7). The cell samples were extracted overnight in 80% aqueous acetone, containing 1% acetic acid and 10 mg/L butylated hydroxytoluene (7). The samples were dried prior to HPLC. If the experiment did not involve labeling with [^{3}H]ABA, an internal standard of (±)-[^{3}H]ABA (16.4 Ci·mmol^{-1}) was added to the extract to determine losses of ABA due to manipulation of the sample. ABA was purified using reverse phase HPLC with a μBondapak C_{18} column (Waters Associates) (7). ABA was eluted from the column with a 0 to 50% linear gradient of ethanol in 1% acetic acid. The samples were methylated with ethereal diazomethane and then quantified by GLC with electron capture detector on a capillary column, Durabond DB-1 (J and W Scientific, Inc.) (7). Ethylated ABA was used as an internal standard for quantification (4). Radioactivity was determined by liquid scintillation counting.

RESULTS

Equilibration of (±)-[^{3}H]ABA or (±)-*t*-ABA in Isolated *Xanthium* Cells. The unique property of *Xanthium* which allows rapid isolation of intact cells (18) was exploited to study the compartmentation of ABA within the cell. These studies enabled us to investigate efflux of preloaded (±)-[^{3}H]ABA and/or endogenous ABA to the free space. Each sample was purified on the HPLC so that the radiolabel recovered in the medium or the cells was only from ABA. Compartmental analysis was used to determine if exogenously supplied (±)-ABA equilibrates with endogenous ABA. The introduction of DMSO to the medium has been used to study the compartmentation of small molecules within the cell (4, 9). The cells were preloaded with (±)-[^{3}H]ABA (1 μCi) for 1 h, were washed, and then were incubated for 0, 1, or 3 h. At the end of the incubation period, the cells were placed in 2.5 or 20% DMSO for a 20 min period. 20% DMSO was previously shown to disrupt the tonoplast while 2.5 and 20% DMSO had little effect on the release of ABA from the chloroplast (4). The specific activity of ABA in the cells and in the medium was determined. The specific radioactivity of ABA in the medium was higher than it was in the cells at all time points (Fig. 1). These results indicate that the (±)-[^{3}H]ABA that was preloaded into the cells was more readily exported to the medium than the endogenous ABA. The addition of 20% DMSO to the medium allowed more efflux of ABA to the medium. With 20% DMSO, the specific activity of ABA in the medium and the cells was less than with 2.5% DMSO, showing that more endogenous ABA was released from the cells with 20% DMSO. The same results were obtained when intact leaves were fed (±)-[^{3}H]ABA and the cells were subsequently analyzed by compartmental analysis. If the pH of the medium was lowered to pH 5.5 and the cells were fed 1 μCi of (±)-[^{3}H]ABA for 2 or 3.5 h, or if 1 μg of unlabeled ABA was supplied to the cells after the feeding of the (±)-[^{3}H]ABA, the results were identical to those shown in Figure 1 (data not shown). Cells were also fed (±)-*t*-ABA (14 μg) for 1 h at pH 5.6. Export of (±)-*t*-ABA to the medium containing 2.5 or 20% DMSO during a 20 min period was measured to determine the equilibration of the (±)-*t*-ABA with the endogenous ABA. The percentage of (±)-*t*-ABA in the medium was greater than that of ABA at 0, 1, and 3 h after preloading (Fig. 2). Therefore, more of the supplied (±)-*t*-ABA than of the endogenous ABA was exported from the cells. When 20% DMSO was included in the medium compared to 2.5% DMSO, a greater percentage of ABA and (±)-*t*-ABA was released into the medium.

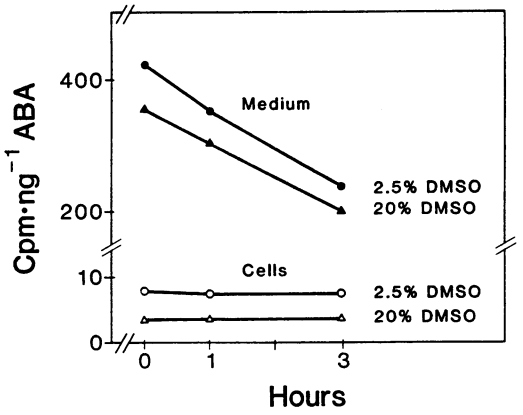

FIG. 1. Equilibration of (±)-[^{3}H]ABA in isolated *Xanthium* cells. Cells were preloaded with 1 μCi of (±)-[^{3}H]ABA for 1 h at pH 7.0 and were thoroughly washed by centrifugation. The cells were aliquoted by volume and were incubated for 0, 1, and 3 h. At the end of each incubation period, DMSO was added to 2.5 or 20% and compartmental analysis was done for 20 min. After preloading and at the beginning of the incubation period (0 h) each aliquot of cells contained 427.9 ± 2.2 ng of ABA and 20,756 ± 1,173 cpm of (±)-[^{3}H]ABA.

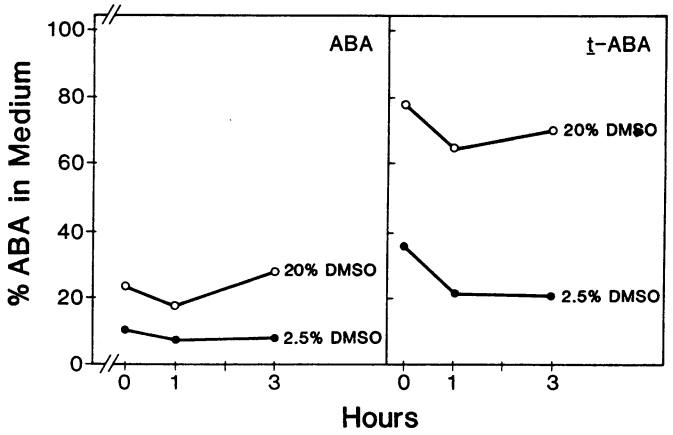

FIG. 2. Equilibration of (±)-*t*-ABA in isolated *Xanthium* cells. Cells were preloaded with 14 μg of (±)-*t*-ABA at pH 7.0 for 1 h and were thoroughly washed by centrifugation. The cells were incubated for 0, 1, and 3 h. At the end of each incubation period compartmental analysis was done for 20 min. The percentage of (left) endogenous ABA and (right) preloaded (±)-*t*-ABA in the medium was measured. Before the compartmental analysis began, each aliquot of cells contained 156.0 ± 15 ng of ABA and 551.8 ± 24 ng of (±)-*t*-ABA. The values of ABA and (±)-*t*-ABA were corrected for recovery of [^{3}H]ABA added as an internal standard. The recovery of ABA was 76.8 ± 2.4%.

Compartmentation of Endogenous ABA in Isolated *Xanthium* Cells. Isolated *Xanthium* cells were used to measure the compartmentation of endogenous ABA. The pH gradients within the cell and from the cytoplasm to the free space were altered to determine if the release of the endogenous ABA from isolated cells was dependent upon pH gradients.

Isolated *Xanthium* cells were incubated in media of different pH for 20 min in 0 or 20% DMSO. When the external pH was 4, 5, or 6, approximately 5% of the cellular ABA was exported to the medium (Fig. 3). When the external pH was greater than

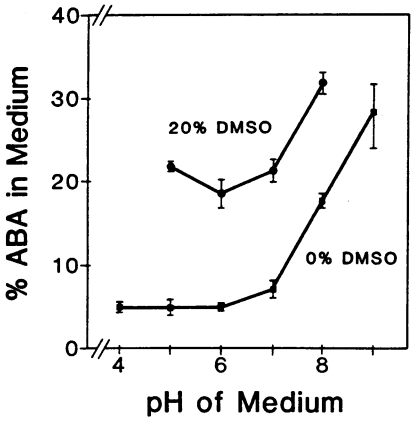

FIG. 3. Effect of pH of the medium on the efflux of ABA. *Xanthium* cells were isolated at pH 7.0 and the pH of the medium was adjusted stepwise to the desired pH. The results of two experiments were averaged. Each aliquot of cells contained 93.8 ± 2.8 ng of ABA before the compartmental analysis was begun. The recovery of ABA was 74.3 ± 3.0%.

Table I. *Effect of KNO_2 on the Distribution of ABA in Isolated Xanthium Cells*

The cells were isolated, aliquoted by volume, and were preincubated for 10 min with or without 8 mM KNO_2. Compartmental analysis was done at pH 8.0 in 2.5% DMSO for 20 min. Each aliquot of cells contained 81.9 ± 5.0 ng of ABA before the compartmental analysis was begun. The recovery of ABA from the samples was 88 ± 3%.

	% ABA Released into the Medium
Control	12.7 ± 0.6[a]
KNO_2	16.9 ± 0.8

[a] Average of three replications ± SE.

Table II. *Effect of Light and Dark on the Compartmentation of ABA in Isolated Xanthium Cells*

The cells were treated in the dark or in the light with 0 or 20% DMSO at pH 7.0 for 20 min. The amount of ABA in the cells and in the medium was analyzed. Each aliquot of cells contained 143.2 ± 4.2 ng of ABA at the time that the compartmental analysis was begun. The recovery of ABA was 73.9 ± 2.7%.

	% ABA Released into the Medium	
	0% DMSO	20% DMSO
Dark	19.0 ± 1.1[a]	25.7 ± 3.4[a]
Light	10.8 ± 0.7[b]	19.7 ± 2.1[b]

[a] Average of two replications ± SE. [b] Average of four replications ± SE.

6 the amount of ABA that was exported to the medium increased. At pH 9, almost 30% of the cellular ABA was released into medium. When release of ABA was measured in 20% DMSO, a greater proportion of the ABA was exported to the medium at each external pH (Fig. 3).

Nitrite has been shown to disrupt the pH gradient between the chloroplast and the cytoplasm when it is included in the medium at pH 7.3 and 7.9 (16). When KNO_2 was included in the incubation medium of the *Xanthium* cells in the presence of 2.5% DMSO at pH 8.0, more ABA was available to the free space (Table I). At pH 5.0, there was also an increased release of ABA to the medium in the presence of KNO_2 (data not shown). With 20% DMSO at pH 8.0, there was no increase in release of ABA to the medium with KNO_2 (data not shown).

In a further experiment, *Xanthium* cells were incubated in the dark or in the light and the efflux of ABA from the cells was determined. In the dark, almost twice as much ABA was released from the cells as when the cells were incubated in the light (Table II). When 20% DMSO was included in the medium, there were no significant differences between the treatments.

Release of ABA from *Xanthium* Cells during Stress. *Xanthium* plants were drought-stressed, the cells were isolated and aliquoted by volume for compartmental analysis. The percentage of total ABA released to the medium from cells that were stressed was almost 2 times that from control cells in 2.5 and 20% DMSO (Table III).

If isolated *Xanthium* cells were stressed for 1 h by the addition of 0.6 M mannitol the percentage of ABA recovered in the medium did not increase (Table IV). The addition of a permeable osmotic agent, ethylene glycol, also did not affect the amount of ABA that was released (data not shown).

Others have found that osmotic stress resulted in a release of ABA (11, 13). Therefore, a time course experiment was done to determine the efflux of applied (±)-[^{3}H]ABA and endogenous ABA during osmotic stress in the isolated cells. Cells were preloaded with (±)-[^{3}H]ABA and the amount of ABA and the radiolabel in ABA in the cells and in the medium was determined at intervals up to 20 min. The percentage of ABA that was released from the cells in 0.6 M mannitol was twice that of the control cells (Fig. 4). Yet, only 4 to 8.5% of the total cellular ABA was released. Of the radiolabel, 20 to 60% was released

Table III. *Effect of Drought-Stress on Release of ABA*

Xanthium plants were subjected to three drought-stress cycles by withholding water until the plants were visually wilted at which time the plants were watered. Cells were isolated from wilted leaves in 0.1 M Hepes (pH 7.0) containing 0.6 M mannitol and from control leaves in 0.1 M Hepes (pH 7.0). The cells were aliquoted by volume. Therefore, the wilted and control cell samples did not contain the same number of cells. Compartmental analysis was done for 20 min with 2.5 and 20% DMSO at pH 7.0. Each aliquot of control cells contained 263.8 ± 5.3 ng of ABA and each aliquot of wilted cells contained 153.3 ± 4.0 ng of ABA before the compartmental analysis was begun. The recovery of ABA was 78.7 ± 1.1%.

	% ABA Released into the Medium	
	2.5% DMSO	20% DMSO
Control	7.1 ± 0.5[a]	10.7 ± 0.9
Wilted	12.6 ± 1.2	20.2 ± 0.2

[a] Average of three replications ± SE.

Table IV. *Effect of Osmotic Stress on the Release of ABA to the Free Space from Isolated Xanthium Cells*

Xanthium cells were isolated and aliquoted by volume. One part of the cells was stressed for 1 h by the addition 0.6 M mannitol to the medium. The control cells were incubated in basal medium for 1 h. The compartmental analysis was done at pH 7.0 in the presence of 2.5 or 20% DMSO for 20 min. The average of three experiments is reported. At the beginning of the experiment, there was 100.2 ± 4.0 ng of ABA in each aliquot of cells, in two of the experiments. There was 27.3 ± 1.4 ng of ABA in each aliquot of cells in the third experiment. The recovery for ABA was 91.4 ± 1.5%.

	% ABA Released into the Medium	
	2.5% DMSO	20% DMSO
Control	22.7 ± 1.2[a]	32.1 ± 1.1
0.6 M mannitol	24.8 ± 1.9	35.0 ± 0.8

[a] Average of three experiments with two replications per treatment in each experiment.

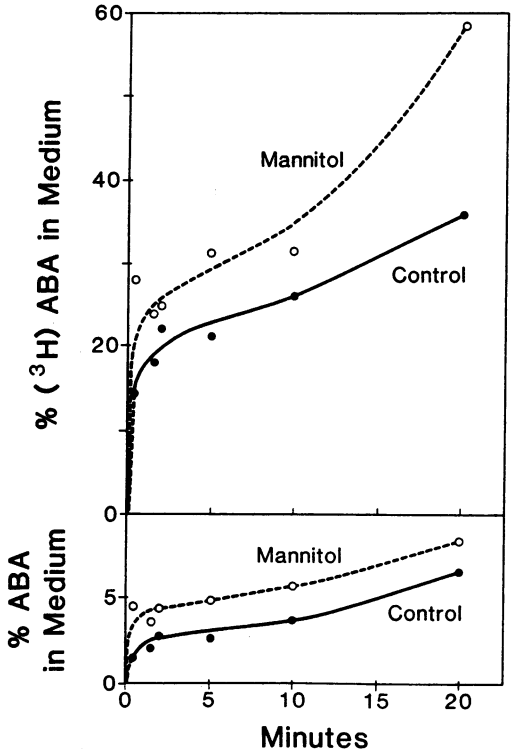

FIG. 4. Efflux of (±)-[^{3}H]ABA and endogenous ABA from control and osmotically stressed *Xanthium* cells. Cells were preloaded with 1 μCi of (±)-[^{3}H]ABA at pH 5.0 for 1 h. The cells were washed and the medium was brought to pH 7.0. At the beginning of the experiment, 0.6 M mannitol or control medium was added to the aliquots of cells. The amount of ABA and [^{3}H]ABA in the cells and the medium was determined at intervals. The data are presented as the percentage of ABA or [^{3}H]ABA that was exported to the medium. Each aliquot of cells contained 65.2 ± 1.8 ng of ABA and 3609.4 ± 80.0 cpm of (±)-[^{3}H]ABA at the beginning of the experiment.

which was almost 10 times more than the percentage of endogenous ABA released. A similar time course for endogenous ABA efflux was observed in the absence of preloaded radiolabeled (±)-ABA (data not shown).

DISCUSSION

Equilibration of ABA in the cell is thought to be dependent upon the protonation of the ABA molecule. Therefore, efflux of ABA to the free space is determined by the pH gradients within the cell and between the cytoplasm and the free space (12, 13). We demonstrated that radiolabeled (±)-ABA did not equilibrate with the endogenous ABA. ABA supplied to cells as a racemic mixture was not released to the medium in the same amounts as the endogenous ABA (Fig. 1). Only 5 to 10% of the endogenous ABA was released into the free space when the external pH was below 8 (Figs. 2 and 3), whereas more than 40% of the radiolabeled (±)-ABA was released (Fig. 4). Kaiser and Hartung (13) also observed a release of 40% of preloaded (±)-[^{14}C]ABA. In addition, exogenously applied (±)-*t*-ABA did not equilibrate with the endogenous ABA (Fig. 2). The release of [^{14}C]ABA was not due to (±)-[^{14}C]ABA being absorbed to the cell wall because the amount of radiolabel within the cell did not decrease when the cells were washed at 0°C (13). The same results were obtained with isolated *Xanthium* cells when whole leaves were fed (±)-[^{3}H]ABA, or when unlabeled (±)-ABA was added to the wash solution.

The inability of the exogenously applied ABA to equilibrate with the endogenous ABA may indicate that there are receptors at the membrane that can distinguish between (+)-ABA and (−)-ABA. It is possible that the [^{3}H]ABA released is the (−)-ABA that was not recognized by the receptor. It has been reported that the uptake of gibberellin A_1 is carrier-mediated (15). Astle and Rubery (2, 3) have described a saturable carrier for ABA uptake in the apical zone of primary roots and suggest that this carrier-mediated uptake is energized by the pH gradients across the plasmalemma.

After finding that (±)-[^{3}H]ABA did not equilibrate with the endogenous ABA it was important to determine if endogenous ABA equilibrates in the cell in response to pH gradients as predicted by experiments in which the release of radiolabel of (±)-ABA was measured (10, 11, 13). In these experiments, the release of endogenous ABA from isolated *Xanthium* cells to the medium was dependent upon the pH external to the cells and the pH gradients within the cells. The release of ABA to the medium was increased by KNO_2, which destroys the pH gradients across the chloroplast membranes (16), increased external pH and placing the cells in the dark. The increased release of ABA to the free space was approximately 10% when the cells were placed in the dark and was approximately 4% when the cells were treated with KNO_2 (Tables I and II). Kaiser and Hartung (13) observed a larger release of ABA due to these conditions which was probably due to the fact that they were following the movement of radiolabeled (±)-ABA instead of endogenous ABA. Our results show that distribution of endogenous ABA in the cell is affected by the pH gradients that are maintained within the cells (13).

Increased efflux of ABA to the free space during periods of drought-stress has been predicted and is thought to result in stomatal closure (6). During drought stress in *Xanthium*, a substantial accumulation of ABA is observed in the leaf tissue (19, 20). The stomatal closure that occurs during drought-stress often precedes this increase in ABA. Raschke (17) concluded that the ABA in the plant was in more than one compartment, because less than 2.5% of the ABA in the cell was necessary to close the stomata. Therefore, the amount of ABA necessary to close the stomata is much less than the total amount of ABA in the leaf. Ackerson (1) measured the amount of ABA in the apoplast and found that stomatal closure may occur prior to accumulation of ABA in the apoplast. On the other hand, Cornish and Zeevaart (5) found that the apoplastic ABA content of *Xanthium* leaves increased before bulk leaf ABA levels rose. In the present experiments, redistribution and release of ABA to the free space in response to 0.6 M mannitol occurred within 30 s. At the end of the incubation period, the amount of ABA released was approximately 8% of that in the cells, which was a 1.3 to 2 times increase over the control. Therefore, immediately upon stress the amount of ABA released into the apoplast was increased. Using the isolated cell system, the free space was much larger than in the intact leaf, which may affect the actual percentage that was released from the cell, but we do not expect that it would alter the relative effects of the treatments. When the osmotic stress in the media was continued for 1 h the difference in release of ABA to the media was diminished (Table IV). Therefore, the increased release of ABA by osmotic stress was a short term effect. In a prolonged stress, the cells adjusted to the stress and the release of ABA to the medium was decreased. This adjustment might occur through changes in the pH of the compartments within the cell. Hartung *et al.* (11) found that 50% of preloaded (±)-[^{14}C]ABA was released from the control leaf slices after 60 min and 70 to 75% from slices incubated in 0.8 M mannitol. Similar trends were observed in our experiments, using isolated *Xanthium* cells that were preloaded with (±)-[^{3}H]ABA. After 20 min, 36 and 58% of the total radioactivity were released from control cells and cells in mannitol, respectively. On the other hand, only 8.5% of the endogenous ABA was released

from cells in mannitol. This clearly demonstrates that applied radiolabeled ABA behaved quite differently from the endogenous ABA.

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