ABA levels in water-stressed tissues are usually 10 to 40 times greater than those found in turgid tissues (21). Several workers (1, 2, 8, 22) have noted that ABA accumulation is dependent on leaf water potential declining below a certain "threshold" level, usually around -1.0 to -1.2 MPa. Pierce and Raschke (16) concluded that turgor is the critical component of plant cell water relations that controls ABA levels, i.e., loss of turgor is the signal that causes ABA accumulation.

We decided to use leaf slices of spinach incubated in various solutes as a way of studying ABA accumulation. Several workers have triggered ABA accumulation by incubating plant tissues in hyperosmotic solutions of mannitol or sorbitol (7, 12, 13, 15, 18, 20). These compounds will cause plasmolysis (loss of turgor). Greenway and Leahy (6) have shown that ethylene glycol, since it rapidly penetrates the cell membrane, will decrease a cell's osmotic potential and cause only a transient loss of turgor. With the use of penetrating and nonpenetrating solutes, one should be able to determine whether or not loss of turgor is important in causing ABA accumulation.

For those cases where mannitol caused increased ABA levels (7, 12, 13, 15, 18, 20), the possibility exists that mannitol may have a perturbing effect on the cell membrane. Rios and Yang (17) used 100 mM mannitol to stimulate ethylene production in Citrus leaf discs. They concluded that mannitol exerted a chemical stress on the membranes since the mannitol concentration used was too low to induce water stress. Other workers (5) have observed that when cucurbit leaf discs were incubated in the presence of 2-propanol, KH2O3, or KCl, the production of ethylene increased. Since ethylene production is a result of membrane damage, addition of these compounds to the medium with spinach leaf slices should indicate if ABA production is caused by a chemical stress to the membranes.

MATERIALS AND METHODS

Plant Material. Spinach plants (Spinacia oleracea cv Savoy Hybrid 612) were grown under SD as described (23) and were transferred to LD for 8 to 14 d. For experiments involving detached leaves, just fully expanded (light green in color) leaves were excised and either placed immediately in plastic bags ('control' leaves), or were allowed to lose 15% of their fresh weight and then placed in plastic bags ('stressed' leaves).

Leaf slices were prepared in the following manner. Just fully expanded leaves were detached, midribs removed, and leaf blades were sliced 1 to 2 mm in width with a sharp razor blade. Slices were placed in BM2 (10 mM Hepes, pH 6.5 with KOH) containing 2.5 g l-1 PVP-40 for 10 min, then rinsed and placed in BM for 10 min. Next, slices were blotted dry and placed in the appropriate incubation medium (10 ml total volume). Total incubation time in most experiments was 4 h. Slices were incubated in 60-ml test tubes under light from a General Electric H400RD3X33-1 mercury-vapor lamp (50 W/m2) which was filtered through a 5-cm layer of distilled H2O. To insure that the slices did not experience anaerobic conditions, air was bubbled into the solution throughout the rinse and incubation period at a rate of approximately 25 ml/min.

All experiments were performed at least twice with two replicates per treatment.

ABA Purification Scheme. After the incubation period, slices and medium were separated. Slices were washed for 10 min with glass-distilled H2O, and the wash was then combined with the incubation medium. Slices were frozen and lyophilized for dry weight determinations, and then extracted as described (4). ABA was purified by HPLC with a C18 µBondapak column (4), using a convex gradient from 0 to 50% ethanol containing 1% acetic acid in aqueous 1% acetic acid. The increase in ethanol with

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2 Abbreviations: BM, basal medium; GC-SIM, gas chromatography-selected ion monitoring; m/z, mass/charge; ECD, electron capture detector; MeABA, methyl ester of ABA.
respect to time (t, mins) may be described as: % ethanol = 50[1-(I/20)^-2]. The fraction containing ABA, which eluted between 18 and 21 min, was dried, methylated, and quantified on a Hewlett-Packard 5840A gas chromatograph (4) equipped with a Hewlett-Packard 7672A automatic sampler. The ethyl ester of ABA was used as an internal standard.

The medium plus wash was acidified with glacial acetic acid to give 1% acetic acid and applied to a C18 Sep-Pak cartridge (Waters Associates) equilibrated with 1% acetic acid. A small amount of (±)-[3H]ABA (16.4 Ci mmol^-1) was added before acidification of the medium to determine losses during the purification procedure. The Sep-Pak was washed sequentially with 2 ml of 1% aqueous acetic acid and 20% ethanol in 1% acetic acid. ABA was eluted with 5 ml of 40% ethanol in 1% acetic acid. The eluate was dried, methylated, and quantified as described above for the extracts from leaf slices.

The percentage recovery of [3H]ABA from tissue was 60% to 80%, while for medium it was 70% to 90%. All data were corrected for losses.

[14C]Mannitol Uptake and Catabolism. Spinach leaf slices (2.1 g fresh weight) were incubated with 0.6 M mannitol plus 1 μCi D-[1-14C]mannitol (45 mCi mmol^-1, New England Nuclear) for 4 h. The tissue was treated as described above and the sugars extracted three times with boiling 80% ethanol. After removal of the ethanol with a stream of N₂, an aliquot of the aequous solution was applied in a narrow band to a 22-cm strip of Whatman 3MM paper and subjected to descending chromatography for 18 h with methyl ethyl ketone:acetic acid:water saturated with boric acid (9:1:1) (10) as solvent. Standards (10 μg each) of sucrose, glucose, fructose, and mannitol were also applied to the origin. Sugars were visualized by spraying with CrO₃-KMnO₄ spray (25). Peaks of radioactivity were detected with a Packard model 7220/21 radiochromatogram scanner.

Water Potential Measurement. Leaf water and osmotic potential were measured with a Wescor HR-337 dew point microvoltmeter equipped with 6 C-52 sample chambers as described (24). Turgor potential was determined by subtraction. The osmotic potential of the incubation medium was measured by placing one drop of medium on a cellulose disc in a C-52 sample chamber for 2 h.

Mass Spectrometry. The GC-SIM response at m/z 190 (dwell time, 100 ms) was monitored as described (4).

RESULTS AND DISCUSSION

Maximum accumulation of ABA occurred when detached spinach leaves were allowed to lose 13% or more of their fresh weight and were then placed in a plastic bag for 4 h (data not shown). A maximum level of ABA (about a 10-fold increase) was reached within 3 to 4 h when such leaves had lost 15% of their fresh weight (Fig. 1). Therefore, in all experiments described here, leaves to be stressed were detached and allowed to lose 15% of their fresh weight. At this point, turgor was zero (Fig. 2).

In a similar manner, ABA accumulation in spinach leaf slices began only when mannitol concentrations greater than or equal to 0.4 M were used, and reached a maximum at 0.6 to 1.0 M (Fig. 2). Concentrations greater than 1.0 M could not be used due to solubility problems. In all further experiments 0.6 M mannitol was used as an internal standard.

![Figure 1](https://via.placeholder.com/150)

**FIG. 1.** Changes in ABA content of detached control (turgid), and in stressed (wilted) spinach leaves. The fresh weight of wilted leaves was reduced by 15%.

![Figure 2](https://via.placeholder.com/150)

**FIG. 2.** ABA accumulation in detached control (C), stressed (S) spinach leaves, and in spinach leaf slices and media. Leaf slices were incubated in increasing concentrations of mannitol (Man). Parameters for cell water relations of detached leaves (insert) and osmotic potentials of the incubation media (ordinate) are indicated.

![Figure 3](https://via.placeholder.com/150)

**FIG. 3.** ABA accumulation in detached spinach leaves, and in spinach leaf slices and media. The leaf slices were incubated in 0.6 mannitol (Man) for the times indicated. In this experiment, the wash (see "Materials and Methods") was analyzed separately from the medium.
LOSS OF TURGOR CAUSES ABA ACCUMULATION

Fig. 4. Levels of ABA in detached spinach leaves, and in spinach leaf slices and media. The leaf slices were incubated in ethylene glycol (EG), EG plus 0.6 M mannitol (Man), 0.6 M mannitol, or Aquacide III (AQ III). was used. When slices were incubated in 0.6 M mannitol, maximum accumulation of ABA in the tissue occurred within 3 h (Fig. 3), while ABA levels continued to increase somewhat in the medium. Some ABA was also found in the wash. This ABA was probably due to efflux from the slices. In addition, if slices were incubated in 0.6 M mannitol for 4 h and were then placed in BM for 4 h, ABA levels in the tissue declined to those found in control leaves or slices incubated in BM for 8 h (data not shown). Thus, with regard to ABA accumulation, spinach leaf slices incubated in mannitol solutions act in a manner similar to a stressed, detached Xanthium leaf (24).

To insure that the measured ECD response of ABA from tissue and medium was due solely to MeABA, equal amounts of ABA (as determined by ECD) extracted from tissue and medium, along with standard (±)-ABA (Sigma) were analyzed by GC-SIM. If the MeABA peak from tissue or medium contained a contaminant, its SIM response would be less than that of the standard. The SIM response at m/z 190 (base peak) of MeABA from tissue, medium, and standard were identical within experimental error (data not shown), indicating that no contaminating peaks cochromatographed with MeABA during the measurement of ABA.

Some variation in the ABA level in turgid leaves and the accumulation of ABA in stressed leaves occurred between experiments. Possible sources of variation include leaf age (leaves were harvested 8 to 14 d after transfer to LD) and the stress history of the plant. To minimize these effects, uniform leaves were selected from groups of plants placed in trays containing deionized H2O for 12 to 16 h before the onset of the experiment.

When leaf slices were incubated in 0.6 M ethylene glycol, no ABA accumulated in either the medium or the tissue (Fig. 4). Similar results have been obtained with thiourea (data not shown), another solute which is able to rapidly penetrate the cell membrane (9). However, when slices were incubated in 0.6 M mannitol or Aquacide III, ABA levels rose in both the medium and tissue. Aquacide III, a highly purified form of polyethylene glycol, average mol wt 18,000, is unable to penetrate the cell wall and causes cytorrhysis (14). Thus, treatment with Aquacide III mimicks the situation found in water-stressed leaves (14). When slices were incubated with ethylene glycol plus mannitol, ABA accumulation was similar to that with mannitol alone. Thus, with regard to ABA accumulation, ethylene glycol was not toxic or injurious to the leaf tissue. Since ABA accumulation occurred only when solutes were used which caused plasmolysis or cytorrhysis, we conclude that ABA accumulation is dependent on the loss of turgor.

Since Rivó and Yang (17) observed uptake and metabolism of mannitol in Citrus leaf discs, we were concerned that mannitol could penetrate the cell membrane in our system and cause osmotic adjustment, or mannitol could be metabolized. Based on radioactivity found in the tissue, 0.73% of the total mannitol found in the medium penetrated the cell membrane. The concentration of mannitol in the tissue was approximately 20 mM, or contributed about -0.05 MPa to the osmotic pressure (assuming a 1.0 M ideal solution is equivalent to -2.4 MPa). This is not enough to cause osmotic adjustment. In addition, contrary to the results that Rivó and Yang obtained with Citrus (17), mannitol was not metabolized to sucrose in spinach (data not shown).

No significant accumulation of ABA occurred when three different concentrations of KHSO4 (5, 15, and 25 mM), 2-propanol (1, 5, and 10%), or KCl (3, 50, and 100 mM) were used (data not shown). These compounds caused the production of ethane (a measure of membrane damage) when incubated with cucurbit leaf discs (5). We conclude, therefore, that the effect of mannitol on leaf slices is a physical one involving water stress, and not a chemical one involving membrane damage.

Of considerable interest is the large accumulation of ABA in the medium (Figs. 2–4; see also Ref. 12). In most experiments, approximately 400 mg fresh weight of slices were used per 10 ml incubation medium, presenting a relatively ‘infinite’ free space to the tissue. A likely explanation is that ABA crosses the plasma membrane and accumulates in the medium where it cannot be catabolized. On the basis of the model for cellular compartmentation of ABA (3), and appropriate values for the relative volumes of free space, tissue, chloroplasts, and vacuoles, one can calculate that most of the ABA will be found in the medium.

Attempts to develop similar systems with slices of bean (Phaseolus vulgaris cv Redkloud) and Xanthium strumarium failed. Accumulation of ABA in slices of these tissues was erratic and irreproducible. Interestingly, Loveys (11) reported higher levels of ABA in Vicia faba mesophyll tissue infiltrated with 0.88 M mannitol than with buffer, but the total ABA in the tissue plus medium was lower than the value reported for nonstressed turgid leaves. Xanthium mesophyll cells have high photosynthetic activity (19), yet do not appear to accumulate ABA when stressed with mannitol (ML Pierce, EA Bray, personal communications) even though stressed, detached leaves of Xanthium accumulate large amounts of ABA (24). When spinach leaf slices were incubated in 0.6 M mannitol along with Xanthium mesophyll cells (19), the amount of ABA that accumulated was reduced (EA Bray, RA Creelman, JAD Zeevaart, unpublished results), indicating the presence of a diffusible inhibitor. This inhibitor is probably nonspecific in action and most likely a result of tissue wounding and cellular leakage. It is also possible that the Xanthium mesophyll cell system was not fully optimized for ABA production.

Pierce and Raschke (16) observed that ABA accumulation occurred when turgor was zero using data obtained from pressure-volume curves. Using spinach leaf slices incubated with plasmolyzing or nonplasmolyzing solutes, we obtained results that support the hypothesis of Pierce and Raschke (16). We conclude, therefore, that turgor is the critical parameter of cell water relations governing stress-induced ABA accumulation.

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