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Regulation of H^+ Excretion[1]

EFFECTS OF OSMOTIC SHOCK

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ABSTRACT

Osmotic shock, a 15-minute plasmolysis followed by a 15-minute rehydration in the cold, is a nondestructive technique which inhibits fusicoccin-stimulated H^+ excretion from oat mesophyll cells (*Avena sativa* L.). Osmotic shock also causes a loss of intracellular solutes and stimulates H^+ uptake, but osmoregulation can still occur, and enhanced H^+ uptake is observed only at low external pH. It is concluded that osmotic shock interferes directly with the excretion of H^+ rather than affecting only H^+ or counter ion uptake.

Plasmolysis alone does not inhibit fusicoccin-enhanced H^+ excretion, and the rehydration step must be rapid and in the cold for maximum inhibition. This suggests that the plasma membrane is perturbed, possibly due to release or rearrangement of membrane protein. Compared to corresponding osmolarities of sorbitol or NaCl, polyethylene glycol 4000 is much less effective during the plasmolysis step; the possibility is discussed that the ineffectiveness of polyethylene glycol 4000 is due to its preservation of plasmodesmata.

The excretion of H^+ is involved in solute flux (23) and aspects of hormone action in higher plants (18), and is governed, at least in part, by events at the plasma membrane. An approach which may be used to understand the regulation of H^+ excretion is to subject cells to osmotic shock—a brief plasmolysis followed by rehydration in the cold. The shock treatment alters fluxes of phosphate (8, 12, 15), glucose (8, 15), and amino acids (1, 9, 20) as well as H^+ (14, 19), but does not markedly affect respiration or protein synthesis (9, 20). Thus, a selective perturbation of the plasma membrane is evoked without recourse to homogenization. Furthermore, protein is released during the rehydration step (1, 8, 12, 15); in microorganisms, this protein is associated with events which were altered by the shock treatment (16).

The data to follow show that osmotic shock inhibits the component of H^+ excretion which is stimulated by the fungal toxin, FC[2]; the shock also causes an increase in permeability to H^+, but it is suggested that the active excretion of H^+ is affected preferentially. The nature of the plasmolysis agent is important, and rapid expansion of the membrane in the cold is required for the maximal effect.

MATERIALS AND METHODS

'Garry' oats (*Avena sativa* L.) were germinated and grown in vermiculite at 23°C under 16-h photoperiods with fluorescent (Econowatt, Westinghouse, Corp.) and incandescent light at 145 $\mu E\ m^{-2}$. One-quarter strength Hoagland solution was applied at time of sowing and on a daily basis after 4 d. The primary leaves were harvested 7 d after sowing, the lower (abaxial) epidermis was peeled away, and one or two 1-cm segments were cut approximately 1 cm below the tip.

Segments to be osmotically shocked were first plasmolyzed on 0.6 M sorbitol, 29 mM sucrose, 5 mM Hepes (pH 7.0) for 15 min. This solution was then aspirated, the tissue moved to an ice bucket, and cold 5 mM Hepes (pH 7.0) was added. After rehydration for 15 min, the sections were transferred to an appropriate medium for estimates of H^+ flux. Unshocked sections were treated identically except that the sorbitol plus sucrose was omitted from the Hepes buffer during the first 15-min treatment. Sucrose alone is without effect, but was included for future comparisons with protoplasts which are suspended in media containing sucrose.

The presumptive net efflux of H^+ was measured by floating five 1-cm peeled leaf segments on 2.5 ml of 1 mM Hepes (pH 6.5) plus 1 mM KCl in 20-ml beakers. To determine the alkalinization of the medium, segments were floated on Hepes-KCl adjusted to pH 4.8 with HCl. For both types of measurements, the beakers were shaken continuously at ambient light and temperature, and the pH of the medium was determined at intervals with a Model 26 Radiometer pH meter equipped with a Radiometer combination electrode. Data were converted to H^+ equivalents with the aid of a titration curve for the solution used. All treatments were run in duplicate and experiments repeated on at least 2 separate d.

Tissue osmolarity was determined by the methods of Van Volkenburgh and Cleland (personal communication). Ten peeled leaf segments were wrapped in aluminum foil after treatment and frozen on dry ice for 1 to 2 h. After thawing for 2 to 5 min, 5 to 10 μl of cytoplasm was expressed and placed on a filter paper disc in a Wescor Model 5100B vapor pressure osmometer. Osmolarities of incubation media were determined by freezing point depression with an Advanced Model 3L osmometer.

For scanning electron microscopy, peeled or unpeeled leaf segments were fixed overnight in osmium vapor and then dehydrated in acetone and critical point exchanged. The tissue was mounted on copper pegs, coated with gold/palladium in a sputter coater, and examined with a JEOL Model JS35 SEM at an accelerating voltage of 25 kev.

RESULTS

Preliminary experiments indicated that leaf tissue must be peeled in order to measure H^+ flux or to perturb the membrane by osmotic shock. To determine if removing the epidermis injures the remaining cells, unpeeled and peeled leaf surfaces were examined with the SEM (Fig. 1). A reticulate cuticle covers the epidermal cells (Fig. 1B). Peeling removes only the epidermis with the cuticle (Fig. 1C), although narrow strips of epidermal cells may remain. The exposed mesophyll cells appear intact and uninjured (Fig. 1D) and have large intercellular spaces which may

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[2] Abbreviations: FC, fusicoccin; SEM, scanning electron microscope; Cl-CCP, carbonylcyanide, *m*-chlorophenyl-hydrazone.

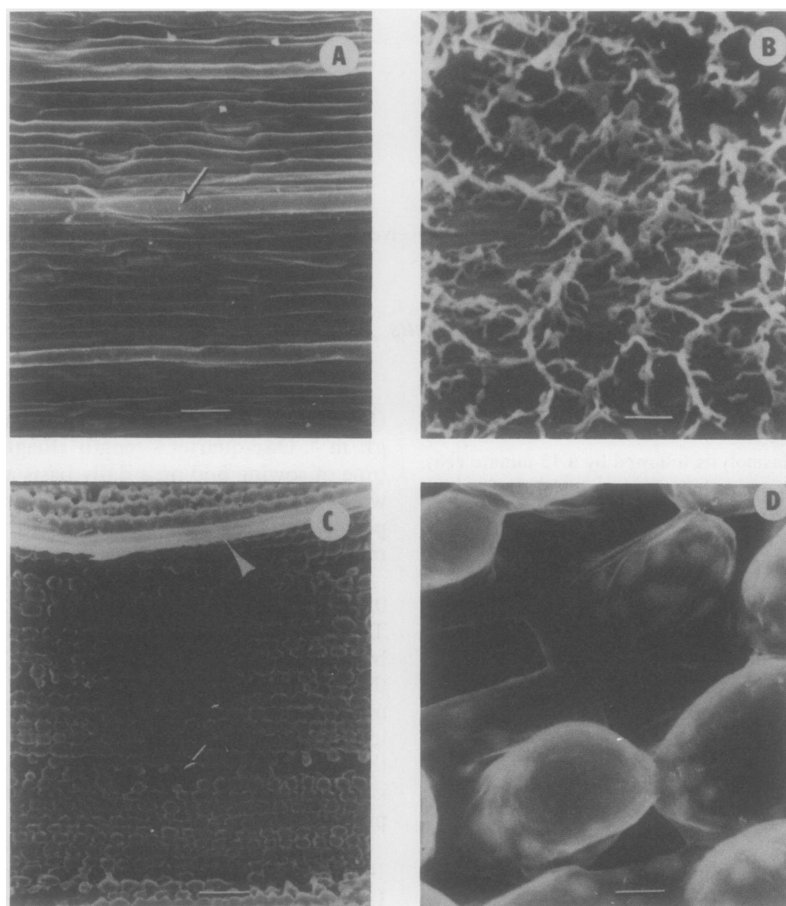

FIG. 1. Effects of removing the epidermis on cell structure of remaining mesophyll cells. A, Abaxial surface of unpeeled leaf with rows of stomata and bulliform cells (arrow), bar = 50 μm; B, a reticulate cuticle covers the unpeeled surface, bar = 1 μm; C, low power view of peeled surface showing rows of intact mesophyll cells and substomatal cavities (arrow), some epidermal cells may remain (arrow head), bar = 50 μm; D, higher magnification of peeled leaf showing mesophyll cells which appear undamaged, chloroplasts are seen within the mesophyll cells, bar = 5 μm.

equilibrate freely with an external medium.

General Characteristics of H^+ Excretion by Oat Leaves and Effects of Osmotic Shock. Based on estimates of membrane potential for oat leaf segments (22) and internal pH (10), the excretion of H^+ by control and FC-treated segments (Fig. 2A) is against an electrochemical gradient. Acidification is not due entirely to respiratory CO_2, since the pH of the medium is not altered more than 0.1 unit when purged for 1 to 2 min with N_2. The apparent excretion of H^+ is inhibited within 15 min by respiratory poisons and by 1 mM Na_3VO_4 (data not shown) as is also found for other tissues (e.g. Ref. 4).

Plasmolysis followed by rehydration in cold buffer (an osmotic shock) decreases FC-enhanced H^+ excretion by 50% or more, while very small stimulations of control rates are routinely seen (Fig. 2). When FC is added to unshocked sections after 2 h, the rate of acidification by the toxin is the same as that occurring initially (Fig. 2A); this is also true for the inhibited rate of shocked tissues (Fig. 2B), so there is no measurable recovery of FC-stimulated acidification by 2 h after shock.

Effects of Osmotic Shock on Membrane Permeability. Endogenous levels of osmotically active solute were estimated by vapor pressure osmometry to assess the extent of alteration of membrane permeability after osmotic shock, and to see if failure to take up K^+ was the reason for inhibitions of H^+ excretion by FC. The data in Table I show that by completion of the rehydration period (0-time), there is a loss of osmotically active solutes compared to unshocked tissue. If the segments are transferred to a buffered medium containing 1 mM KCl, there is little change in tissue osmolarity unless FC is present, in which case tissue osmolarity increases in both unshocked and shocked treatments. If segments are transferred to a medium with 10 mM KCl, both shocked and unshocked tissues without FC show an increase in internal osmolarity; the addition of FC elevates the osmolarity even more. Thus, the concentration of osmotically active solutes of shocked, FC-treated segments floated for 3 h on 10 mM KCl is about the same as unshocked, FC treatments on 1 mM KCl; but, when H^+ excretion is measured, 10 mM KCl (or even 25 mM KCl) is unable to alleviate the inhibitory effect of osmotic shock on FC-enhanced H^+ excretion (data not shown).

Attempts were made to determine the effects of osmotic shock on uptake of H^+ by floating peeled leaf segments on a pH below their equilibrium value and monitoring the resulting increase in pH (Fig. 3). The alkalinization consists of a rapid phase lasting for about 15 min and then a slower phase which approaches an equilibrium pH of about 5.8. These kinetics are not observed unless the segments are peeled, and rates of alkalinization are correlated with tissue mass, not the number of cut surfaces (Fig. 3). Plasmolyzed segments (on 0.6 M sorbitol and 29 mM sucrose) alkalinize the medium at the same rate as turgid segments (data not shown).

The effects of a respiratory poison and osmotic shock on alkalinization are shown in Figure 4. Cl-CCP stimulates the apparent loss of H^+ from the medium by a small amount over the first 15 to 30 min, and an equilibrium is approached which is considerably above that of controls (Fig. 4A). Osmotically shocked segments also alkalinize the medium slightly more rapidly than

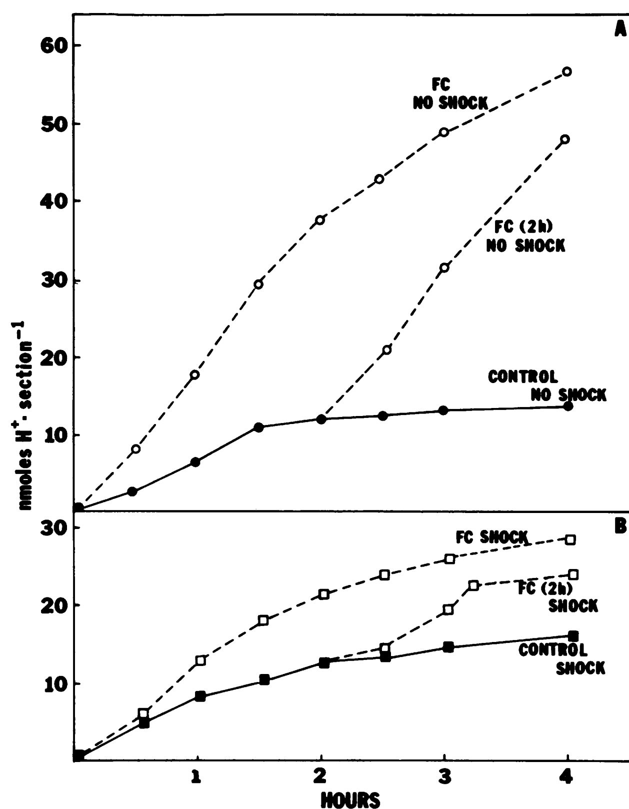

FIG. 2. H^+ excretion by control leaf segments (●, ■) or segments exposed to 0.1 μM FC (○, □). Tissue was unshocked (A) or osmotically shocked (B) before being placed on acidification medium (1 mM Hepes, 1 mM KCl, pH 6.5).

Table I. *Effect of FC and KCl on Osmotically Active Solutes after Osmotic Shock*

Initial osmolarity of expressed sap from freshly harvested leaves was 214 ± 4 mosm/kg. Tissue to be shocked was then given 0.6 M sorbitol, 29 mM sucrose for 15 min followed by cold 5 mM Hepes (pH 7.0) for 15 min; controls were given Hepes at 23°C for 15 min followed by cold Hepes for 15 min. After this 30-min interval, 0-time osmolarities were determined. The remaining tissue was transferred to 1 mM Hepes (pH 6.5) containing 1 or 10 mM KCl with or without 0.5 μM FC. Final readings were taken after 3 h. Data presented as mean ± SD (n = 3).

Treatment	0-time	1 mM KCl		10 mM KCl	
		−FC	+FC	−FC	+FC
		mosm/kg			
No shock	216 ± 4	220 ± 3	235 ± 7	296 ± 7	378 ± 14
Shock	196 ± 6	170 ± 6	192 ± 20	208 ± 3	228 ± 7

controls (by 12 ± 7 nmol/ml; n = 4), but only for about 15 min; from that point, the rate slows and an equilibrium value similar to controls is approached.

A closer inspection of events occurring at the lower pHs was afforded by replacing the bathing medium at 5-min intervals with solutions at the original pH of 4.8 (Fig. 4B). The data show that after 5 min, alkalinization proceeds at a linear rate. The rate is about 1.2 times faster than controls after segments are osmotically shocked and is over 1.3 times faster with 10 μM Cl-CCP in the medium. Thus, osmotic shock can mimic to some extent the effects of a H^+ ionophore, but this stimulation of alkalinization can only be seen at a pH range between 4.8 and 5.1.

Conditions for Inhibition of H^+ Excretion by Osmotic Shock. Plasmolyzed tissue responds to FC in a manner similar to turgid

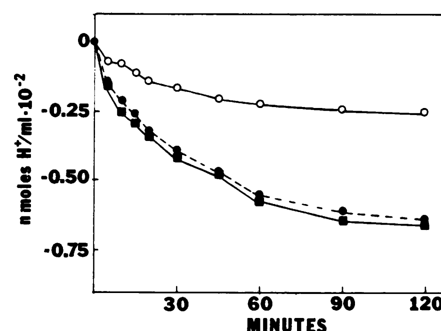

FIG. 3. Effects of peeling and number of cut surfaces on alkalinization of the medium. (●), Five 1-cm peeled segments on 2.5 ml 2 mM Hepes, 1 mM KCl (pH 4.80); after 2 h, pH = 5.34. (○), Five 1-cm unpeeled segments on 2.5 ml 2 mM Hepes, 1 mM KCl (pH 4.80); after 2 h, pH = 4.98. (■), Twenty 0.25-cm peeled segments on 2.5 ml 2 mM Hepes, 1 mM KCl (pH 4.80); after 2 h, pH = 5.45.

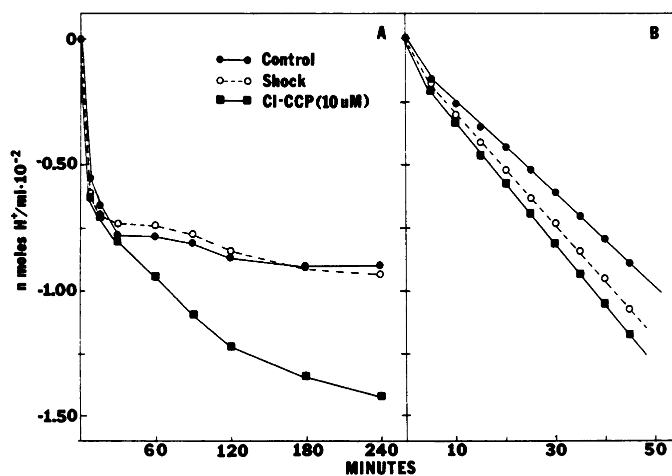

FIG. 4. Effects of Cl-CCP and osmotic shock on alkalinization of the medium. A, Segments floated on 1 mM Hepes, 1 mM KCl (pH 4.80), and pH followed over 4 h; final pH: control = 5.94, shock = 5.99, Cl-CCP = 6.36. B, Segments on identical buffer, but the medium was replaced with fresh at 5-min intervals.

treatments (Fig. 5A). But the nature and osmolarity of the plasmolyzing agent is important for the H^+ excretion which is observed following rehydration. Dilute solutions of sorbitol and NaCl (about 300 mosm/kg) during the plasmolysis step often lead to slight stimulations of acidification, and at higher osmolarities, both are equally capable of reducing FC-enhanced acidification following rehydration in the cold (Fig. 5, B and C); the inhibition is most noticeable when solution osmolarities are 500 mosm/kg or greater. In general, as the osmolarity of the solution increases, the inhibitory response appears sooner after rehydration and the equilibrium value approached is less acidic. PEG 4000, however, is much less effective than sorbitol or NaCl at inhibiting FC-enhanced acidification; for example, 700 mosm/kg PEG 4000 during the plasmolysis step inhibits H^+ excretion following rehydration by 10% (Fig. 5D), while sorbitol at an equal osmolarity results in a 75% inhibition (Fig. 5B).

To see if PEG 4000 plasmolyzed cells to the same extent as sorbitol, the cytoplasmic osmolarity of leaf segments was determined after they had been floated on 5 mM Hepes (pH 7.0) with or without sorbitol or PEG 4000 for 15 min. Table II shows that a concentration of PEG 4000 which, after rehydration, inhibits FC-enhanced acidification less than sorbitol (see Fig. 5, B *versus* D), results in a somewhat greater increase in cell osmolarity than sorbitol. Thus, the less severe inhibition of H^+ excretion by the

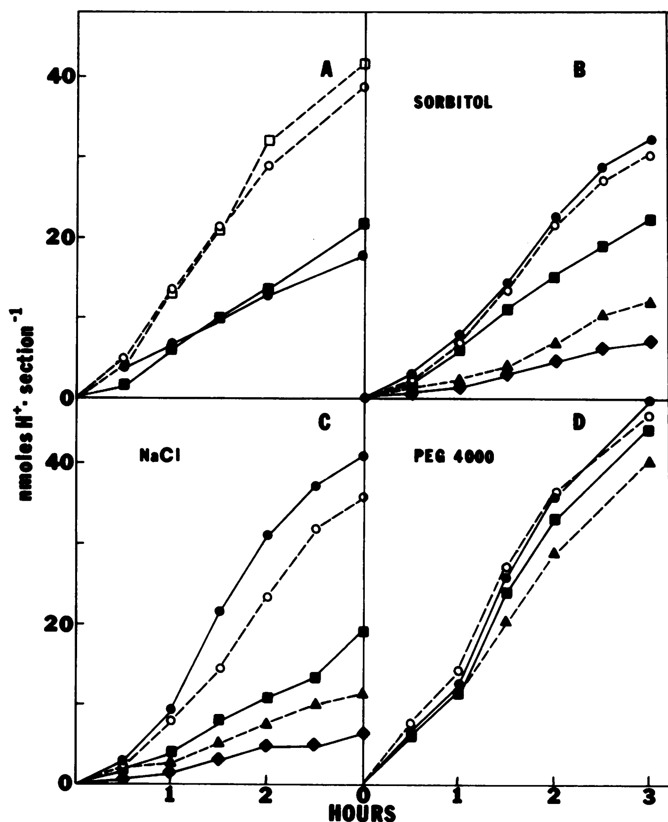

FIG. 5. A, Effect of plasmolysis by 0.6 M sorbitol on acidification. (●), Control; (○), + 0.1 μM FC; (■), + 0.6 M sorbitol, 29 mM sucrose; (□), + 0.1 μM FC; 0.6 M sorbitol, 29 mM sucrose. The acidification medium contained 1 mM KCl, but treatments with sugars were buffered with 1 mM Hepes, and those without sugars buffered with 2 mM Hepes to just offset the small amount of buffering by 0.6 M sorbitol; starting pH = 6.7. B, Effect of various osmolarities of sorbitol during the plasmolysis step on FC-enhanced acidification after rehydration in the cold. Sorbitol solutions also contained 29 mM sucrose and 5 mM Hepes (pH 6.7). (○), 5 mM Hepes; (●): 308 mosm/kg; (■), 518 mosm/kg; (▲), 656 mosm/kg; (◆), 829 mosm/kg. Acidification medium consisted of 1 mM Hepes, 1 mM KCl, 0.1 μM FC (pH 6.7). C, Effect of various osmolarities of NaCl during the plasmolysis step on FC-enhanced acidification after rehydration in the cold. The NaCl was dissolved in 5 mM Hepes (pH 6.7). (○), 5 mM Hepes; (●), 315 mosm/kg; (■), 558 mosm/kg; (▲), 647 mosm/kg; (◆), 938 mosm/kg. Acidification medium same as in B. D, Effect of various osmolarities of PEG 4000 during the plasmolysis step on FC-enhanced acidification after rehydration in the cold. The PEG 4000 was in 5 mM Hepes (pH 6.7). (○), 5 mM Hepes; (●), 244 mosm/kg; (■), 632 mosm/kg; (▲); 900 mosm/kg. Acidification medium same as in B.

Table II. *Effect of Sorbitol or PEG 4000 on Intracellular Osmolarity*

Leaf segments were floated on the specified solution for 15 min. The tissue was then rinsed, frozen, thawed, and osmolarity of the expressed sap was determined. Data presented as mean ± SD (n = 3).

Addition	Osmolarity	
	Solution	Tissue
	mosm/kg	
None	27 ± 1	249 ± 1
Sorbitol	693 ± 5	510 ± 14
PEG 4000	815 ± 30	554 ± 38

polymer is not related to an inability to remove water from the cells.

The rehydration procedure was altered in order to understand what aspects of this step are crucial for the inhibitory effects of osmotic shock on H^+ excretion. Instead of transferring the plasmolyzed tissue directly to a buffer of low osmolarity, a series of less concentrated osmotica were used. As shown in Figure 6A, this gradual rehydration is much less effective than a rapid rehydration at inhibiting the FC-enhanced component of H^+ excretion; in fact, rates of H^+ excretion are similar to the unshocked FC treatment until 90 min after shock.

In order for osmotic shock to inhibit H^+ excretion by FC more completely, it is also necessary to rehydrate plasmolyzed tissue in cold rather than warm buffer (Fig. 6B). Unlike corn roots (7), a cold treatment to oat leaves without prior plasmolysis has no effect (data not shown). Thus, the rate of, and temperature during, rehydration influences the extent of inhibition by osmotic shock. The difference in acidification by unshocked controls in Figure 6, A and B, is a result of normal variability.

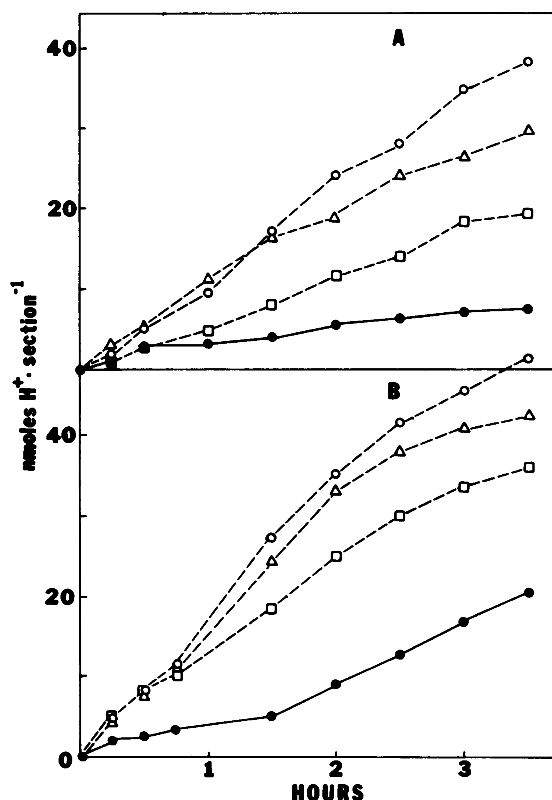

FIG. 6. Effects of rehydration conditions on subsequent excretion of H^+. A, Comparison of gradual *versus* rapid rehydration. (●), No shock, then to acidification medium consisting of 1 mM Hepes, 1 mM KCl (pH 6.65). (○), No shock, then to acidification medium also containing 0.1 μM FC. (□), Plasmolysis with 0.6 M sorbitol, 29 mM sucrose for 15 min followed by immediate transfer to cold 5 mM Hepes for 15 min. Acidification medium also contained 0.1 μM FC. (△), Plasmolysis with 0.6 M sorbitol, 29 mM sucrose for 15 min followed by transfer to 0.45, 0.3, 0.15 M sorbitol for 5 min each in the cold. Acidification medium also contained 0.1 μM FC. B, Effect of rehydration temperature. (●), No shock, then to acidification medium consisting of 1 mM Hepes, 1 mM KCl (pH 6.8). (○), No shock; acidification medium also contained 0.1 μM FC. (□), Plasmolysis with 0.6 M sorbitol, 29 mM sucrose followed by rehydration at 4°C. Acidification medium also contained 0.1 μM FC. (△), Plasmolysis with 0.6 M sorbitol, 29 mM sucrose followed by rehydration at 23°C. Acidification medium also contained 1 μM FC.

DISCUSSION

As in other systems (13), H^+ excretion by oat mesophyll cells proceeds against an electrochemical gradient. Osmotic shock inhibits FC-stimulated H^+ excretion and, unlike amino acid uptake (1, 20), the excretion mechanism does not recover by 2 h. In contrast, acidification by control leaf segments, may be slightly stimulated by shock (Fig. 2), so the shock treatment does not destroy all transport properties of the membrane.

Inhibitory effects of osmotic shock on acidification by coleoptiles is reported by Masuda *et al.* (14), but the tissue is not peeled, and while peeling does not affect the cell structure of coleoptiles (21) or leaves (Fig. 1), it is a requirement for measurements of H^+ extrusion (18) and for the shock effect. Using only 'longitudinal cuts,' Masuda *et al.* (14) report that with auxin, the pH of the medium is 0.2 pH units lower than controls after 3 h, but the usual effect is often 1 or 1.5 units lower (18); furthermore, the reduction of acidification by osmotic shock after 3 h is only 0.07 pH units. This very small effect by osmotic shock on H^+ excretion may be why Masuda *et al.* could not detect a shock-induced inhibition of growth as reported previously (19).

It is important to know whether osmotic shock primarily inhibits the excretion of H^+ or whether a passive influx of H^+ is facilitated. The leakage of solutes (Fig. 5; Refs. 8, 20) points to important changes in membrane permeability, but it is difficult to determine if H^+ uptake and/or excretion is affected by shock because only net flux of H^+ is measured. Reasoning, however, that passive uptake of H^+ would predominate at a low external pH, the effects of osmotic shock and Cl-CCP were observed while cells alkalinized the medium from pH 4.8 to approximately 5.8. Interpretations of these results depend on the alkalinization being due primarily to H^+ uptake at low pH as concluded previously by Cleland (3) and Vanderhoef *et al.* (24), but the phenomenon may also be explained by other factors which control external pH, *e.g.* fluxes of OH^-, organic acids, or dissolved CO_2. It is also impossible to estimate the role of unstirred layers in limiting the alkalinization rate.

The pH increase occurs in two phases (Figs. 3 and 4A). The early, rapid phase probably involves some equilibration with an external compartment, but this is not an explanation for a large portion of the early response, since the alkalinization rate is linear for over 40 min when the pH is maintained at 4.8 (Fig. 4B); it is likely, therefore, that H^+ is diffusing into the cells at that low pH. The presumptive uptake of H^+ at high external H^+ concentrations can be stimulated further by the H^+ ionophore Cl-CCP (Fig. 4).

There is also a small stimulation of alkalinization after osmotic shock (Fig. 4), which may indicate that shock changes the permeability of the membrane to H^+. But the equilibrium pH for shocked tissues is similar to that of unshocked controls (Fig. 4A); thus, at about pH 5.5 and above, shocked cells seem able to compensate for any changes in passive permeability by regulating the rate of H^+ excretion. Inasmuch as the equilibrium for both control and shocked tissues is at or below the pH range where the most rapid acidification by FC occurs (pH 6.5–5.5), it seems likely that the effect of osmotic shock is more on the H^+ efflux mechanism than on H^+ uptake.

Another explanation exists, however, for the inhibition by osmotic shock of FC-enhanced acidification which does not involve a direct effect on an excretion mechanism. It is known that K^+ uptake is required for maximal rates of acidification (13), so the inhibition may be due to a failure to take up counter ions. Osmotic shock does inhibit uptake of phosphate (8, 12, 15) and K^+ leaks from the cells (15, 20), but it is unlikely that shock acts in this manner because the cells can still take up ions against a concentration gradient (Table I). Furthermore, by incubating the tissue on higher concentrations of KCl, the shocked cells can accumulate KCl to a level equal to that of unshocked cells (Table I); yet, this increase in rate of KCl uptake has no effect on the depressed rate of FC-enhanced H^+ excretion after osmotic shock.

The augmentation of osmotically active solutes in mesophyll cells by FC is the opposite of that seen by Lado *et al.* (11). This is because Lado *et al.* (11) chose tissue (root segments) which elongates in response to FC; the uptake of water would lower the internal concentration of solutes. No change in length or fresh weight of oat leaf segments is detected in the presence of FC, presumably because the cells are fully expanded. This tissue was chosen so that interactions of FC with the plasma membrane could be studied without any complicating factors related to cell growth.

In order to inhibit H^+ excretion, osmolarities of sorbitol and NaCl probably must be high enough to cause plasmolysis (Fig. 5, B and C). Similar observations were made for corn root tips by Grunwaldt *et al.* (8). Plasmolysis alone, however, neither inhibits FC-stimulated H^+ excretion (Fig. 5A) nor stimulates H^+ uptake at low pHs, so the membrane alterations occur during the rehydration step. For greatest effectiveness, the rehydration must be rapid, not stepwise (Fig. 6A), and, as with bean leaves (1), the medium must be cold (Fig. 6B). The rapid expansion of the membrane in the cold may result in the breaking of hydrophobic bonds (6) which could lead to the release of regulatory protein.

Curiously, when turgor is reduced by PEG 4000 during the plasmolysis step, the inhibition of FC-stimulated H^+ excretion seen after rehydration is less pronounced than when sorbitol or NaCl is used (Fig. 5), even though the polymer is as effective as sorbitol at removing water from the cells (Table II). Carpita *et al.* (2) have postulated that PEG 4000 does not penetrate the wall; as a result, the wall and protoplast collapse when water leaves the cell as compared to only shrinkage of the protoplast in the presence of sorbitol or NaCl. Cell shrinkage in PEG 4000 has also been observed with oat leaves (M. J. Saunders, B. Rubinstein, P. K. Hepler, unpublished observations). The pressure of the wall on the membrane may protect it from the effects of osmotic shock, or PEG 4000 may interact with and stabilize membrane components.

Another explanation for the ineffectiveness of PEG 4000 is that plasmodesmata may become less altered when the entire tissue collapses. Plasmolysis is known to destroy some plasmodesmata (5, 17), but since plasmolysis alone does not inhibit H^+ flux (Fig. 5A), it is necessary to postulate that sites at plasmodesmata are disturbed only when the protoplast swells. This interpretation implies that the integrity of plasmodesmata is essential for maintaining rates of H^+ excretion in the presence of FC.

Osmotic shock primarily inhibits the H^+ excretion mechanism rather than H^+ uptake, and this occurs as a result of a plasmolysis step which shrinks only the protoplast. The actual inhibition is related to a rapid expansion of the plasma membrane in the cold. The relationship of H^+ excretion and membrane expansion to protein appearing in the rehydration medium is considered in the following paper.

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