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Is Modulation of the Rate of Proton Pumping a Key Event in Osmoregulation?[1]

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ABSTRACT

The net uptake of 3-*O*-methylglucose into leaf segments obtained from *Senecio mikanioides* Otto, and net proton efflux from the segments, were both promoted when the osmotic potential of the medium was decreased by addition of mannitol, sorbitol, or polyethylene glycol (optimal osmolarity, 0.3 Osmolar for mannitol and sorbitol). The effect was not due to promotion of 'aging', since the antibiotic cerulenin suppressed aging without reducing the size of the mannitol stimulation; further, mannitol did not accelerate aging. Neither was the effect ascribable to diminished efflux (*i.e.* reduced 'leak' because: first, visualization of the unidirectional sugar fluxes by double labeling indicated that the effect of added osmoticum was to promote influx rather than to reduce efflux; second, compartment analysis did not suggest any effect of mannitol on the rate constants for efflux from either the slowly equilibrating or more rapidly equilibrating compartment. The effect was not specific to poly-ols since it was also obtained with betaine and choline chloride. Since methyl glucose is not taken up into the phloem it could not be ascribed to a turgor effect on phloem loading. We conclude that the effect may reflect osmoregulation. As the sugar flux is probably driven by protonmotive force, it is likely that the effects on proton flux and on sugar flux are related. We suggest that the plasmalemma-sited proton pump is sensitive to the hydrostatic pressure gradient across the plasmalemma-cell wall complex, and functions both as detector and as effector in osmoregulation.

We have earlier (8, 9) brought evidence which suggests that plasmalemma transport of sugars into pea mesophyll protoplasts in the light (though possibly not in the dark) is driven by pmf[2] as is believed to be the case in many other plant systems (11). Apart from its sensitivity to light, the sugar transport system of leaf tissue also shows sensitivity to turgor. Because experiments in which external osmotic pressure is manipulated in the hypotonic range are not practicable with isolated protoplasts, we have carried out these experiments with leaf segments of *Senecio mikanioides* from which the lower epidermis had been removed. We have observed that a change towards more negative external water potential brings about an increased rate of proton extrusion (also reported by 5, 7) as well as an increase in sugar influx. The observed effect of turgor on sugar transport may thus well be mediated via the proton extrusion pump and the pmf. In a brief preliminary communication (12), we proposed that these responses to low turgor reflected osmotic regulation, and suggested that the proton pump might play a key role in the mechanism of osmotic adaptation. However, a number of alternative explanations for the observed effects suggested themselves, which we thought necessary to investigate. We have presented the results of our investigation in detail.

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[2] Abbreviations: pmf, protonmotive force; MeG, 3-*O*-methyl-D-glucose.

MATERIALS AND METHODS

Leaves which had almost reached their maximum size were collected from plants of *Senecio mikanioides* Otto growing at two sites out-of-doors in Jerusalem. After removal of the lower epidermis, segments approximately 4 mm^2 were excised and 'aged' in 10^{-4} M $CaSO_4$ for about 90 min. Samples weighing 100 mg were then transferred to 480 μl incubation medium containing 10 mM KCl, 0.2 mM $CaSO_4$, 2.5 μM fusicoccin, 2.5 mM Mes (pH 5.8) and other additives as indicated in the text. The fusicoccin was added to promote net outwards H^+ movement in the proton extrusion studies. Experiments (not presented here) were also conducted where fusicoccin was omitted, and the effects of added osmotica were similar to those in the experiments reported below. The osmolarity of the media was determined in a Knauer semi-micro osmometer.

Sugar uptake was initiated with the addition of 10 μl of a labeled solution of MeG, a nonmetabolizable sugar, to a final concentration of 1 mM (radioactivity, 75 MBq per sample). During incubation, the samples were shaken in a bath at 27°C illuminated by a water-cooled General Electric Quartzline lamp, the light intensity at the level of the incubation tubes being 500 μmol m^{-2} s^{-2} in the range of 400 to 700 nm. After the required incubation period, MeG was removed from the free space of the leaves by four successive washes, the first three lasting 30 s each and the third 15 min, in a medium of composition similar to the incubation medium except for the omission of MeG. When various osmotica were added to the incubation medium they were also added to the washing medium in order to avoid osmotic shock (2). The leaf segments were then extracted with 90% ethanol at 60°C, and samples of the extract were counted in a Triton-toluene scintillation fluid.

In certain experiments, the unidirectional fluxes were observed by a double-labeling technique involving use of both [^{14}C]MeG and [^{3}H]MeG as described in "Results.' A curve was constructed to correct for quenching using external standards.

For the compartmental analysis, samples of leaf segments were preincubated for 90 min in a MeG medium of the standard composition but containing 375 MBq per sample, with or without the addition of 0.3 M mannitol. They were then transferred to vials with perforated bases in which they could be rapidly transferred through a series of flasks each containing 5 ml of a solution identical to the incubation medium but not labeled. Samples of 0.5 ml were taken from the wash-out media for counting. At the conclusion of the experiment, the leaf segments were extracted in 90% ethanol for assessment of radioactivity remaining in the tissue.

Changes in pH in the external medium were measured with a

Radiometer electrode BK 2401C connected to an expanded scale Radiometer pH meter. The ratio of tissue to medium in these experiments was 0.5 g to 10 ml. Between readings, the samples were shaken in the illuminated water bath at 27°C. CO_2-free air (saturated with water vapor) was bubbled through the media for 5 min before each reading.

Chemicals. [^{14}C]MeG and [^{3}H]MeG were obtained from Amersham International, U.K.; MeG and betaine from Sigma Chemical Co.; choline chloride and PEG_{6000} from Merck, Darmstadt, W. Germany. Fusicoccin was the kind gift of Professor E. Marrè of the University of Milan.

RESULTS

Figure 1 shows the stimulatory effect of an added osmoticum on MeG uptake. When mannitol or sorbitol was the osmoticum added to the medium, the maximum effect on sugar uptake was obtained at about 0.3 Osm. Higher osmolarity was required when PEG (mol wt 6000) was used as osmoticum.

An example of an experiment in which acidification of the medium was followed with time is shown in Figure 2. Acidification proceeded at a higher rate in the presence of 0.3 M mannitol. Since CO_2-free air had been bubbled through the solutions for 5 min before each reading, acidification is not attributable to respiration. In any event, the net CO_2 flux during incubation was observed to be inward under the conditions of our experiments due to photosynthesis (data not shown). Mannitol thus promoted net H^+ efflux.

Possible Involvement of the 'Aging" Phenomenon. Excised leaf tissue gradually develops uptake capacity during aging in 10^{-4} M $CaSO_4$ (1, 13) and we have investigated the possibility that this development might for some reason be promoted or facilitated when the cells are at low turgor. Since we followed sugar uptake over a period of 1 to several hours, our assessment of uptake would be affected by aging. Two lines of evidence indicate, however, that the mannitol effect does not relate to aging. First,

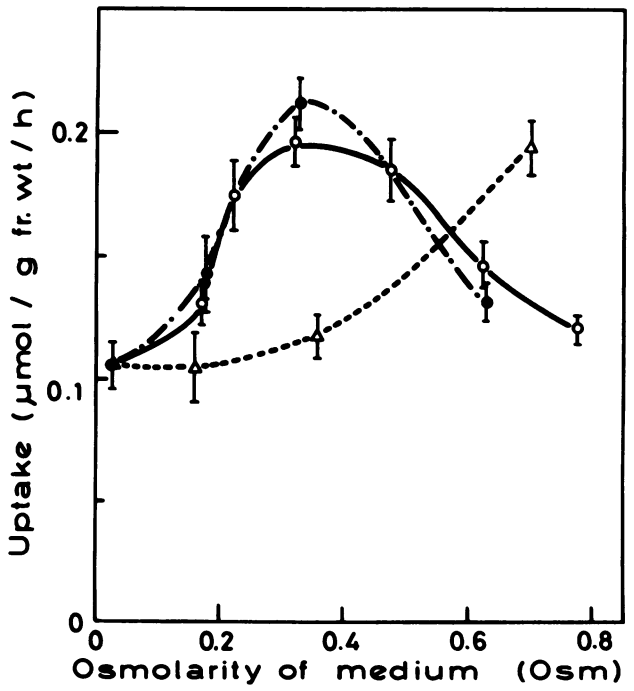

FIG. 1. The dependence of MeG uptake by *Senecio* leaf segments on osmolarity of external medium. The osmoticum added to the medium was mannitol (○), sorbitol (●), or PEG_{6000} (△). Each point represents the mean of triplicates ± SE.

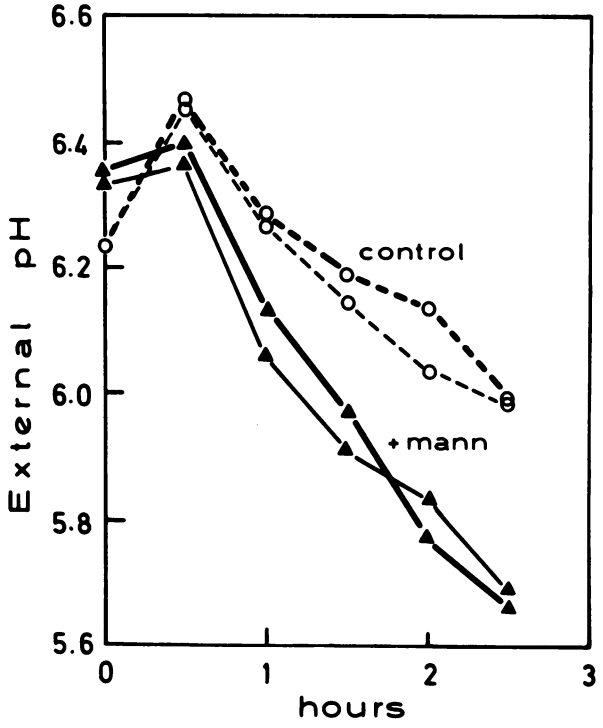

FIG. 2. Time course of acidification of of the external medium by *Senecio* leaf segments in the presence (▲) or absence (○) of 0.3 M mannitol. The two curves for each treatment represent two duplicate samples. The basic medium contained 10 mM KCl, 0.2 mM $CaSO_4$, 2.5 μM fusicoccin and was not buffered. CO_2-free air was bubbled through the media for 5 min before each reading.

we have followed the course of aging in the presence and absence of mannitol but have observed no acceleration of the process in the former case. Figure 3 shows that the course of aging in the presence of mannitol runs parallel to that in its absence. Second, we have made use of the antibiotic cerulenin which inhibits the biosynthesis of membrane lipids and which, as we have reported (1) suppresses aging. Approximately the same percentage stimulation of sugar uptake by mannitol was observed in the absence and presence of a cerulenin concentration which slowed aging very substantially (Fig. 3).

Effect of Adding an External Osmoticum on the Unidirectional MeG Fluxes. We have considered the possibility that more negative external osmotic pressure might exert its effect on net inward sugar flux, not via a stimulating effect on unidirectional influx, but by reducing unidirectional efflux (possibly by diminishing the passive permeability of the membrane and hence reducing outwards 'leak'. Dainty and Ginzburg (4) have observed a decrease in membrane permeability to both water and urea with rising external osmolarity, and have suggested that the permeability changes may relate to changes in the 'packing' of the membrane as it shrinks or swells. This possibility has been explored in two types of investigation.

(a) The unidirectional fluxes were 'visualized' in experiments of the following design. The leaf segments were first loaded with [^{14}C]MeG for 90 min in the presence and absence of mannitol. They were then very briefly rinsed (5 s) and transferred to a MeG solution of the same concentration but now labeled with ^{3}H instead of ^{14}C (again with or without the addition of mannitol, respectively). Loss of ^{14}C by the tissue was followed with time and gave a measure of MeG efflux, while uptake of ^{3}H with time provided a measure of influx (Fig. 4). There is no indication whatsoever in the data shown in Figure 4 that mannitol treatment decreased ^{14}C loss (*i.e.* efflux), although the conclusion cannot

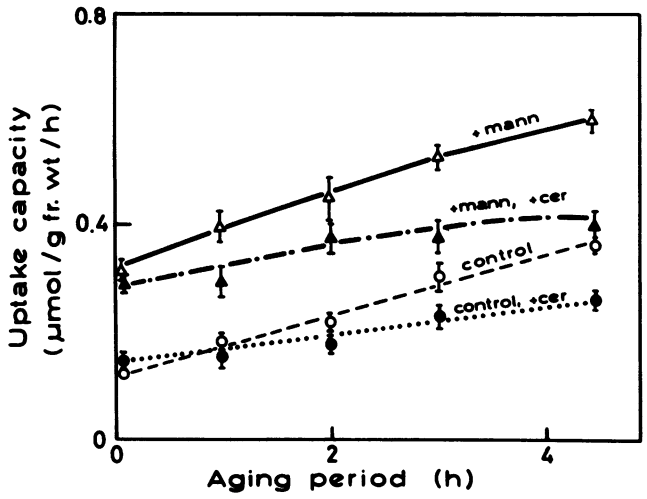

FIG. 3. The effect of 0.3 M mannitol on MeG uptake by *Senecio* leaf segments, and on development of MeG uptake capacity during aging in absence or presence of 35 mg/l cerulenin. After aging, samples were incubated in MeG with or without mannitol for 1 h to determine uptake capacity. Each point represents the mean of triplicates ± SE.

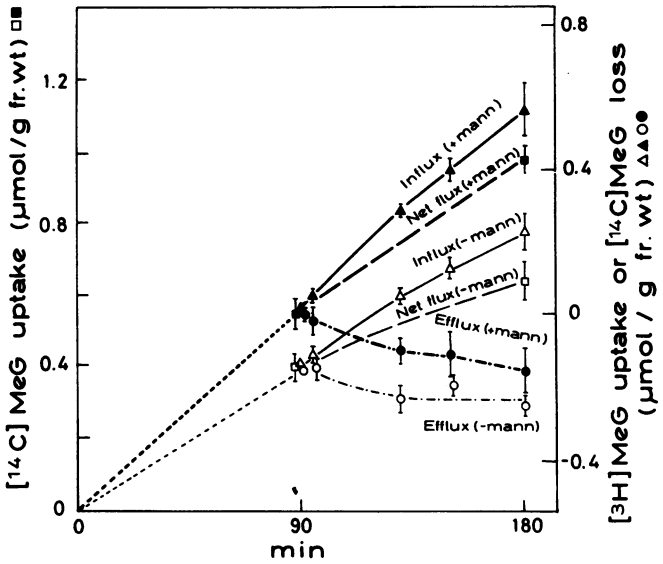

FIG. 4. Net MeG flux inwards (■, □) influx (▲, △), and efflux (●, ○) into and out of *Senecio* leaf segments. In the case of influx and efflux, the leaf segments were first incubated in [^{14}C]MeG (1 mM) for 90 min, then transferred to [^{3}H]MeG at the same concentration. Efflux was followed as loss of ^{14}C from the segments, influx as gain in ^{3}H. In the case of net flux, the segments remained in [^{14}C]MeG throughout the experiment and net flux was assessed as gain in ^{14}C. In each case, the flux is given by the slope of the curve. (■, ▲, ●), + 0.3 M mannitol; (□, △, ○), without mannitol. Each point represents the mean of triplicates ± SE.

be drawn with certainty because of the scatter of the points. On the other hand, there is a clear indication that mannitol treatment increased ^{3}H uptake (influx). The influx curves diverge during the period 90 to 180 min. The increased net flux inwards must therefore be largely attributed to enhanced influx, not to decreased efflux.

(b) Efflux has also been investigated using the techniques of compartmental analysis (see *e.g.* 10). Here the tissue was again first loaded with [^{14}C]MeG, and subsequently passed successively through a series of flasks containing MeG solutions at the same concentration as the loading medium, but not labeled. Exit of previously absorbed labeled MeG was followed with time over a period ranging from 20 s to 3 h. The slopes of the lines shown in Figure 5 and Figure 5 (inset) give the rate constants for efflux from a slowly equilibrating compartment (presumably the vacuole) and a more rapidly equilibrating compartment (presumably the cytoplasm), respectively. There is no indication here that the rate constant for efflux from either compartment was lower in mannitol-treated tissue.

Effect of Osmotica without Poly-ol Groups. Figure 1 showed that the stimulatory effect of added osmoticum was observable whether mannitol, sorbitol or PEG was the osmoticum tested. These substances all contain poly-ol groups. To investigate whether the effect might relate not to osmoregulation, but to some action of poly-ols on the cell membranes, we tested the effect of two substances which do not contain such groups, but which are suitable for use as osmotica since they penetrate the membrane only slowly—choline and betaine. Both these substances when added to the external medium promoted MeG uptake. After 90 min of uptake, betaine and mannitol (at 340 and 325 mOsm, respectively) had stimulated uptake to the same extent (Fig. 6). The fact that after 3 h the point for the betaine treatment lay somewhat below that for the mannitol treatment might possibly be due to a toxic effect of the former, since apparent toxicity was noted in preliminary experiments with choline chloride. For this reason, the highest choline chloride

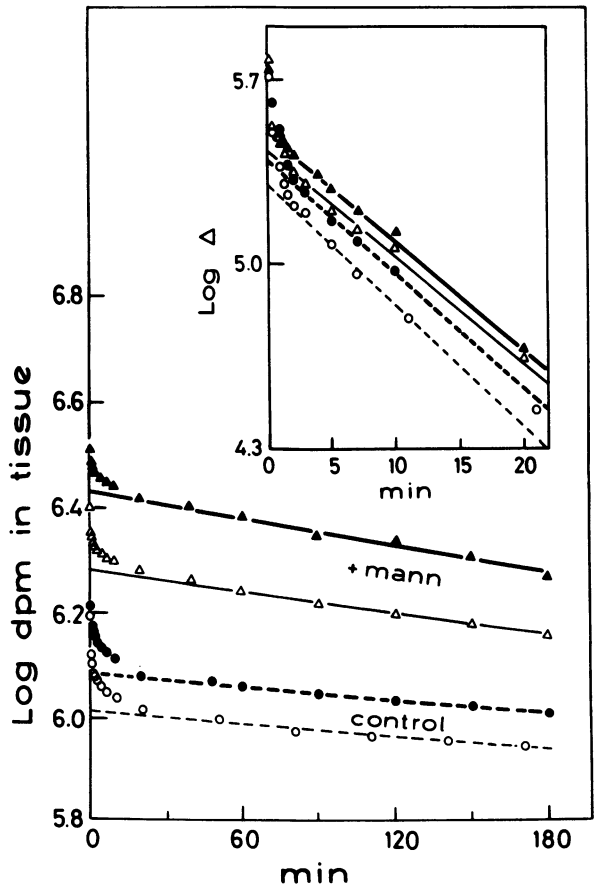

FIG. 5. The effect of addition of 0.3 M mannitol on the time course of exit of labeled MeG from *Senecio* Leaf segments. Preincubation period in labeled MeG in presence or absence of 0.3 M mannitol 90 min in case of three lower curves, 110 min for uppermost curve. Inset: (△), difference between the points shown in Figure 5 and the extrapolated straight lines. (○, ●), Duplicate samples without addition of mannitol; (△, ▲), + mannitol.

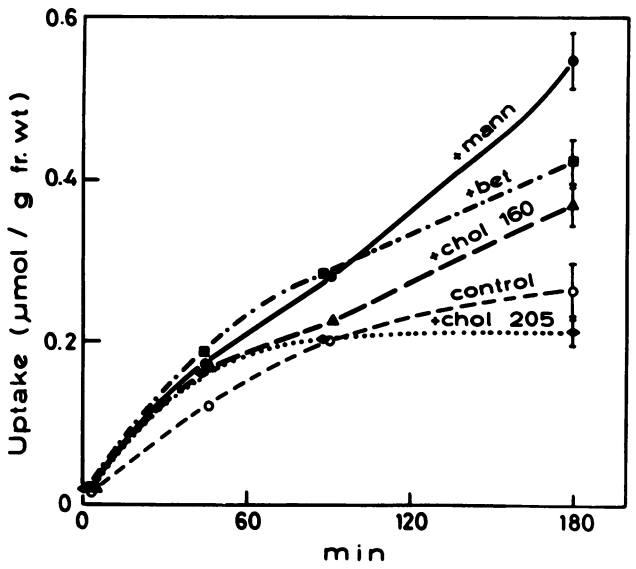

FIG. 6. Progress curve for MeG uptake by *Senecio* leaf segments in the presence and absence of various osmotica. (○), No added osmoticum; (●), + 325 mOsm mannitol; (■), + 340 mOsm betaine; (▲), + 160 mOsm choline chloride; (♦), + 205 mOsm choline chloride. Basic medium as given in "Materials and Methods." Each point represents the mean of triplicates ± SE.

treatment tested in the experiment shown in Figure 6 was 205 mOsm. This concentration slightly depressed uptake below control value after 3 h. Choline chloride at 160 mOsm, however, produced a clear stimulation of uptake. These results rule out the possibility that the basis of the observed effect was some action of poly-ols on the membrane rather than their contribution to the external osmotic potential.

DISCUSSION

The results reported here show that decreasing the external osmotic potential brings about an increase in rate of sugar uptake into leaf cells. It also brings about an increase in rate of proton extrusion (Fig. 2). It is highly likely that the two effects are related, since there is now persuasive evidence (see 11) that in many systems, including leaf mesophyll protoplasts (8, 9), sugar uptake is driven by H^+-symport. From the experiments summarized in Figures 3 to 6, we can conclude that the basis of the effect on sugar flux is unlikely to be promotion of the aging process, suppression of leak (*i.e.* reduction in efflux), or the action of poly-ols.

An additional possibility to be considered is that the cells in the leaf segments which are sensitive to turgor, and thus responsible for enhanced sugar uptake when external osmolarity is raised, are the phloem companion cell-sieve tube complex. Phloem loading may be in part controlled by the turgor of the sieve tubes. However, it is generally accepted that the phloem loading system is highly specific for sucrose. MeG is believed to be taken up only by the mesophyll, not by the phloem (*e.g.* 6). This explanation for the observed effects of osmotica may thus also be rejected.

Exclusion of all the alternative explanations considered above increases the probability that our earlier proposal (12) is valid and that the observed responses to external osmotica relate to osmotic regulation. Bisson and Gutknecht (3) have formulated some key questions that need to be asked with regard to the mechanism of osmoregulation. They concern the identity of the detector which measures turgor, volume, or some related quality; the identity of the effector(s) which brings about changes in internal osmotic potential; and the mode of information transfer from detector to effector.

We propose, on the basis of the evidence discussed here, that the proton extrusion pump may function simultaneously both as detector and as effector. The site of the pump is believed to be the plasmalemma. Its operation may be directly affected by the hydrostatic pressure gradient across the plasmalemma-cell wall complex, possibly because of deformation of the plasmalemma by the cell wall (3). Changes in the rate of proton pumping (as observed by us and by others [5, 7] when the external osmotic concentration is raised) would be expected to be reflected not only in the rate of sugar flux (see 11), as observed here, but in the fluxes of a variety of other solutes currently believed to be driven across the membrane by pmf. Thus, the sensitivity of a single-membrane-sited system to the hydrostatic pressure gradient would lead to changes in the internal concentration of many of the solutes which make up the osmotic potential of the cell. The dual functioning of one system both as detector of changes in cell turgor (or volume) and as effector of changes in internal osmotic potential, would obviate the postulated need for information transfer between detector and effector.

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