Plant Physiol. (1987) 84, 895–899
0032-0889/87/84/0895/05/$01.00/0

Osmotic Dependence of the Transmembrane Potential Difference of Broadbean Mesocarp Cells[1]

Received for publication January 16, 1987

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ABSTRACT

Pod walls of broadbean (*Vicia faba* L. cv Aguadulce) were harvested at the import (S_1), at the transition (S_2) or at the export (S_3) phase for assimilate transport. Measurements of the transmembrane potential difference (PD) of mesocarp cells were made under various osmotic conditions. Internal osmotic potentials and cell turgor were calculated from osmolality measurements of cell saps recovered by freeze-thawing, after correction for the contribution of the free-space solution. Changes in the mannitol concentration of the medium altered the PD within a few minutes, and new stable values of PD were reached within 20 minutes after the osmotic change. With mannitol as the osmoticum, the most negative PD was measured at an external osmotic potential of -0.70 megapascals (MPa) for S_1 and S_2, while the most negative was at -0.40 MPa for S_3. Ethylene glycol, a permeant osmoticum, had little effect on PD, showing that the PD was sensitive to turgor, not to solute potential per se. For S_1 and S_2, the PD was less negative for turgor potentials lower than 0.1 MPa or greater than 0.3 MPa. S_3 samples exhibited a different turgor dependence, with a sharp optimum of the negativity of the PD at 0.3 MPa. The data are consistent with the proposal that the proton pump acts as a transducer of the osmotic conditions. They show that the osmotic sensitivity of the PD of mesocarp cells of broadbean changes with the stage of development of the pod.

Transport processes at the cell level in the sources and in the sinks of a plant depend on the osmotic environment (2–3, 5, 6, 12, 20, 23, 24). The proton-pumping activity of leaf tissues, as measured by the rate of acidification of their incubation medium, is influenced by the osmotic potential of this medium (3, 19, 25). The uptake of exogenous sugars and amino acids by the leaf is also affected by the concentration and the nature of the osmoticum (2, 8, 9). Evidence has been given that, in leaf tissues of *Phaseolus coccineus*, the osmotic conditions altered sucrose uptake, and more particularly its saturable component, via changes in cell turgor (1). Several data suggest that the rate of assimilate transport may also be controlled by the osmotic environment in sink tissues. The rate of photosynthate "unloading" from seed coats of *Phaseolus vulgaris* depends on cell turgor potential (17). In developing seeds from *Vicia faba* and *Pisum sativum*, the release of sucrose and of amino acids from the seed coat is sensitive to the osmolality of the bathing solution while phosphate release is unaffected by these solutions (23, 24). However, the rate of unloading in seed coats of developing soybean seeds is not affected by mannitol concentrations up to 500 mM (17). In sugar beet taproot tissue, the saturable component of sucrose uptake is sensitive to turgor (25).

At the whole plant level, according to the mass-flow model (14), long distance transport also depends on the establishment and on the maintenance of an osmotic gradient between the source and the sink (5). Osmotic control of ion fluxes and of nonelectrolyte solute fluxes thus may help integrate the transport processes at the cellular level into the complex framework of long distance transport in the whole plant. In a recent review, Giaquinta (7) concluded that small changes in the hydrostatic pressure gradient across the phloem plasma membrane are probably too small to affect directly solute transfer, and that these hydrostatic changes were transduced and amplified through the proton pump and membrane potential. Many data show that the uptake of solutes by plant cells occurs with proton symport. The proton motive force affects the kinetics of solute uptake by decreasing the Michaelis constant and/or by increasing the maximum velocity of transport by the carriers (4, 11).

The present paper deals with the osmotic dependence of the electrical component of the proton motive force which energizes the transport of solutes through the plasmalemma. The experiments were conducted with pod walls excised from broadbean fruit, which are a convenient material for electrophysiological studies. Since the pod wall undergoes a relatively rapid import-export transition during its development (13, 16, 20), this material also provides a good example for the study of the changes in membrane transport associated with this transition. Therefore, possible changes of the osmotic sensitivity of the PD[3] during the import/export transition were also examined.

MATERIALS AND METHODS

Plant Material. Broadbean (*Vicia faba* L. cv Aguadulce) plants were grown in a greenhouse where natural light was supplemented for 14 h per d with Osram L-Fluora lights; the temperature was maintained at 25 ± 3°C during the light period and at 15 ± 2°C during the dark period. The flowers were numbered as they appeared, about 6 weeks after planting. Based on previous studies of the nitrogen content of the pod wall, which showed an import-export transition between 36 and 45 DAF (13), three stages of development of the pod wall, each characterizing a given status for membrane transport, were chosen for the present work. Pod walls were harvested respectively between 18 and 22 DAF (S_1), between 36 and 44 DAF (S_2) or between 48 and 60 DAF (S_3). The stages S_1, S_2, and S_3 characterized the import, the transition, and the export phase respectively.

[1] Supported by the Centre National de la Recherche Scientifique (UA 574).
[2] Supported by a grant from the People's Republic of China.

[3] Abbreviations: PD, transmembrane potential difference; DAF, days after flowering; S_1, import phase; S_2, transition phase; S_3, export phase; CCCP, carbonyl-cyanide-*m*-chlorophenyl-hydrazone; FW, fresh weight.

Electrophysiological Measurements. The preparation of pod wall fragments and the equipment used for the measurements of PD have been described elsewhere (13). After removal of exocarp with a razor blade and brief washing, a pod wall fragment was immersed into a 4-ml (1 × 1 × 4 cm) plexiglass chamber. The fragment was cut in such a way that it fitted exactly the size of the chamber and was thus well maintained by the sides and the bottom walls of the chamber. The incubation medium contained 0.5 mM $CaCl_2$, 0.25 mM $MgCl_2$, mannitol as needed and buffered at pH 5.2 with 10 mM Na citrate and 20 mM Na_2HPO_4. The glass micropipette was inserted into a mesocarp cell, through the cut surface, with a mechanical micromanipulator. Measurements of the PD of mesocarp cells were started 30 min after the beginning of incubation. Preliminary experiments showed that just after excision, the PD was only -70 mV (stage S_2) but increased rapidly within 30 min to a value which then remained constant for several h. In some experiments, the plant material was immersed into a flow-through chamber (10 ml) containing either a low mannitol (0 mM) or a high mannitol (500 mM) solution, and connected through a small peristaltic pump to a reservoir containing respectively either a high mannitol (500 mM) or a low mannitol (0 mM) solution. The concentration of the osmoticum in the chamber was increased stepwise from 0 to 500 mM, or decreased stepwise from 500 to 0 mM, by circulation of appropriate volumes from the reservoir solution, and the PD was monitored continuously during this time. The changes in mannitol concentration brought about by the circulation of the solution were calibrated previously by addition of a dye indicator (Evans Blue) in the reservoir and measurements of the optical density in the chamber.

Measurements of the Apparent Free Space. After abrasion of the external epidermis with carborundum 600, pod wall fragments (7 × 12 mm) were floated on the same solution as that described above for PD measurements, but supplemented with [^{3}H]inulin (mol wt = 5,200). The specific and the volumetric activity of [^{3}H]inulin in the incubation medium was 53 GBq·mmol^{-1} and 200 Bq·ml^{-1} respectively. Preliminary experiments showed that the diffusion equilibrium of inulin into the extracellular spaces was reached within the first 25 min of incubation. Since, after this time, the radioactivity retained by the tissues did not increase for up to 60 min, inulin was not hydrolysed and metabolised to small oligosaccharides. Therefore, a 25 min incubation time was routinely used to study the effect of the osmoticum concentration on the volume of the apparent free space. At the end of incubation, the fragments were rapidly rinsed (2 s) in unlabeled medium and combusted in an Oxymat IN 4101 apparatus. The radioactivity recovered as ^3H_2O was counted by liquid scintillation spectroscopy.

Tissue Solute Osmolality. Osmolality of the intracellular sap was estimated by a method adapted from Patrick (17) and from Tomos *et al.* (22). After a 20 min incubation in test solutions, the pod wall fragments were rapidly rinsed (2 s) on a filter paper previously soaked in distilled water, blotted dry, weighed, and frozen. The samples were then thawed at 70°C for 2 min and the sap of the tissues was collected after centrifugation at 2500*g* for 5 min. Osmolality measurements were made with a freezing-point osmometer (Fiske OS 220). Contamination of the collected sap by the free space solutes was estimated on the assumption that the free space pool had equilibrated with the mannitol solution, and corrections were made accordingly when calculating the intracellular osmotic potential.

RESULTS

PD of Mesocarp Cells as a Function of Fruit Development. The PD of mesocarp cells varied markedly depending on the stage of development of the pod wall (Fig. 1). Under our experimental conditions, after an initial decrease from -70 mV to -105 mV between 18 and 30 DAF, the PD remained constant until 48 DAF, and increased to about -55 mV at 63 DAF. This trend validates the selection of the time spans chosen for S_1, S_2, and S_3 (see "Materials and Methods"). The changes in PD which are associated with the development seem to depend on the active component of PD, since the values measured in the presence of 10 μM CCCP remained fairly constant (Fig. 1). In addition to its proton-conducting properties on various membranes (plasmalemma, mitochondria, chloroplasts), side effects of CCCP cannot be excluded since the PD measured in the presence of the uncoupler was less negative than the diffusion potential usually observed in plant cells.

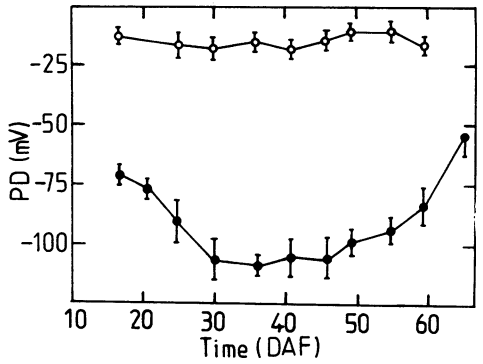

FIG. 1. The PD of mesocarp cells of *V. faba* as a function of fruit development. Control (●); 10 μM CCCP added in the medium (○). All points are means of at least 3 measurements made at 250 mM mannitol (-0.75 MPa) ± SE.

Osmotic Dependence of PD. An example of chart recording (Fig. 2, trace b) shows that the PD of mesocarp cells responded rapidly to changes in the concentration of osmoticum. The electrical response began within 10 min after each change in osmotic concentration and was completed within 20 to 30 min. Control experiments showed that the PD of cells maintained at -0.1 (Fig. 2, trace c) or at -0.75 Mpa (Fig. 2, trace a) remained constant throughout the duration of the recording. Responses similar to that presented on trace b were observed when the mannitol concentration of the medium was decreased from 500 mM to 250 mM (trace d).

Two slowly penetrating osmotica, mannitol and PEG, were tested and the osmotic dependence of PD was also studied as a function of the stage of development of the pod wall (Fig. 3). The PD reached a minimum (most negative) at -0.70 MPa (250 mM mannitol) for S_1 and S_2, whereas the minimum was at -0.40 MPa (150 mM mannitol) for S_3 (Fig. 3A). Experiments with PEG yielded similar trends, although the minimum PD was reached with slightly less negative osmotic potentials, *i.e* -0.60 MPa for S_1 and S_2, and -0.30 MPa for S_3 (Fig. 3B). Also, the decrease in PD occurring under hyperosmotic conditions was more pronounced in mannitol than in PEG (compare the right portions of Figure 3, A and B). The 'passive' component of PD, as measured in the presence of 10 μM CCCP, was not very sensitive to the concentration of PEG (Fig. 3B). Either in mannitol or in PEG, for a given stage of development, the difference between the minimum and the maximum PD reached 50 to 70 mV.

Ethylene glycol is a permeant osmoticum which is expected to decrease the water potential, but not to affect cell turgor (1, 6, 25). Compared to mannitol, changes in the concentration of ethylene glycol had little, if any, effect on PD, suggesting that the proton-pump was sensitive to cell turgor rather than to water potential *per se* (Table I). In this experiment, the tissues were equilibrated for 3 h in ethylene glycol to allow a complete penetration of the compound in the tissues (1). All experiments described in the following paragraphs were performed with mannitol as the osmoticum.

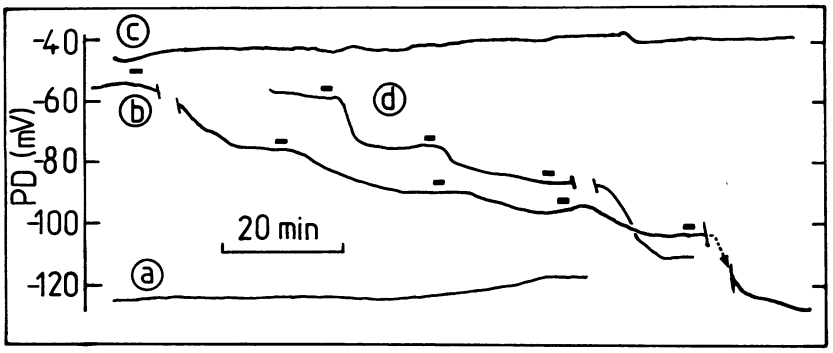

FIG. 2. Short-time adjustments of PD to changes in mannitol concentrations. In b, mannitol concentrations were increased stepwise from 0 mM (beginning of measurement) to 250 mM (end of measurement) by circulation of adequate solutions in a flow-through chamber, as described under "Materials and Methods." Mannitol concentration was increased by 50 mM at each step, when indicated by thick black lines. The samples studied were at the transition phase (S_2). Changes in cell turgor sometimes induced a transient exit of the electrode out of the cell, resulting in a break in the recording. In b, the two breaks lasted 15 and 10 min respectively. In d, the initial mannitol concentration was 500 mM, and was decreased successively to 400, 300, and 250 mM. Break in the recording lasted 10 min. Traces a and c are PD recordings from cells maintained at 250 and 0 mM mannitol, respectively.

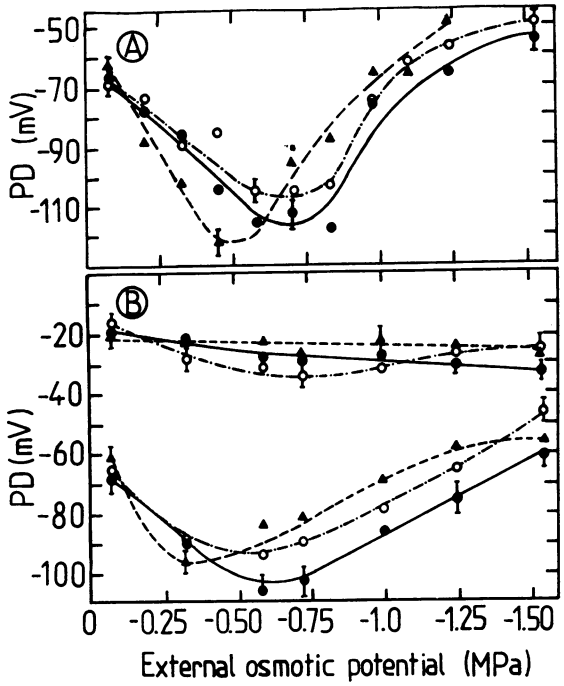

FIG. 3. Osmotic dependence of PD in the presence of mannitol (A) or of PEG (B). The fragments were excised from pod walls at the import phase S_1 (○), at the transition phase S_2 (●) or at the export phase S_3 (▲). In B, the three upper curves were made in the presence of 10 μM CCCP in the medium. PD values are means of 12 to 26 measurements for each point in mannitol and of 6 to 8 measurements in PEG.

Apparent Volumes of Free Space. Hypo-osmotic, iso-osmotic, and hyperosmotic conditions are clearly apparent from the amounts of inulin retained by the tissues (Fig. 4). The low amount of inulin measured in the tissues incubated at osmotic potentials ranging from 0 to -0.30 MPa may be ascribed to the swelling of the symplast, while the increase in label found above -0.75 MPa can be explained by shrinkage of the symplast under hyperosmotic conditions. Under iso-osmotic conditions (-0.50 to -0.75 MPa), the apparent free space volumes measured were 60, 40, and 60μl·g FW^{-1} for S_1, S_2, and S_3, respectively. Therefore, the transition phase S_2 seemed characterized by a smaller apparent free space. The export phase (S_3) is more sensitive to plasmolysis than S_1 and S_2.

Intracellular Osmotic Potential as a Function of Extracellular Osmotic Potential. The volumes of apparent free space estimated by use of labeled inulin were combined to the measurements of the osmolality of the corresponding mannitol solutions (0–500 mM) to calculate the contribution of free space to the total osmolality of the cell sap recovered by freeze-thawing (18). The following equation was used:

$$\Psi\pi_i = \frac{\Psi\pi_t - (\Psi\pi_{fs} \times V_{fs}/V_t)}{V_i/V_t)}$$

where $\Psi\pi_i$, $\Psi\pi_t$, and $\Psi\pi_{fs}$ are the intracellular, total, and free space osmotic potentials, respectively, and V_i, V_t, and V_{fs} are the corresponding volumes.

The internal osmotic potential became more negative with increasing mannitol concentrations (Fig. 5). A break in the lines was found at an external osmotic potential of about -1.0 MPa, and beyond incipient plasmolysis, the points followed a line where $\psi\pi_i = \psi\pi_e$ (external osmotic potential). No significant difference was observed for the internal osmotic potential at different stages of development of the pod (Fig. 5).

Several authors (1, 6, 19, 25) have shown that solute uptake was sensitive to turgor potential rather than to osmotic potential, and our data (Table 1) suggest the same sensitivity for the PD. It was therefore interesting to express the PD as a function of turgor potential. Fresh weight measurements (not shown) indicated that, under our experimental conditions, water fluxes from and into the pod wall cells were at equilibrium within 20 min. Given water-flux equilibrium, the extracellular osmotic potential provides an estimate of the intracellular water potential (12, 18). Using this rationale, turgor pressure can be estimated from the difference between the extracellular and the intracellular osmotic

Table I. *Effect of a Permeant (Ethylene Glycol) and of a Nonpermeant (Mannitol) Osmoticum on the PD*
Data are means of 8 measurements ± SE, except for 0 mM (21 measurements).

	Osmoticum Concentration (mM)				
	0	100	250	550	1000
Osmotic potential (MPa)	−0.08	−0.33	−0.71	−1.60	−2.93
PD recorded in mannitol (mV)	−71.8 ± 3.1	−85.4 ± 3.0	−113.0 ± 4.4	−55.4 ± 3.4	ND[a]
PD recorded in ethylene glycol (mV)		−79.5 ± 3.2	−79.6 ± 5.0	−77.5 ± 5.1	−69.9 ± 5.4

[a] ND, not determined.

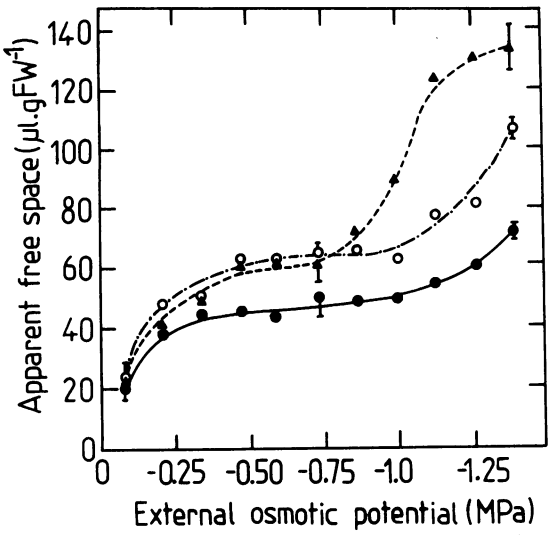

FIG. 4. Osmotic dependence of the apparent free space in fragments of pod wall. S_1 (O); S_2 (●); S_3 (▲). Means of measurements ± SE.

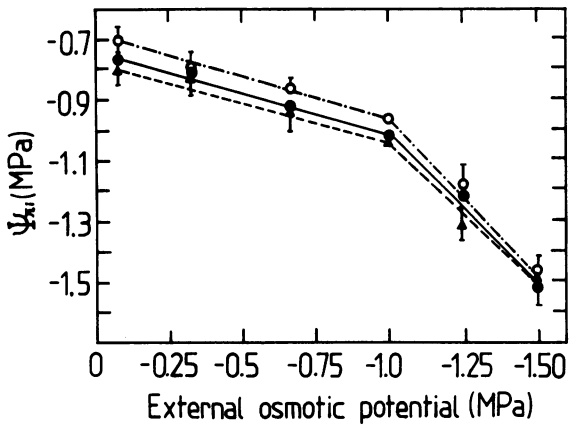

FIG. 5. Internal osmotic potential as a function of external osmotic potential. S_1 (O); S_2 (●); S_3 (▲). Means of 3 measurements ± SE

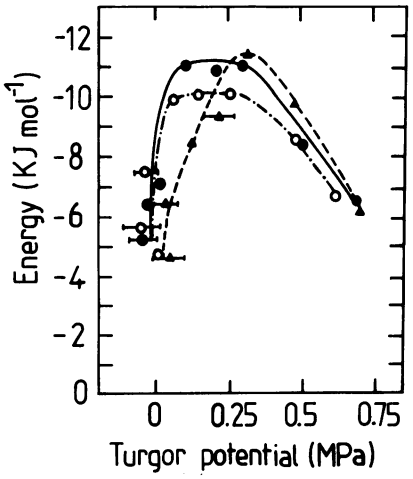

FIG. 6. Turgor dependence of the electrical component of the proton motive force. S_1 (O); S_2 (●); S_3 (▲). The energy associated with PD was calculated as F × PD, with F being the Faraday and PD expressed in volts. Calculations were made from the data in Figures 3 and 5. Turgor values are expressed ± SE, and the SE on energy were smaller than the symbols.

potentials. This estimation was used to draw Figure 6, which shows that, at stages S_1 and S_2, the PD became less negative for turgor potentials lower than 0.1 MPa or higher than 0.30 MPa. A plateau is visible between 0.1 and 0.30 MPa. The turgor dependence of PD was apparent at stage S_3, since no plateau was visible, and there was a sharp optimum at 0.3 MPa (Fig. 6).

DISCUSSION

Osmotic phenomena seem involved in the control of both long distance and membrane transport, and it has been proposed that the proton pump may act as a transducer and amplifier of the osmotic status of the cell (7, 19). Although this possibility has received support from proton fluxes measurements (3, 9, 19, 25), little information is still available on the osmotic dependence of the PD, particularly in higher plant cells (10, 15). Ion fluxes, which may act as compensation charge for H^+ fluxes, may be also affected by osmotic conditions, and it is interesting to know whether and how the changes in proton-pumping activity are translated in terms of PD. The present work provides such data, and also studies the osmotic control of PD in relation to different stages of development corresponding to a definite status for membrane transport at the cell level.

The ability to take up and to retain intracellular solutes depend in part on the PD. In this respect, the marked changes exhibited by the PD of mesocarp cells in the course of development (Fig. 1) may be of physiological significance for transport processes. The PD became more negative during the import phase, and the transition phase was characterized by the maintenance of PD at strongly negative values. The markedly less negative PD measured during the transition + early export phase, combined with strongly negative values of PD in the seed at this time (R El Ayadi, S Delrot, JL Bonnemain, unpublished data) may explain, at least in part, the loss of organic solutes by the pod wall during this stage of development. During the development of the pod, the active component of PD of mesocarp cells depends on the number of proton-pumps in the plasmalemma as well as on numerous parameters (ATP supply, physical or chemical effectors of the pump) which may affect their activity directly or indirectly. Which parameter is determinant for this developmental trend of PD is beyond the scope of this work.

Figures 2 and 3 clearly show that the PD can adjust rapidly to a large range of the osmotic conditions of the medium. Therefore, the osmotic dependence of the extrusion of H^+ previously recorded (3, 9, 19, 25) is not fully compensated by fluxes of other ions and results in changes of PD. Adjustments of PD can be due to changes in H^+ pumping, but also to changes in influx or efflux of charges related to ion channels, or changes in ion-coupled transports. The parallelism between the osmotic dependence of PD and the osmotic dependence of H^+ pumping measured by the rate of acidification of the medium (3, 9, 15, 19) suggests that change of H^+ pumping is the main reason for the osmotic dependence of PD. Besides, when the rate of H^+-coupled transports is changed, the resulting changes in PD and in the external pH are transient (13). Contrarily, changes in osmoticum concentration induced new steady-state values for the PD (Fig. 2). Osmotic changes in ion-coupled solute uptake occur (2, 3, 9), but more likely as a result, not a cause of the changes in PD. The results presented support the idea that the proton pump behaves as a sensor and a transducer of the osmotic conditions, and more precisely of cell turgor (Table I).

The PD, and the energy it can provide for solute uptake, depend markedly on cell turgor, as estimated by indirect measurements (Fig. 6). The optimum PD were found for cell turgors ranging from 0.1 to 0.30 MPa at stages S_1 and S_2, and at 0.30 MPa for S_3. These values are in good agreement for the optimum of acidifying activity recently reported in sugar beet taproot (21), where turgor was measured directly with a pressure probe. When

compared to the import and the transition phases, the export phase seems characterized by a different sensitivity of the PD towards the osmotic conditions (Fig. 6). To maintain maximum PD in the mesocarp cells, a higher cell turgor would be needed at the export phase than at the import or at the transition phase.

In conclusion, the present data shows that the PD is sensitive to its osmotic environment, and that this sensitivity depends on the stage of development of the cell. This sensitivity, as well as the rapidity of the responses observed, give support to the previous suggestion that the proton pump may act as a transducer and amplifier of the osmotic conditions. Whether this osmotic control is of physiological importance *in vivo*, and what are its effects on solute uptake still needs further investigation. Convincing evidence has been presented for the existence of high solute concentration in the apoplast of legume embryos (18, 20). However, we have no information on the apoplast content of the pod wall nor on its variations during the development of the pod. Such work is presently underway to assess more precisely the part played by osmotic control in the developmental trend of PD exhibited by mesocarp cells (Fig. 1).

Acknowledgments—We are grateful to J. Chedhomme and Dr. J. P. Rona (University of Paris VI) for loan of the osmometer.

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