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Effects of Water and Turgor Potential on Malate Efflux from Leaf Slices of *Kalanchoë daigremontiana*[1]

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ABSTRACT

Malate efflux from leaf cells of the Crassulacean acid metabolism plant *Kalanchoë daigremontiana* Hamet et Perrier was studied using leaf slices submerged in experimental solutions. Leaves were harvested at the end of the dark phase and therefore contained high malate levels. Water potentials of solutions were varied between 0 and −5 bar using mannitol (a slowly permeating solute) and ethylene glycol (a rapidly permeating solute), respectively. Mannitol solutions of water potentials down to −5 bar considerably reduced malate efflux. The slowly permeating solute mannitol reduces both water potential and turgor potential of the cells. The water potential of a mannitol solution of −5 bar is just above plasmolyzing concentration. Malate efflux in ethylene glycol at −5 bar was only slightly smaller than at 0 bar, and much higher than in mannitol at −5 bar. Tissues in rapidly permeating ethylene glycol would have turgor potentials similar to tissues in 0.1 mM $CaSO_4$. The results demonstrate that malate efflux depends on turgor potential rather than on water potential of the cells.

In previous reports (15, 18) it has been shown that malate efflux from leaf slices of the CAM[3] plant *Kalanchoë daigremontiana* is greatly reduced when the water potential of the medium is lowered, in the nonplasmolytic range, by addition of mannitol. Slowly permeating mannitol would lower both water and turgor potentials. A distinction between these two parameters of water status can be made by comparing effects of slowly and rapidly permeating solutes (8). In contrast to mannitol, rapidly permeating ethylene glycol will only transiently reduce turgor potential, although the water potential will be lowered. A use of this approach in studies of malate efflux from *K. daigremontiana* leaf slices is reported here. The results suggest that the malate efflux from the leaf slices is affected mainly by turgor potential.

MATERIALS AND METHODS

Maintenance of Experimental Plants. Plants of *K. daigremontiana* Hamet et Perrier were grown in a greenhouse for about 6 to 10 months and then kept in a growth chamber for at least 3 weeks. The growth chamber had 12 hr light/12 hr dark, 25 C during the light phase and 15 C during the dark phase, and 60% relative humidity. Light of 12 klux at the level of the plants was obtained from Philipps HPLS lamps.

Experimental Leaf Material. Leaves were always harvested in the early morning at the end of the dark phase so that they contained high levels of malic acid. Initial malate levels of the tissue are given in the legends of the figures.

Leaf slices, 2 mm wide, were obtained by cutting leaves with very sharp and frequently renewed razor blades. Leaf slices were submerged in experimental solutions (2 g fresh wt/100 ml). All solutions contained 0.1 mM $CaSO_4$ and mannitol or ethylene glycol as indicated in the figures. In a cryoscopic determination the water potential of 0.1 mM $CaSO_4$ gave somewhat less than −0.05 bar. This is referred to as 0 bar in the text.

Experimental solutions with the leaf slices were kept in the dark at 25 C in a shaking water bath.

Analytical Methods. Malate was determined enzymically following the procedure of Hohorst (10). Since leaf slices changed their fresh wt to different degrees in the various solutions used, all results are expressed per g of initial fresh wt of the leaf slices before incubation.

All points shown in the figures are averages of duplicates or triplicates.

RESULTS

Figure 1 shows that malate efflux from *K. daigremontiana* leaf slices is high in solutions of high water potential and low in mannitol solutions of low water potential. This has been demonstrated before (15, 18), but it has been criticized that it might be related to a wounding artifact. Figure 1 proves that this effect is not an immediate consequence of slicing. The rate of malate efflux changes at any time after slicing if the tissue is transferred from a solution of 0 bar to a mannitol solution of −5 bar water potential (Fig. 1), or vice versa (unpublished data).

Figure 2 compares the effects of −5 bar mannitol and ethylene glycol with a solution at 0 bar. As before, malate efflux is low in −5 bar mannitol solution and high at 0 bar water potential. During the first 20 min in −5 bar ethylene glycol solution malate efflux is as small as in mannitol. At longer times, however, malate efflux is much greater in ethylene glycol than in mannitol, although the rate of efflux in solutions at 0 bar is not quite attained. The malate efflux at 0 bar decreases with time presumably due to a combination of an increasingly smaller concentration gradient and a gradual reduction in turgor potential as malate is lost from the tissue. In the ethylene glycol the malate efflux is nearly linear with time, possibly because the tendency to decrease, as observed at 0 bar, is counterbalanced by continued uptake of ethylene glycol retaining a high turgor potential despite a decrease in malate concentration of the tissue. The slower efflux in ethylene glycol at −5 bar than in the control (0 bar) may be due to a slower penetration of ethylene glycol than of water (21) indicated in Figure 3. A simple way to follow water movements into or out of a tissue submerged in a solution is to record tissue weight. At 0 bar, leaf slices rapidly gain weight due to rapid water movement and equilibration. Subsequently there

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[3] Abbreviation: CAM: Crassulacean acid metabolism.

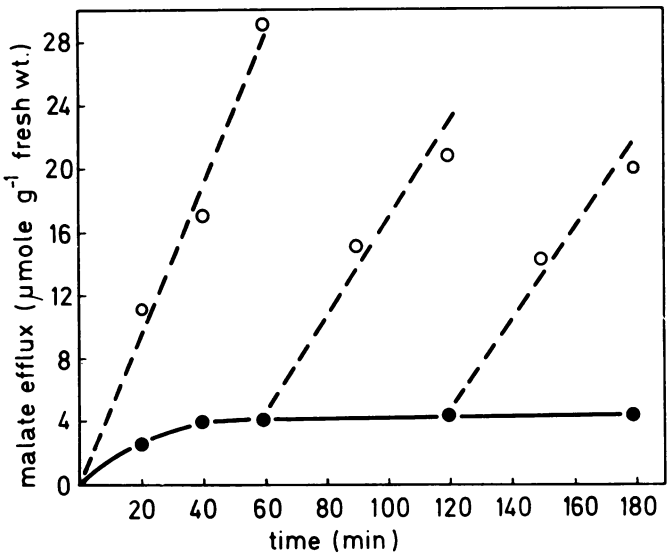

FIG. 1. Malate efflux from leaf slices of *K. daigremontiana*. Initial malate level was 108 μmol malate g^{-1} fresh wt. Leaf slices were incubated in control solution and in mannitol solution immediately after slicing. (O- - -O): times of incubation in control solution (water potential 0 bar); (●—●): times in mannitol solution of −5 bar. Malate efflux was determined by analyses of aliquots of the external medium.

are only small changes in fresh wt. After 90 min some weight is lost again. Overlapping with rapid water uptake in 0 bar solution the tissue gradually loses substantial amounts of malate to the medium (Figs. 1 and 2), the concurrent water flow explaining the loss of weight after 90 min. In −5 bar mannitol, leaf slices rapidly lose a small amount of water and then there is no further change. This agrees with earlier observations (18) that −5 bar mannitol is very close to the isoosmotic point of *K. daigremontiana* leaf tissue, which has accumulated malate at the end of the dark phase. In −5 bar ethylene glycol, there is initially a similar small loss of weight, but then weight is gained, presumably because both ethylene glycol and water are taken up. Even at 200 min, however, the fresh weight of the leaf slices in ethylene glycol at −5 bar is still smaller than in solutions at 0 bar.

Transfer of leaf slices from mannitol to ethylene glycol solution of −5 bar increases malate efflux (unpublished results, see also Fig. 4 legend). Malate efflux is reduced immediately following a return of these leaf slices to mannitol solution (Fig. 4).

DISCUSSION

Mannitol in the external solution reduces both water and turgor potential of the tissue slices. Ethylene glycol, after an initial phase of adaptation, only reduces water potential, because it is rather rapidly taken up. There are no direct measurements on permeation of mannitol and ethylene glycol relative to each other in *K. daigremontiana*. In the small cells and the soft tissue of *K. daigremontiana* leaves, pressure probe and pressure bomb techniques cannot be used to measure water relation parameters directly. However, Figure 3 supports the supposition that ethylene glycol is taken up much more rapidly than mannitol, so that an initial reduction of turgor is relieved during longer periods of incubation in ethylene glycol but not in mannitol. Mannitol is known to permeate plant cells only to a very limited extent. By contrast, ethylene glycol appears to permeate rather rapidly in a large variety of algal cells and higher plant tissues (*Chara* [ref. 2] *Chlorella pyrenoidosa* [ref. 7], tomato roots [ref. 20], maize roots [ref. 8], cotton leaf slices [ref. 13]). In maize roots the half-time of exchange of ethylene glycol between the tissue and the external medium is about 60 min (6).

Malate efflux is large in external media of 0 bar as well as in ethylene glycol solutions of −5 bar, but small in mannitol solutions of −5 bar. This suggests that malate efflux is determined mainly by turgor rather than by water potential. The efflux can be changed rather rapidly by transfer from solutions of 0 bar or −5 bar ethylene glycol to −5 bar mannitol and vice

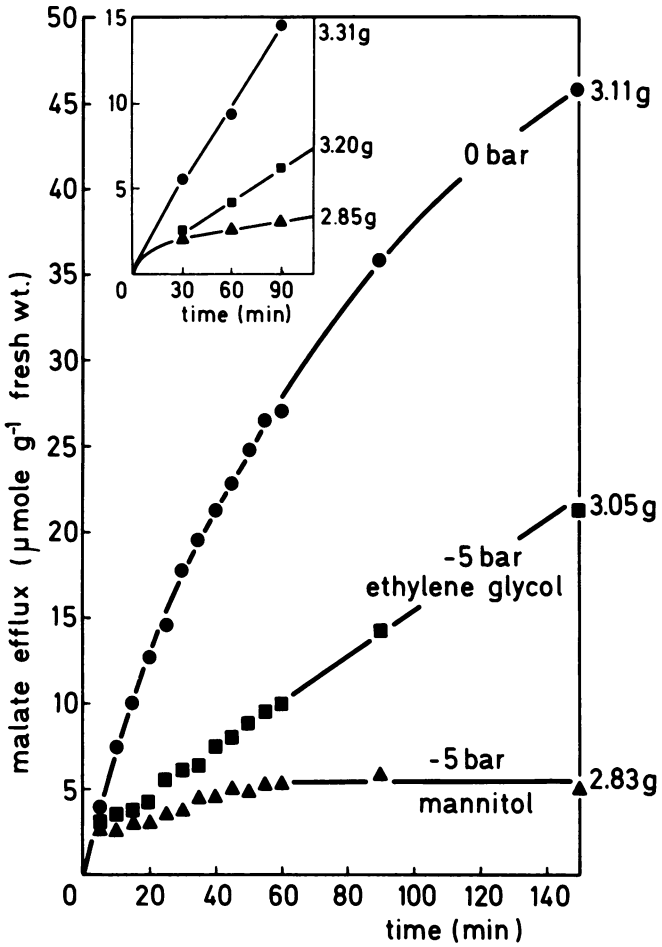

FIG. 2. Malate efflux from leaf slices of *K. daigremontiana* in control solution of 0 bar and in ethylene glycol and mannitol solutions of −5 bar water potential; measured by analysis of the external solution. Inset and main graph represent two separate experiments. Symbols of inset refer to similar solutions as indicated on curves of main graph for same symbols. Initial malate level of the tissue: inset, 97 μmol g^{-1} fresh wt; main graph, 140 μmol g^{-1} fresh wt. Initial fresh wt of samples was 3 g. Data on rim of graphs give final fresh wt of samples (inset, after 180 min; main graph, after 150 min).

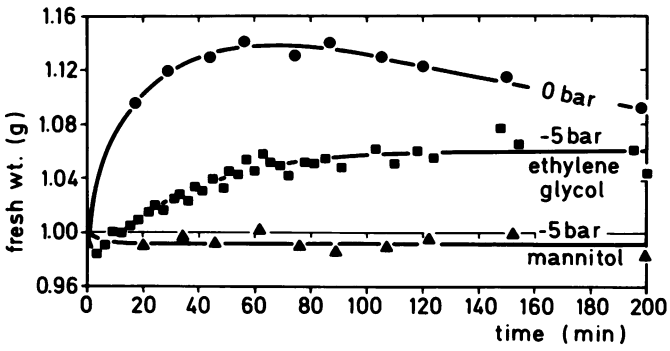

FIG. 3. Time course of fresh wt changes of *K. daigremontiana* leaf slices in control, ethylene glycol, and mannitol solutions. Initial malate level of tissue was 120 μmol g^{-1} fresh wt.

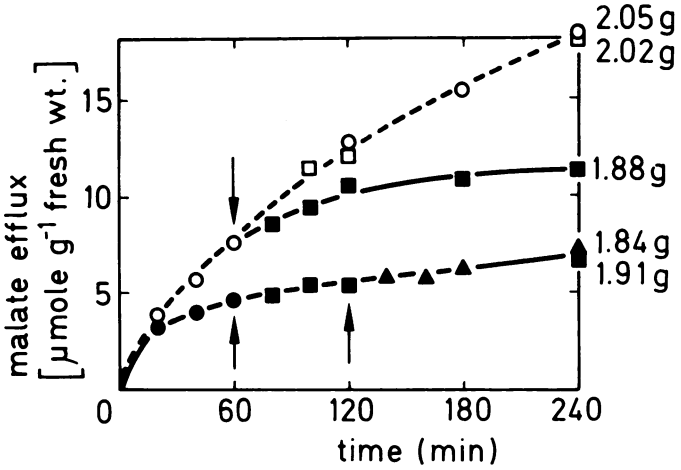

FIG. 4. Malate efflux from leaf slices of *K. daigremontiana* in ethylene glycol (- - -) and in mannitol solutions (——) of −5 bar water potential, measured by analyzing aliquots of the external solution. Initial fresh wt of samples was 2 g; final fresh wt was as indicated on rim of graph. Initial malate level was 117 μmol g^{-1} fresh wt. Arrows plus change of symbols indicate transfer of leaf slices to new solution: slices kept for the first 60 min in −5 bar ethylene glycol (○) were transferred to new ethylene glycol solution (□) or to −5 bar mannitol solution (■); slices kept in −5 bar mannitol from the beginning of the experiment (●) were transferred twice to fresh mannitol solution (after 60 min: ■; after 120 min: ▲). An experiment similar to that of Figure 1 was also performed using −5 bar ethylene glycol instead of 0 bar control solution (unpublished data). Transfer of leaf slices from −5 bar mannitol to −5 bar ethylene glycol 60 min or 120 min after commencement of the experiment resulted in rapid malate efflux just as shown for transfer to 0 bar control solution in Figure 1.

versa (Figs. 1 and 4). Thus, the efflux of malate depends on the turgor potential existing at any one time, *i.e.* transient fluctuations in turgor potential cannot act as a trigger for longer term changes in malate efflux. In CAM cells the bulk of the malate is stored in the vacuoles (1, 14, 18); this presents the question of whether turgor pressure can exert an effect at the tonoplast (18). Recent investigations by Coster *et al.* (3) clearly suggest that turgor can affect membrane processes acting as an absolute pressure which would also affect the tonoplast. Turgor affects membranes not merely as a pressure gradient which could exist only across the plasmalemma and not across the tonoplast.

An effect of the reduced activity of water in solutions of low water potential on membranes and enzymes in most biological systems has been considered unlikely by Hsiao (11) for quantitative reasons. This had been previously confirmed experimentally for respiration (8) and protein and sucrose synthesis (7), but not for processes like solute fluxes through membranes. As argued above, the present experiments show that malate efflux from leaf slices of CAM plants is mainly dependent on turgor rather than on water potential. That the efflux in ethylene glycol remained somewhat smaller than in the controls may be due to incomplete penetration of ethylene glycol either in the bulk of the cells or into all molecular structures of membranes. Alternatively, low water potentials may have a small effect on malate efflux.

From inhibitor and kinetic studies (16, 17) we have good reason to assume that malate efflux is a passive process. Nevertheless, it is of interest whether turgor potential affects metabolic reactions involved in CAM, as well as malate efflux from the cells. This can be assessed from the malate degradation in leaf slices during experiments like that shown in Figures 1, 2, and 4. Metabolic malate degradation can be calculated as follows:

$$\text{malate}^{\text{initial}}_{\text{tissue}} - (\text{malate}^{\text{end}}_{\text{solution}} + \text{malate}^{\text{end}}_{\text{tissue}})$$

In the light this is about 6 to 9 μmol hr^{-1} g^{-1} fresh wt, which is similar to the average rate of about 10 μmol hr^{-1} g^{-1} fresh wt estimated for leaves of intact plants. In the dark this degradation is less than one-third of that in the light, which appears reasonable since photosynthetic reactions will utilize malate more rapidly than mechanisms operating in the dark. There is little effect on this of mannitol at −5 bar (15), demonstrating that in contrast to malate efflux metabolism of malate is not affected by turgor potential.

These responses of malate in CAM leaf slices to water and turgor potentials are consistent with effects in other species. Reductions in water potential without concurrent changes in turgor potential have little or no effect on metabolism (7, 8, 19). Metabolic reactions within the cytoplasm, such as respiration (8) and sucrose and protein synthesis (7) are not affected appreciably even when turgor as well as water potentials are lowered. In contrast, turgor potential does affect reactions on the membrane-cell wall interface, such as ion fluxes (4, 9, 22), sucrose fluxes (12), urea permeability (5), cell extension (11, 19), and in the present case, malate efflux.

LITERATURE CITED

1. BUSER C, P MATILE 1977 Malic acid in vacuoles isolated from *Bryophyllum* leaf cells. Z Pflanzenphysiol 82: 462-466
2. COLLANDER R 1949 The permeability of plant protoplasts to small molecules. Physiol Plant 2: 300-311
3. COSTER HGL, E STEUDLE, U ZIMMERMANN 1976 Turgor pressure sensing in plant cell membranes. Plant Physiol 58: 636-643
4. CRAM WJ 1973 Internal factors regulating nitrate and chloride influx in plant cells. J Exp Bot 24: 328-341
5. DAINTY J, BZ GINZBURG 1964 The permeability of the cell membranes of *Nitella translucens* to urea, and the effect of high concentrations of sucrose on this permeability. Biochim Biophys Acta 79: 111-121
6. GREENWAY H 1974 Effects of slowly and rapidly permeating osmotica on permeability of excised roots of *Zea mays*. Aust J Plant Physiol 1: 247-257
7. GREENWAY H, B LANGE, M LEAHY 1972 Effects of rapidly and slowly permeating osmotica on macromolecule and sucrose synthesis. J Exp Bot 23: 459-468
8. GREENWAY H, M LEAHY 1970 Effects of rapidly and slowly permeating osmotica on metabolism. Plant Physiol 46: 259-262
9. GUTKNECHT J 1968 Salt transport in *Valonia:* inhibition of potassium uptake by small hydrostatic pressures. Science 160: 68-70
10. HOHORST HM, 1970 L(−)-Malat, Bestimmung mit Malatdehydrogenase und NAD. *In* HU Bergmeyer, ed, Methoden der enzymatischen Analyse, Vol II. Verlag Chemie, Weinheim pp 1544-1548
11. HSIAO TC 1973 Plant responses to water stress. Annu Rev Plant Physiol 24: 519-570
12. HUMPHREYS TE 1973 Sucrose transport at the tonoplast. Phytochemistry 12: 1211-1219
13. JONES HG 1975 Photosynthesis by thin leaf slices in solution. II. Osmotic stress and its effect on photosynthesis. Aust J Biol Sci 26: 25-33
14. KLUGE M, CB OSMOND 1972 Studies on phosphoenolpyruvate carboxylase and other enzymes of Crassulacean acid metabolism in *Bryophyllum tubiflorum* and *Sedum praealtum*. Z Pflanzenphysiol 66: 97-105
15. LÜTTGE U, E BALL 1974 Proton and malate fluxes in cells of *Bryophyllum daigremontianum* leaf slices in relation to potential osmotic pressure of the medium. Z Pflanzenphysiol 73: 326-338
16. LÜTTGE U, E BALL 1977 Concentration and pH dependence of malate efflux and influx in leaf slices of CAM plants. Z Pflanzenphysiol 83: 43-54
17. LÜTTGE U, E BALL, H-W TROMBALLA 1975 Potassium independence of osmoregulated oscillations of $malate^{2-}$ levels in cells of CAM-leaves. Biochem Physiol Pflanzen 167: 267-283
18. LÜTTGE U, M KLUGE, E BALL 1975 Effects of osmotic gradients on vacuolar malic acid storage. A basic principle in oscillatory behavior of Crassulacean acid metabolism. Plant Physiol 56: 613-616
19. ORDIN L 1960 Effect of water stress on cell wall metabolism of *Avena* coleoptile tissue. Plant Physiol 35: 443-450
20. PERRY MW, H GREENWAY 1973 Permeation of uncharged molecules and water through tomato roots. Ann Bot 37: 225-232
21. STEIN W 1967 The movement of molecules across cell membranes. *In* Theoretical and Experimental Biology Vol 6. Academic Press, New York
22. ZIMMERMAN U, E STEUDLE 1974 The pressure dependence of the hydraulic conductivity, the membrane resistance and membrane potential during turgor pressure regulation in *Valonia utricularis*. J. Membr Biol 16: 331-352