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Induced Changes in Permeability of Plant Cell Membranes to Water

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ABSTRACT

The half-time for THO equilibration was three times longer for a living carrot (*Daucus carota* L.) cylinder than for a dead one. Furthermore, the energy of activation of THO flux was more than twice as high for the living cylinder. Passage through living membranes thus constitutes a rate-limiting step for THO flux in carrot tissue.

CO_2 increased the half-time ($t_{1/2}$) for THO equilibration. Treatment with abscisic acid or with tertiary butanol decreased $t_{1/2}$. In neither case was the selective permeability of the membrane destroyed.

***p*-Chloromercuribenzoate and carbonyl cyanide *m*-chlorophenylhydrazone, if supplied together with abscisic acid or tertiary butanol, abolished their action. If abscisic acid or butanol was first allowed to act alone, its effect was stable to subsequent treatment with the inhibitors. *p*-Chloromercuribenzoate and carbonyl cyanide *m*-chlorophenylhydrazone at concentrations at which they affected abscisic acid and butanol action, did not influence THO flux in control tissue. At considerably higher concentrations, however, 2,4-dinitrophenol and carbonyl cyanide *m*-chlorophenylhydrazone raised $t_{1/2}$. The CO_2 effect was very rapidly reversible. Full reversal of the butanol effect required 3 hours, and that of abscisic acid required 4 days.**

This paper extends our earlier studies (6) on reversible changes in permeability of plant cells membranes to water. We previously drew attention to the scantiness of the literature on this subject. The number of investigations into factors which influence water permeability remains meager in comparison with the literature on permeability to solutes. However, it has recently been enlarged by a series of interesting papers on maize roots (4, 9, 17). The striking finding reported in these papers, that permeability to water in this tissue depends on metabolic energy, underlines the fact that water permeability is a complex property which is as yet very poorly understood.

In our earlier studies we observed hydraulic conductivity. Here we report on labeled water exchange, *i.e.*, diffusional permeability. This technique avoids the complications due to the possibly rate-limiting role played by solute diffusion in the osmotic studies (15). We bring evidence for a metabolically dependent action of both abscisic acid and tertiary butanol which leads to substantially increased permeability to water without inducing leakage of solutes.

MATERIALS AND METHODS

Root storage tissue of *Daucus carota* L. was used in these experiments. Cylinders of xylem parenchyma 6.2 mm in diameter and 20 mm in length were excised and washed in running tap water for 3 hr. They were then transferred to aerated tap water at 18 C for 1 to 4 days until required for the experiments. During this period the intercellular spaces became injected.

When dead cylinders were investigated, the cells were killed by exposing to chloroform vapor or by freezing and thawing. In order that the final dimensions of living and dead cylinders should be equal in the experiments in spite of the lack of turgor and consequent shrinkage of dead tissue, the initial dimensions of the cylinders to be killed were made somewhat larger (diameter 6.7 mm, length 21.5 mm).

THO exchange between the tissue and the medium was observed by measuring flux from the tissue after prior equilibration in tritiated water, specific radioactivity 50 μc/ml, for at least 3 hr (not less than 10 $\times$ $t_{1/2}$). Pilot experiments showed that the tissue water had the same specific radioactivity as the incubation solution after 2 hr immersion at 27 C and that 2 additional hr did not make any change. In each experiment it was checked that the tissue water had in fact reached equilibrium with the medium. The THO flux between the tissue and the medium was followed by transferring each cylinder sequentially through a series of six test tubes containing 10 ml of H_2O, at selected time intervals. The test tubes were held in a temperature-controlled bath, and their contents were stirred by a stream of air bubbles. The time intervals were selected, after pilot experiments, in such a way that each washout volume contained 15% to 20% of the total radioactivity of the cylinder (see Fig. 1). The sixth washout period lasted for 16 hr.

Samples of 1 ml from each test tube were transferred to 15 ml of scintillation fluid (containing 4 g of PPO, 200 mg of POPOP, 60 g of napthalene, 20 ml of ethylene glycol, 100 ml of methanol, made up to 1 liter with *n*-dioxane) and were counted in a 2311 Packard liquid scintillation spectrometer. The total radioactivity in the cylinder at the end of the equilibration period was calculated from the counts in the series of six test tubes.

The percentage of radioactivity remaining in the tissue at the end of each washout period was then calculated. The results were plotted as shown in Figure 1. The time at which $A_t/A_0 = 0.50$ indicates the half-time for THO flux.

The substances under investigation were applied either before or during THO equilibration. In order to investigate reversibility of the effect, cylinders were transferred to water for various lengths of time after treatment. They were subsequently

equilibrated in THO. Flux measurements always followed immediately after THO equilibration.

Weight of cylinders ranged between 590 and 630 mg. The fresh weight of each cylinder was determined before immersion in the treatment solution and at the end of the flux measurement. Except where otherwise stated the difference between these two weights was less than 1%.

All treatments and flux measurements were in general carried out at 27 C unless otherwise indicated. In those experiments carried out at 15 and 5 C the equilibration time with THO was 6 and 12 hr respectively.

All substances used were analytical reagent grade. CO_2 treatments were applied as described previously (5, 6).

RESULTS

Control of THO Flux by Living Membranes in Carrot Tissue. There is little point in attempting to demonstrate effects on permeability of plant cell membranes to water in cases where THO flux is rate-limited, not by the membranes, but by diffusion through the "unstirred layer" and extracellular resistances. Kohn and Dainty (12) have reported that in beet root and artichoke discs extracellular resistances are in fact rate-limiting. Effects of various factors on the membranes would thus be masked. We therefore conducted preliminary experiments to test whether the controlling effect of living membranes is detectable in carrot tissue. Our evidence that the latter is the case may be summarized as follows:

1. The half-time for THO equilibration is three times as long in a living carrot cylinder as in one that has been killed in chloroform vapor or by freezing (Fig. 1). It should be emphasized that in these experiments the final radius of the dead cylinders, in spite of their lack of turgor and consequent shrinkage, was equal to that of the living ones (see "Materials and Methods"). It should also be emphasized that the living and dead cylinders were comparable from the point of view of diffusional pathway and compartment size, since the intercellular spaces were injected in both cases.

2. The temperature dependence of THO flux in living and dead cylinders was widely different. Again the cylinders were cut so as to allow for the shrinkage on killing, and the final radius of the dead and the live cylinders was equal. Estimation of energy of activation (ΔE) for THO exchange was obtained from the slope of the curve relating ln $^1/t_{1/2}$ to the reciprocal of absolute temperature according to the Arrhenius equation (see ref. 3). Half-time is a function of apparent diffusion coefficient (13). Figure 2 shows that over the range of 5 to 25 C this relationship was linear. The energy of activation for THO exchange in dead tissue was found to be 4.4 kcal·mole^{-1} which is very close to that of self-diffusion of water (3), whereas in living cylinders it was 10.7 kcal·mole^{-1}. The relatively high value for ΔE for live cylinders, as compared with dead ones, supports the view that living membranes control, at least partially, water flux.

Effect of Various Treatments on THO Flux. Table I summarizes experiments in which the effects of a number of substances on THO flux were investigated.

CO_2 markedly prolonged the half-time for THO equilibration. In previous studies (5, 6), we reported that CO_2 depressed osmotic water flux both into and out of plant cells and concluded that CO_2 affected L_p, the coefficient of hydraulic conductivity. The present finding extends this conclusion to P_d, the coefficient of diffusional permeability to water. Further, it resolves any lingering doubt that in the osmotic flux experiments the CO_2 might in some way have been influencing the diffusion of the osmoticum up to or away from the selectively permeable membranes. (The latter has been indicated as the rate-limiting factor in similar experiments [15]).

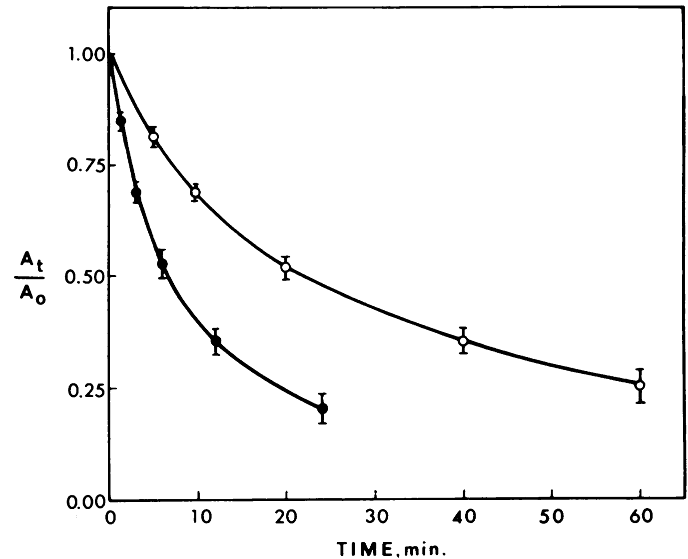

FIG. 1. The course of labeled water flux from cylinders of live and dead carrot storage tissue. Living tissue (○); dead tissue (●). A_0 = total radioactivity in the cylinder at beginning of washout. A_t = radioactivity remaining in the tissue at a given time. Temperature was 15 C. Each point represents the mean ± SE of six determinations.

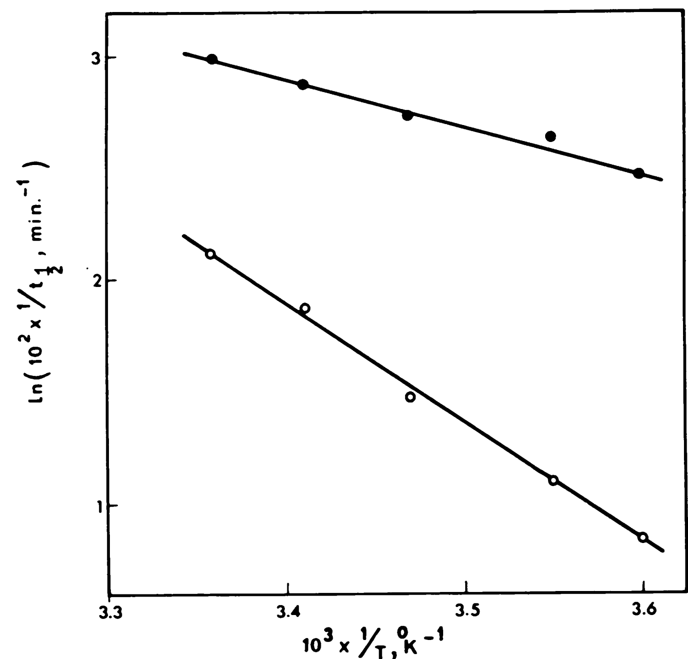

FIG. 2. Relationship between the natural logarithm of the reciprocal of half-time for THO flux from live and dead carrot cylinders and the reciprocal of the absolute temperature. The reciprocal of $t_{1/2}$ is a function of apparent diffusion coefficient. Living tissue (○); dead tissue (●). Each point represents the mean of six determinations.

DNP[1] at concentrations up to 0.1 mM had no effect. This result stands in marked contrast to the observations of other investigators using whole maize roots (4, 9, 17). The uncoupler,

[1] Abbreviations: CCCP: carbonyl cyanide *m*-chlorophenylhydrazone; DNP: 2,4-dinitrophenol; PCMB: *p*-chloromercuribenzoate.

Table I. *Effects of Various Treatments on Reversible Changes in the Half-Time for Labeled Water Efflux from Cylinders of Carrot Storage Tissue*

The half-times are given as means ± standard errors of six determinations.

Treatment	Optimal[1] Length of Treatment	Half-Time	Minimal Time[4] to Obtain Full Reversibility
		min	
H_2O		11.8 ± 0.5	
CO_2[2]	30	16.2 ± 0.7	less than 12 min
DNP, 0.1 mM, pH 6.1[3]		13.2 ± 1.0	
DNP, 1 mM, pH 6.1	180	18.2 ± 1.6	2 hr
CCCP, 3 μM[3]		13.0 ± 0.7	
CCCP, 10 μM	60	14.9 ± 0.9	2 days
CCCP, 0.1 mM	60	19.4 ± 1.2	3 days
PCMB, 0.3 mM, pH 7.2[3]		11.4 ± 0.8	
ABA, 20 mg/liter	180	7.2 ± 0.6	4 days
tert-Butanol, 0.3 M	120	8.1 ± 0.5	3 hr

[1] Causing the maximal effect that could still be reversed.
[2] CO_2 stream continued during efflux measurement.
[3] Treated for 4 hr.
[4] After cessation of treatment.

Table II. *Effects of Combined Treatments of Tert-Butanol or ABA with PCMB or CCCP on Labeled Water Efflux from Cylinders of Carrot Storage Tissue*

Duration of treatment 2 hr. The half-times are given as means ± standard errors of six determinations.

Treatment	Half-Time
	min
H_2O	12.2 ± 0.4
PCMB, 0.3 mM	12.0 ± 0.7
CCCP, 3 μM	13.0 ± 0.9
tert-Butanol, 0.3 M	8.4 ± 0.6
tert-Butanol, 0.3 M + PCMB, 0.3 mM	12.2 ± 1.2
tert-Butanol, 0.3 M + CCCP, 3 μM	12.8 ± 1.1
ABA, 20 mg/liter	8.2 ± 0.5
ABA, 20 mg/liter + PCMB, 0.3 mM	11.8 ± 1.0
ABA, 20 mg/liter + CCCP, 3 μM	12.6 ± 1.3

Table III. *Effect of Treatments with Tert-Butanol or ABA Followed by CCCP on Labeled Water Efflux from Cylinders of Carrot Storage Tissue*

Results given as half-times in minutes (means ± standard errors of six determinations).

First Stage (2 hr)	Second Stage (1 hr)		
	0	3 μM CCCP	10 μM CCCP
H_2O	12.0 ± 0.6	12.8 ± 0.8	15.2 ± 1.0
tert-Butanol, 0.3 M	8.3 ± 0.4	8.2 ± 0.7	8.6 ± 1.0
ABA, 20 mg/liter	8.8 ± 0.7	8.9 ± 1.2	9.2 ± 0.9

CCCP, was also without effect in our tissue at concentrations up to 3 μM, though this concentration strongly affected water flux in maize roots (4, 17). If applied in considerably higher concentrations both DNP and CCCP appeared to reduce water permeability (Table I).

It has been suggested that the sensitivity of apparent water permeability in whole maize roots to uncouplers may indicate that convective flow of the cytoplasm is required to lessen the cytoplasmic resistance to water flux (17). In view of the relative stability to uncouplers of water flux in our tissue we investigated the effect of PCMB which is known as a potent inhibitor of cyclosis (see ref. 10). Table I shows that it, too, was without effect at a concentration which is known to stop completely protoplasmic streaming in *Nitella* (11).

A significant increase in apparent permeability to THO was observed as a result of treatment with ABA, as we have briefly reported earlier (7). In view of reported effects of various alcohols on permeability to betacyanin (8, 16), we also tested several alcohols in our system. Methanol, ethanol, *n*-propanol, *n*-butanol, and *sec*-butanol all shortened the half-time for THO equilibration, but this effect was in all cases "toxic," *i.e.*, involved irreversible destruction of the selective permeability of the membrane as judged by continuous loss of weight by the tissue even after transfer to water. Concentrations lower than those producing this "toxic" effect were without any observable effect on permeability. Certain concentrations of tertiary butanol, on the other hand, increased water permeability without any "toxic" effect, *i.e.*, without causing any loss in the fresh weight of the tissue. Table I shows that 0.3 M *tert*-butanol raised permeability to THO to about the same extent as did ABA.

Reversal of the effects noted in Table I was relatively slow except in the case of CO_2. Unless CO_2 treatment was continued during the actual efflux measurement, no CO_2 effect could be observed, since full recovery was obtained in far less than the 12 min required for half-time measurement. This matches the speed of the reversal of the CO_2 effect on L_p, which was achieved within 2 min (6). Full reversal of the stimulatory effect of *tert*-butanol on water flux required 3 hr and of that of ABA, 4 days. This was determined by comparison with controls which had remained in water for the appropriate period.

Effects of Combinations of ABA or *tert*-Butanol and Metabolic Inhibitors. An interesting finding was that concentrations of inhibitors which were without effect on control tissue did have an effect when supplied in combination with ABA or *tert*-butanol. Table II summarizes an experiment where butanol and ABA again decreased $t_{1/2}$ for tritium equilibration by a third. PCMB and CCCP had no effect on the control tissue, but raised $t_{1/2}$ in the presence of either butanol or ABA.

There are two ways of interpreting this result. First, treatment with butanol and ABA may have caused the tissue to become sensitive to the inhibitors. Or, secondly, the inhibitors may have prevented ABA and butanol from raising permeability. In order to decide between these two possibilities, two stage treatments were applied to carrot cylinders. In the first stage they were treated with ABA or butanol, in the second with the inhibitor, after which $t_{1/2}$ for tritium equilibration was determined.

Table III shows that where ABA and butanol were allowed to act first alone, without interference from an inhibitor, exposure to an inhibitor subsequently was without effect. In fact, even a concentration of CCCP high enough to raise $t_{1/2}$ in control tissue was without effect on tissue pretreated with butanol or ABA (Table III, last column). One may conclude, therefore, that the second of the two interpretations suggested above is the more likely to be correct, *i.e.*, that the action of ABA and butanol in raising permeability requires metabolic energy.

In view of this rather surprising energy requirement for the action of *tert*-butanol we investigated whether the toxic effects of the alcohols described above were also sensitive to meta-

bolic inhibitors. A range of concentrations of alcohols was applied including concentrations both below and above the threshold for toxic action. Table IV shows such an experiment for *tert*-butanol. CCCP showed no tendency to prevent membrane damage as assessed by fresh weight change either at 3 μM or at 10 μM, though these concentrations prevented the reversible effect on water permeability (Table II). The highest butanol concentration tried reduced fresh weight by 20%, *i.e.*, to the level of that of tissue killed in chloroform vapor.

Table IV. *Effect of Prior Treatment with Tert-Butanol and CCCP, Alone and in Combination, on Fresh Weight of Tissue after Re-equilibration in Water*

After the various treatments (3 hr) the samples were returned to H_2O for 90 min before assessment of fresh weight. The figures give drop from initial fresh weight. The data are the means ± standard errors of 10 determinations.

Tert-Butanol	0	3 μM CCCP	10 μM CCCP
M		*mg*	
0	2 ± 4	0 ± 3	1 ± 6
0.3	0 ± 2	3 ± 6	2 ± 4
0.6	18 ± 8	16 ± 6	24 ± 10
0.9	36 ± 11	50 ± 12	41 ± 8
1.5	121 ± 10	116 ± 8	120 ± 14
Killing in chloroform vapor	118 ± 8	122 ± 7	117 ± 13

DISCUSSION

"Unstirred layers" and extracellular resistances may frequently be so large in comparison with the resistance of living membranes to isotopic water exchange that the effect of the membranes cannot be detected (see ref. 12). While the contribution of the membranes to the total resistance to water flux in the carrot cylinders studied here may possibly not be large enough to answer Kohn and Dainty's (12) purpose, which was to measure diffusional permeability, we nevertheless show that passage through the living membranes constitutes a rate-limiting step. This is indicated by the much longer half-time required for THO equilibration in living as compared with dead tissue, and by the higher values observed for ΔE in the former case. The degree of control which the membranes exert is sufficient to allow changes in their permeability to be detected. To the extent that flux is also under the control of extracellular resistances such as the unstirred layers (2), our results will underestimate the magnitude of the changes in the membranes.

Our experiments have shown that permeability to water is substantially increased by treatment with ABA. The physiological implications of this effect, and of the opposite effect of kinetin, have already been pointed out (7). Permeability was also increased by *tert*-butanol. In neither case was this increase associated with a marked change in permeability to solutes, since had this been the case the internal osmotic concentration (π_i) would have fallen. This would have been reflected in a drop in fresh weight of the tissue. No significant drop in fresh weight was noted, even over the 4 days during which the tissue was observed in the experiments on the reversibility of the ABA effect. The ABA-treated samples displayed raised permeability to water for at least 3 days without detectable fall in π_i.

The metabolic inhibitors, PCMB and CCCP, when supplied together with ABA or butanol, abolished their action. CCCP is among several which decrease the permeability of maize roots and other tissues to water, an effect which has led to the suggestion (13, 17) that an ATP-requiring process is required for maintaining permeability. A possibility to be considered, therefore, was that ABA and butanol—or at any rate the hormone—was stimulating this ATP-requiring process or the supply of ATP. Had this been so, however, the inhibitors would have abolished the ABA and butanol effects even when supplied *after* the latter substances. This was not the case. If ABA and butanol were allowed to act first, their effects were stable to the inhibitors. Furthermore, the metabolic inhibitors themselves, unless supplied in high concentrations, had no effect on the permeability of our tissue. It must be concluded that the ATP requirement in our case was for the action of ABA and butanol.

That metabolic processes should be involved in hormone action may not be surprising. It is even possible that uptake into the tissue depends on metabolism. It is perhaps more surprising that metabolic processes should be required to potentiate the action of butanol. Various workers (1, 8, 16) have brought evidence that alcohols act directly on cell membranes, possibly by altering their water content. It has been suggested that they effect the removal of the calcium linking adjacent phospholipid molecules (1) or antagonize the stabilizing and compacting influence of sterols on the lipid layer (8). Such actions do not, on the face of them, suggest dependence on metabolic energy. It is possible, however, that the effect of *tert*-butanol on water permeability reported here is qualitatively different from that studied by the workers quoted above. Siegel and Halpern (16) found that branching on the alcoholic C-1 reduced or abolished the power of the alcohol to induce leakage of betacyanin from beet root tissue. We have observed the same phenomenon for the "toxic" effect on our tissue (irreversible destruction of selective permeability). *Tert*-Butanol at the concentrations used had no "toxic" effect. Normal butanol was either toxic or, at lower concentrations, without any effect at all. Further, while the effect of *tert*-butanol on water permeability could be prevented by metabolic inhibitors, its "toxic" effect could not. Thus, the difference between the two effects may be not merely of degree but of kind.

The fact that metabolic inhibitors had little effect on the water permeability of carrot cylinders when supplied in concentrations which are known to decrease the permeability of whole maize roots (17) or maize root sleeves (4) poses the question of the nature of the difference between these systems. It is unlikely to lie in a lesser degree of sensitivity to the inhibitors, since these concentrations were effective in preventing ABA and butanol action in carrots. If the suggestion (17) that cyclosis is the ATP-requiring process involved in the case of maize roots is correct, then a possible explanation is at hand. Where water is moving appreciable distances through the symplast, as may well be the case in whole roots or sleeves, where water must pass from cell to cell, cyclosis may play a much more important role in lessening resistance of the complex "membrane" than where, as in our carrot tissue, the ambient solution is virtually surrounding each cell.

In striking contrast to the lack of effect of the metabolic inhibitors, CO_2 increased the half-time for THO equilibration. This indicates that the effect of CO_2 on water permeability is not analogous to that of the inhibitors and is due neither to the depressing effect of CO_2 on cyclosis (10) nor to interference with ATP supply. This conclusion is supported by the fact that the effect of CO_2, but not that of CCCP, is apparent when the experiment is carried out at 2 C. It is probably that CO_2 is displaying narcotic action (*i.e.*, its effect is "physical" rather than "chemical" and is probably exerted directly on the membrane

itself). The very ready reversibility of the CO_2 effect is also characteristic of narcotics.

The nature of the changes which ABA and tertiary butanol produce and which allow greater permeability to water without appreciable leakage of solutes, even as measured over several days, invites further investigation.

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