Reduction in Turgor Pressure as a Result of Extremely Brief Exposure to CO_2 [1]

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Summary. CO_2 depresses water influx into sunflower hypocotyl segments of low water potential; by contrast, it stimulates flux into segments of high water potential. When segments of high potential were placed in a series of mannitol concentrations and allowed to achieve steady rates of water uptake, influx into CO_2-treated tissue in a solution of 3 atm equalled that into control tissue in water. Reasons are given for deducing that a change in internal osmotic concentration (π_i) of the order of 40 % would be necessary to account for this result on the basis of π_i. Direct measurements (by cryoscopy and by the minimum volume method) detected no difference in the steady state value for π_i as between CO_2-treated and control tissue. It was therefore concluded that CO_2 had caused some reduction in turgor pressure.

Water uptake into tissue treated with CO_2 for only the first 2 minutes of a 30-minute period was equal to that into tissue treated continuously with CO_2, i.e. 3 times the control value. Ten seconds' CO_2 treatment produced a significant stimulation. When the cycles of treatment were repeated the samples receiving flashes of CO_2 maintained a rate of water uptake superior to that of the control, whereas influx into continuously treated tissue fell below the control value after 1 hour.

CO_2 treatment applied in a moist air chamber stimulated subsequent water influx when the tissue was transferred to water. Fifteen seconds' treatment was sufficient to produce a marked effect. Even when a transition period of 30 minutes in the moist chamber was interposed between CO_2 treatment (5 minutes) and transfer to water, a stimulation was observed. The CO_2 effect could be achieved at zero degrees; 5 minutes' treatment in the moist chamber at zero degrees, followed by a 15-minute transition period at the same temperature, substantially increased subsequent water uptake at 25°.

Introduction

We recently described and analyzed a depressive effect of CO_2 on water flux both into and out of segments of sunflower hypocotyl, carrot discs, and other plant tissues (9, 10). The effect, which was shown to be due to a decrease in the coefficient of hydraulic permeability of the cell membranes, was observed, in the case of influx, when the water potential of the cells was relatively low. In subsequent experiments to be described below, where water potential was relatively high, we noted an opposite effect of CO_2: water influx was enhanced. This effect recalls the reported stimulation in extension growth of *Avena coleoptiles* (5, 11, 22). In the present paper we analyze the effect, demonstrate that treatment for only a few seconds is sufficient to produce it, that it can be obtained at zero degrees, and show that it is due to an extremely rapid decrease in turgor pressure brought about by CO_2.

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Materials and Methods

The sunflowers (*Helianthus annuus* L. var Jupiter) were grown in sterile vermiculite at 26° in darkness except for occasional red light. Segments 1 cm long were cut from the hypocotyls of 6-day-old seedlings at a distance of 1 cm below the crook. After cutting, the segments were first washed in distilled water for 1 hour. Samples of 32 segments (approx. 1 g) were then carefully blotted to remove surface moisture and weighed on a balance sensitive to 0.5 mg. Each sample was placed in a small glass vessel with a floor made of stretched muslin. The vessels, which were subsequently placed in the various experimental solutions (50 ml), facilitated rapid transfer of the segments. Water influx was determined by further weighings. CO_2 treatment was applied by leading the gas stream either through a solution in which the segments were immersed (see 9) or through a moist chamber, as indicated in the text. Unless otherwise stated, experiments were carried out at 25°.

Results

Figure 1 defines the conditions under which CO_2 is inhibitory to water influx, and those under which, by contrast, it is stimulatory. The continuous curve shows the course of water influx into hypocotyl segments which had previously lost 15 % of their H_2O content by drying in air at room temperature. Treatment with CO_2 was applied at 2 points on this uptake curve. At the first point the water potential (M, see ref. 7) of the tissue was relatively low; at the second point it was high. It will be seen that the effect produced by CO_2 at the first point was qualitatively the reverse of that produced at the second point.

That the CO_2 stimulation was not due to the low pH of CO_2-saturated water (pH 4.1) is shown in figure 4, where the curve for influx from CH_3COOH at the same pH is given. Although slightly above the control curve (cf. 2) it lies far below the curves for the CO_2 treatments. Neither could the stimulation be explained on the basis of a possibly greater degree of infiltration of intercellular air spaces in CO_2-treated tissue, since tissue which had previously been completely infiltrated under reduced pressure responded just as strongly to subsequent CO_2 treatment.

Water influx into this tissue under our conditions is a function of M, which is the driving force effecting the entry of water; of the hydraulic permeability of the membranes: and of σ, the reflection coefficient (see 7, 10). At the second arrow in figure 1 maintenance of a positive driving force depends on certain metabolic reactions which result in the growth of the tissue. Since these reactions might well be rate-limiting or nearly so with regard to H_2O influx, one might not expect the effect of CO_2 on hydraulic permeability (9, 10) to be noticeable at this point as at the first arrow, where the large driving force is principally due to the air-drying treatment. The CO_2 effect, however, was not merely diminished at the second arrow; it was reversed. This result strongly suggests that under these conditions CO_2 was also influencing some quantity other than hydraulic permeability.

Evidence that the CO_2 Effect is Exerted on Turgor Pressure. The equation describing net influx into the tissue is

$$J_v = L_p (\sigma \Delta \pi - \Delta P) \qquad \text{I}$$

where J_v = volume flow, L_p = coefficient of hydraulic permeability, $\Delta\pi$ is the difference between internal and external osmotic concentrations, σ is the reflection coefficient of the membrane, and ΔP is the hydrostatic pressure difference across the membrane (see 7, 10). The vastly greater permeability of this tissue to water than to the cells' internal solutes justifies the assumption that σ is close to 1 under our conditions. An increase in σ of sufficient magnitude to explain the increase in J_v produced by CO_2 in tissue of high water potential can therefore be ruled out. L_p is known to be depressed. The CO_2 effect must therefore be due to an increase in the quantity $(\Delta\pi - \Delta P)$, the driving force, i.e. either to a rise in π_i (the internal osmotic concentration) or to a fall in P_i (turgor pressure). Since further experiments showed that the increased rate of water uptake by CO_2-treated tissue is maintained for at least 40 minutes, determinations made during this steady-state period should reveal a higher π_i if the latter is responsible for the increased driving force. The accuracy of π_i determinations is recognized to be low (6). We therefore first inquired whether the change in π_i necessary to account for the observed change in influx would be large enough to be readily detectable by available methods.

To make this calculation it was necessary to obtain an estimate of the size of the change in $(\Delta\pi - \Delta P)$ brought about by CO_2. To this end we examined by how much the external osmotic concentration (π_e) had to be raised in order to depress influx into CO_2-treated tissue to the level of that into untreated tissue immersed in H_2O.

Tissue samples were placed in a range of mannitol concentrations. As Ray and Ruesink (18) have pointed out, when plant tissue is transferred to a mannitol solution, an interval is required before a steady state of influx is achieved. Under our conditions approximately 1.5 hours has to elapse before the rate of water uptake becomes constant. We therefore began CO_2 treatment 2 hours after the transfer to mannitol and measured after a further 30 minutes.

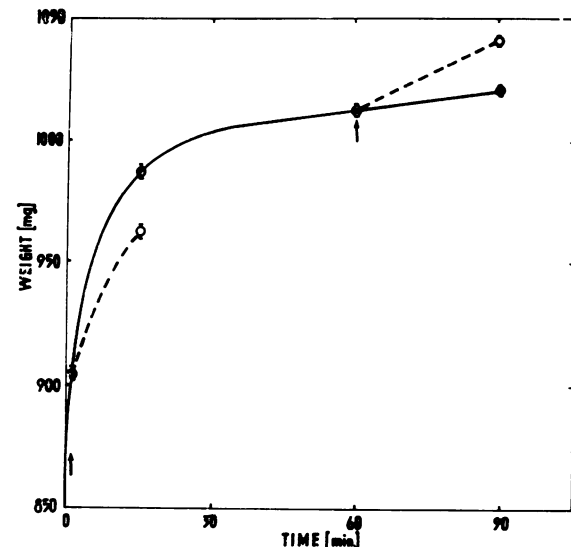
The results showed that a CO_2-treated sample placed in a mannitol solution of 3 atm. (A in fig 2) would take up water at the same rate as untreated tissue in distilled water (B in fig 2). Taking into account the depressive effect of CO_2 on L_p (10), $\Delta \pi - \Delta P$ at A must therefore be at least as big as at B. Since π_e, however, is 3 atm higher in the case of A, π_i for this sample must be at least 3 atm higher than that for B, if the CO_2 effect is to be explained on the basis of π. This increase is at least 40 % of the control value for π_i (see below). Though the size of the increase might conceivably be somewhat different for a CO_2-treated sample in water rather than in mannitol solution, an effect of this order of magnitude should certainly be detectable by available methods.

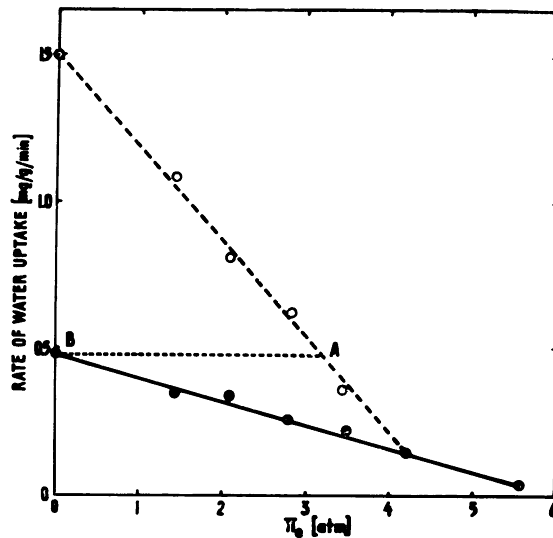
Determinations of π_i were consequently carried out, firstly by the cryoscopic method (21), and secondly, as an additional check, by the minimum volume method (20). The first method indicated 6.5 atm and 6.4 atm for the control and the CO_2-treated samples respectively (SE $\pm$ 0.2 atm). Changes in weight rather than changes in volume were followed in the second method, since under these conditions weight changes are virtually equivalent to volume changes. Figure 3 shows that the curves for both CO_2-treated and control tissue change direction at approximately the same point, i.e. at π_e = 9.2 atm. This indicates a very similar π_i in the 2 cases, thus supporting the cryoscopic determinations. Allowing for the volume difference between full turgor and

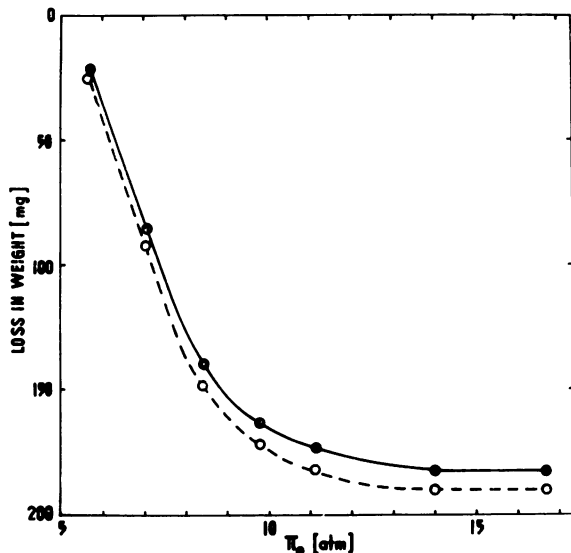
incipient plasmolysis, a value of 7.4 atm is obtained for π_i. The higher π_i indicated by plasmolytic as compared with cryoscopic methods is in line with numerous other investigations (1).

The change in driving force thus cannot be accounted for on the basis of π_i, and must therefore be due to a fall in P_i.

Efficacy of Brief Flashes of CO_2. Figure 4







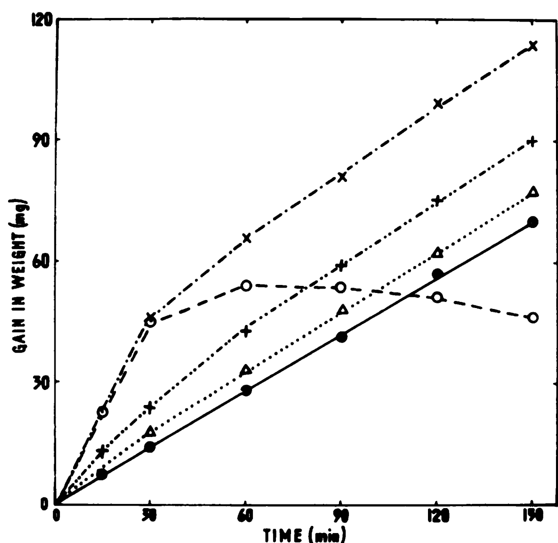


FIG. 1 (*top left*). The effect of CO_2 at 2 points on the curve giving the course of water influx into sunflower hypocotyl segments. The latter had previously lost 15 % of their initial water content by drying in air. Initial weight of samples 1 g. The arrows indicate the 2 points at which CO_2 treatment was applied. ●, Control; ○, CO_2 treated. In the figures each point represents the mean of triplicates. Their range is indicated where this extended beyond the symbol drawn.

FIG. 2 (*top right*). The effect of external osmotic pressure on the rate of water entry into CO_2-treated and control segments of sunflower hypocotyl. The tissue had been immersed in the appropriate mannitol solution for 2 hours before the start of the experiment. ●, Control; ○, CO_2-treated. For explanation of A and B see text.

FIG. 3 (*bottom left*). The relationship between loss in weight of sunflower hypocotyl segments and external osmotic pressure. Loss in weight determined after 5 hours in mannitol solution. ○, Pretreated with CO_2 for 20 minutes; ●, pretreated with air.

FIG. 4 (*bottom right*). Effect of discontinuous CO_2 treatment on water uptake by sunflower hypocotyl segments. — — ○ — —, Continuous CO_2 treatment; · — × — ·, 2 minutes CO_2 repeated every 30 minutes; · · — + — · ·, 10 seconds CO_2 repeated every 30 minutes; —●—, control-aerated H_2O; . . . . △ . . . ., pH control-CH_3COOH pH 4.1 Segments treated discontinuously were transferred to aerated H_2O in between CO_2 treatments.

shows that the continuous supply of CO_2 is not necessary for the achievement of the full stimulatory effect. After 30 minutes, water uptake by tissue treated with CO_2 for only 2 minutes at the beginning of this half-hour period, was equal to uptake by tissue treated continuously, i.e. more than 3 times that of the untreated control. Further, figure 4 demonstrates the remarkable finding that a 10-second flash of CO_2 produced a statistically significant stimulation. The curves for treatment periods intermediate between 10 seconds and 2 minutes have been omitted from figure 4 in the interests of clarity. They lie between the curves for these 2 extremes.

The effect of repeated half-hour cycles of treatment is also shown in figure 4. The curve for tissue treated continuously with CO_2 declined with time. This was not the case, however, for the discontinuous CO_2 treatments.

Intermediate measurements made during the first 30 minutes indicated a steady rate of uptake during this period. (fig 4).

Separation in Time between Exposure to CO_2 and the Expression of its Effect on Influx. Since the previous experiment showed that exposure to CO_2 for 2 minutes affected water influx for the subsequent 30 minutes, we next examined whether treatment applied under conditions where there was no accompanying H_2O uptake would influence water uptake at a later period.

Hypocotyl segments were treated for various periods up to 5 minutes with CO_2 or with air while in a moist chamber. They were then transferred to water, and influx was measured over the following 30 minutes. Figure 5 shows, firstly, that CO_2 treatment for 5 minutes in the moist chamber, followed by immediate transfer to water, was just as effective as treatment while the tissue was immersed. It further shows that exposure to CO_2 in the moist chamber, even for as brief a period as 15 seconds, produced a marked effect on subsequent water uptake. The curve reaches a level value after about 2 minutes' treatment.

We next investigated the effect of interposing a transition period in moist air between CO_2 treatment in the moist chamber and transfer to water. With increasing length of transition period efficacy of treatment declined (fig 6a), the decline being steepest during the first 7.5 minutes. Even after a transition period of 30 minutes, however, influx into treated tissue was higher than into the control.

Efficacy of Brief CO_2 Treatment at Zero Degrees. Figure 6b demonstrates that the CO_2 effect can be achieved, not merely without accompanying water uptake, but also at zero degrees. In this experiment the tissue samples were first pretreated in water at zero degrees for 2 hours, and were then transferred to moist air chambers at the same temperature where they were treated with CO_2 or with air for 5 minutes. As in the last experiment a transition period of varying duration (in this case also at zero degrees) intervened before their subsequent transfer to water at 25°. The curves for water influx are very similar to those obtained when CO_2 treatment was applied at 25° (cf. fig 6a) though the level of influx into both treated and control tissue was much lower than into segments not previously chilled. Brief application of CO_2 at zero degrees thus substantially increased water uptake even after an intervening period of 15 minutes in air at the same temperature. If influx into CO_2-treated segments is expressed as a ratio of that into the control, the effect is much larger than that at room temperature (compare figs 6a and 6b).

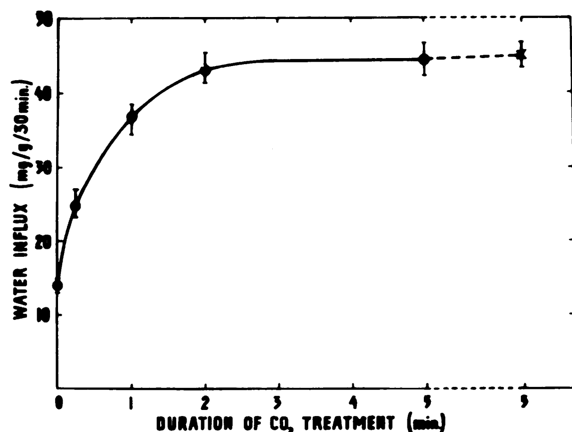


FIG. 5 (*upper*). Effect of prior CO_2 treatment in a moist chamber on subsequent water influx into segments of sunflower hypocotyl. The cross gives the value for tissue treated for 5 minutes while immersed in H_2O.

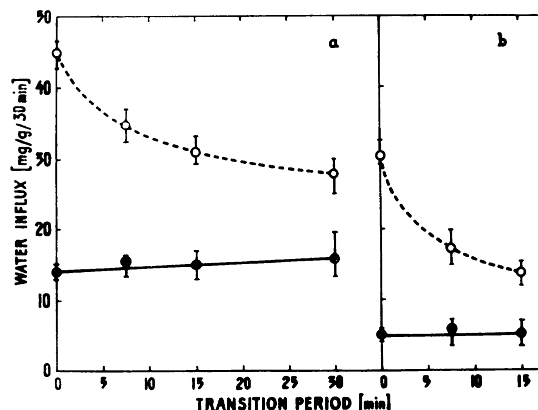


FIG. 6 (*lower*). Effect of length of transition period, interposed between CO_2 treatment and transfer to water, on water influx into sunflower hypocotyl segments. ○, Treated with CO_2 for 5 minutes in moist chamber; ●, treated similarly, but with air. The transition period was spent in the moist chamber. a) Experiment carried out at 25°. b) Treatment and transition period at zero degrees; water influx at 25°. The segments were cooled at zero degrees for 2 hours before treatment.

Discussion

The results presented here have shown that, when observations on water influx are made on tissue of high water potential, the depressive effect of CO_2 on the hydraulic permeability of the cell membranes (9) is marked by a second and opposite effect; that on the driving force bringing about water entry. From figure 2 it was estimated that, if the CO_2 effect was to be explained on the basis of a change in the steady-state concentration of osmotic particles within the cell, π_i for the sample in 3 atm mannitol must have risen by at least 40 %. This is a considerable change; and though the rise might possibly be somewhat different for a CO_2-treated sample in water our determinations should nevertheless have revealed it. Since no appreciable difference between π_i for CO_2-treated and for control tissue was observed during the period when different rates of water uptake were steadily maintained, it is reasonable to deduce that CO_2 brings about a reduction in turgor pressure.

The difference between π_i (control) and π_i (CO_2-treated) due to the greater dilution of osmotically active solutes by the greater water influx in the latter case (46 mg H_2O/g tissue as compared with 14 mg/g) would be too small (about 3 %) for our methods to detect with statistical certainty.

Extensive studies have been reported on an inhibitory effect of CO_2 on extension growth of *Avena* coleoptiles (5, 11, 14, 15, 16, 17). Several workers have, however, also noted a stimulatory effect on coleoptile elongation under certain conditions (5, 11, 22). Our work extends their observations, in particular in that it demonstrates the extreme brevity of exposure period sufficient to produce the effect. Harrison (11) and Cockshull and Heath (5) both noted that the stimulation produced by CO_2 declined with time and was followed by inhibition. Our experiments demonstrate that water uptake will continue at a stimulated rate (though the size of the stimulation declines) if CO_2 treatment is discontinuous. A regime of 2 minutes' CO_2, followed by 28 minutes' air, produced a rate of water uptake which exceeded that of the control for the period of the experiment (2.5 hours); whereas influx into tissue treated continuously with CO_2 declined to a value below that of the control after 1 hour.

The injurious effects of continuous CO_2 treatment may be related to the irreversible damage to the cell membranes previously shown (9) to result after approximately 40 minutes. The fact that, during the first half-hour, 2 minutes' CO_2 + 28 minutes' air are as effective as 30 minutes' CO_2, suggests that a chain of reactions is involved: that the action of CO_2 is followed by a series of reactions or events which are independent of the presence of CO_2. Two minutes' CO_2 treatment is apparently sufficient to activate these subsequent reactions for at least 30 minutes.

An alternative explanation might be that the continued effect of CO_2 after transfer to air is due to the continued presence of CO_2 within the tissue. Contrary evidence is provided, however, by the experiments where an interim period in a moist air chamber was interposed between CO_2 treatment and transfer to water. An interim period of 30 minutes did not abolish the effect of 5 minutes' prior exposure to CO_2. The high rate of diffusion of CO_2 through cell membranes (13) makes it most unlikely that residual free CO_2 remained within the tissue after this period.

The fact that the effect described here can be achieved within 10 seconds, and moreover can be accomplished at zero degrees, points to the possibility that CO_2 causes some physical change. It has frequently been suspected that the effect of CO_2 on a number of physiological processes may be due to its activity in lowering intracellular pH. The finding that various other acids at the same pH are far less effective than CO_2 has been attributed to the ease and rapidity with which CO_2 penetrates cellular barriers (13). If, in the present investigation, CO_2 had penetrated a hydrophobic barrier to some critical position in the cell wall not accessible to H ions or to CH_3COOH, if, for instance, it had penetrated the hydrophobic portions of a protein, then the consequent pH change might have brought about an alteration in the wall matrix in that region. Ginzburg (8) has provided evidence for the presence of a protein gel in the wall; a drop in pH to a value near the isoelectric point of the protein would bring about a decrease in viscosity, since viscosity is at a minimum at the isoelectric point (19).

The moist chamber experiments demonstrate unequivocably that the initial reaction, at any rate, of CO_2 can be effected without accompanying water uptake. This result, together with the fact that the effect disappears above a certain critical value for π_e (see figure 2) and that it can be effected at zero degrees, recalls the action of IAA (3, 4, 12). According to Yamaki (22) the stimulatory effect of CO_2 on coleoptile extension can be observed in the presence of IAA; according to Cockshull and Heath (5), only in its absence. The question as to whether there is in fact any basic similarity of action between these 2 substances must await further study.

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