PHOTOSYNTHESIS BY THIN LEAF SLICES IN SOLUTION

II.* OSMOTIC STRESS AND ITS EFFECTS ON PHOTOSYNTHESIS

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Abstract

The effects of osmotic stresses on photosynthetic $^{14}CO_2$ fixation by cotton leaf slices were investigated. Using mannitol and sodium chloride as examples of osmotica which entered leaf cells fairly slowly, and ethylene glycol as a rapidly permeating osmoticum, the relative importance of different components of tissue water status in inhibiting carbon fixation was studied.

A 5 min treatment in a −17 bar solution of slowly permeating osmoticum inhibited $^{14}CO_2$ fixation by 25%, while significant effects were observed at −4 bars. The degree of inhibition increased twofold after a further 160 min treatment. Recovery was rapid and complete from milder levels of stress, but there was little recovery from stresses greater than −10·8 bars. Treatment with ethylene glycol solutions down to potentials of −17 bars, however, did not cause significant inhibition of ^{14}C fixation rate, even after 165 min treatment.

It was concluded that intracellular process of photosynthesis in leaf slices of cotton responded rapidly to small changes in leaf water status. The observed inhibition of $^{14}CO_2$ fixation, at least at the milder stresses, appeared to be related to cell volume or turgor changes, and not to the total water potential. More severe stresses gave rise to a comparatively irreversible inhibition of $^{14}CO_2$ fixation, which could have been related to structural changes associated with plasmolysis.

I. INTRODUCTION

Much of the effect of water stress on reducing leaf photosynthesis has been ascribed to stomatal closure (Troughton 1969; Troughton and Slatyer 1969; Boyer 1970; Kreideman and Smart 1971), although there is increasing evidence that changes in the intracellular processes may also be important (Boyer 1965, 1971*a*, 1971*b*; Redshaw and Meidner 1972). A useful method for artificially inducing water stress is to immerse tissues in osmotic solutions. This method has been widely used for studying the effects of water stress on the metabolism of plant tissues, including studies on photosynthesis (Brilliant 1924; Glinka and Katchansky 1970). In addition, large numbers of studies have been done on whole plants with their roots immersed in osmotic solutions (e.g. Slatyer 1961).

In this paper the leaf-slice method described in an earlier paper (Jones and Osmond 1973) was used to investigate the short-term effects of osmotically induced

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water stress on the intracellular processes of photosynthesis in cotton leaf slices. The effects of rapidly and slowly permeating osmotica (Greenway and Leahy 1970) were compared in an attempt to investigate the mechanism whereby water stress affects the intracellular processes of photosynthesis.

II. Materials and Methods

(*a*) *Plant Material*

The plant material used was cotton (*Gossypium hirsutum* L. cv. Deltapine Smoothleaf) grown as described in Jones and Osmond (1973). All material was taken from the youngest fully expanded leaf.

(*b*) *Preparation of Leaf Slices and Photosynthesis Assays*

Leaf slices (400 μm thick) were prepared and stored as previously described (Jones and Osmond 1973). All experiments were performed at 25°C, and photosynthesis was assayed by $^{14}CO_2$ fixation. The conditions were as described in Jones and Osmond (1973), where a saturating CO_2 concentration (15 mM $KHCO_3$ at pH 7·6) and a saturating light intensity (120 W m^{-2}, 400–700 nm) were used throughout. The specific activity of the bicarbonate was approximately 0·04 μCi μmole^{-1}. The control rates of ^{14}C fixation were between 32 and 46 nmoles (mg chlorophyll)$^{-1}$ s^{-1}.

(*c*) *Production of Stress*

The osmotica used to induce water stress in the leaf slices were mannitol, sodium chloride, and ethylene glycol. The mannitol and sodium chloride were A.R. grade, while the ethylene glycol was L.R. grade. Osmotic potentials of the various solutions were calculated from tables given by Weast (1969) and Lang (1967). The osmotic potential of the assay solution was −2·0 bars, as measured using a vapour pressure osmometer (Mechrolab model 301A). The solution potentials given throughout are those due to the added osmoticum alone.

(*d*) *Stress Experiments*

Samples of 20 slices were preincubated for varying times in the light in osmotic solutions before the $KH^{14}CO_3$ was added to commence the assay. Recovery was studied by returning the samples to the control buffer, after 165 min treatment in osmoticum, before assay. All times are given as the preincubation period alone, excluding the 5-min assay period. Solution changes were made by direct transfer of slices. In short-term experiments with sodium chloride or mannitol, optimum ^{14}C fixation rates were obtained with between 0 and −2·0 bars of added osmoticum.

Fixation of $^{14}CO_2$ was also studied at lowered water potentials in the presence of bovine erythrocyte carbonic anhydrase (Sigma) at a concentration of 0·2 mg ml^{-1} or increased bicarbonate concentrations (20 mM).

(*e*) *Mannitol Uptake*

The rate of mannitol uptake under the usual assay conditions was followed using D-[U-^{14}C]-mannitol (obtained from I.C.N., California, U.S.A.). Slices were incubated as for the ^{14}C fixation experiments, (but with unlabelled $KHCO_3$) in labelled mannitol solutions of either −5 or −17 bars, at specific activities of approximately 0·02 μCi μmole^{-1}. After the incubation, the slices were washed in three changes of unlabelled mannitol (5 min in each), blotted, and dried in scintillation vials at 70°C. The samples were counted in a Beckman LS-150 scintillation counter in 5 ml dioxane (containing 5 g 2,5-diphenyloxazole and 100 g napthalene per litre). Aliquots of the labelled mannitol solutions were counted and rates of uptake estimated on the basis of external specific activity.

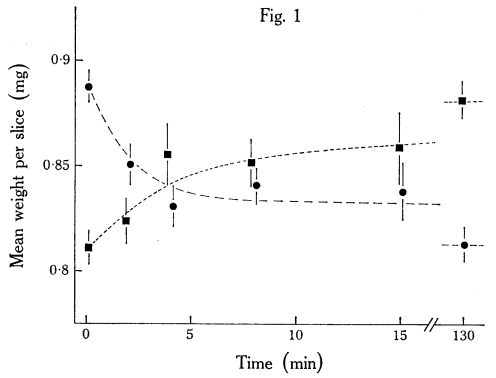
(*f*) *Water Exchange*

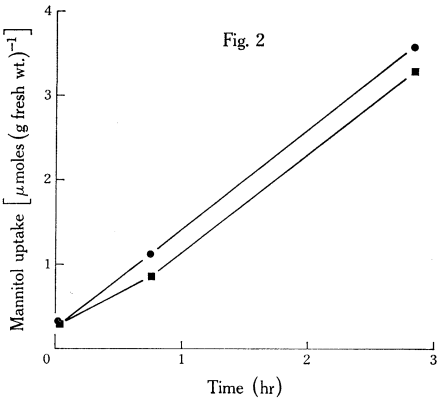
The rate of water exchange on changing the potential of the external solution was followed by weighing the slices. The slices were preincubated either in distilled water, or in −10·8 bars mannitol for 2 hr, then the changes in weight were followed on transferring the samples to the alternate solution. The slices were gently blotted with tissues twice before weighing, then the number of slices was counted. Each sample contained from 30 to 40 slices. To avoid tissue damage each sample was weighed only once.

III. Results

(*a*) *Water Relations*

The changes in weight on transferring slices between distilled water and −10·8 bars mannitol are shown in Figure 1. The initial rates of water exchange were less than 2 g (g fresh wt.)$^{-1}$ hr^{-1}. Although the standard errors are quite large, the half time for weight changes (assumed to be equivalent to water exchange, since changes due to mannitol exchange should be comparatively small) was between 1 and 4 min. This time is greater than the theoretical value of about 30 s expected for isolated cells (Philip 1958; Slatyer 1967), and is probably related to unstirred-layer effects in the cell walls of the tissue. It is apparent, however, that water equilibration should have been nearly complete in all the experiments presented.





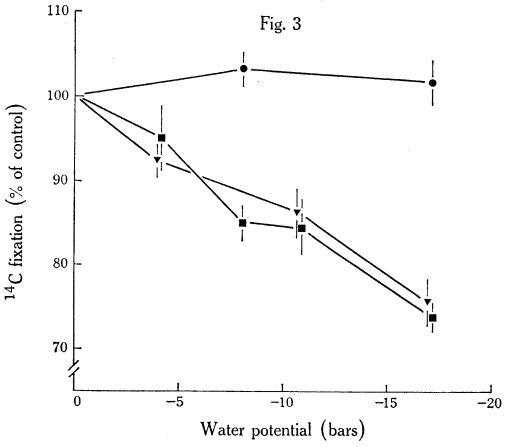


Fig. 1.—Time course of weight changes on transferring leaf slices from distilled water to −10·8 bars mannitol (■) and vice versa (●). Each point is the mean of five replicates with its standard error.
Fig. 2.—Uptake of label from [U-^{14}C]-mannitol solutions by leaf slices in the light. ● From −5 bar solution. ■ From −17 bar solution.
Fig. 3.—Inhibition of ^{14}C fixation by slices after 5 min preincubation in sodium chloride (▼), ethylene glycol (●), or mannitol solutions (■) of various osmotic potentials. Each point represents the mean of from 6 to 10 replicates with its associated standard error.

Incipient plasmolysis, as determined microscopically on thin slices immersed in a range of mannitol solutions, occurred at between −11 and −14 bars. The results were similar if sodium chloride solutions were used. If, however, ethylene glycol solutions were used instead of mannitol, plasmolysis was not apparent, even transiently, with a solution potential as low as −24 bars.

(*b*) *Mannitol Uptake*

Figure 2 shows the rate of uptake of ^{14}C from labelled mannitol solutions with mannitol concentrations equivalent to −5 and −17 bars. The apparent rate of uptake of mannitol was calculated as approximately 1·1 μmoles mannitol (g fresh wt.)$^{-1}$ hr^{-1}, from both solutions. This rate is similar to the rates of active uptake of Na^+, Cl^-, and oxalate, observed in *Atriplex* leaf tissue (Osmond 1968), and also K^+ uptake in maize leaves (Rains 1968). Although this rate is significant, it is only equivalent to an increase in the average internal mannitol concentration of 5–10 μM (−0·1 bars) in 3 hr. Concentrations in specific intracellular compartments, however, may have been higher.

The rate of uptake was linear over the 3 hr of the experiment. Also, the uptake was apparently into the cells, since free space material should have been lost in the washing-out period used, and the uptake extrapolated to close to zero at zero time. Less than 0·1% of the added label was taken up by the tissue during the 3-hr incubation period, and it is possible that the labelled species taken up was a high specific activity contaminant other than mannitol, even though the sample was apparently pure, as judged by paper chromatography.

(*c*) *Water Stress*

The effects on ^{14}C fixation of treating leaf slices for short periods in various osmotic solutions are shown in Figure 3. The slices were all pretreated for 5 min in the osmotic solutions before assay, and all rates are expressed as percentages of control values. The effects of sodium chloride and mannitol solutions of equal osmotic potentials were apparently similar over the concentration ranges studied, with the inhibition of ^{14}C fixation being approximately linearly related to the osmotic potential of the solution. The ^{14}C fixation rates in the −17 bar treatments were about 75% of the control rates. Ethylene glycol, over the same range of water potentials, caused a slight, but non-significant stimulation of photosynthesis.

TABLE 1

EFFECTS OF INCREASED CO_2 AVAILABILITY ON THE MANNITOL INHIBITION OF ^{14}C FIXATION IN COTTON LEAF SLICES

Values are the means with their standard errors, for four replicates, expressed as percentages of controls

Mannitol concn. (bars)	$[HCO_3^-]$ (mM)	Carbonic anhydrase (at 0·2 mg ml^{-1})	Rate (% of control)
0	15	−	100
0	20	−	102 (±2·1)
−10·8	15	−	84 (±1·8)
−10·8	20	−	85 (±2·7)
−10·8	15	+	82 (±3·2)

The results in Table 1 indicate that the reduced ^{14}C fixation, at least in the mannitol treatments, was not due to a reduction in the availability of CO_2. The inhibition of ^{14}C fixation by a 5 min preincubation in −10·8 bars mannitol was not

relieved by increasing the CO_2 supply during assay. The CO_2 supply was increased, either by raising the bicarbonate concentration to 20 mM, or by including carbonic anhydrase (concn. 0·2 mg ml^{-1}) in the medium.

The effects of longer pretreatments (165 min) in mannitol solutions, and of recovery, are shown in Table 2. For all the mannitol concentrations used, there was a 1·5- to 2-fold increase in the degree of inhibition of the ^{14}C fixation rate between the 5 min and the 165 min sample points. By contrast, it was found that none of the ethylene glycol solutions caused significant inhibition of the ^{14}C fixation rate, even after 165 min pretreatment (not shown). The recovery of photosynthetic ability was rapid and complete for the −4 and −8 bar mannitol treatments, while there was partial recovery from a stress of −10·8 bars. There was, however, only slight recovery from the −17 bar stress. Much of the effect of the severe treatment was irreversible during the one hr recovery period. It can be seen from Table 2 that the major portion of the mannitol effect on photosynthesis was manifest in less than 10 min, and that similarly much of the recovery in activity also occurred rapidly, at least in the milder treatments.

TABLE 2

TIME COURSE OF THE EFFECTS OF TREATMENT WITH VARIOUS CONCENTRATIONS OF MANNITOL, AND SUBSEQUENT RECOVERY, ON RATES OF ^{14}C FIXATION BY COTTON LEAF SLICES

Values are expressed as percentages of controls, and are given as the means with their standard errors. There were six replicates for the 5-min treatments, and four for each of the others

Period of stress (min)	Mannitol strength (bars)			
	−4·0	−8·0	−10·8	−17·0
5	95·0 (±3·5)	84·7 (±3·3)	84·0 (±3·1)	74·0 (±1·7)
165	92·3 (±1·0)	74·5 (±1·5)	67·9 (±1·5)	53·8 (±2·2)
165+10 min recovery	97·3 (±1·6)	98·3 (±2·8)	85·3 (±2·0)	63·1 (±2·1)
165+60 min recovery	102·3 (±1·3)	99·0 (±2·5)	87·9 (±3·0)	68·4 (±1·2)

IV. DISCUSSION

The results presented above provide evidence that moderate osmotic stresses can significantly inhibit the rates of carbon assimilation by cotton leaf tissue. Mannitol and sodium chloride were used as examples of relatively slowly permeating osmotica, while ethylene glycol was used as an example of an osmoticum which easily entered leaf mesophyll cells.

The reflection coefficient (σ), which gives an indication of the cell permeability, was estimated for mannitol using the following modification of the method given by Kedem and Katchalsky (1958):

$$\sigma = 1-(k_s/L_p)(\overline{V}_s/\boldsymbol{R}T),$$

where (k_s/L_p) is the ratio of the solute permeability coefficient to the hydraulic conductivity of the membrane (bars), $\overline{V}_s$ is the partial molar volume of solute (cm^3 $mole^{-1}$), T is the absolute temperature (°K), and $\boldsymbol{R}$ is the universal gas constant (bars cm^3 $°K^{-1}$ $mole^{-1}$). Both k_s and L_p were measured in terms of a given fresh weight of

tissue, which was assumed proportional to the cell surface area for the uniform slices used, and were obtained from Figures 2 and 1, respectively. Using this equation, σ for mannitol was greater than 0·99. This contrasts with values between 0·76 and 0·90 obtained by Slatyer (1966), for cotton leaves. Slatyer's estimates, however, were obtained from the discrepancies between vapour equilibration and plasmolytic estimates of tissue water potential, and are open to other explanations. For instance, the immersion of tissue in solution may have affected the tensions in the cell walls (Weatherley 1970). The other experiments quoted by Slatyer did not give the initial rates of exchange, so do not give reliable estimates of σ.

The evidence therefore suggests that mannitol can be used successfully as a relatively slowly permeating osmoticum for periods less than 3 hr. Ethylene glycol, however, is a much more rapidly permeating osmoticum, and has been used in such a capacity for studies on corn root tissue (Greenway and Leahy 1970). The observation that mannitol plasmolysed the leaf slices at about −12 bars, while even a −24 bar ethylene glycol solution did not cause transient plasmolysis, indicates that σ for ethylene glycol was less than 0·5, since the effective osmotic potential of a solution divided by its true water potential, is a measure of σ (Dainty 1963). The similarity between the effectiveness of mannitol and sodium chloride solutions in inducing plasmolysis indicates that σ for sodium chloride is close to that for mannitol.

The effects of osmotica on carbon assimilation are unlikely to have been due to specific inhibitory effects of the osmotica used. In the first place, the short-term effects of mannitol and sodium chloride were similar, which tends to eliminate the possibility of salt effects. In addition, the kinetics of inhibition by mannitol solutions did not correspond with the kinetics of mannitol uptake, and the rapid initial inhibition was apparent whether the osmoticum was mannitol or sodium chloride. It is clear that ethylene glycol was not an inhibitor, though it could have had stimulatory effects, which countered any osmotic effects on photosynthesis. This possibility was not ruled out.

Another possibility is that the reduced ^{14}C fixation in osmotic solutions may have been due to a reduced CO_2 availability. For instance, the osmotica may have been blocking the CO_2 uptake pathways, binding the CO_2, or they may have even altered the solution viscosity enough to reduce the availability of CO_2. The results shown in Table 1, however, preclude these possibilities.

The results provide some evidence regarding the mechanism whereby water stress influences physiological processes. The mannitol experiments alone cannot be used to determine the relative importance of the various water status parameters: ψ (total cell water potential); P (pressure difference between the interior and exterior of the cells); π (cell osmotic potential); and V (cell volume). Since σ for mannitol is approximately 1·0, at equilibrium all these parameters will vary in a manner dependent on the elasticity of the cell walls. The ethylene glycol treatments, however, suggest that neither ψ nor π are of major importance, since these parameters were both expected to alter by the same, or greater, amounts than in the corresponding mannitol treatments, while neither P nor V should have been significantly affected. The reason that the ethylene glycol treatments should be expected to have little effect on P or V, is that the cells are permeable to ethylene glycol, so that, at equilibrium, the control turgor and volume relations should have been regained. In the present

experiments, turgor and cell volume always covaried, so that it was not possible to distinguish their effects. In future work these effects could be separated by experimentally manipulating the cell wall elasticity.

There is little evidence that physiological activities can be regulated by absolute pressure. Flowers and Hanson (1969), for instance, did not detect any effect of total pressure on mitochrondrial respiration. Although both inhibition and enhancement of germination by hydrostatic pressures have been observed (Vidaver and Lue-Kim 1967; Strebeyko and Rufelt 1971), these workers did not eliminate possible structural effects of the high pressures used, since air spaces may have been present in the tissue. Turgor pressures, however, can affect physiological processes. Stomatal operation, for instance, is dependent on turgor (Meidner and Mansfield 1968), as are extension growth (Green 1968) and related metabolic processes (Ordin 1960; Hsaio 1970; Hsaio *et al.* 1970). In addition, Gutknecht (1968) demonstrated that salt transport in *Valonia* is regulated by small changes in turgor.

The importance of cell volume changes in regulating cell metabolism is also well documented. The obvious and often irreversible damage associated with plasmolysis, has been associated with shrinkage of the cytoplasm away from the cell walls (Dessimoni Pinto and Flowers 1970; Greenway and Leahy 1970), and other structural effects of cell shrinkage (Nir *et al.* 1970*a*, 1970*b*). Such mechanisms could account for the effects of severe stresses observed in the present work. It is also possible that there may be more subtle effects of cell volume changes, since, as the cell volume decreases, cell sap concentrations would rise, intermolecular distances between proteins would be reduced, and water structure might change. These changes could lead to "salting out", pH changes, and other effects, which in turn could affect protein conformation and activity. Analogous solute redistribution effects have been observed during the freezing of artificial systems (Taborsky 1970), while Meryman (1970) has shown, by means of freezing experiments, that cells apparently have a minimum volume below which they cannot be reduced without damage. Changes in water structure could conceivably act as metabolic controls (Crafts 1968), since some enzymes, including those inactivated by cold (Peat and Soderwall 1971), may be sensitive to the entropic state of water. At this stage, however, these suggestions must be regarded as purely speculative.

The conclusion that neither ψ nor π are critical parameters for control of photosynthesis are consistent with several reports in the literature. Greenway and Leahy (1970), who also used ethylene glycol as an osmoticum, demonstrated that 10 bar decreases in either ψ or π had little effect on several metabolic processes in corn roots. The observation that osmotic adjustment can occur in whole plants (e.g. Slatyer 1961), is also consistent with this conclusion. Studies on freezing injury to chloroplasts, where cell water is lost to the cell exterior (Williams and Meryman 1965, 1970), also indicate that water stress injury is not necessarily related to water activity.

There was a significant inhibition of ^{14}C fixation rate at potentials as high as -4 bars, which is a potential which might be expected commonly in the field (Slatyer 1967). The intracellular photosynthetic processes in cotton leaf slices therefore show greater sensitivity to water stress than has been observed, using whole leaf gas-exchange techniques, for several species (Troughton and Slatyer 1969; Boyer 1970,

1971*b*). Redshaw and Meidner (1972), however, observed significant increases in the intracellular resistance to photosynthesis in tobacco leaves at what were probably comparable water deficits. Using tobacco leaf disks with an epidermis removed, Graziani and Livne (1971) found that ^{14}C fixation was not affected until 50% of the leaf water was lost; while Glinka and Katchansky (1970) found that the CO_2 compensation points of osmotically stressed maize and sunflower leaf disks were similarly insensitive to stress. These latter workers, however, observed that the CO_2 compensation points of similar disks were sharply increased if they had been air-dried to about −10 bars. Some of these apparent contradictions may be due to the techniques used, although genotypic differences may also be important.

As well as being extremely sensitive to stress, ^{14}C fixation in leaf slices also showed a rapid response to changes in solution water potential, much of the response being manifest within 10 min. It is therefore possible that changes in photosynthetic potential could occur during natural stomatal cycling, since such oscillations commonly have a period of about 30 min, and the leaf water potential may fluctuate by 10 bars or more (Barrs and Klepper 1968). These changes were faster than has previously been detected using gas-exchange methods (Laisk and Oja 1971; Redshaw and Meidner 1972).

In conclusion, this paper presents evidence that the intracellular processes of photosynthesis in cotton respond very rapidly to small changes in leaf water status. The observed inhibition of carbon fixation, at least at moderate stresses, appears to be related to cell volume or turgor changes, and not to the total water potential. More severe stresses gave rise to a comparatively irreversible inhibition of ^{14}C fixation, which could have been related to structural changes associated with plasmolysis. Although the precise relationship of these results to the *in vivo* situation is not clear, the evidence discussed in Part I (Jones and Osmond 1973) suggests that the tissue slices do behave comparably to the intact tissue.

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