PHOTOSYNTHESIS BY THIN LEAF SLICES IN SOLUTION

I. PROPERTIES OF LEAF SLICES AND COMPARISON WITH WHOLE LEAVES

By H. G. Jones*† and C. B. Osmond*

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Abstract

The preparation and photosynthetic properties of thin leaf slices from several plants were examined. Photosynthesis was measured either as oxygen evolution in a polarographic electrode, or as $^{14}$CO$_2$ fixation. Oxygen uptake in the dark gave a measure of the dark respiration rate.

The maximum photosynthetic rates obtained with such a system were similar to those which have been obtained with whole leaf gas-exchange techniques. The use of thin slices reduced diffusion limitations, and, with the addition of carbonic anhydrase to the assay solution, it was possible to reduce the apparent Michaelis constant with respect to CO$_2$ to values similar to those found in other photosynthetic systems. Evidence was presented which indicated that appropriately prepared leaf slices were photosynthetically comparable to whole leaves, at least for short-term experiments. The pH response of photosynthesis provided evidence that free CO$_2$ is the species of CO$_2$ utilized by the C$_3$ species, cotton.

The main advantages of the technique lie in its suitability for the routine estimation of the maximum potential photosynthetic rate on large numbers of small samples. Also, the incubation of leaf tissue in solution provides opportunities for the precise control of tissue environment, and for using inhibitors for studies into photosynthetic mechanisms.

I. INTRODUCTION

Photosynthetic measurements in leaves of higher plants have been facilitated in recent years by the development of leaf-chamber methods involving measurements of gas and water vapour exchange (see Sestak et al. 1971). For many purposes these techniques are unnecessarily elaborate and they are quite inappropriate for studies of inhibitor effects on photosynthesis, for example. At the same time, preparations of isolated chloroplasts (Jensen and Bassham 1966; Gibbs et al. 1967) and whole cells (Jyung et al. 1965; Gnanam and Kulanideivelu 1969; Jensen et al. 1971) show many physiological inadequacies. The cell-isolation procedure adopted by Jensen et al. (1971), for instance, involved a plasmolysis step. Another difficulty is that these techniques are not equally applicable to all species.

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In this paper we describe a detailed investigation of the photosynthetic properties of leaf slices in solution. Leaf-slice techniques were developed for the study of the ionic relations of leaf cells (Smith and Epstein 1964; Osmond 1968; Rains 1968) and more recently, Lütge et al. (1971) have used such a system for assaying photosynthesis in a number of species. We believe that this method largely bridges the gap between whole leaf and isolated chloroplast measurements and permits further insights into the processes limiting photosynthesis. This paper describes the preparation and photosynthetic properties of leaf slices from plants which photosynthesize via the C₃ and C₄ pathways.

II. Materials and Methods

(a) Plant Material

Cotton (Gossypium hirsutum L. cv. Deltapine Smoothleaf) was used for most experiments. The plants were grown from seed for between 4 and 5 weeks in a controlled-environment cabinet at a day temperature of 30°C and a night temperature of 25°C. The photoperiod was 12·5 hr, of which 1 hr was at a low light intensity provided by incandescent lamps, and 11·5 hr was at 95 W m⁻² (400–700 nm) produced by fluorescent tubes supplemented by incandescent lamps. The plants were grown in aerated Hoaglands solution. In some experiments, 3-week-old, greenhouse-grown seedlings of Sorghum sudanense, and mature leaves of Atriplex spongiosa and A. hastata were also used.

(b) Preparation of Slices

The procedure was similar to that described by Osmond (1965). Strips of leaf tissue, 1 cm wide, were cut from the youngest fully expanded leaf, omitting the major veins, using two razor blades separated by a 1 cm spacer. From 8 to 10 of these strips were then placed between two appropriately hollowed pieces of fresh carrot, and then cut into slices 400 or 500 μm thick using a Reichert sliding microtome. To avoid damage to the slices, it was found necessary to change the cutting edge after every 20 slices (approximately 200 slices) for cotton, or more frequently for a coarser tissue such as sorghum. The slices were washed in several changes of 0·5 mM CaSO₄ and stored in a similar solution. They were used between 1 and 8 hr after sectioning and were pre-illuminated for at least 30 min at between 40 and 60 W m⁻² (400–700 nm) with a Phillips 400 W HPLR lamp, immediately before use.

(c) ¹⁴CO₂ Fixation

Assays were performed in 5 ml glass weighing bottles in a shaker bath at 25°C. The light intensity at the sample level was approximately 120 W m⁻² (400–700 nm). Light was provided by a Phillips 400 W HPLR lamp. In the standard procedure, samples of 20 slices were incubated in 2·5 ml of 50 mM HEPES (N-2-hydroxyethylpiperazine-N'2-ethanesulphonic acid) at pH 7·6. Fixation of ¹⁴CO₂ was initiated by adding 50 μl KH¹⁴CO₃ solution of known specific activity (to make a final concentration of 15 mM), and was stopped after 5 min by transferring the slices to 10 mM formic acid on a planchet. The slices were then dried under an infra-red lamp and counted with a Nuclear-Chicago model 470 gas flow counter. In certain experiments the pH or the bicarbonate concentration was varied. The rate of ¹⁴C fixation was linear for assay periods between 2 and 20 min. The counting efficiency of the system was 11%, and therefore the absolute fixation rate could be calculated. The standard deviation of replicate estimates was less than 8%.

(d) Oxygen Exchange

Oxygen exchange was measured in a Clark-type polarographic electrode (Rank Bros. Ltd., Bottisham, Cambridgeshire, England) at 25°C. The sample size was 20 slices and the total solution volume was 4·0 ml 50 mM HEPES at pH 7·6. The solution was stirred magnetically, and damage to the slices was prevented by a disk of nylon netting which separated them from the stirrer. To calibrate the electrode, buffer in equilibrium with air at 25°C was assumed to contain 251 nmoles
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oxygen per millilitre (Hodgman et al. 1959), while a zero oxygen concentration was obtained by adding a small quantity of sodium dithionite to the solution. The relation between oxygen concentration and electrode output was assumed to be linear between these points. Illumination was provided by a quartz–iodine slide projector lamp with a Schott KG2 filter, the intensity inside the cuvette being 150–200 W m⁻² (total incident energy as measured with a YSI radiometer, model 65).

The experimental procedure was to partially deoxygenate the buffer solution by bubbling with nitrogen, add the slices, and finally the KHCO₃ (to make 15 mM final concentration). The cuvette was darkened for 10–15 min to allow for temperature equilibration and to obtain an estimate of the dark respiration rate (oxygen uptake). It was then illuminated to determine the rate of oxygen evolution in the light. The rate measured was the initial rate, and transients of less than 30 s were ignored. To obtain a response curve relating photosynthesis to CO₂ concentration, the procedure was similar, though 1m KHCO₃ was added in steps from a 10-μl gas chromatography syringe. In some experiments purified bovine erythrocyte carbonic anhydrase (Sigma) was added to the buffer. The standard deviation of replicate estimates of photosynthesis was less than 15%.

(e) Chlorophyll Assays

Chlorophylls were estimated by the method of Bruinsma (1963). Samples of 20 slices were blotted and weighed (to obtain an estimate of their fresh weight). These samples were then homogenized in 80% acetone in a glass homogenizer and centrifuged for 10 min at full speed on a bench centrifuge. Total chlorophyll content of the sample was determined from the absorbances at 645 and 663 nm obtained as spot readings on an Hitachi 101 spectrophotometer.

III. RESULTS

(a) Characteristics of Oxygen Exchange in Leaf Slices

The oxygen electrode was used to investigate the effects of slicing on the properties of cotton leaf slices. It was found that the tissue was damaged if the slices

![Fig. 1](image)

Fig. 1.—Kinetics of oxygen exchange for cotton leaf slices 400 μm (—) and 150 μm (— —) thick over a dark–light–dark cycle. Rates were obtained from the derivative of the oxygen electrode output.

![Fig. 2](image)

Fig. 2.—Ratio of the rate of oxygen evolution in the light to oxygen uptake in the dark (P/R) for cotton slices of different thicknesses. Measurements were made in either 15 mM bicarbonate (○) or 20 mM bicarbonate (▼). Each point is the mean of five replicates with its associated standard error.

were cut thinner than about 300 μm. The oxygen exchange characteristics of such tissue were similar to those of tissue which had been damaged by lightly rolling with
a pencil or by sectioning with a blunt blade. Damaged tissue characteristically showed higher dark respiration rates and lower photosynthetic rates than carefully treated control tissue. Sections thinner than 150 μm showed a light-stimulated oxygen uptake. The kinetics of oxygen exchange for leaf slices 150 and 400 μm thick are compared, for a dark–light–dark cycle, in Figure 1.

Figure 2 gives the ratio of oxygen evolution in the light to uptake in the dark for slices of different thicknesses, measured either in 15 mM or in 20 mM KHCO₃, at pH 7·6. Optimum values for this ratio were obtained with slices between 300 and 600 μm thick. The difference between the rates obtained with slices 400 μm and 1 mm thick could be eliminated by using a 20 mM bicarbonate solution.

The prepared slices could be stored in 0·5 mM CaSO₄ solution for at least 12 hr with no significant changes in the rates of photosynthesis (¹⁴C fixation or oxygen evolution) or respiration (oxygen uptake in the dark), after the first hour. A pre-incubation period of 30 min at a light intensity of at least 30 W m⁻² was found to be essential for maximum photosynthetic rates, and to eliminate the lag in oxygen evolution observed on illuminating the slices.

<table>
<thead>
<tr>
<th>Leaf No.*</th>
<th>Area (cm²)</th>
<th>Wᵢ/C†</th>
<th>Wᵢ/A‡ (mg cm⁻²)</th>
<th>P‡</th>
<th>R‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>76</td>
<td>570</td>
<td>30</td>
<td>12.8</td>
<td>-1.0</td>
</tr>
<tr>
<td>3</td>
<td>128</td>
<td>340</td>
<td>24</td>
<td>16.5</td>
<td>-1.2</td>
</tr>
<tr>
<td>5</td>
<td>152</td>
<td>350</td>
<td>20</td>
<td>20.5</td>
<td>-1.5</td>
</tr>
<tr>
<td>6</td>
<td>117</td>
<td>380</td>
<td>19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>101</td>
<td>400</td>
<td>19</td>
<td>29.2</td>
<td>-2.2</td>
</tr>
<tr>
<td>8</td>
<td>48</td>
<td>425</td>
<td>16</td>
<td>26.1</td>
<td>-2.4</td>
</tr>
<tr>
<td>9</td>
<td>17</td>
<td>640</td>
<td>12</td>
<td>22.0</td>
<td>-3.5</td>
</tr>
</tbody>
</table>

* Numbered from the cotyledonary node, leaf 9 being the youngest.
† Wᵢ = fresh weight (mg); C = chlorophyll content (mg);
A = area (cm²).
‡ P, rate of net photosynthesis; R, respiration rate. Units for both variables are nmoles per mg chlorophyll per second. Results given are averages for three replicate samples.

The variation in rates of photosynthesis and respiration (measured as oxygen exchange) between the different leaves of one cotton plant is shown in Table 1. The rates are very dependent on the leaf sampled, though there was least sensitivity to leaf age around leaf number 7, which was used for all other work.

(b) Photosynthetic Responses of Leaf Slices in Solution

Owing to difficulties in the measurement of the light incident on, or absorbed by, the leaf slices, precise light response data were not obtained. The use of only 20 slices per sample meant that there was little self-shading. Light saturation for the ¹⁴C fixation experiments was less than 100 W m⁻², while in the oxygen electrode,
light saturation was between 130 and 150 W m⁻². The difference in light saturation for the two systems may have been due to different spectral characteristics of the light sources, or to the method of measurement. Dark fixation of $^{14}$CO₂ was less than 1% of the light rate in most experiments.

The pH responses of photosynthesis and respiration were determined in the oxygen electrode. Using bicarbonate concentrations which saturated photosynthesis, oxygen exchange was pH-independent between pH 6·0 and 8·0. As the pH was decreased below pH 6·0, oxygen uptake in the dark increased slightly, and then fell, while oxygen evolution in the light decreased. Both respiration and photosynthesis decreased with increasing pH above pH 8·0. All further experiments were done at between pH 7·1 and 7·6, so that the solution might have a high CO₂-buffering capacity for the carbonic anhydrase additions (see below).

![Graph showing response of photosynthetic oxygen evolution in cotton leaf slices to added CO₂ at pH 7·1 and pH 7·6](image)

The response of photosynthetic rate, measured either as $^{14}$C fixation or as oxygen evolution, to non-saturating bicarbonate concentrations was pH-dependent as is shown for the oxygen exchange of cotton leaves in Figure 3. Figure 3 also shows the results from the same experiment, when expressed in terms of free CO₂ concentration. The free CO₂ in solution at any pH was calculated according to Buch (1960). Since the response of photosynthetic rate to free CO₂ was very similar at the two pH values used, this suggests that free CO₂ is the "species" utilized by the leaf slices.

The concentration of free CO₂ required to give half maximum photosynthetic rates (the apparent Michaelis constant, $K_{\text{app}}^m$), when measured either as oxygen evolution or as $^{14}$C fixation, ranged from 60 to 110 μM for several experiments on
cotton, with a mean of 80 \( \mu \text{M} \) (see Table 2). At 25°C this is equivalent to a concentration of CO\(_2\) in the air of about 3500 p.p.m. Table 2 shows that \( K_{\text{m}}^{\text{app}} \) was generally lower for the C\(_4\) plants tested than for the C\(_3\) plants.

<table>
<thead>
<tr>
<th>System</th>
<th>Species</th>
<th>( K_{\text{m}}^{\text{app}} ) (( \mu \text{M} ))</th>
<th>( P_{\text{m}} )†</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolated chloroplasts</td>
<td>Spinach</td>
<td>12</td>
<td>43</td>
<td>Jensen and Bassham (1966)</td>
</tr>
<tr>
<td></td>
<td>Spinach</td>
<td>6</td>
<td>65</td>
<td>Gibbs et al. (1967)</td>
</tr>
<tr>
<td>Leaf slices (oxygen exchange)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cotton</td>
<td>60–110</td>
<td>32 (25–42)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sorghum</td>
<td>40–60</td>
<td>52 (42–67)</td>
<td></td>
</tr>
<tr>
<td>+ Carbonic anhydrase</td>
<td>Cotton</td>
<td>8–15</td>
<td>33 (27–42)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sorghum</td>
<td>7–15</td>
<td>54 (42–70)</td>
<td></td>
</tr>
<tr>
<td>Leaf slices (( ^{14}\text{C} ) fixation)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cotton</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Atriplex</td>
<td>80–110</td>
<td>38 (30–45)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>spongiosa</td>
<td>90</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td></td>
<td>hastata</td>
<td>200</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>Leaf gas exchange</td>
<td>Cotton</td>
<td>4‡</td>
<td>46 (43–50)</td>
<td>Jones and Slatyer (1972)</td>
</tr>
</tbody>
</table>

* With respect to free CO\(_2\) in system.
† Units as for \( P \) in Table 1. Values in parenthesis indicate the range.
‡ With respect to cell wall concentration of free CO\(_2\).

The absolute rates of net oxygen evolution at saturating CO\(_2\) concentrations (\( P_{\text{m}} \)) are also given in Table 2, and were comparable to, though slightly lower than, rates obtained from whole leaf gas-exchange or isolated chloroplasts. The photosynthetic rates of the C\(_4\) plants sorghum and *Atriplex spongiosa* were higher than those of the C\(_3\) plants cotton and *A. hastata*. In cotton the rates of \(^{14}\text{C} \) fixation were generally about 20% higher than the corresponding rates of net oxygen exchange.

Carbonic anhydrase was added to the buffer during the determination of some CO\(_2\) response curves. The effects of two concentrations of carbonic anhydrase on the CO\(_2\) response curves for oxygen evolution are shown in Figure 4. Using carbonic anhydrase at a concentration of 0·2 mg/ml, the \( K_{\text{m}}^{\text{app}} \) with respect to CO\(_2\) for leaf slices in solution was approximately 10 \( \mu \text{M} \), which is similar to values found for other photosynthetic systems (Table 2). Slight further reductions down to about 7 \( \mu \text{M} \) could be obtained with higher carbonic anhydrase concentrations. The addition of carbonic anhydrase to the assay solution did not generally affect estimates of \( P_{\text{m}} \), though it did allow the maximum rate to be attained at bicarbonate concentrations as low as 2 mM. An interesting point is that the maximum photosynthetic rate decreased slightly if large amounts of CO\(_2\) were available to the cells; similar apparent substrate inhibition was observed at very high bicarbonate concentrations in the absence of added carbonic anhydrase.

The utility of the leaf slice system for inhibitor studies was tested with dichlorophenylhydantoins (DCMU), \( p \)-trifluoromethoxy carbonyl cyanide phenylhydrazone
(FCCP), and methylamine. Penetration of these inhibitors was found to be rapid, with the maximum effects being achieved with incubation periods of less than 30 min.

![Graph 4](image4)

**Fig. 4.**—Effect of carbonic anhydrase at concentrations of 0.0625 mg/ml (□) and 0.2 mg/ml (○) on CO₂ response curves for oxygen evolution in cotton leaf slices. ● Control.

**Fig. 5.**—Effect of DCMU (▲), FCCP (□), and methylamine (●) on ¹⁴C fixation by cotton leaf slices.

Figure 5 gives the rates of ¹⁴C fixation as percentages of control values, in the presence of different concentrations of DCMU, FCCP, and methylamine. Each point is the average of three experiments, with all samples being pre-incubated in the presence of the inhibitor for 30 min in the light. The concentrations which inhibited photosynthesis by 90% were similar to those which inhibit chloroplast photosynthesis to the same level (Avron and Shavit 1965; Miflin and Whittingham 1966; Raven 1967, 1969, 1970b). These results are comparable with those of Lütte et al. (1971), who studied the effects of DCMU and FCCP on various species, using a similar system.

**IV. Discussion**

The CO₂ response curves at different pH values provide evidence that the species of "CO₂" assimilated by cotton leaf tissue was free CO₂. Raven (1970a) reviewed similar experiments to determine the species of "CO₂" used by various plants. Among the algae there are species apparently utilizing both CO₂ and bicarbonate. Ruttnert (1947) demonstrated that the aerial leaves of Beta vulgaris and Sambucus nigra (both C₃ plants) could not use bicarbonate, which is in agreement with the present data. The results are of particular interest in view of the evidence that CO₂ is the substrate for ribulosediphosphate carboxylase (Cooper et al. 1969). The situation in C₄ plants is not so clear, but it is likely that the substrate for the primary carboxylase in these plants (phosphoenolpyruvate carboxylase) is bicarbonate (Cooper and Wood 1971). It might be expected, therefore, that C₄ plants would be able to use exogenous bicarbonate.

A major disadvantage of incubating slices in solution is that such a procedure can reduce the supply of CO₂, since it first has to diffuse through the unstirred layer of solution, then through the cut edges of tissue (Weatherley 1955), and either via the liquid phase in the cell walls, or via the gas phase in the intercellular spaces, up
to the photosynthetic cells. During photosynthesis the extracellular diffusion resistance will tend to reduce the concentration of CO$_2$ at the surface of the plasmalemma below that in the bulk solution. This could account for the high $K_{m}^{\text{app}}$ observed for leaf slices in solution, which was nearly an order of magnitude higher than corresponding values obtained for isolated chloroplasts (Jensen and Bassham 1966; Gibbs et al. 1967) or whole leaf gas exchange (Goldsworthy 1968; Björkman et al. 1970; Jones and Slatyer 1972), as shown in Table 2. Other workers have used leaf disk or segment techniques for long- and short-term measurements of photosynthesis (see Bartos et al. 1960; Goldsworthy 1971; Lawes and Treharne 1971; Setlik and Sesták 1971); however, diffusion problems, which are even more severe in such systems, severely restrict the replicability of results, and make the interpretation of comparative studies more difficult.

Evidence that the high $K_{m}^{\text{app}}$ of the leaf slice system is due to a diffusion limitation comes from the experiments where added carbonic anhydrase reduced the $K_{m}^{\text{app}}$. This enzyme catalyses the reversible hydration of CO$_2$ and is thought to facilitate CO$_2$ transfer in solution (Longmuir et al. 1966; Enns 1967; Ward and Robb 1970) and in membranes (Broun et al. 1970). In cotton, carbonic anhydrase could be expected to reduce the resistance to CO$_2$ transfer in the extracellular pathway in the ratio of the total concentration of all CO$_2$ species to that of free CO$_2$ (which is approximately 25 at pH 7.6). Since the resistance due to the unstirred layer outside the cells will be large (Raven 1970a), it is not possible to obtain accurate estimates of the CO$_2$ concentration at the plasmalemma, even with carbonic anhydrase present. The $K_{m}^{\text{app}}$ with respect to the CO$_2$ concentration at the plasmalemma, therefore, will always be overestimated.

In the absence of carbonic anhydrase, the $K_{m}^{\text{app}}$ for C$_4$ plants was consistently lower than that for C$_3$ plants, though this difference was eliminated in the presence of the enzyme. The additional effect of carbonic anhydrase on leaf slices from C$_3$ plants may reflect the inability of these plants to use bicarbonate.

Leaf slices were very susceptible to damage during cutting, though they could be stored successfully, under appropriate conditions, for moderately long periods. Not only was dark respiration higher in very thin slices (which were subject to greater rolling during cutting), but in extreme cases they even showed a light-stimulated oxygen uptake. This could be interpreted in terms of a Mehler-type reaction (Mehler and Brown 1952; Whitehouse et al. 1971). The optimal thickness, at least for cotton leaf slices, was around 400 μm. Above this value diffusion limitations probably became important as is suggested by the data in Figure 3.

Rungie and Wiskich (1972) have suggested that hydrolytic enzymes are released when storage tissue is sectioned. Although we did not eliminate this possibility in leaf slices, the oxygen exchange and $^{14}$C fixation rates were similar to those expected for intact leaves. In addition, the characteristic changes in respiration rate observed on slicing plant storage tissues (ap Rees 1966) were not detected in this system. It seems reasonable to suggest that leaf mesophyll cells are well adapted for gas exchange and for withstanding damage, whereas cells in storage tissue, or other large plant organs are more protected and probably occur in environments which are relatively low in oxygen. Leaf tissue, therefore, might be expected to show less response than storage tissue to slicing and subsequent incubation in aerated solutions.
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The cell environment was probably similar to that in vivo, since in the natural situation the cell walls are close to water saturarion (Gaff and Carr 1961; Weatherley 1970). Therefore, at least at high water potentials, the internal water relations of tissue slices in solution may be similar to those of an aerial leaf in air, particularly since injection of the intercellular spaces was not observed with the present technique. The studies on \( P_m \) and \( K_m^\text{pp} \) and the other evidence presented above all indicate that the photosynthetic behaviour of slices in solution is comparable to that of whole leaves in their natural environment, and that the results obtained with slices 400 or 500 \( \mu \text{m} \) thick should be representative of the intact tissue.

The major application of the leaf-slice technique to photosynthetic studies is in the measurement of the maximum potential photosynthetic rate (\( P_m \)). This important parameter has rarely been measured in the past, since the usual gas-exchange techniques are limited by stomatal closure (Jones and Slatyer 1972). The use of thin slices means that stomatal control of photosynthetic rate should be eliminated. The \( ^{14}\text{C} \) fixation method is particularly suitable for the routine estimation of \( P_m \) because of its simplicity, accuracy, and speed. In addition, large numbers of samples can be studied, and each sample requires only a small amount of tissue. Although the oxygen-exchange method has a disadvantage in that only a relatively small number of samples may be handled, it is particularly useful for studies on the gas-exchange behaviour of leaf slices. In conjunction with inhibitor studies (see also Lützge et al. 1971) both methods can be used for studies of photosynthetic mechanisms. The leaf slice system is also suitable for the precise control of tissue environment, and a subsequent paper (Jones 1973) describes the use of the system to investigate the effects of osmotically induced water stress on photosynthesis.

V. ACKNOWLEDGMENTS

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VI. REFERENCES


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