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Effect of Vacuum Infiltration on Photosynthetic Gas Exchange in Leaf Tissue

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ABSTRACT

Using a manometric method, photosynthetic oxygen evolution and $^{14}CO_2$ fixation have been determined for leaf tissue of *Triticum aestivum* L., *Hordeum vulgare* L., *Phaseolus vulgaris* L., and *Lemna minor* L. Approximately similar values in the range 0.2 to 0.4 millimoles·grams fresh weight^{-1} hour^{-1} were obtained for both gases. In tissue subjected to vacuum infiltration, O_2 evolution and $^{14}CO_2$ fixation were barely measurable. It is considered that the elimination of photosynthetic gas exchange results from a decreased supply of CO_2 to the chloroplasts. Chopping wheat laminae also leads to a reduction in photosynthetic gas exchange, slices 1 millimeter or less giving only 10 to 20% of the value for whole tissue. Respiration is unaffected by either treatment. Carbonic anhydrase did not improve photosynthetic gas exchange in infiltrated tissue. The use of sliced or vacuum-infiltrated leaf tissue in photosynthetic studies is discussed.

Vacuum infiltration of leaf tissue is resorted to from time to time to facilitate the diffusion of compounds into the leaf, the cuticle of which resists the penetration of hydrophilic substances. Recently the technique has been used in studies of leaf respiration (22), ion absorption (11, 24), phosphate metabolism (13), proline metabolism (21), and in a number of photosynthetic studies (2, 4, 15, 17, 23). The assumption underlying the use of this technique is that water injection of the air spaces in leaf tissue facilitates diffusion of solutes, neither injuring the cell membranes nor altering the cellular metabolism, and evidence supporting this has been cited for leaf tissue (11, 22) and root tissue (14).

While facilitating solute diffusion, water injection would be expected to impede gaseous diffusion in leaf tissue. A study (12) on light-stimulated absorption of Cl^- by infiltrated wheat laminae suggested that the uptake was linked to a photochemical reaction and this encouraged the thought that photosynthesis was unimpaired by vacuum infiltration. Measurements of photosynthetic gas exchange in infiltrated leaf tissue have now established that it is virtually obliterated by this treatment. Chopping the leaf tissue, the alternative approach to overcoming the problem of cuticular impermeability, can be equally damaging.

MATERIALS AND METHODS

Seeds of wheat, (*Triticum aestivum* L. cv. Capelle) were germinated in damp horticultural peat. The seedlings were grown in the dark at 22 C for 6 days and then greened by exposure to light for 2 or 3 days. The lower (proximal) half of the terminal 50 mm of the first leaf was used as the basic unit of experimentation. Tissue to be sectioned was cut using a hand microtome (American Optical Co., Buffalo, N. Y.). A number of laminae were grouped together and supported vertically between two pieces of carrot root tissue. Tissue chopped with a single-edge razor blade gave similar results. Vacuum infiltration was accomplished by submerging the tissue in glass-distilled water in a vacuum desiccator; a grid was used to prevent the tissue from rising out of the solution. The desiccator was evacuated to a pressure of 20 cm Hg using a water pump. Sudden release of the vacuum, once or twice, led to injection of the air spaces. Successful infiltration could be checked visually.

Seeds of barley (*Hordeum vulgare* L. cv. Golden Promise) and bean (*Phaseolus vulgaris* L. cv. Canadian Wonder) were also germinated in damp peat and used after 8 to 10 days growth. Barley laminae were used in the same way as wheat. Gas exchange in bean leaf was determined with 1-cm disks punched so as to avoid the ribs of the leaf. *Lemna minor* L. was grown in a nutrient culture and several fronds were used for each determination.

Oxygen Uptake Measurement. Tissue respiration was determined in the dark by manometry using a Braun VL 85 Warburg apparatus. Each treatment was run in quadruplicate, each vessel containing either 8 × 25 mm sections from 8 laminae (0.09 g, approximately), or disks or fronds as appropriate to the tissue, with 2 ml of H_2O in the outer ring and 0.15 ml of 10% KOH in the center well. The 25-mm sections were halved for easy insertion into the Warburg flasks.

Oxygen Evolution Measurement. Braun photosynthetic conical reaction flasks having a large auxiliary vessel and side arm (total capacity approximately 38 ml) were used. The conical compartment contained one 25-mm section (or the equivalent subdivided) of a lamina floating on 1.5 ml of H_2O. A constant CO_2 pressure was maintained by having a suitable buffer mixture in the auxiliary vessel. Normally the buffer used was diethanolamine prepared according to Krebs (8). Each vessel contained 1.5 ml of 4 M diethanolamine containing 0.1% thiourea. The buffer was equilibrated with air before use. It was established that the O_2 evolution of the weight of tissue used was not rate-limited by the capacity of the diethanolamine to supply CO_2. Oxygen evolution was also followed using a solution of sodium bicarbonate-sodium carbonate containing 0.19 M bicarbonate and 10 mM carbonate to maintain a constant CO_2 tension. This method yielded similar values for O_2 evolution. Each vessel was illuminated from below by a 40-w incandescent bulb yielding a light intensity of approximately 18,000 lux (70 w m^{-2}) at the base of the Warburg flask. Oxygen evolution determined in the light was corrected for O_2 uptake determined in the dark.

Table I. *Effect of Vacuum Infiltration on Respiration Rate of Leaf Tissue*

Oxygen uptake from 0.1 to 0.2 g of tissue was determined at 25 C in the dark over 4 hr.

	O_2 Absorbed	
	Control	Infiltrated
	μmoles g fresh wt^{-1}·hr^{-1}	
Wheat	10.0	9.4
Barley	9.9	8.6
Bean	11.9	12.7
Lemna	10.2	13.8

Table II. *Effect of Subdivision on Gas Exchange of Wheat Laminae*

Gas exchange was determined at 25 C. O_2 uptake was measured in the dark. Segments of varying length were cut using a microtome. Total length for photosynthesis 30 or 32 mm, for respiration 240 mm.

Length	O_2 Absorbed	O_2 Evolved	$^{14}CO_2$ Fixed
mm	*μmoles g fresh wt^{-1}·hr^{-1}*		
0.5	13.4	6	65
0.75	13.4	74	73
1.0	13.6	87	68
1.5	13.0	159	172
2.0	13.0	191	264
4.0	12.4	224	429
12.0		371	

Carbon Dioxide Fixation. One 25-mm length (or equivalent subdivided) of lamina was floated on 1.8 ml of 5.5 mM phosphate buffer (pH 6.1) contained in the main compartment of a Warburg photosynthetic reaction flask. The side arm contained 0.2 ml of 37.5 mM $NaH^{14}CO_3$ (specific radioactivity 1 μCi/0.1 ml). The flask was equilibrated for 30 min in the light or dark according to the experimental conditions before the contents of the side arm were tipped in. The addition of the bicarbonate raised the pH to 6.75 and under these conditions the CO_2 contributed approximately 0.4% of the gas phase. At the end of the fixation period the tissue was removed from the flask, rinsed in iced H_2O for 2 to 3 min, and then extracted in boiling 80% ethanol. Radioactivity was determined in the alcohol-soluble and insoluble fractions separately. After decanting the hot ethanol extract, the tissue was then macerated in 80% ethanol using an Ultra-Turrax blender to give a fine suspension. Five-milliliter aliquots of both fractions were counted by scintillation counting using Instagel and the external standard ratio channel which corrects for the quenching due to the extract color and suspended particles. Standards for the original $NaH^{14}CO_3$ were counted in the same way. Counting efficiency was about 65%. Values quoted in the text represent the total activity in the tissue. About 25% of this was present in the alcohol-insoluble fraction.

RESULTS

Oxygen Uptake. Vacuum infiltration has a negligible effect on the respiration rate of leaf tissue (Table I) even though it brings about the water injection of the air space in the leaf. Slicing the tissue either with a microtome or a razor blade also tends to bring about the water injection of air space in the tissue proximate to the cut edge. The shorter the segments the greater is the proportion of tissue exhibiting the characteristics of water injection. Even so, the O_2 absorbed by wheat laminae is unaffected by the degree of subdivision of the lamina (Table II).

Oxygen Evolution. Values of O_2 evolution for leaf tissue of four species are given in Table III. Vacuum infiltration, however, virtually eliminated O_2 evolution from all the tissues investigated. Infiltration of wheat laminae with bicarbonate solution did not significantly raise the level of O_2 evolved either with or without the addition of carbonic anhydrase (Table IV) which might be expected to facilitate CO_2 transfer. A study of the effect of slicing on the rate of O_2 evolution by wheat laminae showed a close relationship: the narrower the segments the lower the rate of gas evolution (Table II).

Carbon Dioxide Fixation. $^{14}CO_2$ fixation by wheat laminae as a function of bicarbonate concentration is shown in Figure 1. A plateau is reached at about 2.5 mM which, under the conditions employed, corresponds to a CO_2 level of less than 0.3% of the gas phase. Routinely, $^{14}CO_2$ fixation was determined over 30 min at a bicarbonate concentration of 3.75 mM. Uptake was linear during this time.

Values for $^{14}CO_2$ fixed by three species are given in Table III. Dark fixation of $^{14}CO_2$ and zero time fixation were always less than 1% of the light value. As with O_2 evolution, $^{14}CO_2$ fixation values for infiltrated tissue were minimal and the addition of carbonic anhydrase did not increase the fixation rate significantly (Table V). Similarly, subdivision of the laminae resulted in a massive decrease in the rate of fixation (Table II).

DISCUSSION

Notwithstanding certain advantages, the use of leaf tissue samples in the study of photosynthesis has not enjoyed much popularity although the method has been commended (20) and shown to yield values comparable with whole attached

Table III. *Comparison of Photosynthetic Rate of Different Tissues at 25 C as Determined by $^{14}CO_2$ Fixation or O_2 Evolution*

Tissue	O_2 Evolved	$^{14}CO_2$ Fixed
	μmoles·g fresh wt^{-1}·hr^{-1}	
Wheat	345	382
Barley	328	270
Bean	235	349
Lemna	211	

Table IV. *Effect of Vacuum Infiltration with H_2O and $KHCO_3$ on Oxygen Evolution of Wheat Laminae at 25 C before and after Addition of 0.1 mg/ml Carbonic Anhydrase*

O_2 evolution was determined from 2 × 12.5 mm segments at 25 C for 2 hr before addition of enzyme and for a further 2 hr thereafter. Enzyme was dissolved in 5.5 mM phosphate buffer pH 6.5.

Infiltration Solution	O_2 Evolved	
	Before enzyme addition	After enzyme addition
	μmoles g fresh wt^{-1}·hr^{-1}	
None	487	
H_2O	1	11
1 mM $KHCO_3$	18	16
5 mM $KHCO_3$	20	23
25 mM $KHCO_3$	19	18

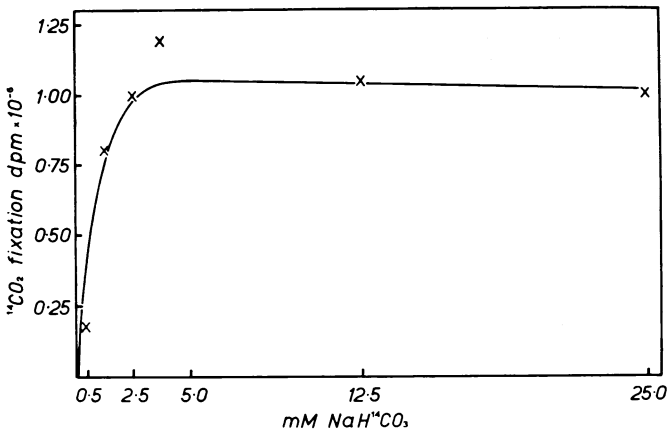

FIG. 1. $^{14}CO_2$ fixation by wheat lamina as a function of bicarbonate concentration. Each point represents the total activity obtained from 1 × 25 mm lamina in 5.5 mM phosphate buffer at 25 C for 30 min with varying concentrations of $NaH^{14}CO_3$.

Table V. *Effect of Carbonic Anhydrase on $^{14}CO_2$ Fixation by Wheat Laminae*

$^{14}CO_2$ fixation was followed over 30 min at 25 C using 2 × 12.5 mm segments. The enzyme was dissolved in phosphate buffer pH 6.5.

Concentration Carbonic Anhydrase	$^{14}CO_2$ Fixed	
	Control	Infiltrated
mg/ml	*μmoles g fresh wt^{-1} · hr^{-1}*	
0	501	8
0.01	501	10
0.1	526	13
0.2	462	16

leaves (25). Values similar to those reported in the literature have been obtained with 25 mm or 12.5 mm lengths of laminae in this study (*e.g.* for wheat about 0.4 mmole $^{14}CO_2$ fixed g fresh weight^{-1} hr^{-1} or 0.7 mmoles dm^{-2} hr^{-1}). This is the length of cereal laminae commonly taken by those who favor the use of leaf tissue samples (16, 25). Where disks of dicotyledonous leaves are employed the diameter is usually 1 cm or more (1, 3). Setlik *et al.* (19) showed that decreasing the diameter of the disks brought about a decline in the rate of photosynthetic dry matter accumulation per unit area. With chopped wheat lamina, photosynthetic rate is clearly a function of the lamina length (Table II), 1-mm segments giving only 10 to 20% of the $^{14}CO_2$ fixation of longer segments.

There are a few reports in the literature of photosynthetic studies using leaf tissue strips of narrow width. Lüttge *et al.* (9), working with 0.5-mm segments of *Zea mays*, cite a value for O_2 evolution of 25 μmoles g fresh weight^{-1} hr^{-1}. For 1-mm slices of barley and maize, values of 19 and 28 μmoles O_2 g fresh weight^{-1} hr^{-1}, respectively, are given (10). Robertson and Laetsch (18), working with 1-mm strips of barley, quote values of about 75 μmoles O_2 g fresh weight^{-1} hr^{-1} and 20 μmoles CO_2 g fresh weight^{-1} hr^{-1}. These are similar to the values obtained with 1-mm segments in this study (Table II). Taken together, they suggest that 1-mm slices are unsuitable for photosynthetic studies.

A contrary view, however, is presented by Jones and Osmond (6) who studied the photosynthetic properties of leaf slices in solution and concluded that the method bridges the gap between whole leaf and isolated chloroplast studies. Working mainly with cotton leaf tissue they found that the photosynthetic rate was constant down to a slice thickness of 300 μm. However, damaged tissue gave a lower photosynthetic rate and to avoid damage they found it necessary to change the cutting edge of their microtome after 20 cuts for cotton and more frequently for sorghum. The ratio of photosynthetic O_2 evolution to respiratory O_2 uptake quoted by Jones and Osmond (6) for cotton suggests that their sliced tissue retained the photosynthetic capability of the whole leaf. Clearly then, if leaf tissue is cut with sufficient care it is possible to obtain suitable material for photosynthetic studies, but the preparation of chopped tissue in the hands of many workers is similar to that employed in this study and has given similar results. It may be that the parallel venation of cereal leaves being more resistant to cutting gives rise to more widespread injection of water in the region of the cut edge.

Since the maximum amount of damaged tissue in 1-mm slices is 25% (11), a decrease in photosynthetic activity of 80% or more (Table II) cannot be accounted for in terms of cellular damage. The fact that photosynthesis is much more severely affected than ion uptake, which is relatively unaffected (12), also argues against damaged tissue as a sufficient explanation. On the other hand, if the decrease in photosynthesis with decreasing segment length or disk diameter is to be attributed to water injection of the cut edge, the effect of vacuum infiltration, which brings about the occlusion of all the intercellular air space, would be expected to be even more extensive and the absence of any significant photosynthetic gas exchange is in keeping with the prediction.

The photosynthetic values reported in the literature for vacuum-infiltrated leaf tissue support the conclusion that such tissue is incapable of normal photosynthetic activity. Nadler *et al.* (15) quote 0.66 nmole O_2/min as being a maximum value for a 25-mm segment of vacuum-infiltrated barley leaf. This is only 1% of the O_2 evolution of noninfiltrated barley leaf reported in Table III and is similar to the miniscule values for infiltrated tissue obtained in this study. Similarly, Oelze-Karow and Butler (17) obtained values for O_2 evolution of greening *Phaseolus* leaf ranging from 29 to 329 μmoles g dry weight^{-1} hr^{-1}. This also is one to two orders of magnitude lower than the values quoted in Table III (assuming dry weight to be 10% of fresh weight).

The causative factor in the elimination of photosynthetic gas exchange in infiltrated tissue and the corresponding reduction in sliced tissue is most probably the decreased supply of CO_2 to the chloroplast. Glinka and Meidner (3) found that the rate of CO_2 fixation in tobacco disks was reduced by 50% when floated on water and they attributed this to the decreased diffusion of CO_2 in water. Jones and Slatyer (7) consider that the major intracellular limitation to photosynthesis is the transport of CO_2 and since the diffusion coefficient of CO_2 in the liquid phase is appropriately 10^4 lower than that in the gas phase, any increase in the length of the diffusion path in the liquid phase will result in a massive decrease in the CO_2 supply to the chloroplast (5).

The limitation thus imposed on CO_2 diffusion by water injection applies equally to O_2 diffusion and yet the respiration of infiltrated tissue is unaffected. Three factors may be invoked to account for the indifference of O_2 uptake to the altered conditions. First, the K*m* value for O_2 uptake permits respiration to proceed at near normal rates in low O_2, while photosynthesis shows greater sensitivity to low CO_2. Figure 1 indicates a K*m* for CO_2 fixation of approximately 3 × 10^{-5} M CO_2 whereas the K*m* for leaf respiration is of the order of 2.5 × 10^{-6} M O_2. Second, water has dissolved in it a larger

quantity of O_2 (2.5×10^{-4} M) when in equilibrium with air than it has CO_2 (9×10^{-6} M). Third, the tissue requirement for O_2 to maintain its normal respiratory rate is a mere 5% or less of its normal photosynthetic CO_2 requirement. The net effect of the interaction of these factors is to maintain O_2 uptake unimpaired under conditions which severely militate against photosynthesis.

An additional factor further restricting the diffusion of gases in infiltrated tissue is that the stomata of treated tissue as judged by silicone rubber micro relief impressions do not open normally and at best can only open narrowly to a width of 1 to 2 μm (12). Since the cuticle is impervious to the diffusion of CO_2 and O_2, this effect on stomatal opening must aggravate the difficulty of gas diffusion.

Unpublished experiments on the Hill activity of chloroplasts isolated from spinach leaves have shown that vacuum infiltration of the leaf tissue before plastid extraction does not impair the ability of the plastids to reduce 2,6-dichlorophenolindophenol. This indicates that vacuum infiltration does not in itself damage the chloroplast (at least with respect to its capacity for electron transfer), a view supported by Hernandez-Gil and Schaedle (4), who found that vacuum infiltration of cottonwood leaves with the isolation medium before chloroplast extraction increased the rate of cell-free photosynthesis by 100 to 200%.

While vacuum-infiltrated tissue can carry out many of the processes associated with the leaf, *e.g.* the greening and unrolling of the leaf blade proceeds normally in the light, it cannot be maintained that the method is not injurious to the normal functioning of the leaf metabolism (11). Photosynthetic studies would seem to be particularly inappropriate. The fact that the photosynthetic activity of chopped tissue may be similarly affected indicates the need for care in the preparation of leaf segments for photosynthetic studies. Vacuum-infiltrated tissue, no less than chopped tissue, exhibits a light-enhanced ion uptake (12).

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