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The Simultaneous Measurement of O_2-Evolving and CO_2-Fixing Activities in Fresh Leaves

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A simple method for measuring simultaneously O_2-evolving and CO_2-fixing activities in fresh leaves was developed combining oxygen and pH electrodes, and results obtained were compared with those by the methods of CO_2 gas analysis and radio active $^{14}CO_2$ fixation.
Photosynthetic activity was measured changing $NaHCO_3$ concentration, pH and pre-illumination time. In most tree leaves, the evolved oxygen amount determined with oxygen electrode was equal to the fixed CO_2 amount determined with pH electrode. However, the ratio of CO_2 to O_2 was larger than unity in leaves of some trees. This suggests that there is some difference in photosynthetic metabolism (sugar and organic acid syntheses) depending on tree species. The metabolic difference was examined with various plant leaves, and was discussed by determining their assimilation quotients.

INTRODUCTION

There are many methods for measuring photosynthetic activity of leaves, but they need expensive instruments and/or skilled techniques. So, we tried to find a new method for measuring simultaneously O_2-evolving and CO_2-fixing activities with a cheep, handy instrument.

Oxygen electrode, a handy instrument is commonly used for studying chloroplast Hill reaction. This instrument is designed to measure O_2-evolving activity in aqueous phase. However, by comparing Hill reaction activity of leaves in aqueous phase with their CO_2-fixing activity in air phase, the activity obtained with oxygen electrode was confirmed by us to give nearly the same value as that in air phase.

When a leaf (10×10 cm^2) fixed 22 mg CO_2/100 cm^2·hr in 100 ml $NaHCO_3$ solution, the activity was calculated to be 500 μmoles CO_2/100 ml·hr or 5 mM/hr. The change of 10 μM in CO_2 or O_2 concentration was easily measured with pH or oxygen electrode.

In the present paper, photosynthetic activities, O_2-evolution and CO_2-fixation, in various tree leaves were measured simultaneously with an apparatus

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consisting of oxygen and pH electrodes under various experimental conditions to obtain further detailed information on photosynthesis in tree leaves. Results obtained were discussed in relation to the metabolic pathway.

MATERIALS AND METHODS

The apparatus for measuring photosynthetic activity of leaf is schematically shown in Fig. 1. A fresh leaf held between two plastic frames was placed in a reaction cell. The reaction cell, made by transparent plastic plate, has the inner dimension of $6\times4\times2\,cm^3$, and provides three holes at the top of the cell for inserting oxygen and pH electrodes. The cell was filled with 30 mM potassium phosphate buffer and 20 mM $NaHCO_3$ solution for measuring O_2-evolution alone. However, for the simultaneous measurement of O_2-evolution and CO_2-fixation, 7.5 mM potassium phosphate buffer and 20 mM $NaHCO_3$ solution were used. The solution was stirred with a magnetic stirrer attached behind the leaf.

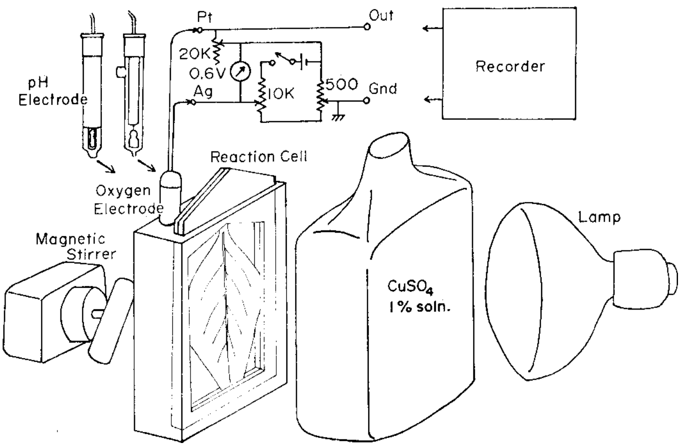

Fig. 1. Schematic *diagram* of the instrument for measuring O_2-evolving and CO_2-fixing activities simultaneously in aqueous phase.

The leaf was illuminated for 10 min with the light (40 klux) from a 300 W tungsten lamp, filtered through a 7cm layer of a 1% $CuSO_4$ solution in 1*l* Roux flask. All the experiments were carried out at room temperature.

The sensitivity of oxygen electrode was determined using a distilled water with known O_2 concentration. The photosynthetic activity of leaf is usually expressed by the unit of mg $CO_2/100\,cm^2\cdot hr$. O_2-evolving activity obtained with oxygen electrode is converted to CO_2-fixing activity from the correspondence of one mole O_2-evolution to one mole CO_2-fixation.

The decrease of $NaHCO_3$ by CO_2 fixation (leading to the evolution of NaOH) was quantitatively determined from the pH rise in the solution, by comparing it with the NaOH titration curve (Fig. 2).

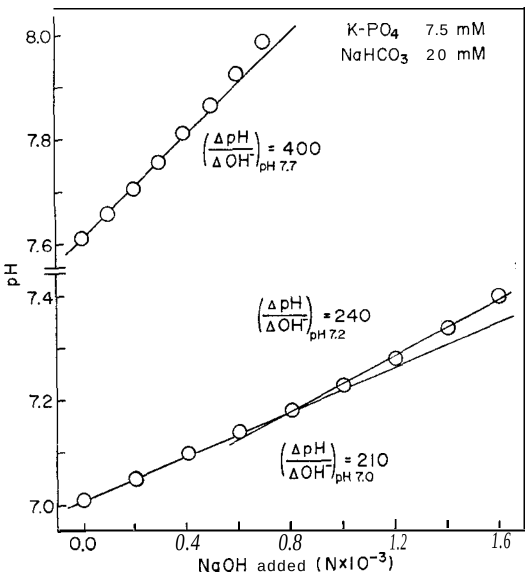

Fig. 2. The pH titration curve of 20 mM $NaHCO_3$–7.5 mM K-PO, solution with NaOH. pH was measured at room temperature (about 25°C).

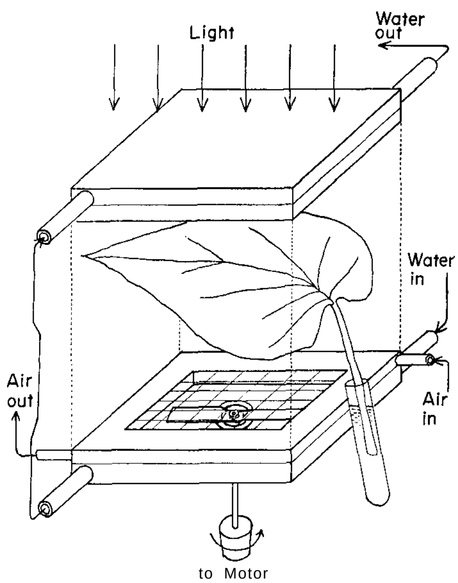

Fig. 3. Assimilation box to measure CO,-fixing activity in gas phase with CO, gas analyzer. The assimilation box ($20\times20\times2\,cm^3$) was opened or closed by moving the upper part up- or down-ward along the broken line to insert leaf in the inner space of the assimilation box. CO,-containing air was introduced into the box through the inlet (Air in) and stirred with a fan. The outlet (Air out) was connected with a CO, gas analyzer.

The incorporation of radioactive $^{14}CO_2$ from $NaH^{14}CO_3$ (2×10^7 c. p. m./25 ml) was investigated with 10 min illumination using a smaller cell. The ^{14}C-containing products were analyzed by the method of Imai *et al.* (1971). Amino acids and other organic acids were adsorbed on Dowex-1 (CH_3COO^-) column to separate neutral sugars from them, then they were eluted out with 1 N HCl (20 ml). Ethanol-insoluble residuals were hydrolyzed by boiling them with 6 N HCl (50 ml) for 30min. The solution (2 ml) of radioactive products was mixed with PPO-toluene-Triton X-100 solution (15 ml), and its radioactivity was measured with a scintilation counter. The components of PPO-toluene-Triton X-100 mixture were 400mg PPO, 100 ml toluene and 50ml Triton X-100.

Photosynthetic CO_2 fixation in air was measured with a CO_2 gas analyzer which provided a leaf holder in a box. The box was cooled by running water (Fig. 3).

RESULTS

Increase in photosynthetic activity by pre-illumination of leaf in solution

Usually, fresh leaves harvested in the field exhibited very low photosynthetic activity. The illumination time for the activity measurement was 10 min. But, repeating the activity measurement several times, the activity gradually increased to a maximum value. This activity increase was larger in young-

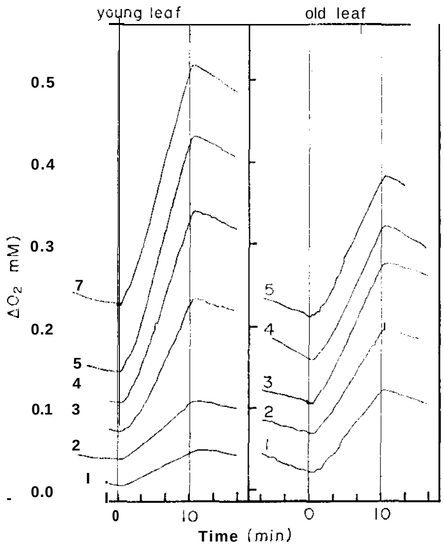

Fig. 4. Increase in photosynthetic O_2-evolving activity by repeating measurement. O_2-evolving activity was measured with young and old leaves of *Pseudosasa japonica* in the $NaHCO_3$ solution (20 mM) 7 and 5 times, respectively. The reaction conditions were the same as for those described in Materials and Methods. The number at the left side of the curve indicates the experimental run with the same leaf. The $NaHCO_3$ solution was replaced at every experimental run.

er leaf than in older one. This is shown in *Pseudosasa japonica* leaf (**Fig.** 4). Therefore, in the following experiments, photosynthetic activity was recorded after the maximum activity was obtained with sufficient pre-illumination.

Effects **of light intensity, pH and $NaHCO_3$ concentration on leaf photosynthesis in solution**

As shown in Fig. 5, photosynthetic activity of most plant leaves was saturated at the light intensity of about 20 klux. But, dark-green, thick leaf of ***Catalpa bignonioides*** seems to require more light than other plant leaves for reaching the activity saturation. So, we used the light intensity of 40 klux for the activity measurement.

Photosynthetic activity of leaf in solution varied with the pH value of NaHCO, solution, and the optimum pH value differed depending on plant species (Fig. 6). Most plants have the optimum point in the alkaline pH range. But, *Populus nigra* showed a higher activity in the lower pH range, exceptionally. Therefore, it is important to find the optimum pH and to measure photosynthetic activity at this pH.

The activity of leaf photosynthesis in the solution varied with NaHCO, concentration and pH. Photosynthetic activity of ***Catalpa bignonioides*** saturated at about 20 mM NaHCO, (pH 7. 6), but that of *Pleioblastus hindsii* reached the maximum at about 10 mM NaHCO, (pH 7. 6), as shown in Fig. 7. The optimum

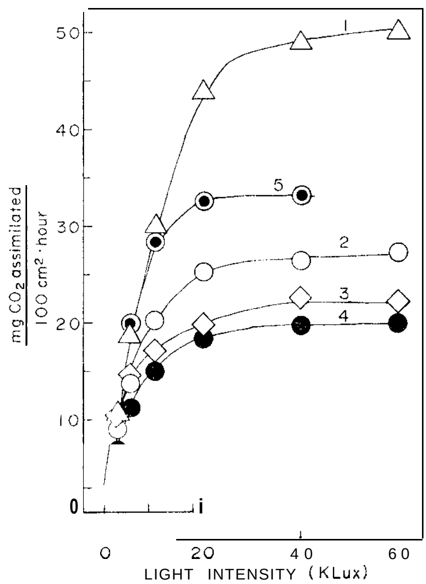

Fig. 5. Effect of light intensity on the photosynthetic O_2-evolving activity. The activity was measured in the same way as described in Materials and Methods except for the light intensity. 1 : —△—, *Catalpa bignonioides* (at pH 7.8) ; 2 : —○—, *Populus nigra* (at pH 7.0) ; 3 : —◇—, *Morus alba* (at pH 7.8) ; 4 : —●—, *Pleioblastus hindsii* (at pH 7.8) ; 5 : —⊙—, *Pscudososa* japonica (at pH 7.8) .

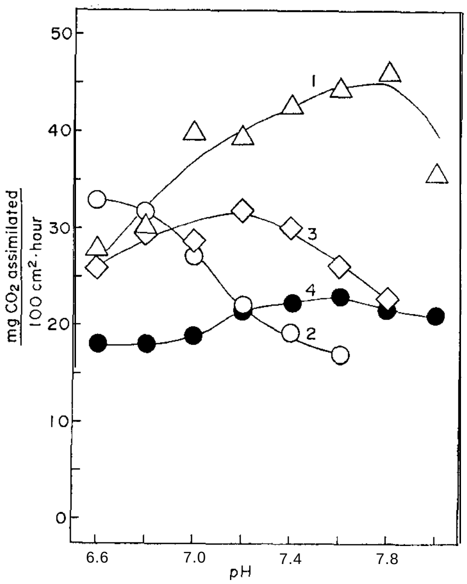

Fig. 6. Effect of pH on photosynthetic O_2-evolving activity of tree leaves in NaHCO, solution. The reaction conditions were the same as for those described in Materials and Methods except for pH. The number and symbol of the curve correspond to those in Fig. 5, respcctivcly.

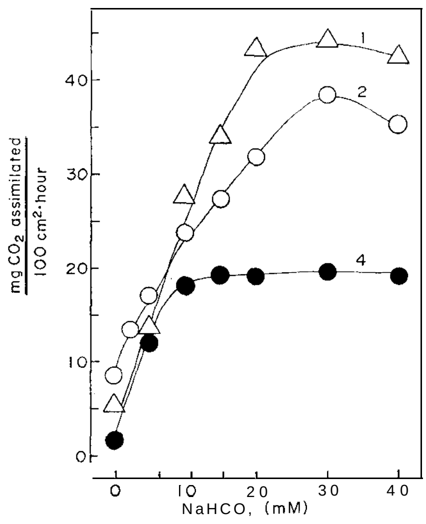

Fig. 7. Effect of NaHCO, concentration on photosynthetic O_2-evolving activity of tree leaves. The reaction conditions were the same as for those described in Materials and Methods except for NaHCO, concentration. The number and symbol of the curve correspond to those in Fig. 5, respectively.

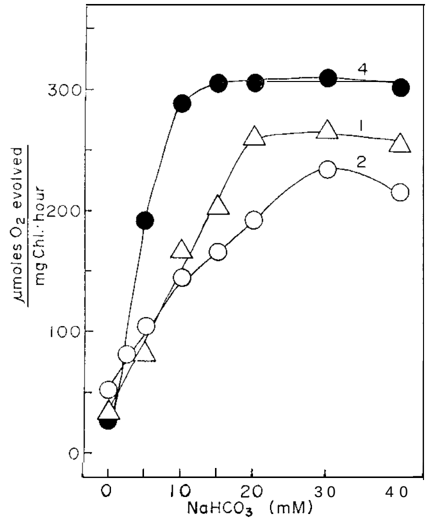

Fig. 8. Photosynthetic O_2-evolving activities of tree leaves on the basis of mg chlorophyll. The reaction conditions were the same as those described in Fig. 7. The number and symbol of the curve correspond to those in Fig. 5, respectively.

pH for leaf of *Populus nigra* shifted to lower pH range than others. The CO_2 concentration may increase in the solution at pH 6.8 than at pH 7.6. But, leaf of this plant required 30mM $NaHCO_3$ to saturate photosynthetic activity at the optimum pH 6.8.

The result obtained above was usually expressed on the basis of leaf area (mg CO_2/100 cm^2•hr). But, when the activity was expressed on the basis of chlorophyll (mg CO_2/mg Chl•hr), photosynthetic activities of different plant leaves approached each other as shown in Fig. 8.

The amount of $NaHCO_3$ consumed photosynthetically for 10 min was much lower than 1 mM, as shown in Figs. 9 and 10. Therefore, the $NaHCO_3$ concentration of 20 mM, which we used as the standard experimental condition, was high enough for this experiment. The $NaHCO_3$ concentration of 30 or 40 mM injured the leaf activity on repeating the measurement several times.

Simultaneous measurement of **O_2-evolution** and CO_2-fixation of leaf in solution

Ordinarily, photosynthetic reaction is summarized as follows.

$$CO_2 + H_2O \longrightarrow CH_2O + O_2$$

Consequently, the amounts of evolved O_2 and fixed CO_2 should be equal when carbohydrate is the only product. But, when the photosynthetic metabolism proceeds to produce organic acids or amino acids instead of sugar, the amount of CO_2 fixed is to be larger than that of O_2 evolved. So, the following experi-

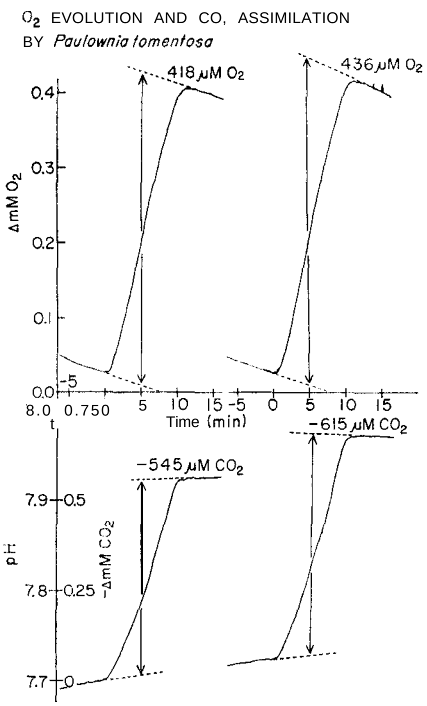

Fig. 9. Simultaneous measurement of O_2-evolving and CO,-fixing activities of *Paulownia tomentosa*. Experimental conditions are described in Materials and Methods.

ment was carried. out to examine the CO_2/O_2 ratio (assimilation quotient) for several species of plants.

As shown in Figs. 9 and 10, the illumination of leaf was started at the time 0 min and was turned off at the time 10 min. The base and response lines decreased or increased with time owing to the respiration or other reason. Therefore, extending these lines, we estimated the averaged amount of net response from the difference between the two extended broken-lines at 5 min.

The photosynthetic response of *Paulownia tomentosa* leaf is shown in Fig. 9. In this case, leaf fixed more CO, than O_2 evolved. But, in the case of *Morus alba* (Fig. 10), the fixed amount of CO, was slightly less than the amount of O_2 evolved. Such O_2-evolving and CO,-fixing properties were studied with several species of plants. Results obtained are given in Table 1. Some plants tended to fix more CO, than O_2 evolved. The assimilation quotient (CO_2/O_2) is given as B/A in Table 1.

Next, we compared photosynthetic activity in aqueous solution with that in air. The activity of a fresh leaf was first measured with a CO, gas analyzer in air, then the leaf was put into $NaHCO_3$ solution to measure its activity with oxygen and pH electrodes. Results are given in Table 2. There is

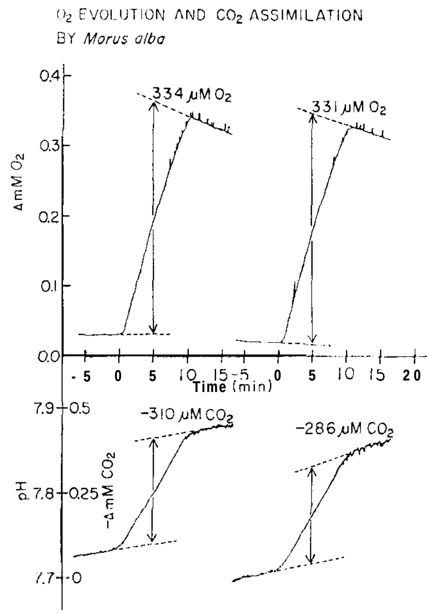

Fig. 10. Simultaneous measurement of O_2-evolving and CO,-fixing activities of *Morus alba.* Experimental conditions arc described in Materials and Methods.

some difference between the activities measured with CO, gas analyzer and electrodes. But, the value obtained with electrodes is reliable because the essential difference in photosynthetic activity among plants was also clearly obtained with electrodes as with CO, gas analyzer.

The reliability of pH electrode method was further ascertained by $^{14}CO_2$-fixation analysis. Photosynthetic activity of a *Paulownia tomentosa* leaf was surveyed by the electrode method and "CO,-fixation analysis. Results given in Table 3 demonstrate that the response of pH electrode was well coupled with CO_2-fixation and the amount of photosynthetic CO_2-fixation surpassed the amount of O_2-evolution in this leaf.

DISCUSSION

Photosynthetic O_2-evolving activity of leaf has been measured with oxygen electrode in $NaHCO_3$ solution (Yamashita et al., 1972; Jones and Osmond, 1973; Pitman *et al., 1975* and Ishii *et al.,* 1977).

In the present investigation, we combined O_2 electrode with pH electrode to measure O_2-evolution and CO_2-fixation simultaneously. To prepare such an instrument, it is most important to increase the sensitivity. For this reason, we must use large leaf area and small volume of $NaHCO_3$ solution as much as we can. Then, concentration changes of O_2 and $NaHCO_3$ in the solution caused

Table 1. Photosynthetic activities of leaves measured simultaneously with oxygen and pH electrodes in aqueous phase, and assimilation quotients.

	A (O_2 electrode) $\left(\frac{mg\ CO_2\ fixed}{100\ cm^2 \cdot hr}\right)$	B (pH electrode) $\left(\frac{mg\ CO_2\ fixed}{100\ cm^2 \cdot hr}\right)$	Ratio $\left(\frac{B}{A}\right)$	Temp. (°C)	Date of measurement
Paulownia tomentosa	17.2 26.9?	23.36. 75	1.1.36 38	22 28. 5	June 21 2 (1972) July (1972)
	23. 7	31. 6	1.33	24	Sept. 12 (1972)
Catalpa *bignonioides*	24.3	42. 5	1.75	28	Aug. 20 (1971)
	19.9	36. 9	1.85	21	May 25 (1972)
	22. 5	28. 4	1.26	28. 5	July 21 (1972)
Pseudosasa japonica	33. 2	44. 0	1.33	26	Aug. 20 (1971)
	24. 7	23. 0	0. 93	26	June 16 (1972)
	37. 8	34.0	1.22	28	July 7 (1972)
Ailanthus altissima	22. 7	25. 2	1.11	26	July 4 (1972)
	16. 4	23. 4	1.43	23	Sept. 19 (1972)
Morus alba	16. 6	15. 3	0.92	26	Aug. 20 (1971)
	20. 5	17. 8	0. 87	28	July 28 (1972)
	17.0	13. 8	0. 81	23. 5	Sept. 22 (1972)
Liliodendron tulipifera	18. 0	19. 8	1. 10	27	Aug. 24 (1971)
	15. 9	13. 0	0. 81	22	June 7 (1972)

Photosynthetic activity was measured as described in Materials and Methods. A, CO_2-fixing activity converted from O_2-evolving activity which was measured by oxygen electrode, assuming that 1 mole O_2 evolution corresponded to 1 mole CO_2 fixation; B, CO_2-fixing activity measured by pH electrode ; B/A, assimilation quotient.

'Table 2. Photosynthetic activities of leaves measured simultaneously with oxygen and pH electrodes in aqueous phase and with CO_2 gas analyzer in gas phase.

Plants	O_2 electrode $\left(\frac{mg\ CO_2\ fixed}{100\ cm^2 \cdot hr}\right)$	pH electrode	CO_2 gas analyzer	Temp. (°C)	Date of measurement
Paulownia tomentosa	24. 2	43. 2	35. 5	33	July 25 (1973)
	27. 5	41.7	34. 1	33	July 26 (1973)
Catalpa bignonioides	21. 2	21. 9	18. 4	33	July 26 (1973)
	28. 4	26. 0	29.0	'9	July 30 (1973)
Morus alba	36. 5	29. 8	31. 1	32	Aug. 29 (1973)
Populus nigra	23.8	17.4	26. 5	32	Aug. 29 (1973)
Ailanthus altissima	10. 0	6. 3	17. 8	32	Aug. 29 (1973)

The photosynthetic activity was measured as described in Materials and Methods.

Table 3. Photosynthetic activities of *Paulownia tomentosa.*

Methods of measurements	$\left(\frac{\text{mg } CO_2 \text{ fixed}}{100\ \text{cm}^2\cdot\text{hr}}\right)$
O_2 electrode	20. 1
pH electrode	27. 2
Radio isotope (^{14}C)	
Sugar	10.6
Organic acids	5.7
Polymer	9.5
Total	25. 8

Photosynthetic activities were measured as described in Materials and Methods.

by photosynthesis can be enlarged. When the leaf area in the reaction cell was 10 cm^2, the photosynthetic activity 22 mg CO_2/100 $cm^2\cdot$hr and the inner dimension of the cell $6\times4\times2$ cm", the CO_2 concentration change in the cell was 50 μmoles CO_2/48 ml$\cdot$hr, namely 1. 04 mM CO_2/hr or 0. 17 mM CO_2/10 min. This order of change in CO_2 or O_2 amount in solution is easily detected with pH or oxygen electrode.

The increase in photosynthetic activity by repeated illumination might hc derived from the activation of enzymatic reaction by reduction or from the opening of stomata caused by diminution of CO_2 concentration in the leaf through photosynthesis.

As usual, younger leaf was more active for photosynthesis as confirmed with a leaf of *Pseudosasa japonica.* Tree leaf is usually believed to have low photosynthetic activity (about 10~20 mg CO_2/100 $cm^2\cdot$hr). Rut, the young, fresh tree leaf used in our investigation showed high activity comparable to the activity of grass plants. On the other hand, old leaf of *Pseudosasa japonica*, for example, had only a fraction of the initial activity as a result of senescence.

The optimum pH value depended on plant species, which is difficult to explain from the present experimental results alone. If $NaHCO_3$ solution could not penetrate into leaf tissue, the cause of this pH effect may be restricted merely to the concentration change of CO_2 in solution and the physiological activity of epithelium, especially that of stomata. Plant leaf contains carbonic anhydrase, of which activity mny also affect the pH curve of photosynthesis in $NaHCO_3$ solution according to the rapid equilibrium of the following reaction.

$$CO_2 + H_2O \rightleftarrows H_2CO_3 \rightleftarrows H^+ + HCO_3^-$$

When this enzyme is active enough, plant leaf can obtain ample CO_2 even at higher pH's where HCO_3^- ion is predominant in the solution.

Photosynthetic activity of *Pleioblastus hindsii* leaf was saturated at lower $NaHCO_3$ concentration (10 mM). This is interesting because this plant can grow in a bush where CO_2 concentration might be lower than that in the place of *Paulownia tomentosa* or *Populus nigra* standing.

The assimilation quotient may demonstrate that the photosynthetic metabolism of most plant leaves is arranged to synthesize organic acids as well as sugars, as shown in Tables 1–3. This metabolic activity may be related to synthesize amino acids or to store CO, in the leaf for photosynthesis. But, it is not known yet what kind of metabolic pathway, TCA cycle or dicarboxylic acid cycle (Kortschak et al., 1965; Hatch and Slack, 1966), is operative for production of such organic acids in tree leaf.

Our instrument is simple, but it gave us many reliable information about the photosynthetic metabolism. For this reason, the present method is expected to be used for many photosynthetic experiments and also to be applied to other analytical measurements besides photosynthesis.

ACKNOWLEDGEMENTS

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