USE OF A GLASS ELECTRODE FOR MEASURING RAPID CHANGES IN PHOTOSYNTHETIC RATES*

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Recent measurements of photosynthesis in intermittent illumination with a period of 1 or 2 minutes revealed a hitherto unobserved phenomenon. These studies, carried out with algal suspensions in conventional manometer vessels, showed an extremely rapid initial apparent rate of photosynthesis in the light and a correspondingly rapid initial rate of respiration in the dark (Burk, 1953; Burk and Warburg, 1951). Some criticism of these experiments has been expressed on the grounds that the period of intermittency is not long enough for the establishment of a steady state transport of carbon dioxide and oxygen across the liquid-gas interface (Brown and Frenkel, 1953). These difficulties could be obviated by using specific liquid phase analytical procedures for either oxygen or carbon dioxide. Polarographic methods have been used successfully to follow the concentration of dissolved oxygen in photosynthesizing suspensions (Petering and Daniels, 1938; Blinks and Skow, 1938 *b*; Brackett *et al.*, 1953; Damaschke *et al.*, 1953). Several authors have followed the concentration of carbon dioxide by taking advantage of its effect on the acidity of relatively unbuffered solutions. Osterhout and Haas (1918) added an acid-base indicator to a medium containing photosynthesizing cells and followed acidity changes colorimetrically. Blinks and Skow (1938 *a*) used a glass electrode inserted into an algal suspension as an indicator of rapid changes in carbon dioxide content. The glass electrode was used incidentally by Österlind (1949) to determine slow changes in carbon dioxide content during the growth of green algae. This paper will describe the calibration of a glass electrode apparatus designed for the quantitative determination of rapidly changing carbon dioxide concentrations in solution.

The Apparatus.—The reaction cell, illustrated in Fig. 1, is a lucite cuvette, 3 cm. diameter by 5 mm. inside thickness, with flat windows. A Beckman

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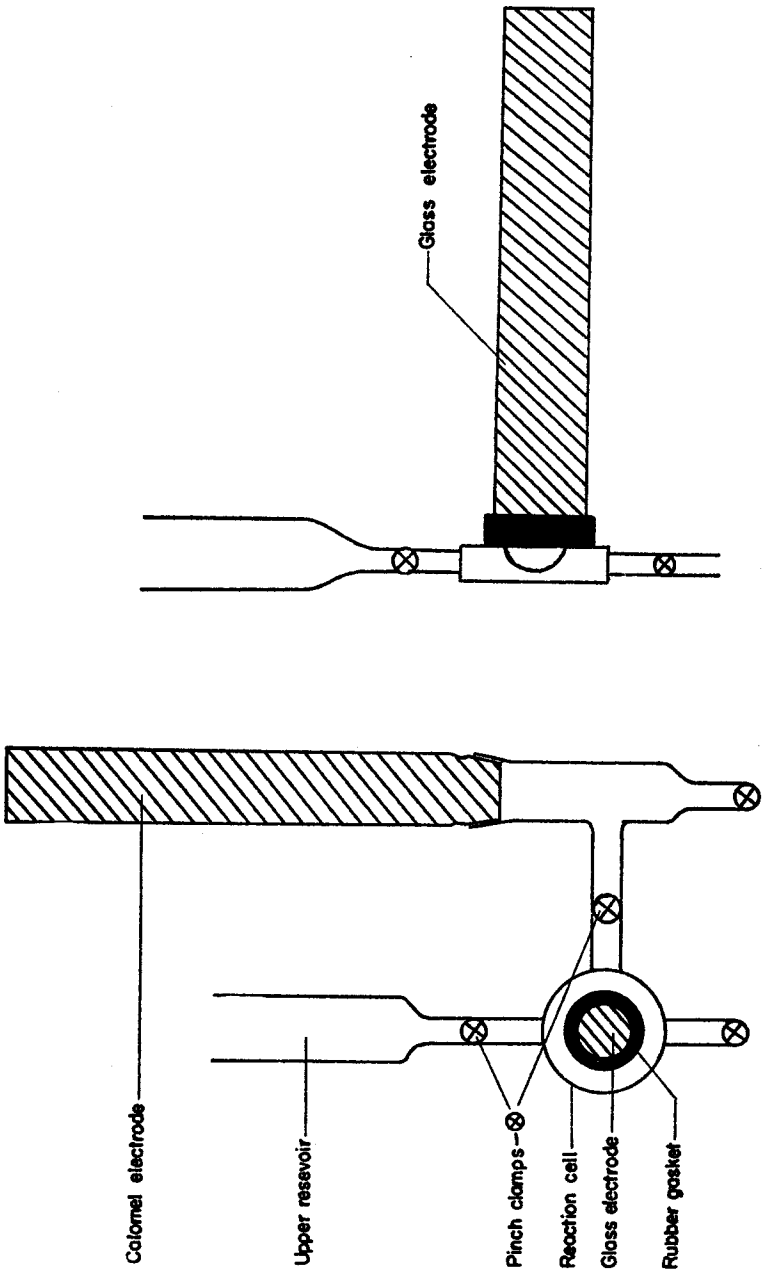

FIG. 1. Glass electrode apparatus.

glass electrode, model 1190-80, is sealed into the center of the reaction cell so that the tip of the electrode is 2 to 3 mm. from the inside of the front window. At least one glass electrode examined for this study was photosensitive in that its potential, when immersed in a non-reacting solution, shifted by several millivolts in the light. This response, undoubtedly a property of the silver chloride reference electrode built into the glass electrode, was not investigated. Problems arising from the effect were avoided in the present research by the selection of electrodes which did not show a response to the light.

The side vessel, containing the reference saturated calomel electrode, is connected to the reaction cell by a capillary side arm. Light from a projector lamp is focused on the reaction cell and is uniform over the whole area of the cell. Filters, including infrared absorbers, can be inserted in the optical path as required. The electrodes are connected to a Beckman model R pH meter, the output of which drives a Brown recorder. The meter output is selected so that the 10 inch chart paper of the recorder covers a span of 2.00 pH units. The usable span can be set for the range 6.00 to 8.00 or for any other desired region of the pH scale by manipulating a standard control on the pH meter. The apparatus responds to a sudden change in light intensity within 1 second. The recorder is fast enough to follow the most rapid changes needed for this work, 0.1 pH per second.

The experimental procedure is as follows: A sample of algae is centrifuged lightly from its nutrient medium, washed, and then resuspended in a sodium bicarbonate solution of concentration, c_0, up to 0.01 molar. The cell proper and the connecting side vessel are filled with the suspension so as to overflow into the upper reservoir and out of the side vessel. With the side vessel clamped off, the suspension in the cell is saturated with several per cent carbon dioxide in air by bubbling up from the bottom arm; or alternately, the contents of the upper reservoir are saturated by bubbling the gas through the open top, and the freshly saturated suspension is allowed to flow down into the cell. The upper and lower pinch clamps are then closed and the side clamp is opened; thus the reaction cell and the connecting side vessel are completely filled with liquid and there is no gas phase. The pH meter is standardized and the continuous trace of the pH on the recorder is begun. The projector lamp is turned on and the event is noted on the chart. When the light is turned off to discontinue photosynthesis, the event is again noted. The cell contents are stirred occasionally during an experiment by pinching a rubber tubing connection at one of the side arms. If the portions of the pH curve before and after pinching are continuous, the suspension is regarded as sufficiently uniform. (The apparatus was not designed for constant stirring because of mechanical difficulties.) Young algae harvested from a thin culture resist settling around the electrode in the course of a 20 minute experiment. Older algae settle rapidly and cannot be used.

Since there is no gas phase in equilibrium with the reaction medium, and since low bicarbonate concentrations are used, the length of a photosynthesis period is limited in part by the availability of inorganic carbon sources in the suspension. After the available carbon dioxide has been exhausted by photosynthesis, it can be replenished either by respiratory production in the dark or by resaturation of the suspension by a fresh gas stream. In either method the pH can be used as a continuous monitor of the carbon dioxide concentration.

Principle of the Method.—The basic assumption underlying the method is that the buffering properties of the cell suspension depend only on the carbon dioxide–bicarbonate-carbonate equilibria. A change in pH in such a closed system can then be related unambiguously to a change in the amount of carbon dioxide and its related species.

The following notation will be used:

$[i]$ = concentration of i in moles/liter

a_i = activity of $i = \gamma_i[i]$

$[C] = [CO_2] + [H_2CO_3] + [HCO_3^-] + [CO_3^=]$

= total concentration of inorganic carbon

c_0 = molar concentration of Na^+ added as $NaHCO_3$

K_1 = apparent first ionization constant of CO_2

K_2 = second ionization constant of CO_2

$$S = \frac{[CO_3^=]}{[HCO_3^-]} = \frac{\gamma_{HCO_3^-} \times K_2}{\gamma_{CO_3^=} \times a_{H^+}}$$

$$T = \frac{[CO_2] + [H_2CO_3]}{[HCO_3^-]} = \frac{a_{H^+} \times \gamma_{HCO_3^-}}{K_1}$$

$P = -\frac{d[C]}{dt}$ in a light period

= net rate of photosynthesis in moles/liter/second

$R = +\frac{d[C]}{dt}$ in a dark period

= rate of respiration

μ = ionic strength

$M = -0.51\sqrt{\mu}$ = Debye-Hückel limiting law expression for $\frac{\log \gamma_i}{z_i^2}$

$\left(\frac{d[C]}{d\,\mathrm{pH}}\right)_0$ = value of $\frac{d[C]}{d\,\mathrm{pH}}$ in a pure bicarbonate solution

$\left(\frac{d[C]}{d\,\mathrm{pH}}\right)_f$ = value of $\frac{d[C]}{d\,\mathrm{pH}}$ in a bicarbonate solution containing additional acids or bases

The rate of pH change, $\frac{d\,\mathrm{pH}}{dt}$, can be measured as the tangent to the experimental curve. This slope can be converted into R or P by the multiplying factor, $\frac{d[C]}{d\,\mathrm{pH}}$. A calculated value of this factor can be derived by considering the first and second dissociations of carbon dioxide and the maintenance of electrical neutrality in the solution. Equation (1) represents the case

$$\frac{1}{c_0}\left(\frac{d[C]}{d\,\mathrm{pH}}\right)_0 = \frac{2.3}{1+2S}\left[T\left(\frac{dM}{d\,\mathrm{pH}}-1\right)+S\left(1-3\frac{dM}{d\,\mathrm{pH}}\right)-\frac{2S(1+S+T)}{1+2S}\left(1-3\frac{dM}{d\,\mathrm{pH}}\right)\right] \quad (1)$$

in which the Debye-Hückel limiting law is used to evaluate the activity coefficients. It is apparent from this equation that, except for terms involving the activity coefficients, $\frac{1}{c_0}\left(\frac{d[C]}{d\,\mathrm{pH}}\right)_0$ is independent of c_0. In Table I values of $\frac{1}{c_0}\left(\frac{d[C]}{d\,\mathrm{pH}}\right)_0$ are tabulated for $c_0 = 0.002$ moles per liter.

The numerical values 6.36 and 10.33 were used for pK_1 and pK_2 respectively at 25° (Harned and Davis, 1943; Harned and Scholes, 1941). Table I values can be used to better than 5 per cent accuracy for bicarbonate solutions less than 0.002 molar over the whole pH range. Within the same accuracy they can be used for $c_0 = 0.004$ below pH 8.7, and for $c_0 = 0.01$ below pH 7.6. Values of $\frac{1}{c_0}[CO_2]$, calculated from equation (2), are also tabulated in Table I for 0.002 molar bicarbonate. This function is also almost independent of bicarbonate concentration.

$$[CO_2] = \frac{c_0 T}{1+2S} \quad (2)$$

A special protractor was constructed which reads slopes of experimental curves directly as $\frac{1}{c_0}\frac{d[C]}{dt}$. Values of $\frac{1}{c_0}\left(\frac{d[C]}{d\,\mathrm{pH}}\right)_0$ were taken from Table I for the construction of the protractor.

Other Buffering Salts.—Two conditions can be found in equation (1) and Table I for increased apparatus sensitivity, at which a given change in inorganic carbon will cause a large change in pH. (1) The bicarbonate concentration, c_0, should be as small as possible. (2) The working pH region should be near 8.3, the point of poorest bicarbonate buffering. It is equally apparent that the conditions for increased sensitivity are also the conditions for the most likely breakdown of the basic assumption of the method. The less the

buffering power of the bicarbonate systems, the greater the chance that foreign acidic or basic substances will contribute to the pH of the solution. These foreign substances may be residual salts from the culture medium that have not been completely washed out, salts added to the suspension medium, cell substances, or impurities.

Residual salts can be removed by repeated washings. The principal foreign ion in this category for our cultures is phosphate. In the category of salts

TABLE I

Calculated Equilibrium Values for $\left(\frac{d[C]}{d\ pH}\right)_0$ *and* $[CO_2]$ *in Bicarbonate Solutions*

pH	$-\frac{1}{c_0}\left(\frac{d[C]}{d\ pH}\right)_0$	$\frac{[CO_2]}{c_0} \times 10^2$	pH	$-\frac{1}{c_0}\left(\frac{d[C]}{d\ pH}\right)_0$	$\frac{[CO_2]}{c_0} \times 10^2$
5.5	15.8	688	7.7	0.106	4.32
5.6	12.6	546	7.8	0.087	3.43
5.7	10.0	434	7.9	0.073	2.72
5.8	7.95	345	8.0	0.062	2.15
5.9	6.30	274	8.1	0.055	1.70
6.0	5.00	217	8.2	0.051	1.35
6.1	3.98	173	8.3	0.049	1.07
6.2	3.16	137	8.4	0.050	0.85
6.3	2.51	109	8.5	0.053	0.67
6.4	2.00	87	8.6	0.061	0.53
6.5	1.59	69	8.7	0.067	0.41
6.6	1.26	55	8.8	0.077	0.32
6.7	1.00	43	8.9	0.091	0.25
6.8	0.80	34.5	9.0	0.106	0.20
6.9	0.63	27.4	9.1	0.127	0.15
7.0	0.50	21.7	9.2	0.149	0.12
7.1	0.400	17.3	9.3	0.173	0.09
7.2	0.320	13.7	9.4	0.196	0.07
7.3	0.254	10.9	9.5	0.213	0.05
7.4	0.203	8.7	9.6	0.249	0.04
7.5	0.163	6.9	9.7	0.267	0.03
7.6	0.131	5.5			

The values were calculated from equations (1) and (2), for $c_0 = 0.002$ M.

added to the suspension medium would be cyanide or other ions added for inhibition studies. If the amount of phosphate, cyanide, or any other specific ion were known, it would be possible to calculate the effect on the pH by solving simultaneously the equations for the ionization of carbon dioxide and the foreign substance. A new set of solutions, $\left(\frac{d[C]}{d\ pH}\right)_f$, would be found, which could be compared with the $\left(\frac{d[C]}{d\ pH}\right)_0$ values of equation (1) and Table I in three distinct regions.

(a) $pH \ll pK_f$: $$\left(\frac{d[C]}{d\,pH}\right)_f = \left(\frac{d[C]}{d\,pH}\right)_0 \frac{c_0 + f_b}{c_0}, \quad (3)$$

in which K_f is the acid dissociation constant of the foreign substance and f_b is the amount of the foreign system initially present in basic form, in molar units.

(b) $pH \sim pK_f$: The general effect is for $-\left(\frac{d[C]}{d\,pH}\right)_f$ to be much greater than $-\left(\frac{d[C]}{d\,pH}\right)_0$ because of the buffering action of the foreign system.

(c) $pH \gg pK_f$: $$\left(\frac{d[C]}{d\,pH}\right)_f = \left(\frac{d[C]}{d\,pH}\right)_0 \frac{c_0 - f_a}{c_0} \quad (4)$$

in which f_a is the amount of the foreign system initially present in the acid form.

The specific effects of phosphate and cyanide have been evaluated over the intermediate pH range. Fig. 2 illustrates the pH ranges for applicability of the limiting cases for phosphate initially present as $HPO_4^=$ and for cyanide salts. The region (a) approximation applies to within 10 per cent at all pH values below the solid curves and the region (c) approximation to within 10 per cent at all pH values below the broken curve. The pK_a values for $H_2PO_4^-$ and HCN used in the calculations are 7.2 and 9.2 respectively. It is apparent from the curves that a phosphate/bicarbonate ratio of less than 0.025 is desirable if Table I is to be used to within 10 per cent over the whole pH range; and a cyanide-bicarbonate ratio of less than 0.1 is to be used for pH values less than 7.8.

Supernatant phosphate determinations were made after centrifuging many typical algal suspensions prepared as for a glass electrode experiment. The amount of total phosphorus in the supernatant was within the above limits for inorganic phosphate. Some of the phosphate may not have been inorganic. Since most organic phosphate esters of biological significance have pK_2 values less than orthophosphoric acid (Kumler and Eiler, 1943) the errors could not be greater than predicted for an equivalent inorganic phosphate contamination at pH values above 7.

Elementary analyses cannot be used to study interferences in the category of cell substances, since the nature of the interfering substances is not known. A more general treatment is the determination of total acids and bases by titration. The titre, which may be regarded as a blank for the carbon dioxide determinations, is determined on a sample of algae suspended in CO_2-free distilled water. The assumption is made that the contribution of a given number of cells to the titre of the medium, whether as diffusible material or as the cell membrane, is the same in the blank as in dilute bicarbonate. Suspensions of *Chlorella* and *Scenedesmus* in distilled water always had pH values below the range used for photosynthetic studies with the glass electrode. Thus it was sufficient to determine the blank titration curve on the alkaline side

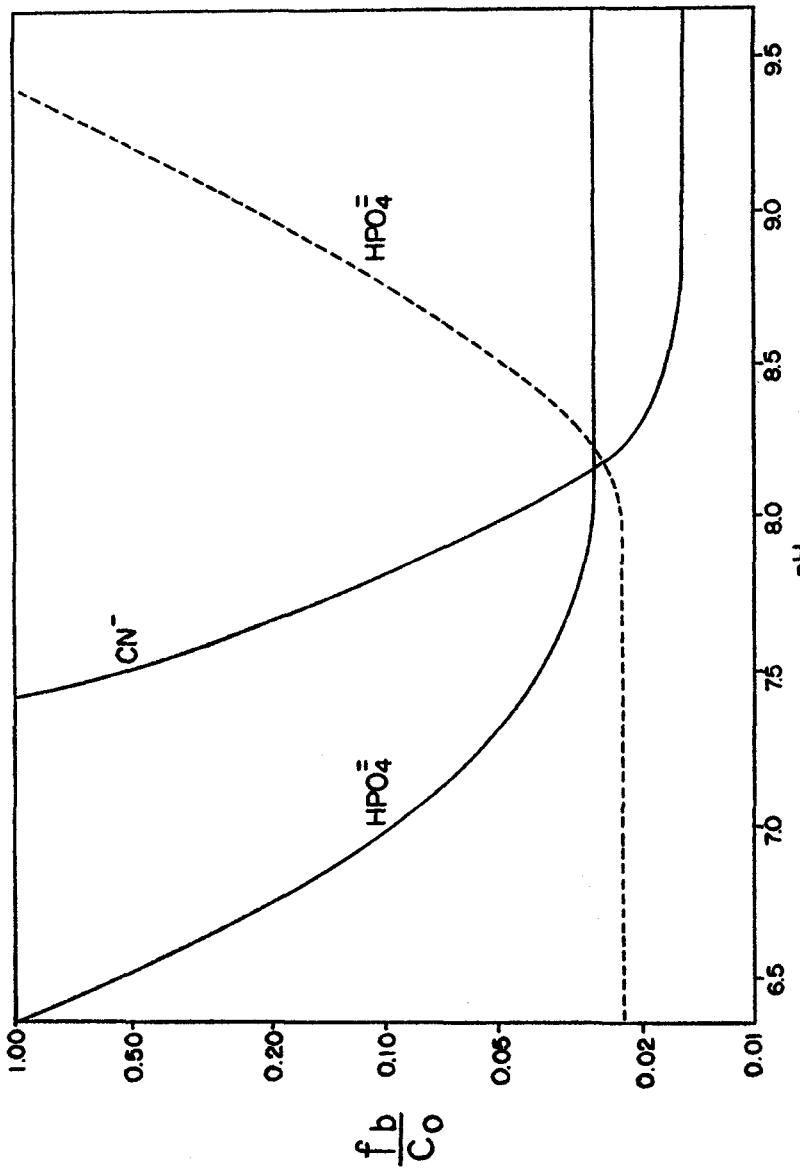

FIG. 2. Effect of added $HPO_4^=$ and CN^- on the pH of a bicarbonate buffer. This figure shows the conditions of applicability of the limiting forms of the interference equations. Equation (3) is valid within 10 per cent in regions below the solid curves. Equation (4) is valid within 10 per cent in the region below the broken curve.

only. From the titration curve, t_B, the titre with respect to alkali in equivalents per liter, and D_B, defined as $\frac{dt_B}{d\,\mathrm{pH}}$, can be obtained as functions of pH. Then it can be shown that if activity coefficient corrections are neglected,

$$\left(\frac{d[C]}{d\,\mathrm{pH}}\right)_f = \left(\frac{d[C]}{d\,\mathrm{pH}}\right)_0 \frac{(c_0 - t_B)}{c_0} - \frac{D_B}{1 + 2S}(1 + S + T), \tag{5}$$

in which $\left(\frac{d[C]}{d\,\mathrm{pH}}\right)_0$ is again the idealized value from equation (1) and Table I, and the other terms are as defined previously.

In practice the blank titration was determined on a concentrated suspension. The measured titre was reduced by a concentration factor in order

TABLE II

Limits Imposed by Cellular Interferences in 0.5 Per Cent Unwashed Chlorella Suspensions

c_0	$pH_{max.}$
0.001	7.2
0.002	7.5
0.003	7.7
0.005	7.9

$pH_{max.}$ is the pH value below which there is less than 10 per cent error in applying equation (1). The values are based on experimental titrations of *Chlorella* suspensions. A similar table can be constructed for cell concentrations other than 0.5 per cent by the following consideration. The c_0 value corresponding to any tabulated value of $pH_{max.}$ is directly proportional to the cell concentration. Thus, for a 0.2 per cent suspension, a c_0 value of 0.002 corresponds to a $pH_{max.}$ of 7.9.

that t_B and D_B in equation (5) correspond to the blank titres at the working cell densities in the glass electrode apparatus. Alkaline titrations were done on several unwashed *Chlorella* suspensions in the dark. A correction for respiratory CO_2 production during the titration was approximated. This correction amounted to between 20 and 50 per cent of the total titre. The titration data and the estimated corrections are summarized in Table II, in which the approximate limits of applicability of equation (1) are given as a function of the bicarbonate concentration.

Carbon Dioxide Limitations.—A necessary condition for this method is the small reservoir of available inorganic carbon. As the pH rises during an assimilation period, the carbon dioxide concentration falls. Table I can be used to relate the equilibrium carbon dioxide concentration to the instantaneous pH. For example, the carbon dioxide concentration in 0.001 M bicarbonate is reduced to 1×10^{-5} M, the concentration in a solution equilibrated with normal air, at pH 8.3. On the other hand, if the pH is

lowered much below 6.5, in the presence of bicarbonate the equilibrium carbon dioxide concentration becomes too high for the algae. There is thus a narrow pH range of roughly 6.5 to 8.5, within which most experiments must be performed. Some of the other limitations discussed above may narrow this range further.

In addition to the equilibrium carbon dioxide limitations there may also be some kinetic limitations. The uncatalyzed reactions,

(A) $$H_2CO_3 \xrightarrow{k_1} CO_2 + H_2O \qquad k_1 = 10 \text{ sec.}^{-1}$$

(B) $$CO_2 + H_2O \xrightarrow{k_2} H_2CO_3 \qquad k_2 = 0.02 \text{ sec.}^{-1}$$

(C) $$HCO_3^- \xrightarrow{k_3} CO_2 + OH^- \qquad k_3 = 0.47 \times 10^{-4} \text{ sec.}^{-1}$$

(D) $$CO_2 + OH^- \xrightarrow{k_4} HCO_3^- \qquad k_4 = 2 \times 10^3 \text{ liter mole}^{-1} \text{ sec.}^{-1}$$

are not instantaneous but occur at finite rates determined by the rate constants (summarized, Rabinowitch, 1951, p. 907). The possibility thus exists that $[CO_2]$ may be less than its equilibrium value in a suspension from which carbon dioxide is being rapidly withdrawn by photosynthetic assimilation. If photosynthesis requires the direct utilization of unhydrated carbon dioxide, a limitation might occur at a pH lower than would correspond to the limiting equilibrium carbon dioxide concentration found in Table I. Also calculations of photosynthetic rates based on equation (1) would be incorrect for the non-equilibrium case. This type of carbon dioxide limitation is more serious here than in a manometric apparatus. In the latter either there is a large reservoir of CO_2 in the gas phase for acid buffer measurements or the large concentration of bicarbonate in a medium like Warburg's buffer 9 enables reaction (C) to produce CO_2 rapidly.

An estimate of the maximum deviation of $[CO_2]$ from its equilibrium value can be made by solving equation (6), the equation of conservation for CO_2.

$$k_1[H_2CO_3] = k_2[CO_2] + P + \frac{d[CO_2]}{dt} \tag{6}$$

Reactions (C) and (D) can be neglected below pH 8. An approximate transformation can be made from the variable t to the variable pH.

$$[CO_2] - [CO_2]_{eq.} = -\frac{Pc_0S}{k_2(1 + 2S)^2} \frac{1}{\frac{d[C]}{d\,\text{pH}}} \tag{7}$$

Equation (7), in which $[CO_2]_{eq.}$ is the equilibrium value from Table I, has been solved numerically for several constant values of P and c_0. The solutions

are given in Table III for the pH range 7 to 8. In this range the percentage deviation of $\frac{d[C]}{d\,\mathrm{pH}}$ from its equilibrium value is of the same order of magnitude as the deviation of $[CO_2]$. A P value of 5×10^{-6} moles liter^{-1} sec.$^{-1}$ corresponds to the highest photosynthetic rates encountered in normal experiments with green algae; it represents a CO_2 assimilation of 40 cell volumes per hour by a 1 per cent suspension. It can be seen from the tables that the slow dehydration of H_2CO_3 might become limiting above pH 7.5. Experimental attempts were made to correlate some observed decreases in rate with this type of limitation. A crude carbonic anhydrase preparation was added to catalyze reactions (A) and (B). Unfortunately an amount of the preparation

TABLE III

Maximum Fractional Deviation from Equilibrium Due to the Slow Dehydration of Carbonic Acid

pH	$c_0 = 0.001$ M			$c_0 = 0.002$ M
	$\frac{[CO_2]_{eq.} - [CO_2]}{[CO_2]_{eq.}}$			$\frac{[CO_2]_{eq.} - [CO_2]}{[CO_2]_{eq.}}$
	$P = 1 \times 10^{-6}$	$P = 3 \times 10^{-6}$	$P = 5 \times 10^{-6}$	$P = 5 \times 10^{-6}$
7.4	0.009	0.030	0.048	0.02
7.5	0.016	0.050	0.080	0.03
7.6	0.034	0.092	0.146	0.08
7.7	0.064	0.169	0.258	0.14
7.8	0.115	0.298	0.451	0.25
7.9	0.214	0.522	0.775	0.42
8.0	0.351	0.825		0.70

The numerical values were calculated on the basis of equation (7).

sufficient to give the desired enzymatic activity contained enough acidic substance to interfere with the interpretation of the results.

Changing Environment.—It should be emphasized that the algae are not kept in a constant medium during the course of a glass electrode experiment. The pH changes continuously; if it did not there would be nothing to measure. This fact alone probably makes the method useless for many biological systems. Another environmental factor which might limit the application of the method to other systems is the low required ionic strength. The background of years of manometric experience of many investigators with *Chlorella* and *Scenedesmus* led us to believe that these organisms would photosynthesize at a rate almost independent of pH over a wide range and would tolerate the low salt concentration of the bicarbonate medium.

Diffusion.—The question of diffusion limitations arises since the suspensions are not stirred. Suspensions were examined microscopically for clumping. Clumped preparations were discarded so that diffusion blocks could

not occur around cell clusters. An exact calculation of the rate of diffusion of carbon dioxide into isolated cells was not attempted. The situation can be approximated, however, by the simple mathematical model of diffusion into a spherical sink. Each cell may be regarded as a sink, at the surface of which an incoming carbon dioxide molecule has a certain probability of being absorbed. If the sink diameter is much larger than a molecular dimension, the rate of diffusion, j, into a single cell of radius r, is given by equation (8), in which D is the diffusion coefficient and $[CO_2]$ is the over-all concentration in the free liquid (Collins, 1950).

$$j = 4\pi Dr\,[CO_2] \tag{8}$$

The total diffusive flux, J, in moles per liter per second, into all the cells of suspension of volume fraction v, is

$$J = \frac{3vD[CO_2] \times 10^3}{r^2} \tag{9}$$

The numerical value of J for a 1 per cent suspension of *Chlorella* is $\sim 10^4[CO_2]$ moles/liter/sec. Even when $[CO_2]$ is 3.3×10^{-6}, calculated by equation (9), corresponding to a partial pressure of 0.01 per cent atmosphere, the diffusive flux is almost four orders of magnitude greater than the saturation rate of photosynthesis. It is therefore fair to conclude that the extracellular diffusion of carbon dioxide does not become limiting until the external concentration becomes so low that a true intracellular equilibrium carbon dioxide limitation has already set in.

An Example.—A typical experimental record is shown in Fig. 3. The two curves are tracings from the original strip charts. They represent two separate samples of *Chlorella* from the same harvest. The two curves are juxtaposed to demonstrate the reproducibility of the apparatus. Photosynthetic rates, computed from slopes measured at various points along the curves, are plotted in Fig. 4. The following features of the experiment may be pointed out. (1) The rate of photosynthesis rises gradually during the first few minutes of each light period. (2) After the induction period, the rate of photosynthesis is fairly constant until the pH exceeds 7.1. Over the pH interval 6.5 to 7.0 the experimental slope varies by a factor of four, but the calculated rate varies by less than 10 per cent. (3) The average rate in the constant region, 3.0×10^{-6} moles/liter/sec., corresponds to 25 cell volumes of CO_2 per hour, a value consistent with manometrically determined rates. (4) Although the rate falls off sharply with increasing pH, the points throughout the light period are fairly reproducible. (5) The rate of respiration is less reproducible than the rate of photosynthesis. This corresponds to the well known fact that the respiratory rate depends very much upon the previous schedule of light and dark periods.

The decrease in rate above pH 7.1 was observed in over a hundred separate

experiments. The actual pH at which the decline sets in is not constant from experiment to experiment but depends in a complicated manner on the bicarbonate concentration of the medium, the cell concentration, and the light intensity. This phenomenon occurs at too low a pH to be explained by limitations in the external diffusion rate or in the rate of dehydration of H_2CO_3. Another possible explanation is that the equilibrium carbon dioxide concentration in the region of declining rates is not enough for maximum photosynthetic

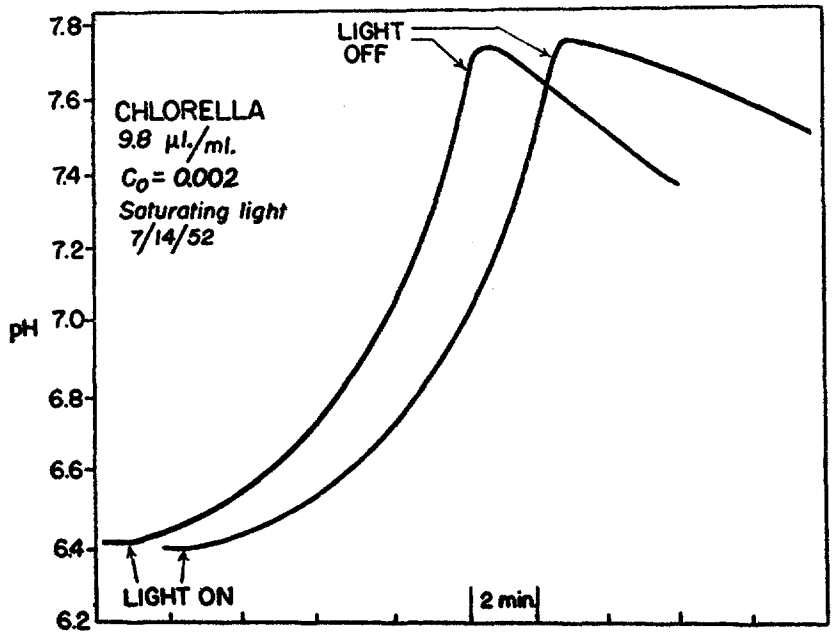

FIG. 3. Experimental photosynthesis and respiration curves. The experiments represented by the two curves were performed several hours apart on separate samples of algae taken from the same harvest. The curves are tracings from the original recorder charts. The relative lateral spacing of the two curves is arbitrary.

efficiency. It is well known that the rate of photosynthesis depends on the carbon dioxide concentration, but it had been believed that limitations in the green algae occur only at carbon dioxide pressures below a thousandth of an atmosphere (Emerson and Green, 1938; further references in Rabinowitch, 1951, chapter 27). In our experiments with the glass electrode, however, the decrease in rate occurs at carbon dioxide concentrations corresponding to as much as 1 per cent of an atmosphere.

The explanation of the phenomenon could lie in several possible categories. (1) There is some systematic error in the glass electrode experiments which has

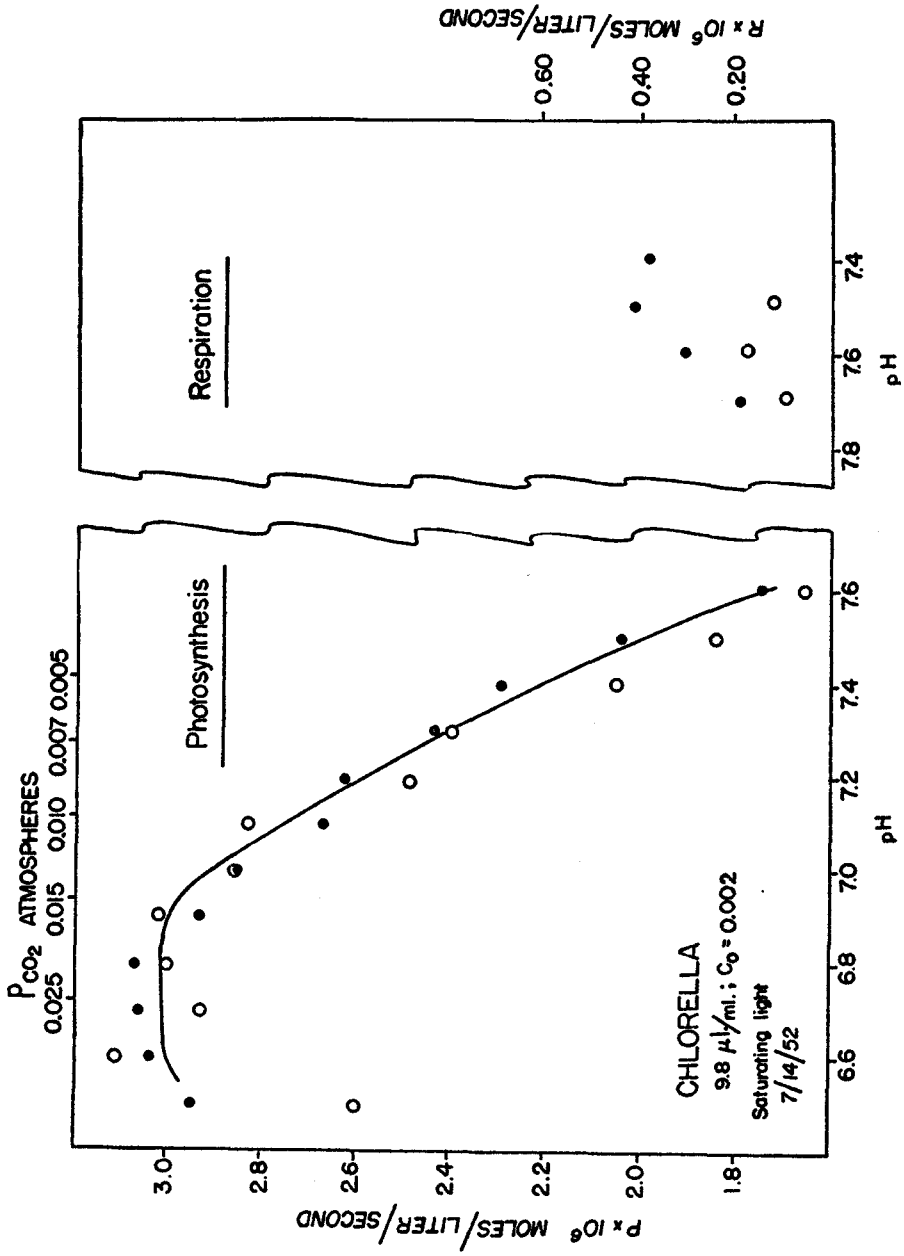

FIG. 4. Calculated photosynthetic and respiratory rates. The points marked on this figure were calculated from slopes measured at various points along the curves of Fig. 3. The solid line represents both sets of points for photosynthesis fairly well. No common line could be drawn for respiration. The filled circles refer to the left-hand curve in Fig. 3; the open circles refer to the right-hand curve.

not yet been recognized. (2) The effect is real for these experiments but would not be observed in steady state experiments. The pH changes continuously during a glass electrode measurement. The algae for some reason do not adapt quickly enough to their changing environment, and they show their inaptitude in the form of decreasing carbon dioxide uptake. (3) The effect is due to an intrinsic carbon dioxide limitation and could be observed even in a steady state measurement. This phenomenon is peculiar to media low in both carbon dioxide and bicarbonate, and it would therefore not be observed in the usual manometric experiments in which the concentration of either carbon dioxide or bicarbonate ion is high.

Steady State Measurements.—In order to test these various possibilities an independent method was used for measuring steady state rates of photosynthesis in media of low bicarbonate content and of fixed partial pressure of carbon dioxide. The method consists of passing a carbon dioxide–air mixture through an algal suspension at a fixed rate and of analyzing the exit gas stream for carbon dioxide. The analysis was performed by determining the pH of a solution of sodium bicarbonate equilibrated with the gas stream. Higgins and Marriott (1917) used such a method for analyzing the carbon dioxide content of air. They determined the pH colorimetrically by adding an acid-base indicator to the bicarbonate solution. The substitution by Wilson *et al.* (1932) of a glass electrode for the color indicator was employed in the present research.

A schematic flow diagram of the apparatus is shown in Fig. 5. The gas mixture is saturated with water, passed through a capillary flow meter, bubbled up through a 1 cm. coarse Corning sintered glass disc into the algal suspension, and then bubbled up through another sintered glass disc into the electrode chamber. The algal vessel may be by-passed so that the original gas mixture may be analyzed. The transfer lines were made of capillary tubing in order to keep the hold-up time at a minimum. The reaction cell holds 4 ml. of algal suspension. It was illuminated by white light from a projector bulb. The cell was not thermostatically controlled, but the room temperature was maintained at 25 ± 1°. The electrode chamber holds about 1.5 ml. of electrolyte. A Beckman glass electrode, 4990-83, was used. The calomel electrode was prepared in place. The electrolyte was 0.02 M KCl and 0.02 M $NaHCO_3$, and bathed both the calomel and glass electrodes. The absence of a barrier between the calomel electrode and the bubbling chamber avoided the problem of liquid junction potentials. The hydrogen ion diffusion potential arising from the non-saturation with CO_2 of that portion of the electrolyte closest to the calomel electrode was negligible because of the very low concentration of hydrogen ions relative to the other ions in solution. The use of equivalent amounts of chloride and bicarbonate for cells of this type was proposed by MacInnes and Belcher (1933) to minimize the activity coefficient correction. The electrodes were connected to a Beckman model R pH meter and the cell

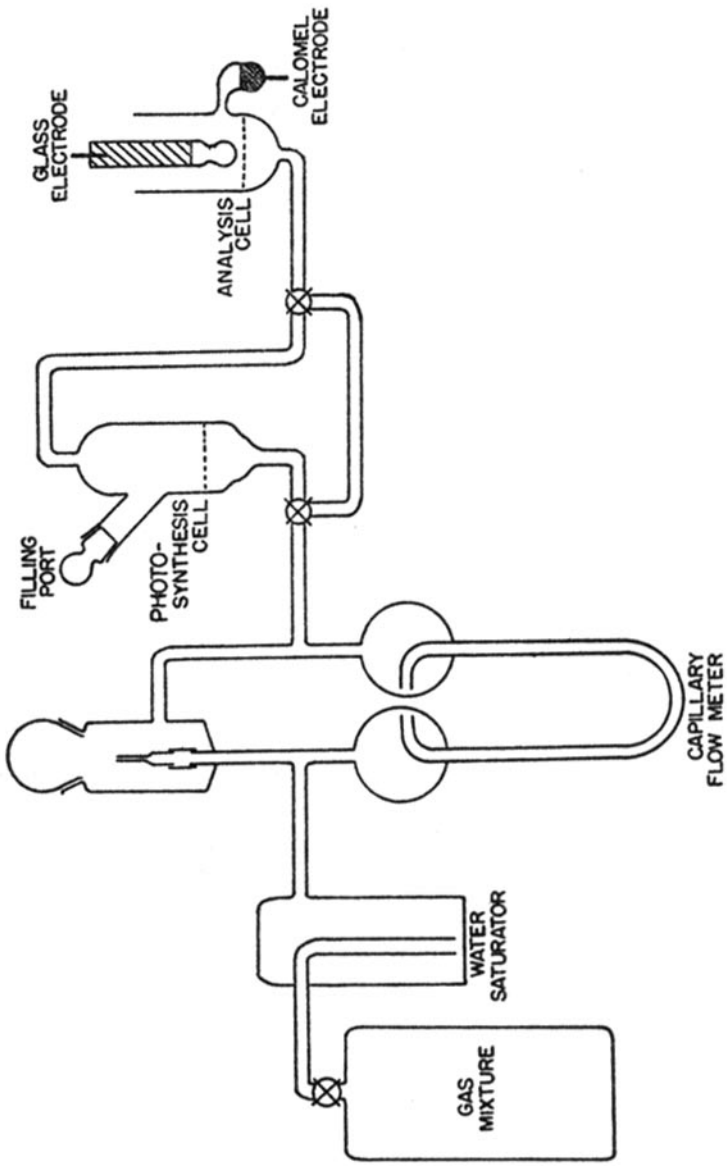

FIG. 5. Schematic flow diagram of apparatus for steady state measurements.

potential was actually read on a pH scale. Equation (10) relates the pH to p, the partial pressure of CO_2.

$$\log p = A - \text{pH} \tag{10}$$

The constant A was eliminated by a differential technique in which the ratio of two partial pressures, y, was related by equation (11) to the difference of the two corresponding pH values.

$$\log \frac{p_1}{p_2} = -\log y = \text{pH}_2 - \text{pH}_1 = \Delta\text{pH} \tag{11}$$

TABLE IV

Relationship between pH Changes and Partial Pressure of Carbon Dioxide

$\text{pH}_2 - \text{pH}_1$	$1 - y$	$\text{pH}_2 - \text{pH}_1$	$1 - y$
0.01	0.023	0.16	0.308
0.02	0.045	0.17	0.324
0.03	0.067	0.18	0.339
0.04	0.088	0.19	0.354
0.05	0.109	0.20	0.369
0.06	0.129	0.21	0.383
0.07	0.149	0.22	0.397
0.08	0.168	0.23	0.411
0.09	0.187	0.24	0.425
0.10	0.206	0.25	0.438
0.11	0.224	0.26	0.450
0.12	0.241	0.27	0.463
0.13	0.259	0.28	0.475
0.14	0.276	0.29	0.487
0.15	0.292	0.30	0.499

This table is based on equation (11) in the text. $(1 - y) = \frac{p_1 - p_2}{p_1} = \frac{\Delta p}{p_1}$.

Photosynthetic rates were measured as the difference between light and dark readings.

$$\Delta p_{\text{photosynthesis}} = p_{\text{dark}} - p_{\text{light}} = (1 - y)p_{\text{dark}}$$

The rate of photosynthesis in cubic centimeters of CO_2 per unit time is then $v\Delta p$, in which v is the flow rate of the gas in cubic centimeters per unit time and Δp is the partial pressure difference in atmospheres. A tabulation of values of $(1 - y)$ as a function of pH, calculated from equation (11), is given in Table IV.

The model R pH meter was calibrated every few weeks against the more accurate battery-operated model G Beckman meter. The two meters agreed to within 0.01 pH unit. The linear dependence of the pH upon $\log p$, demanded

by equation (10), was verified by using known gas mixtures of 0.25 to 1.75 per cent CO_2 in air. The mixtures were analyzed manometrically in Warburg flasks by the iodide-permanganate technique (Umbreit 1945). Because of the satisfactory agreement of these experiments with theory, it was possible to rule out, at least for these differential measurements, disturbances due to bubbling of the gas around the glass electrode.

The E.M.F. of the cell was found to be slightly dependent upon the flow rate of the gas. The maximum variation over the range of flow rates 2 to 10 ml./min. was about 0.02 pH unit. In any given experiment, the flow rate was maintained at a constant value for both the dark and the light measurements.

The response time of the apparatus depends on the equilibration of the gas stream with the solutions in the reaction and analysis cells. The equilibration time decreases as the flow rate is increased. On the other hand, for a given rate of photosynthesis, as the flow rate is increased the magnitude of the observable pH change is lowered and the sensitivity of the method is decreased. The actual choice of flow rate is a compromise between the two demands, a measurable change in pH and a reasonably short equilibration time. For the experiments described in this paper the flow rates were from 2 to 10 ml./min. and the average time for equilibration after each change in light intensity was about 20 minutes. The range of observed ΔpH was 0.11 to 0.27. Part of the equilibration time is the induction period in which the rate of photosynthesis readjusts to a change in light intensity. This period cannot be shortened by altering the flow rate of the gas. An estimate of the non-biological contributions to the equilibration time was made by filling the algal vessel with a cell-free bicarbonate solution. After a new gas was introduced at the beginning of the flow train at a rate of 5 ml./min., about 5 minutes were required for the pH to change by 90 per cent of its ultimate increment.

From equation (11) it is seen that the fractional change in p_{CO_2} determines the observed change in pH. Thus the method is most sensitive in detecting small absolute changes, Δp, at low partial pressures. The over-all reliability of the observations is about 0.01 pH unit. Hence, at the highest p_{CO_2} values used in this work, 1.3 per cent, the limit of detection is $\Delta p_{CO_2} = 0.003$ atmosphere. At these higher values of p_{CO_2} the flow rate was usually decreased in comparison with the rates for experiments with 0.25 per cent CO_2.

The concentration of carbon dioxide in the flowing gas stream decreases gradually as the gas bubbles up through the suspension. The concentration of carbon dioxide in the liquid phase, however, is made uniform from top to bottom by the violence of the bubbling. Whatever concentration gradients exist in the liquid occur only at the interfacial films surrounding the bubbles. If equilibration were instantaneous at the interface and if diffusion in the interfacial film were infinitely fast, then the carbon dioxide in the liquid would be in equilibration with the exit gas stream. For example, a gas containing 0.25

per cent CO_2 is passed through a sample of algae and the exit gas is found to contain 0.13 per cent CO_2. Then the amount of CO_2 available to the algae corresponds to 0.13 per cent CO_2, if there are no diffusion limitations. The maximum concentration gradient of CO_2 across the interfacial films was con-

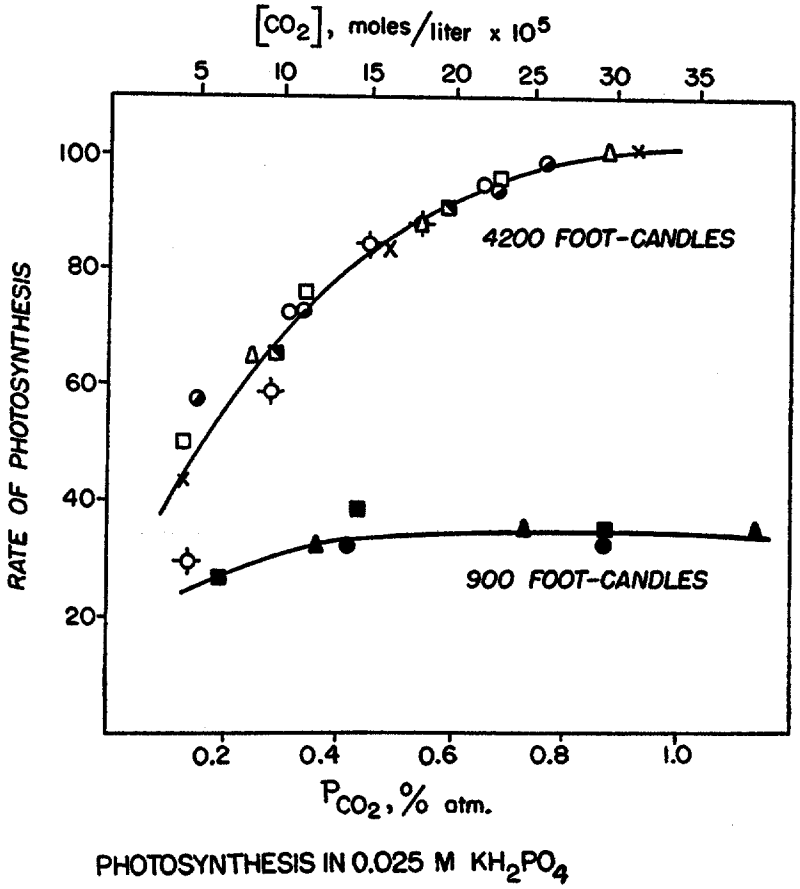

FIG. 6. Photosynthesis of *Chlorella* in 0.025 M KH_2PO_4. Average density of cells, 10 μl. per ml.

servatively estimated from diffusion theory to be 0.03 per cent. Since the calculated effect of diffusion is so small, the assumption will be made that diffusion is not limiting in these experiments, and the liquid will be considered to be in equilibrium with the exit gas stream.

Most of the measurements were made with suspensions of *Chlorella pyrenoidosa* (strain Emerson) in either 0.025 M KH_2PO_4 or in 0.002 M $NaHCO_3$.

The algae were grown in large surface culture flasks at about pH 7 with air containing several per cent carbon dioxide bubbling through the medium. The results for two light intensities are summarized in Figs. 6 and 7. The high light is saturating and the low light allows about one-third of the maximum

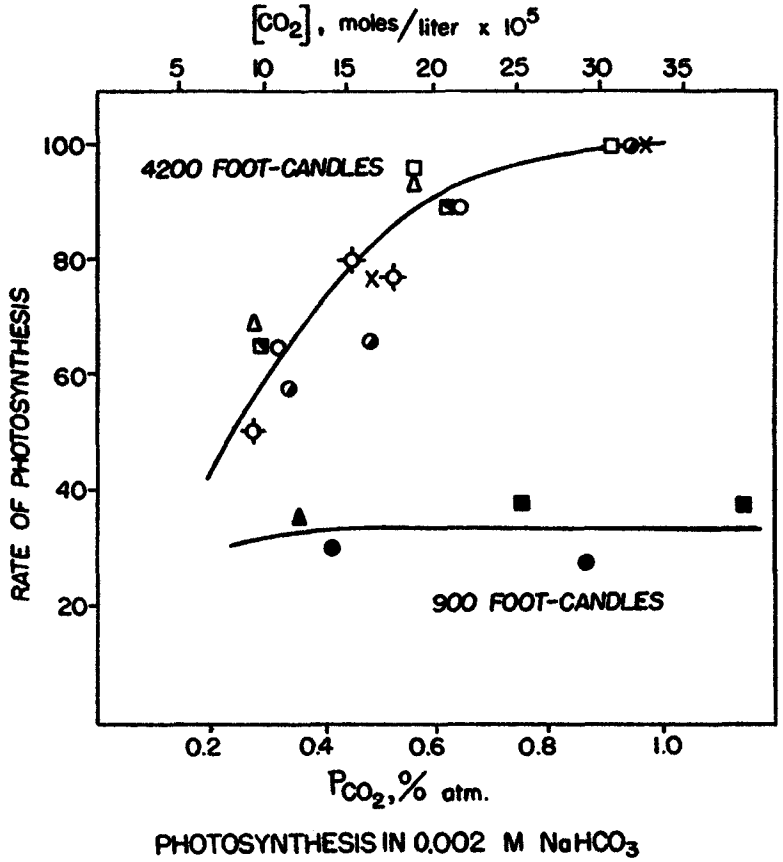

FIG. 7. Photosynthesis of *Chlorella* in 0.002 M $NaHCO_3$. Average density of cells, 10 μl. per ml.

photosynthetic rate. In each suspending medium seven series of experiments are reported, each series on a different batch of algae. The cell concentration varied from 9 to 11 μl. algae per ml. suspension. The average maximum rate of the fourteen suspensions was 28 $\pm$ 4 μl. CO_2 per μl. algae per hour. In order to compare all the data, the series of experiments with each suspension were normalized at one point so as to fit an average curve at the highest carbon dioxide pressure. The smoothed average curves of Figs. 6 and 7 were obtained

in this manner. Carbon dioxide limitations amounting to 10 per cent inhibition set in at partial pressures as high as 0.6 per cent for saturating light. This limitation occurs both in acid phosphate medium and in 0.002 M $NaHCO_3$. At lower light intensities, the limitations occur at much lower partial pressures. Preliminary experiments indicated that at high light the inhibition was removed by higher concentrations of bicarbonate, but the effect of bicarbonate was not studied systematically. The experiments reported here do not support the criticism of the use of carbonate-bicarbonate buffers for measurements of the quantum yield of photosynthesis (Warburg *et al.*, 1951). Although 0.23 per cent atmosphere, the carbon dioxide partial pressure in the familiar 0.1 M Warburg buffer 9, may be insufficient to support the maximum rate of photosynthesis at saturating light intensities, it seems to be adequate for the maximum rate attainable at low intensities. From the lower curve in Fig. 7, 0.35 per cent CO_2 is sufficient at 900 foot-candles, at which the photosynthetic rate is about eight times compensation. As the light intensity is lowered further to the compensation point, the region in which quantum efficiencies are determined, even smaller partial pressures of CO_2 would be adequate.

These results confirm the validity of the glass electrode apparatus. Also, some of the carbon dioxide limitations observed in the glass electrode apparatus are shown to be not merely transient effects. Although the few experiments reported in this paper are not sufficient basis for a discussion of the mechanisms of carbon dioxide limitations in photosynthesis, they add additional evidence that the maximum rate under steady illumination may require several tenths of a per cent of carbon dioxide. The large accumulation of conflicting data in the literature makes it certain that carbon dioxide limitations depend sensitively on the details of the experimental environment and of the previous history of the preparation. Information is unfortunately lacking from many of the published papers with regard to pertinent experimental factors, like cell density, culturing conditions, light intensity, absolute rates, duration of the experiment, and previous scheduling of light and dark. Future systematic investigations will require close control of these, and perhaps of other variables.

The application of the glass electrode apparatus to the study of transient effects in photosynthesis is reported by Gaffron (1954).

SUMMARY

1. A continuously recording glass electrode apparatus has been described for measuring carbon dioxide concentration changes in solution. The limits of applicability of the apparatus have been analyzed.

2. The glass electrode apparatus has been used for the measurement of transient rates of photosynthesis by algal suspensions.

3. The decline of the photosynthetic rate in high light at carbon dioxide

partial pressures less than 0.5 per cent atmosphere, observed in the glass electrode apparatus, has been confirmed by steady state experiments in which flowing gas streams were analyzed.

The use of the glass electrode was first proposed for this research by Dr. Hans Gaffron. The analysis of the method described in this paper was undertaken at his suggestion and was the outgrowth of many hours of discussion with him.

The author is indebted to Mr. Charles Soderquist for the construction of the lucite reaction cell, and to Mr. Isamu Iwaoka for help with the electrical circuit. The experiments were performed with the technical assistance of Mrs. Gladys Benedek, Mrs. Yü Liang Chou Cha, and Mr. A. Daniel Williams.

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