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Photosynthesis and Inorganic Carbon Transport in Isolated *Asparagus* Mesophyll Cells[1]

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ABSTRACT

The possibility of HCO_3^- transport into isolated leaf mesophyll cells of *Asparagus sprengeri* Regel has been investigated. Measurement of the inorganic carbon pool in these cells over an external pH range 6.2 to 8.0, using the silicone-fluid filtration technique, indicated that the pool was larger than predicted by passive $^{14}CO_2$ distribution, suggesting that HCO_3^- as well as CO_2 crosses the plasmalemma. Intracellular pH values, calculated from the distribution of $^{14}CO_2$ between the cells and the medium, were found to be higher (except at pH 8.0) than those previously determined by 5,5-dimethyl[2-^{14}C]oxazolidine-2,4-dione distribution. It is suggested that the inorganic carbon accumulated above predicted concentrations may be bound to proteins and membranes and thus may not represent inorganic carbon actively accumulated by the cells, inasmuch as in a closed system at constant CO_2 concentration, the photosynthetic rates at pH 7.0 and 8.0 were 5 to 8 times lower than the maximum rate which could be supported by CO_2 arising from the spontaneous dehydration of HCO_3^-. Furthermore, CO_2 compensation points of the cells in liquid media at 21% O_2 at pH 7.0 and 8.0, and the $K_{1/2}$ CO_2 (CO_2 concentration supporting the half maximal rate of O_2 evolution) at 2% O_2 at pH 7.0 and 8.0 are not consistent with HCO_3^- transport. These results indicate that the principal inorganic carbon species crossing the plasmalemma in these cells is CO_2.

The chemical species of inorganic carbon which crosses the plasmalemma of the photosynthetic cells of higher plants is not known with certainty, but has generally been assumed to be CO_2. Based on the photosynthetic response of thin leaf slices in media at various pH values and inorganic carbon concentrations, Jones and Osmond (13) and Ullrich-Eberius *et al.* (27) have concluded that only CO_2 and not HCO_3^- is utilized in photosynthesis. This conclusion is also supported by the observation that carbonic anhydrase stimulated photosynthesis in media of alkaline pH (13, 27). In contrast, Volokita *et al.* (28), on the basis of experiments with isolated pea protoplasts, suggest that the HCO_3^- ion can cross the protoplast membrane and that the passage is mediated by a transfer mechanism. Volokita *et al.* (28) ascribe the discrepancy between the results of protoplast and leaf slice experiments to the bulkiness of thin leaf slices which impede the diffusion of HCO_3^- through the free space of the tissue.

Investigations of inorganic carbon transport in algae, particularly the cyanobacteria (3, 9, 15, 20, 21), have provided considerable evidence to indicate that these organisms can actively transport HCO_3^- across the plasmalemma. Raven (24) has suggested that HCO_3^- transport is implied if the rate of algal photosynthesis is substantially higher in an alkaline medium compared to that in an acidic medium under the condition of constant CO_2 concentration. Other workers (3, 9, 15, 19–21) have used more exacting criteria to establish active HCO_3^- transport, which in algal cells has the following characteristics: that the observed rate of photosynthesis, in alkaline medium, is substantially greater than the rate of photosynthesis which could be supported solely by the spontaneous dehydration of HCO_3^- to CO_2, within a closed system (19, 21), and there is an accumulation of a large, acid-labile, inorganic carbon pool within the cell which occurs against concentration and pH gradients (3, 9, 15, 20).

In the present study, we have applied the criteria established for HCO_3^- transport in algae, to test for the presence of inorganic carbon transport in isolated mesophyll cells of *Asparagus sprengeri* Regel. These cells can be obtained quickly and in large quantities by a simple mechanical isolation technique (10), thus eliminating the potential hazards associated with enzymic isolation (11, 22) and the deleterious effects of osmotic stress on photosynthesis (10, 22). *Asparagus* mesophyll cells can maintain high rates of photosynthesis for a prolonged period of time (10) and their robust nature permits vigorous stirring of the reaction media and reduction of the surface boundary layer without significant damage to cellular integrity or loss of photosynthetic activity.

MATERIALS AND METHODS

Asparagus sprengeri Regel mesophyll cells were isolated as described previously (10). All experiments were conducted at 25°C and a light fluence which was saturating for photosynthesis (300 wm^{-2} [10]). Cells were suspended in 50 mM K^+-phosphate, of appropriate pH, and contained an inorganic salts mixture (12), except for the determination of Γ[2] where the salts mixture was not used. The Chl content of the cell suspensions was determined by the method of Arnon (1).

The rate of photosynthesis was determined by measurement of O_2 evolution in a Clark-type O_2 electrode as described previously (10). The substrate concentration at which one-half the maximum rate of whole-cell photosynthesis ($K_{1/2}$) occurred was determined from double reciprocal plots of photosynthetic rate *versus* substrate (CO_2 and DIC) concentration by linear regression analysis. The compensation point of *Asparagus* cells in liquid medium was determined by the gas-chromatographic technique of Birmingham and Colman (5) and was measured directly as the compensation concentration of total dissolved inorganic carbon (Γ [DIC]) in the medium. The values of Γ (CO_2) were calculated from Γ (DIC) values by applying the equations of Buch (7). The rate of spon-

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[2] Abbreviations: Γ, compensation point; DIC, dissolved inorganic carbon; DMO, 5,5-dimethyloxazolidine-2,4-dione; pH_e: pH of external medium; pH_i; overall intracellular pH; RuBP, ribulose-1,5-bisphosphate; $K_{1/2}$, substrate concentration which elicits one-half the maximum rate of photosynthesis.

taneous dehydration of HCO_3^- (*i.e.* maximal CO_2 supply rate), in a closed system and given DIC concentration, was calculated by the procedure of Miller and Colman (21) which is a modification of the procedure described by Lucas (19).

Uptake and Accumulation of Inorganic Carbon. The assay for the uptake and accumulation of inorganic carbon was conducted as described by Miller and Colman (20) with modifications as noted below.

Isolated *Asparagus* cells were resuspended in N_2-gassed K^+-phosphate containing an inorganic salts mixture (12), sealed within the O_2 electrode chamber, and allowed to reach Γ at saturating light intensity. Depending upon pH, this took 3 to 10 min. Light intensity was then reduced to a low level, such that O_2 evolution or consumption remained at zero, a low DIC concentration was achieved and photoinhibition was avoided. Control experiments with N_2-gassed buffer in the sealed chamber showed that invasion of the system by O_2 (and presumably CO_2) did not occur for periods of time in excess of 60 min.

Fifty μl cell suspension (50–60 μg Chl ml^{-1}) was removed from the chamber and preilluminated (300 w m^{-2}), in the pipette tip, for 30 s. The cells were then layered over 60 μl silicone fluid (AR20, Wacker Chemie, Munich, F. R. G.) and 100 μl of a 10% (v/v) methanol solution in 2 N KOH all contained in a 400-μl microcentrifuge tube. The tube was positioned in the head of the microcentrifuge (Eppendorf No. 5412), illuminated (300 w m^{-2}) from above and the carbon uptake experiment was initiated by the injection of $NaH^{14}CO_3$ (0.15 μCi μmol^{-1}; final concentration, 8.3 mM) into the cell suspension. At timed intervals, the reactions were terminated by centrifugation of the cells through the silicone fluid into the basic terminating solution at reduced light intensity (room light). Then the tubes were frozen quickly in a dry ice-methanol solution. Total and acid-stable carbon associated with the cell pellet was determined as described previously (9, 20). For determination of the inorganic carbon pool size at various pH_e, an incubation time of 45 s was used. Intracellular pH was calculated from the distribution of CO_2 between the medium and the intracellular pool using the Henderson-Hasselbach equation, assuming that, the concentration of CO_2 was equal on both sides of the membrane, and employing a pK_a of 6.35 for H_2CO_3 at 25°C (7). The proportion of CO_2 in solution at various pH values of the medium was calculated according to Buch (7). The intracellular volume was determined using the [^{14}C]sorbitol-^3H_2O method (12).

RESULTS

The pH dependence of isolated *Asparagus* cell photosynthesis, at a constant 10 μM CO_2 concentration, is shown in Figure 1 for two O_2 concentrations (21% and 2%). Under these conditions, the optimum for photosynthesis is pH 7.0. The rate of photosynthesis was reduced by 21% O_2 at all pH values examined. The degree of inhibition was least at pH 7.0 (64%) rising to 69% and 75% at pH 6.2 and pH 8.0, respectively. The rate of photosynthesis at 2% O_2 and constant CO_2 declines in both acid and alkaline media; by 43% at pH 6.2 and 26% at pH 8.0. At 21% O_2, the pattern of pH inhibition of whole-cell photosynthesis is similar except that the magnitude of inhibition increased to 52% (pH 6.2) and 45% (pH 8.0). The CO_2-saturated rate of photosynthesis, at pH 7.2, was 44 μmol O_2 mg Chl^{-1} h^{-1}.

From the data in Figure 1, the observed rates of photosynthesis in terms of nmol O_2 ml^{-1} min^{-1} were calculated and, from the concentration of DIC in the closed O_2 electrode chamber, the rate of dehydration of HCO_3^- was also calculated (Table I). At all pH values examined, and at both O_2 concentrations, the maximum CO_2 supply rate greatly exceeded the observed rate of photosynthesis. At the pH optimum for photosynthesis (pH 7.0), the rate of photosynthesis is only 21.7% of the maximum calculated rate which could be supported by the rate of formation of CO_2 from HCO_3^-.

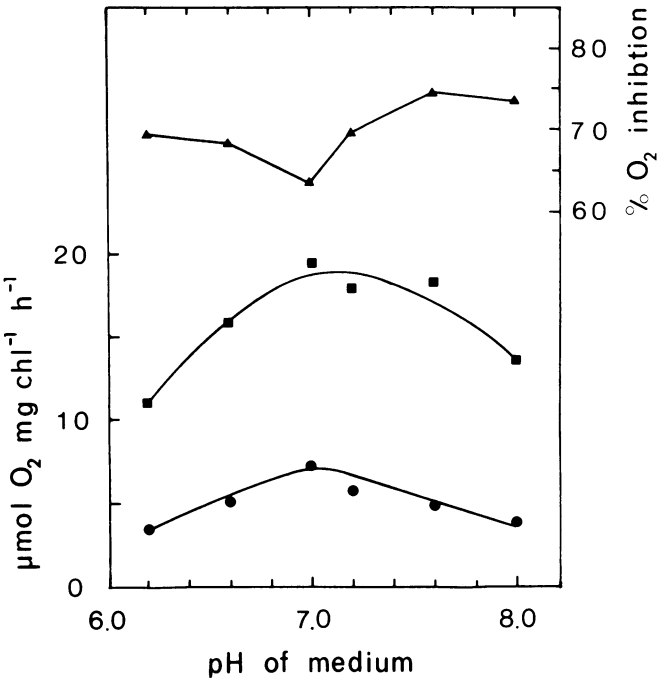

FIG. 1. The pH dependence of *Asparagus* cell photosynthesis in 50 mM K^+-phosphate containing 2% O_2 (■) or 21% O_2 (●) and 10 μM CO_2. O_2 inhibition of photosynthesis (▲) is expressed as a percentage of the rate of photosynthesis in 2% O_2, at a particular pH. Chl concentration was 19.2 μg ml^{-1}.

Inasmuch as photosynthesis reduces the external concentration of DIC, it could be correctly argued that the rate of spontaneous dehydration given in Table I is a maximum value and decreases as photosynthetic consumption of CO_2 reduces the concentration of DIC in the medium. Thus, at a much lower DIC concentration, the rate of photosynthesis may exceed the rate of spontaneous dehydration. Figure 2A shows the time course of O_2 evolution which could occur from utilization of all the available CO_2 in the medium while the observed rate of O_2 evolution is shown for comparison. At pH 8.0, the dehydration of HCO_3^- could support a rate of photosynthesis substantially larger than the observed rate (Fig. 2A). The observed rate of photosynthesis is only a small fraction of the maximum predicted rate based on spontaneous dehydration of HCO_3^- over the time course of the experiment. In the presence of 21% O_2, the observed rate of O_2 evolution is considerably less than that supportable by bicarbonate dehydration (Table I). In fact, at a DIC concentration of approximately 107 μM (2409 μl L^{-1}) the observed rate of photosynthesis is zero (Table II). At pH 7.0 (Fig. 2A), the inorganic carbon in the medium should be depleted after 4 min of photosynthesis if the cells used all the CO_2 as fast as it became available. The observed rate of photosynthesis does not fit this predicted rate and a much lower rate of photosynthesis is observed. The rate of depletion of DIC with time, calculated from the measured photosynthetic rate using a 1:1 stoichiometry between O_2 evolution and CO_2 fixation, is much lower than the calculated maximum rate of CO_2 formation (Fig. 2B).

In these experiments, the rate of photosynthesis is determined as O_2 evolution and may not exactly represent the rate of CO_2 fixation. However, our experience with *Asparagus* cells indicates that the rate of O_2 evolution reflects very closely the rate of CO_2 incorporation (data not shown). Indeed, for the observed rate of photosynthesis to equal the rate of spontaneous dehydration of HCO_3^-, it would be necessary that the rate of O_2 evolution underestimate the rate of CO_2 fixation by 80 to 88% (Table I).

Several physiological characteristics of whole-cell photosyn-

Table I. *Rate of Photosynthesis and the Rate of Spontaneous Dehydration of HCO_3^- in a Closed System.*

The observed rates of photosynthesis were calculated from the data contained in Figure 1. Chl concentration of cell suspensions was 19.2 $\mu g\ ml^{-1}$.

pH of Medium	[DIC][a]	(A) Observed Rate of Photosynthesis		(B) Rate of Spontaneous Dehydration	A/B		Rate of Photosynthesis Theoretically Supportable by Spontaneous Dehydration of HCO_3^-
		2% O_2	21% O_2		2% O_2	21% O_2	
	μM	*nmol O_2 ml^{-1} min^{-1}*		*nmol CO_2 ml^{-1} min^{-1}*	*ratio*		*μmol O_2 mg^{-1} Chl h^{-1}*
6.2	17.05	3.55	1.09	28.1	0.126	0.039	87.9
6.6	27.72	5.08	1.60	28.1	0.181	0.057	87.9
7.0	54.50	6.23	2.27	28.6	0.217	0.079	89.5
7.2	80.52	5.62	1.73	28.8	0.195	0.060	90.1
7.6	187.52	6.00	1.53	30.1	0.199	0.051	94.2
8.0	454.55	4.70	1.25	33.2	0.142	0.038	103.9

[a] These DIC concentrations generate an equilibrium CO_2 concentration of 10 μM.

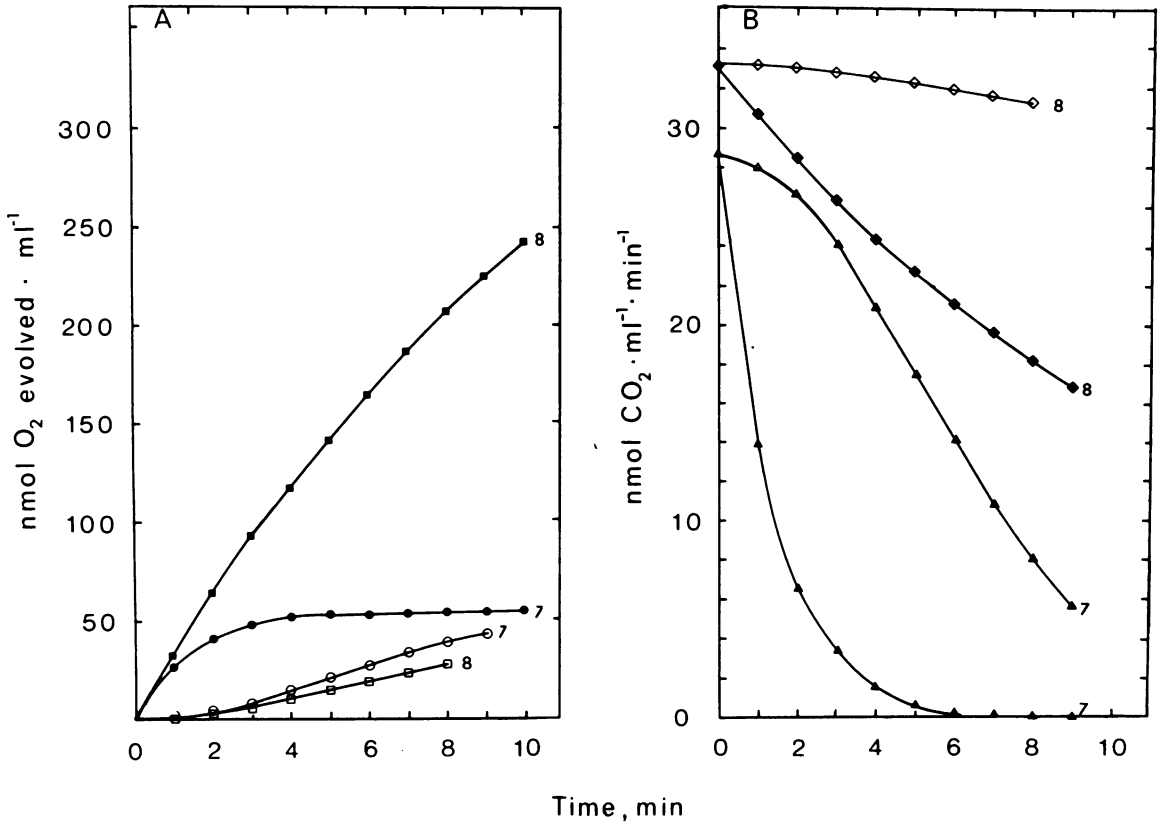

FIG. 2. A, Time course of O_2 evolution by *Asparagus* cells at pH 7.0 (○) and pH 8.0 (□) compared to that which could be theoretically supported by CO_2 supplied from the spontaneous dehydration of HCO_3^- at pH 7.0 (●) and pH 8.0 (■). B, The observed rate of dehydration as a function of time and decreasing DIC concentration was calculated from the measured photosynthetic rate, using a 1:1 stoichiometry between O_2 evolution and CO_2 fixation, at pH 7.0 (△) and pH 8.0 (◇). The maximum predicted change in the dehydration rate, at pH 7.0 (▲) and pH 8.0 (◆), as a function of time, assuming that CO_2 is removed as fast as it is formed. Experiments were conducted in a closed O_2-electrode and Chl concentration was 19.2 $\mu g\ ml^{-1}$.

thesis are shown in Table II. The $K_{1/2}$ (CO_2) of photosynthesis (2% O_2) is 15.5 μM at pH 7.0 and is approximately double that value at pH 8.0. The $K_{1/2}$ (DIC) at pH 7.0 is 84.5 μM and increases by a factor of 16.2 at pH 8.0. The value of Γ (CO_2) (21% O_2) of the isolated cells, in liquid medium, are typical of values obtained for the leaves of C_3 plants (29) by IR gas-exchange analysis and show some sensitivity to the pH of the medium (Table II). Γ (DIC) shows a considerable dependence upon pH, increasing by 9-fold between pH 7.0 and 8.0 (Table II).

Inorganic Carbon Uptake and Accumulation. To obtain additional information on the identity of the inorganic carbon species taken up by leaf mesophyll cells, the effect of pH_e on the rate of uptake and accumulation of inorganic carbon by *Asparagus* cells was investigated.

The time course of carbon accumulation within the cells, at pH 8.0 (Fig. 3B) indicates that the accumulated carbon occurred in two distinct fractions. One fraction consisted of acid-stable products of photosynthesis, whereas the other was acid-labile and represents an inorganic carbon pool. The increase in acid-stable carbon was linear with time and no detectable lag in CO_2 fixation

Table II. *The Effect of pH on Some Kinetic Parameters of Whole-Cell Photosynthesis*

pH of Medium	Γ (CO_2)[a]	Γ (DIC)	$K_{1/2}$ (CO_2)	$K_{1/2}$ (DIC)	K_m (CO_2) RuBP Carboxylase[b] / $K_{1/2}$ (CO_2) photosynthesis
	$\mu l\ l^{-1}$		μM		*ratio*
7.0	49.0 ± 0.9	267.0 ± 4.8	15.5	84.5	1.67
8.0	52.8 ± 1.8	2,409.0 ± 8.7	30.2	1,372.7	0.83

[a] At Γ, the O_2 concentration in the buffer was equivalent to air containing 25% O_2 (pH 7.0) and 28% O_2 (pH 8.0).

[b] A value of 25 μM CO_2 was used for the K_m (CO_2) of RuBP carboxylase from soybean at 25°C (16).

was observed under these conditions (Fig. 3B). The uptake of inorganic carbon has two phases, an initial rapid uptake followed by one which closely parallels the increase in acid-stable carbon. An acid-labile inorganic carbon pool is quickly established, the ratio of internal to external inorganic carbon being 2 in this experiment.

Similar experiments conducted at pH 7.0 (Fig. 3A) showed that the time course of carbon uptake is similar to that observed at pH 8.0 (Fig. 3B). The increase in acid-stable carbon was linear with time and no lag in fixation was observed. The magnitude of the inorganic carbon pool was, however, higher than at pH 8.0 and was 5.8 times the external DIC concentration. The initial rates of carbon uptake, computed over the first 15 s of these experiments were 6.8 μmol C mg^{-1} Chl min^{-1} at pH 7.0 and 2.3 μmol C mg^{-1} Chl min^{-1} at pH 8.0.

The intracellular pH (pH_i) of isolated *Asparagus* cells calculated on the basis of CO_2 distribution between the medium and the intracellular pool is shown in Figure 4 for five values of pH_e. For comparative purposes, the pH_i obtained from DMO distribution, which we have reported previously (12), is also shown. Although the pH_i values computed on the basis of CO_2 distribution show more variability than those obtained with DMO (12), it is clear that the pH_i values obtained from CO_2 distribution are considerably higher than those which would be predicted by the passive distribution of CO_2 between the cells and the medium, except at pH 8.0. The intracellular pools of inorganic carbon exceeded those predicted solely on the basis of CO_2 distribution following a pH gradient, by a factor of 2.8 at pH 8.0 (Fig. 3B) and 17.7 at pH 7.0 (Fig. 3A).

DISCUSSION

In this study, we have attempted to identify the species of inorganic carbon (CO_2, HCO_3^-) which crosses the plasmalemma of higher plant leaf mesophyll cells. The basis of these experiments is the model developed for algae, which possess a highly efficient active HCO_3^- transport mechanism (3, 9, 15, 19, 20). Following Raven's (24) general criteria, Figure 1 indicates that HCO_3^- transport by *Asparagus* cells is very unlikely since the observed rate of photosynthesis at pH 7.0 exceeds that observed in alkaline media in the presence of a constant concentration of CO_2. The decrease in photosynthetic rate in alkaline media has previously been correlated with an increase in the intracellular pH of the *Asparagus* cells and a change in the direction of the pH gradient between the cells and the medium (12).

Data comparing photosynthetic rates and the maximal CO_2 supply rate, in a closed system (Table I; Fig. 2), show that the observed rate of photosynthesis can be adequately explained on the basis of CO_2 supply and uptake and that mediated transfer of HCO_3^- across the plasmalemma need not be invoked. Indeed, the CO_2 supply rate could theoretically support rates of photosynthesis 5 to 8 times higher than those observed (Table I; Fig. 2A).

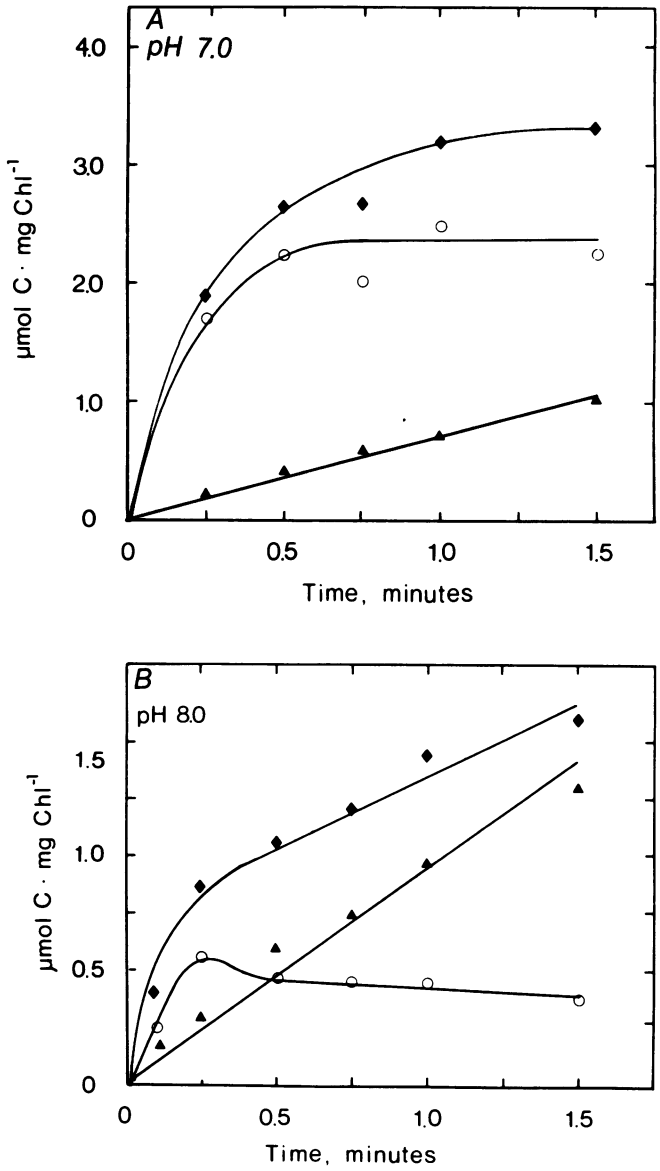

FIG. 3. Time course of inorganic carbon accumulation by illuminated *Asparagus* cells at pH 7.0 (A) and pH 8.0 (B) in 50 mM K^+ phosphate buffer containing an initial inorganic carbon concentration of 8.3 mM. Total carbon accumulated (◆); carbon assimilated into acid stable products (▲); and acid-labile inorganic carbon within the cell (○). Each point is the average of triplicate samples.

A consequence of the action of an efficient HCO_3^- transport mechanism in algae is that the inorganic carbon concentration around RuBP carboxylase/oxygenase is saturating except when the DIC concentration in the medium is extremely low. As a result algae have C_4-like photosynthetic characteristics such as very low Γ (CO_2) and Γ (DIC) values (5, 18), Γ shows little or no sensitivity to O_2 and temperature (5, 8, 18), there is little apparent O_2 inhibition of photosynthesis (8, 18) and photorespiration is minimal or absent (6, 18). The intervention of a HCO_3^- transport mechanism, presumably located at the plasmalemma, of *Coccochloris peniocystis* and *Anabaena variabilis* has a profound effect on the kinetics of whole-cell photosynthesis (3, 8) in comparison to the kinetics of isolated RuBP carboxylase/oxygenase (2, 14) obtained from these organisms. The ratio of the apparent K_m (CO_2) of RuBP carboxylase/oxygenase: $K_{1/2}$ (CO_2) of whole-cell photosynthesis (K_m [CO_2]:$K_{1/2}$[CO_2]) is approximately 860 and 975 for *Coccochloris* and *Anabaena*, respectively. If RuBP carboxylase/

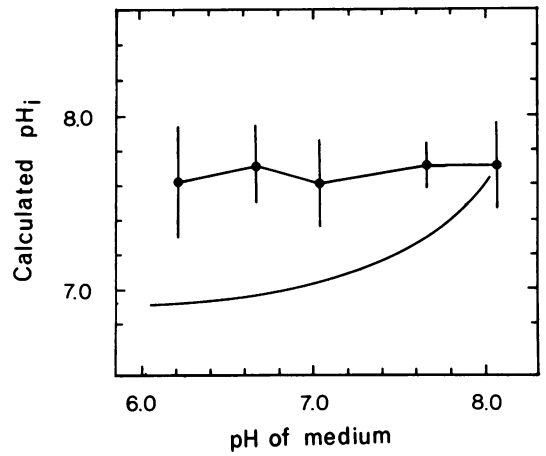

FIG. 4. Intracellular pH of *Asparagus* cells calculated from the distribution of CO_2 between the medium and the intracellular, acid-labile, inorganic carbon pool as a function of the pH of the medium (●). Experiments were conducted in CO_2-free (2% O_2) 50 mM K^+-phosphate containing 8.3 mM $NaHCO_3$ as described in "Materials and Methods." Incubation time was 45 s. Error bars represent the SD of four separate experiments each done in quadruplicate. The lower line represents the intracellular pH of *Asparagus* cells determined by 5,5-dimethyl[2-^{14}C] oxazolidine 2,4-dione distribution reported in reference (12).

oxygenase was the major determinant of the apparent CO_2 affinity of the cell during photosynthesis, a K_m (CO_2):$K_{1/2}$ (CO_2) ratio of 1 would be expected.

The $K_{1/2}$ (CO_2) of *Asparagus* cell photosynthesis (Table II) is similar to published values (*e.g.* 16) of the apparent K_m (CO_2) of C_3 RuBP carboxylase/oxygenase. Although the K_m (CO_2) of *Asparagus* RuBP carboxylase/oxygenase is not known at present, using a value of K_m (CO_2) of 25 μM (soybean 25C, 16) results in K_m (CO_2):$K_{1/2}$ (CO_2) ratio which deviates only slightly from 1 (Table II). This suggests that the kinetic properties of *Asparagus* cell photosynthesis are largely determined by the kinetic properties of RuBP carboxylase/oxygenase and indicate the absence of an intervening HCO_3^- transport mechanism, which would be expected to significantly increase the K_m (CO_2):$K_{1/2}$ (CO_2) ratio. Estimating $K_{1/2}$ (CO_2) of pea protoplast photosynthesis from CO_2 response curves given in Volokita *et al.* (28) (12–40 μM), result in a K_m (CO_2):$K_{1/2}$ (CO_2) ratio which ranges from 0.6–2.1.

Experiments with isolated soybean mesophyll cells (25) indicated that $K_{1/2}$ (CO_2), Γ (CO_2) and the magnitude of O_2 inhibition of photosynthesis decreased with increasing pH. However, in these experiments CO_2 equilibration between the aqueous and gaseous phases in the reaction vials was not taken into account (23). Subsequent work (23) has shown that the magnitude of O_2 inhibition of soybean cell photosynthesis increases with increasing pH, results which are in agreement with our present findings with *Asparagus* cells (Fig. 1).

In light of the other physiologic consequences of active HCO_3^- transport, it is very unlikely that *Asparagus* cells possess an efficient transport mechanism at the plasmalemma. The O_2 inhibition of photosynthesis (Fig. 1) and the high value of Γ (CO_2) and Γ (DIC) (indicating substantial photorespiration, Table II) show that the intracellular conditions are such that the oxygenase reaction of RuBP carboxylase/oxygenase is competing very favorably with the carboxylase reaction. This situation occurs when the intracellular concentration of CO_2 is low and O_2 is high. Thus, it is unlikely that there is a large intracellular pool of CO_2 available for fixation generated by an inorganic carbon accumulation mechanism.

If a HCO_3^- transport mechanism is present at the plasmalemma of *Asparagus* cells, the K_m must be considerably higher then 2,409 μl L^{-1} DIC (107.5 μM) at pH 8.0 (Table II) and similar to the $K_{1/2}$ (DIC) (1373 μM) of whole-cell photosynthesis (Table II). At pH 8.0, 98% of the DIC is HCO_3^-, yet the *Asparagus* cells are unable to carry out net photosynthesis in the presence of abundant HCO_3^- (Γ = 105.1 μM HCO_3^- + 2.4 μM CO_2; Table II). In contrast, at pH 7.0 and 54.5 μM DIC (18.3% is CO_2) net photosynthesis does occur (Fig. 1; Table I) and ceases only when the DIC concentration is 267 μl L^{-1} (11.9 μM, = 9.7 μM HCO_3^- + 2.2 μM CO_2): that is, when the equilibrium concentrations of CO_2 at pH 7.0 and pH 8.0, in a closed system, are approximately equal. Previously, we have shown (10) that at constant DIC concentration (350 μM), the rate of photosynthesis is drastically reduced at alkaline pH, where HCO_3^- is the predominant form of DIC.

The results of the inorganic carbon uptake experiments (Fig. 3) and determination of intracellular pH by CO_2 distribution (Fig. 4) are contradictory to the data presented in Figures 1 and 2 and Tables I and II in that they suggest that HCO_3^- is transferred across the plasmalemma, because the intracellular pool of inorganic carbon is considerably higher than that which would be expected from passive CO_2 distribution along a pH gradient. The inorganic carbon accumulated above the expected level implies the existence of a transport mechanism, as the accumulation of inorganic carbon would be against its electrochemical gradient (assuming an inside negative membrane potential). These data are consistent with the results of Volokita *et al.* (28). The pH_i determined for *Asparagus* cells (Fig. 4) and pea protoplasts (28), by CO_2 distribution, are similar in value, but, pH_i determined by DMO distribution (12, Fig. 4) is considerably lower in pea protoplasts and are similar to values obtained with *Acer pseudoplatanus* suspension culture cells (17).

The use of weak acids or bases as a chemical probe to determine pH_i assumes that the compound is in free aqueous solution within the cell (12, 17). Intracellular binding of a weak acid probe (*i.e.* CO_2, DMO) will result in a value of pH_i which over-estimates the actual pH_i. Binding of DMO in *Asparagus* cells has been shown to be minimal (12), but binding of CO_2 within the cell to such possible binding sites as, the α and ε amino groups of amino acids and peptides (4), the CO_2 activating site of RuBP carboxylase/oxygenase, and thylakoid membranes (26), may be considerable (4). In the absence of a reliable assay for bound inorganic carbon, the interpretation of inorganic carbon accumulation data and estimates of pH_i from CO_2 distribution must be made with caution and in conjunction with other data. Estimates of inorganic carbon pools presented here and elsewhere (3, 9, 15, 20, 28) do not take bound inorganic carbon into account and consequently may significantly over-estimate the amount of inorganic carbon which is free in aqueous solution and thus over-estimate the value of pH_i. This does not necessarily invalidate the conclusion that algae can actively transport HCO_3^-, inasmuch as other independent lines of evidence also support this conclusion. However, the data (Figs. 1 and 2; Tables I and II) for *Asparagus* cells strongly indicate that CO_2 is the only form of inorganic carbon which crosses the plasmalemma and consequently the evidence which is suggestive of HCO_3^- transport (Figs. 3 and 4) may be an artifact of the assay in this system.

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