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pH homeostasis of the chloroplast stroma can protect photosynthesis of leaves during the influx of potentially acidic gases

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Photosynthesis is sensitive to acidification of the chloroplast stroma. Accordingly, potentially acidic gases such as CO_2 and SO_2 can inhibit photosynthesis. Whereas CO_2-dependent inhibition was not reversed in time in isolated chloroplast, partial or full reversal was observed in isolated protoplasts. Reversal is caused by readjustment of the stroma pH which does not require light. After leaves were given time to compensate the acidification of the chloroplast stroma by a high concentration of CO_2, sudden removal of the CO_2 resulted in the alkalization of the stroma which was slowly reversed. Maximum rates of compensatory proton removal and proton production or uptake were 10 and 5 μequiv. (mg chlorophyll)$^{-1} \cdot h^{-1}$, respectively. The results are discussed in relation to the impact on leaves of atmospheric pollution with potentially acidic gases.

Introduction

In photosynthesis, light is used to transfer electrons from water to an intermediary acceptor, $NADP^+$. Simultaneously, ATP is synthesized. Reduced $NADP^+$ and ATP reduce CO_2 to the sugar level in a complicated set of enzymic reactions commonly described as the Calvin cycle or photosynthetic carbon cycle. Some of the enzymes involved in the carbon cycle are sensitive to changes in pH. This is particularly true for fructose bisphosphatase and sedoheptulose bisphosphatase, which are localized in the chloroplast stroma. These enzymes are subject to light regulation [1]. When activated by the chloroplast thioredoxin system, they are catalytically active at pH 8, but not at pH 7 [2].

The substrate of photosynthesis, CO_2, can react with water. The hydration product is an acid. Acids inactivate photosynthesis if their concentration is high enough to decrease the pH of the chloroplast stroma. As a matter of fact, it has been shown that photosynthesis of chloroplasts and mesophyll protoplasts is inhibited by high concentrations of CO_2 [3,4]. Loss of photosynthesis in high CO_2 is caused by inhibition of fructose bisphosphatase and possibly also of sedoheptulose bisphosphatase [5]. In air, the concentration of CO_2 is low. Under these conditions, CO_2 does not decrease the cytoplasmic pH. However, CO_2 is not the only potentially acidic component of the atmosphere which is capable of penetrating biomembranes and of acidifying alkaline compartments such as the chloroplast stroma. In 1982, the burning of fossil fuels caused in the F.R.G. the release of $3 \cdot 10^6$ tons of SO_2 into the atmosphere [6]. This corresponds to an average of more than 180 mmol $SO_2 \cdot m^{-2}$ soil area. The release of nitrogen oxides was even higher than that of SO_2. After having entered the intercellular space of leaves, these gases diffuse across the plasmalemma of leaf cells and are hydrated in the cytoplasm [7]. As the hydration products are acids, acidification could endanger photosynthesis. In spinach, generation of about 1 mmol $H^+ \cdot m^{-2}$ leaf area in the cytoplasm of mesophyll cells would effectively inhibit photosynthesis by acidification if protons could not be removed by neutralization or transfer to compartments which tolerate acidity [8]. The aim of the present work is to investigate whether and to what extent green leaf cells are capable of countering effects of acidification which threaten photosynthetic productivity.

Materials and Methods

Spinach was grown in a greenhouse and barley and sunflowers in climatic chambers. Chloroplasts were isolated from spinach leaves by a procedure similar to that

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described in Ref. 9. The percentage of chloroplasts which had retained their envelopes during isolation was determined by the ferricyanide method [10]. Photosynthetic oxygen evolution was recorded in the electrode described in Ref. 11. The isotonic reaction medium was adjusted to pH 7.3, and catalase was added [12]. Bicarbonate concentrations were as indicated. The pH increase caused by bicarbonate was compensated by acid. Very similar experiments were performed with mesophyll protoplasts which had been isolated from young barley leaves [13]. The reaction medium was adjusted to pH values between 5 and 7, and bicarbonate was added to produce CO_2 concentrations as indicated. The pH increase caused by bicarbonate was measured and taken into account to calculate CO_2 concentrations.

After sunflower leaves were gassed with air containing different CO_2 concentrations, they were transferred to CO_2-free air, and CO_2 liberated from the leaves was measured by infrared gas analysis [14]. In order not to overload the gas analyzer, a suitable dilution system had to be used after a leaf had been loaded with 9.75% CO_2. In this case, measuring times were long (3 min). After loading a leaf with 0.2% CO_2, no dilution was necessary, and the main part of the CO_2 was evolved after transfer to CO_2 free air within less than 20 s [14].

Results and Discussion

Fig. 1 shows photosynthetic oxygen evolution from CO_2 of isolated spinach chloroplasts. The chloroplasts were first illuminated with rate-saturating light in the presence of 2 mM bicarbonate. At pH 7.3, the equilibrium concentration of CO_2 is 160 μM, or somewhat less than 0.5% in the gas phase, if the pK of CO_2 is 6.2 [15]. After a characteristic lag phase during which light-regulated enzymes are photo-activated and pools of photosynthetic intermediates are filled [16], oxygen was evolved at a steady rate. Addition of more bicarbonate not only did not increase the rate of oxygen evolution but actually decreased photosynthesis. At a bicarbonate concentration of 40 mM (equilibrium concentration of CO_2 about 9% in the gas phase), inhibition of photosynthesis was essentially complete (Fig. 1A). Once photosynthesis was inhibited, it could be restarted by increasing the pH, i.e., by decreasing the CO_2 concentration (Fig. 1B). Therefore, inhibition could not be attributed to osmotic action of the added bicarbonate (see also Ref. 17). Rather, it was caused by the acidification of the stroma pH which was produced by high CO_2 concentrations. When the bicarbonate concentration (and therefore also the CO_2 concentration) is low, the stroma pH of illuminated chloroplasts is higher than the pH of the medium outside the chloroplasts [18]. This is explained by the light-dependent pumping of protons not only from the stroma into the intrathylakoid space but also from the stroma into the medium [19]. This maintains a proton gradient across the chloroplast envelope. However, the photosynthesis inhibition by high CO_2 shown in Fig. 1 indicates that proton extrusion from the chloroplasts is not effective enough to readjust the stroma pH to a value suitable for photosynthesis, once it has decreased so as to inhibit photosynthesis.

However, isolated protoplasts are capable of compensating acidification by CO_2. This is shown in Fig. 2. The experiment was similar to that performed with isolated spinach chloroplasts. When protoplasts of barley mesophyll cells were illuminated with sufficient bicarbonate to adjust the concentration of CO_2 in the reaction mixture to 200 μM CO_2 (corresponding to about 0.6% CO_2 in the gas phase), respiratory oxygen uptake was replaced by photosynthetic oxygen evolution. When bicarbonate was added to increase the concentration of CO_2 to 3.79 mM (corresponding to about 11% CO_2 in the gas phase, a concentration sufficient to

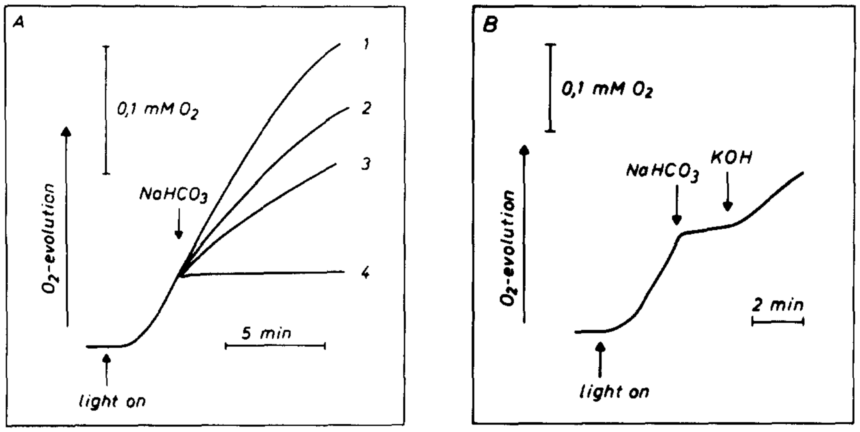

Fig. 1. Assimilatory O_2 evolution of isolated spinach chloroplasts and inhibition of photosynthesis by excess CO_2. The initial CO_2 concentration was 160 μM. (A) CO_2 levels were increased by adding bicarbonate as constant pH (7.3). Bicarbonate additions (1) 0 mM (2) 10 mM (3) 20 mM and (4) 40 mM. 40 mM bicarbonate corresponds to about 9% CO_2 in air. (B) Addition of $NaHCO_3$ inhibited photosynthesis, and KOH relieved inhibition by decreasing the CO_2 concentration according to $CO_2 + OH^- \rightarrow HCO_3^-$.

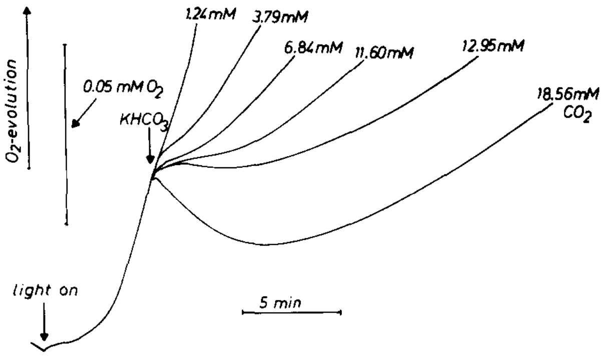

Fig. 2. Assimilatory oxygen evolution of isolated barley protoplasts. The initial CO_2 concentration was 200 μM. The transient inhibition caused by addition of $KHCO_3$ is partially reversed within a few minutes. The time-span of recovery as well as its extent depend on the final CO_2 concentration which is indicated.

inhibit photosynthesis of isolated chloroplasts completely, see Fig. 1A), photosynthesis decreased fairly rapidly, but recovered later partially. Inhibition increased as the CO_2 concentration was increased. After raising the concentration of CO_2 to 18.56 mM (corresponding to about 54% CO_2 in the gas phase), inhibition of photosynthesis was essentially complete initially. However, appreciable recovery was observed later on, even under these conditions. After not much more than 8 min exposure of the protoplasts to the high concentration of CO_2, photosynthesis had reached a steady, although reduced, rate. The experiments show that protoplasts, but not isolated chloroplasts, are capable of compensating imposed acidification rapidly. Apparently, cytosolic reactions are involved in the readjustment of the pH of the chloroplast stroma. However, incomplete recovery of photosynthesis at high CO_2 concentrations indicates that there are limits to the extent of readjustment. Excessive acidification cannot be fully compensated.

The interpretation of the photosynthesis inhibition shown in Fig. 2 as being caused by acidification of the stroma pH, and of the recovery of photosynthesis as being due to a subsequent increase in the stroma pH, is indirect. It is based on earlier work on the importance of the stroma pH for photosynthesis of isolated chloroplasts [3,5,20]. More direct evidence is required. If the pH of the chloroplast stroma is really increased in protoplasts (Fig. 2), but not in chloroplasts (Fig. 1), after high CO_2 concentrations have caused acidification, solubilization of CO_2 should also increase. In C_3 plants such as spinach, barley or sunflower, the chloroplast stroma contains carbonic anhydrase which catalyzes rapid solubilization of CO_2 according to

$$CO_2 + OH^- \Leftrightarrow HCO_3^- \qquad (1)$$

At a low pH, Eq. 1 is shifted to the left, and at a high pH to the right. Accordingly, chloroplasts freed of CO_2 and bicarbonate should after admission of CO_2 absorb more CO_2 when the stroma pH is high than when it is low. Likewise, more CO_2 should be released into a CO_2-free medium from chloroplasts containing CO_2 and bicarbonate in equilibrium according to Eqn. 1, if the stroma pH is high than when it is low. In the experiment of Fig. 3 which is represented by the lower, 'stress' curve, a leaf of *Helianthus annuus* whose stomata had been opened in a CO_2-free atmosphere was gassed for 30 s in the dark with air containing 9.75% CO_2. This was sufficient to equilibrate the chloroplasts in the mesophyll with the high atmosphere CO_2 concentration. Subsequently, the leaf was switched back for 3 min to a CO_2-free atmosphere, and the CO_2 liberated from the leaf into this atmosphere was measured by infra-red gas analysis. About 140 nmol CO_2/cm^2 leaf area were released. The leaf was then exposed again to the high CO_2 concentration, and the procedure was repeated after different times of exposure to high CO_2. Fig. 3 shows that solubilization of CO_2 increased with time until it was more than doubled after only 20 min of exposure to 9.75% CO_2. It should be noted that the 3

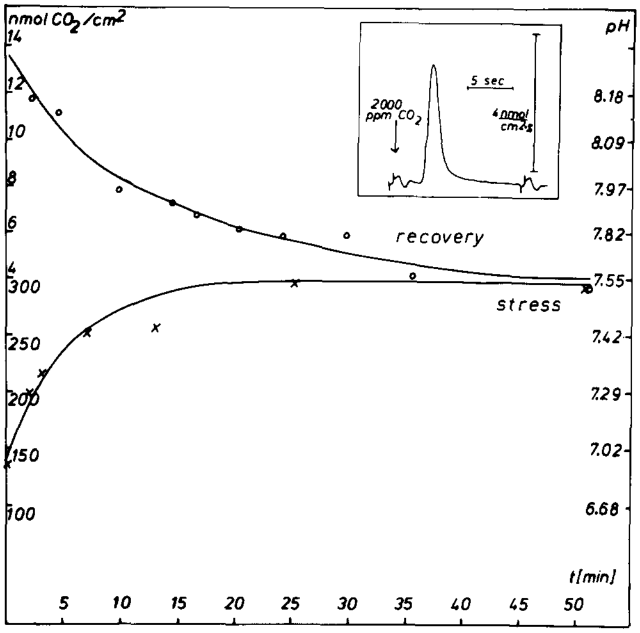

Fig. 3. Release of CO_2 from a sunflower leaf into CO_2-free air after the leaf had taken up CO_2 from air containing either 9.75 or 0.2% CO_2. Left ordinate: CO_2 release in nmol·cm^{-2} leaf area; right ordinate: calculation of the pH of the chloroplast stroma from the amount of CO_2 released. In the stress experiment, which involved measuring CO_2 release over a time-span of 3 min, CO_2 originating from cytosolic bicarbonate was included in the calculation. In the recovery experiment, measuring times were short and cytosolic bicarbonate was not considered. For further details see text. Lower curve: CO_2 released after uptake from air containing 9.75% CO_2 as a function of exposure time to 9.75% CO_2. Upper curve: the leaf was first exposed to 9.75% CO_2 for 30 min. At zero time, the leaf was switched to a CO_2-free atmosphere. At the times indicated by experimental points, the leaf was exposed to 0.2% CO_2 in air for 30 s. Subsequently, CO_2 solubilized in the leaf during this time was released into CO_2-free air and measured. Inset: Kinetics of CO_2 solubilization in a leaf. Unloading is the mirror image of solubilization.

min periods necessary for diluting and measuring CO_2 liberated from the leaf may have affected adaptation of the leaf to the high CO_2 concentration, as it finds expression in the increase of solubilization of CO_2. During this time, the leaf was in an atmosphere devoid of CO_2. If such long periods of interrupting acidification had not been made necessary by the experimental set-up, solubilization would have increased more than it actually did.

In a second experiment (upper curve in Fig. 3), a leaf was first stressed in the dark with 9.75% CO_2 for 30 min. Subsequently, the leaf was gassed with CO_2-fee air. At the times indicated by experimental points in the figure, CO_2-free air was replaced for 30 s by air containing 0.2% CO_2. This was sufficient to dissolve CO_2 in the leaf and to equilibrate the chloroplasts with bicarbonate. After 30 s solubilization, the leaf was switched back to CO_2-free air and the CO_2 liberated from the leaf was measured. Kinetics of solubilization are shown in the inset of Fig. 3. The kinetics of CO_2 release are the mirror image of those of loading with CO_2. The procedure of loading and unloading was repeated several times during adaptation of the acid-stressed leaf to the CO_2-free atmosphere. In this way changes in solubilization of CO_2 could be measured as a function of time. During recovery from acid stress, CO_2 solubilization decreased. After 40 min gassing with CO_2-free air it was smaller by a factor of about 3 than immediately after stress was relieved.

The data of Fig. 3 can be used to calculate the pH of the chloroplast stroma [21]. Calculation according to the Henderson-Hasselbalch equation.

$$\mathrm{pH} = \mathrm{p}K + \log\frac{[\mathrm{HCO_3^-}]}{[\mathrm{CO_2}]} \qquad (2)$$

requires knowledge of the concentrations of CO_2 and bicarbonate in the chloroplast stroma. The solubility of CO_2 in aqueous solutions is known. The volume of the aqueous phase of the *Helianthus* leaf was 20 $\mu l \cdot cm^{-2}$ leaf area. The stroma volume was 1.32 $\mu l \cdot cm^{-2}$, if it is assumed that chloroplasts containing 1 mg chlorophyll occupy a volume of 40 μl [14,21]. The volume of the cytosol was assumed to be similar to the chloroplast volume. The CO_2 liberated rapidly from the leaf during degassing consisted mainly of CO_2 previously dissolved in the aqueous phase and of bicarbonate which had been in contact with carbonic anhydrase, i.e., in the stroma. Bicarbonate dissolved in the cytosol and, to a much smaller extent, in the slightly acidic vacuole was released only slowly during degassing. Fast and slow release could be distinguished kinetically [14]. Calculation of the CO_2-release recorded in Fig. 3 reveals a stroma pH of 7.0 shortly after the leaf was transferred from air to 9.75% CO_2. With increasing exposure time to 9.75% CO_2 the stroma pH recovered from the decrease it had suffered during the transition from air to an atmosphere loaded with 9.75% CO_2. After about 20 min exposure to the high CO_2 concentration, the calculated pH of the stroma was 7.55. Thus, the acid stress produced by 9.75% CO_2 decreased the stroma pH transiently by about 0.6 pH units. This acidification could be reversed by a reaction which appeared to be triggered by acid stress.

When CO_2 was released from an acid-stressed leaf which had been permitted to adjust to the stress, the expected over-shoot in pH was observed. Calculation of the CO_2 liberated shortly after acid stress and after the leaf had been briefly loaded with 0.2% CO_2 revealed a pH of about 8.2 (Fig. 3). 40 min later the calculated pH was only 7.56. In this case, the pH decreased by 0.65 pH units after termination of the acid stress which had induced an alkalization response.

In addition to permitting calculations of the stroma pH, the data of Fig. 3 also give information on the buffering capacity of chloroplasts. In the stress experiment of Fig. 3, the calculated bicarbonate concentration was 27 mM in the chloroplast stroma ($\mathrm{p}K_a(\mathrm{CO_2}) = 6.1$ because of the high ionic strength [15]) which had a pH of 7.0 immediately after uptake of CO_2 had caused acidification. The corresponding CO_2 concentration was 3.34 mM (22.5 °C). Thus, about 27 μmol H^+ had been consumed per ml stroma to decrease its pH from about 7.55 to 7.0, i.e., by 0.55 pH units. This corresponds to a buffering capacity of about 50 mM/pH unit. However, this figure includes the buffering capacity of the bicarbonate buffer which had been introduced into the leaf during acidification. The buffering capacity of the HCO_3^-/CO_2 system is defined by

$$\beta = \frac{2.3[\mathrm{HCO_3^-}][\mathrm{CO_2}]}{[\mathrm{HCO_3^-}]+[\mathrm{CO_2}]} \qquad (3)$$

The corresponding value at pH 7.0 is about 7 mM/pH unit. It must be subtracted from the calculated value of 50 mM/pH unit so that the corrected chloroplast value is 43 mM/pH unit. In comparison, direct titration of chloroplasts isolated from barley leaves has yielded a buffering capacity of the chloroplasts of about 45 mM/pH unit [8]. Knowledge of the buffering capacity permits calculation of the maximum rate of proton compensation in the chloroplast stroma after acidification (Fig. 3, lower curve) to about 10 μequiv. H^+/mg chlorophyll per h and of the proton production which compensates the alkaline overshoot documented by excessive solubilization after acid stress had been relieved (Fig. 3, upper curve), which was about half that figure.

Comments

Four aspects of this work require comment.

(i) For purely experimental reasons, we have used chloroplasts of spinach, protoplasts of barley and leaves of sunflower to demonstrate effects of acidification by high concentrations of CO_2. SO_2 was not used in this work because it is highly reactive. We wished to separate acidification from other effects of potentially acidic gases such as SO_2 which may interfere with or obscure acidification. Qualitative experiments with spinach leaves resulted in observations which were similar to those made with sunflower leaves. The data shown in Fig. 3 are therefore of general significance. However, preliminary data indicate that the capacity for adaptation is different in different plant species.

(ii) Measurements of the capacity for photosynthesis of leaves are often performed in a closed system with an oxygen electrode [22,23]. If no gaseous CO_2 is available, a small volume of 1 M $NaHCO_3$ or $KHCO_3$ is recommended as a source of CO_2 [23]. Depending on temperature, the CO_2 concentration of the atmosphere in equilibrium with 1 M bicarbonate may be 12% or more [24]. At this concentration of CO_2, photosynthesis is partially inhibited in sensitive plant species. The danger of acidification can be minimized by using carbonate/bicarbonate mixtures as a source of CO_2 [24] when gaseous CO_2 of the desired concentration is unavailable.

(iii) At a buffer capacity of about 43 mM/pH unit, the acid-induced pH increase in the chloroplast stroma of about 0.5 pH units (Fig. 3, lower curve) and the readjustment of pH by about 0.55 pH units (Fig. 3, upper curve) correspond to a production of base or the removal of acid of about 0.9 μequiv.$\cdot$(mg chlorophyll)$^{-1}$ in the stroma. Reduction of nitrate at the expense of respiratory sugar degradation produces base according to

$$C_6H_{12}O_6 + 2O_2 + 2NO_3^- \rightarrow 6CO_2 + 2NH_4^+ + 4OH^- \qquad (3)$$

Alternatively, acidification by an inorganic acid can be compensated by degradation of an organic acid. A dominant organic acid is malic acid. It is known to play an important role in cellular pH control [25]. However, neither reduction of cytoplasmic nitrate nor the degradation of cytoplasmic malic acid would be sufficient to explain the observed pH shifts in the chloroplast stroma. Moreover, preliminary data on changes in the malate and nitrate content of leaves during short-term adaptation to acid stress do not support the notion that alteration of these solutes is significant in relation to the observed pH shifts. The most likely alternative is removal of H^+ by transport. As a matter of fact, proton pumping is known to occur at the level of the chloroplast envelope [19], the tonoplast and the plasmalemma [26].

(iv) As has already been mentioned in the Introduction, human activity causes massive release of SO_2 and nitrogen oxides into the atmosphere. Insofar as these potentially acidic gases are not neutralized in the atmosphere or washed out by rain, they enter leaves and are absorbed in the mesophyll. As concentrations in air are low, diffusion gradients are small and fluxes correspondingly slow. The fast compensation of the massive proton production in the chloroplast stroma which is produced by loading a leaf with a high CO_2 concentration shows that the immediate danger of acidification by the slow intake of air pollutants is negligible. However, the incomplete recovery of photosynthesis after acidification shown in Fig. 2 indicates that the capacity of the leaf for absorbing protons is limited. When the accumulation of protons in the leaf during prolonged exposure to atmospheric pollutants surpasses the capacity for proton storage or proton neutralization, damage is unavoidable.

Acknowledgments

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References

1 Buchanan, B.B. (1980) Annu. Rev. Plant Physiol. 31, 341–374.
2 Baier, D. and Latzko, E. (1975) Biochim. Biophys. Acta 396, 141–148.
3 Werdan, K., Heldt, H.W. and Milovancev, M. (1975) Biochim. Biophys. Acta 283, 430–441.
4 Kaiser, G. and Heber, U. (1983) Planta 157, 462–470.
5 Enser, U. and Heber, U. (1980) Biochim. Biophys. Acta 592, 577–591.
6 Kalmbach, S. (1985) in Die Sache mit dem Wald. Aktuelles Waldwissen der Allg. Forst-Zeitschrift (Bauer, F., eds.), pp. 162–163, BLV-Verlagsgesellschaft, Munich.
7 Pfanz, H., Martinoia, E., Lange, O.L and Heber, U. (1987) Plant Physiol. 85, 922–927.
8 Pfanz, H. and Heber, U. (1986) Plant Physiol. 81, 597–602.
9 Jensen, R.G., and Bassham, J.A. (1966) Proc. Natl. Acad. Sci. USA 56, 1095–1101.
10 Heber, U. and Santarius, K.A. (1970) Z. Naturforsch. 25b, 718–728.
11 Delieu, T. and Walker, D.A. (1972) New Phytol. 71, 201–225.
12 Heber, U., Neimanis, S. and Dietz, K.J. (1988) Planta 173, 267–274.
13 Kaiser, G., Martinoia, E. and Wiemken, A. (1982) Z. Pflanzenphysiol. 107, 103–113.
14 Laisk, A., Oja, V., Kiirats, O., Raschke, K. and Heber, U. (1989) Planta 177, 350–358.
15 Yokota, A. and Kitaoka, S. (1985) Biochem. Biophys. Res. Commun. 131, 1075–1079.
16 Edwards, G. and Walker, D.A. (1983) C_3, C_4; Mechanisms, and Cellular and Environmental Regulation, of Photosynthesis. Blackwell, London.
17 Kaiser, W.M. and Heber, U. (1981) Planta 153, 423–429.
18 Heldt, H.W., Werdan, K., Milovancev, M. and Geller G. (1973) Biochim. Biophys. Acta 314, 224–241.
19 Heber, U. and Krause, G.H. (1971) in Photosynthesis and Photorespiration (Hatch, M.D., Osmond, C.B. and Slatyer, R.O., eds.), pp. 218–225, Wiley Interscience, New York.

20 Purczeld, P., Chon, C.J., Portis, A.R., Heldt, H.W. and Heber, U. (1978) Biochim. Biophys. Acta 501, 488–498.

21 Oja, V., Laisk, A. and Heber, U. (1986) Biochim. Biophys. Acta 849, 355–365.

22 Delieu, T. and Walker, D.A. (1981) New Phytol. 89, 165–175.

23 Walker, D.A. (1987) The Use of the Oxygen Electrode and Fluorescence Probes in Simple Measurements of Photosynthesis, The University of Sheffield, Sheffield.

24 Umbreit, W.W., Burris, R.H. and Stauffer, J.F. (1957) Manometric Techniques, Burgess, Minneapolis.

25 Raven J.A. (1986) New Phytol. 104, 175–206.

26 Sze, H. (1985) Annu. Rev. Plant Physiol. 36, 175–208.