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pH and buffer capacities of apoplastic and cytoplasmic cell compartments in leaves

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Abstract. After opening the stomata in CO_2-free air, darkened leaves of several plant species were titrated with CO_2 at concentrations between 1 and 16%, in air in order to reversibly decrease cellular pH values and to calculate buffer capacities from pH changes and bicarbonate accumulation using both gas-exchange and fluorescence methods for analysis. After equilibration with CO_2 for times ranging between 4.4 and 300 s, fast CO_2 release from bicarbonate indicated catalysis by highly active carbonic anhydrase. Its time constant was below 2.5 s. Additional CO_2 was released with time constants of about 5, 15 and approximately 300 s. With CO_2 as the acidifying agent, calculated buffer capacities depend on assumptions regarding initial pH in the absence of an acid load. At an initial stroma pH of 7.7, the stromal buffer capacity was about 20 mM pH-unit^{-1} in darkened spinach leaves. At an initial pH of 7.5 it would be only 12 mM pH-unit^{-1}, i.e. not higher than expected solely on the basis of known stromal concentrations of phosphate and phosphate esters, disregarding the contribution of other solutes. At a concentration of 16%, CO_2 reduced the stromal pH by about 1 pH unit. Buffering of the cytosol was measured by the CO_2-dependent quenching of the fluorescence of pyranine which was fed to spinach leaves via the petiole. Brief exposures to high CO_2 minimized interference by effective cytosolic pH regulation. Cytosolic buffering appeared to be similar to or only somewhat higher than chloroplast buffering if the initial cytosolic pH was assumed to be 7.25, which is in accord with published cytosolic pH values. The difference from chloroplast pH values indicates the existence of a pH gradient across the chloroplast envelope even in darkened leaves. Apoplastic buffering was weak as measured by the CO_2-dependent quenching of dextran-conjugated fluorescein isothiocyanate which was infiltrated together with sodium vanadate into potato leaves. In the absence of vanadate, the kinetics of apoplastic fluorescence quenching were more complex than in its presence, indicating fast apoplastic pH regulation which strongly interfered with the determination of apoplastic buffering capacities. At an apoplastic pH of 6.1 in potato leaves, apoplastic buffering as determined by CO_2 titration with and without added buffer was somewhat below 4 mM pH-unit^{-1}. Thus the apoplastic and cytosolic pH responses to additions of CO_2 indicated that the observed cytoplasmic pH regulation under acid stress involves pumping of protons from the cytosol into the vacuole of leaf cells, but not into the apoplast.

Key words: Apoplast – Buffer capacity – Chloroplast – Cytosol – pH regulation

Introduction

A main function of leaves is photosynthesis. Photosynthetic electron transport is coupled to vectorial proton transport. The resulting pH gradient across thylakoid membranes drives chloroplast ATP synthesis. In other biomembranes such as the plasmalemma and the tonoplast ATP is used to energize transport of protons from one to another cellular compartment. Established pH gradients are used as a source of energy for transmembrane ion and metabolite transport. Thus, proton fluxes are bound to influence cellular pH values (Kurkdjian and Guern 1989). In this situation, functional metabolism requires cytoplasmic pH regulation. Enzymes such as phosphoenolpyruvate carboxylase or nitrate and nitrite reductases are known to be involved in a biochemical pH-stat. However, a more direct way of pH regulation is either to actively export excess protons

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Abbreviations: FITC = fluorescein isothiocyanate; NMR = nuclear magnetic resonance

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from the cytoplasm of compartmented cells into acidic external or internal spaces such as the apoplast or vacuoles, or to import protons from outside. In leaves, transport of protons across biomembranes appears to be the dominant mode of pH regulation under acid stress (Heber et al. 1994; Bligny et al. 1997). Under strong acid loads, surprisingly fast pH regulation was observed. It compensated for the acidification of the chloroplast stroma only in intact leaves, but not in isolated chloroplasts (Wagner et al. 1990). Calculated buffer capacities were unusually high, suggesting very fast export of excess protons out of acidified chloroplasts, particularly in the light (Hauser et al. 1995a,b). However, controversy exists regarding the measurement of buffer capacities in different cellular compartments of plant cells (Schönknecht and Bethmann 1998; Frohnmeyer et al. 1998). Chemical buffering by cellular solutes needs to be distinguished from the apparent buffering caused by proton transfer across biomembranes. Correct values for chemical buffering must be known for a proper calculation of rates of transmembrane proton fluxes under acid stress.

In the present work, we were trying to improve the gas-exchange method used earlier (Oja and Laisk 1995a,b; Hauser et al. 1995a,b) to measure pH, and to obtain reliable information on chemical buffering in the chloroplast stroma of intact leaves. In addition, we wished to extend information on pH and buffering also to the extrachloroplastic cytoplasm and to the apoplast because regulatory responses are expected to be triggered by acid loads in leaves not only in the chloroplasts but also in other cellular compartments.

We also wished to know whether protons are pumped not only into vacuoles but also into the apoplast of leaves during cytoplasmic pH regulation under acid stress. As before, we used CO_2 as acidifying agent in intact leaves. At concentrations of CO_2 in air between 1 and 16% we measured deflections of cellular pH values from their steady states in vivo. For the measurement of chemical buffering, we tried to minimize interference by active cellular pH regulation.

Material and methods

Plants. *Spinacia oleracea* L. cv. Polka F1 RS (Wagner, Heidelberg, Germany), *Solanum tuberosum* L. cv. Desirée (Fritz Lange Saatzucht, Bad Schwartau, Germany) and *Helianthus annuus* L. (bird feed, from a local source) were grown in a greenhouse in a day/night cycle of 11/13 h. Leaves of *Mercurialis perennis* L. were from a shaded site in a local beech forest.

Carbon dioxide-solubilization experiments. Mature leaves were used for CO_2 titrations with concentrations of CO_2 up to 16% in air. A specially constructed apparatus was used to minimize time delays (Oja and Laisk 1995a,b; Laisk and Oja 1998). Attached or detached leaves were cut so that they fitted into the inside of a $4.4 \times 4.3 \times 0.3$ cm^3 square cuvette. Only the petiole extended to the outside to facilitate uninterrupted water supply. Effective gas exchange was possible through open stomata on both sides of amphistomatal leaves and also through cut surfaces. The cuvette was connected to either one of two open gas channels so that by switching from one to the other channel the gas composition in the cuvette could be changed very rapidly. Gas flow rates were 0.87 l min^{-1}. One of the channels was connected to an oxygen analyzer (Ametek S-3a; Process & Analytical Instruments Division, Pittsburgh, Penn., USA) and a CO_2/H_2O analyzer (Li 6262; LiCor, Lincoln, Neb. USA) as described in detail by Oja and Laisk (1995a), whereas the other channel served to load the cuvette with concentrations of CO_2 between 1 and 16% for times ranging between 4.5 and 300 s. As CO_2 entered the cuvette with the gas stream in the beginning of a CO_2-loading experiment, it dissolved in the different leaf cell compartments both as CO_2 and, after hydration and dissociation of the resulting carbonic acid, as HCO_3^-. The resulting transient decrease in its concentration in air produced a corresponding increase in the concentration of oxygen which could measured by the oxygen analyzer. Desolubilization of the CO_2 upon switching the gas stream to CO_2-free air during an unloading experiment released CO_2 thereby decreasing the oxygen concentration. This was also measured by the oxygen analyzer (Hauser et al. 1995a). As the initially rapid CO_2 release became slower during unloading experiments, the less-sensitive indirect recording of CO_2-dependent changes by the oxygen concentration was replaced by infrared recording of CO_2 (cf. Fig. 1). Details of the measurements during CO_2 titrations and of data processing have been reported by Oja and Laisk (1995b). After the experiments, water contents were determined and related to leaf area by weighing the leaves before and after drying. Before and during CO_2 titration experiments, care was taken to ensure that stomata were sufficiently open. They were opened in a CO_2-free atmosphere either in the dark or under illumination. Transpiration was measured as described in Hauser et al. (1995a).

Monitoring changes in compartmental pH values in intact leaves by fluorescence. The fluorescent pH indicator pyranine, a marker emitting fluorescence mainly from the cytosol and the nuclei, was fed to excised leaves via the petiole. The pyranine concentration was 1 mM. Feeding times of less than 1 h were sufficient when open stomata permitted transpiration. To remove pyranine from the apoplast, pyranine solution was either replaced by water for several hours, or the leaves were infiltrated with water and the infiltrate was removed by centrifugation for 5 min at 450 *g*. Gas flow, fluorescence excitation and emission were as described in Yin et al. (1990). The dextran conjugate of fluorescein isothiocyanate (FITC; MW 4 kDa), was infiltrated into the apoplast of leaves using the technique described by Jakob and Heber (1998). Excess infiltrate was removed by centrifugation. Fluorescence of FITC was excited

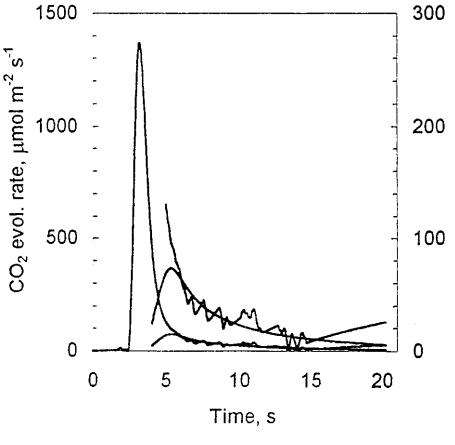

Fig. 1. Rates of CO_2 release from a spinach leaf after loading the leaf with 16% CO_2 in air for 20 s. Indirect recordings by an oxygen analyzer (large peak, left side of figure, *left ordinate*; or noisy line, upper right-hand recordings, *right ordinate*) and direct measurements by the infrared recording of a CO_2 analyzer (tail of large peak, *left ordinate*; or drawn line, upper right hand recordings, *right ordinate*). The CO_2 analyzer was activated 2 s after starting CO_2 release

through an 489-nm interference filter (Schott, Mainz, Germany). The half-bandwidth of transmission was 11 nm. Additional filters were Nos. 9782 and 5030 from Corning Glass Works (Corning, N.Y., USA). The photon-flux density of the exciting beam was 2 μmol m^{-2} s^{-1}. Fluorescence emission was measured after passage through two No. 9782 filters (Corning Glass Works) an OG 515 cutoff filter (Schott) and a K55 broad-band interference filter (Balzers, Liechtenstein). The half-bandwidth of transmission was between 518 and 533 nm. After the CO_2 titrations, dye fluorescence was fully quenched in order to obtain corrrect base lines. Quenching was achieved by exposing the leaf to acetic acid vapor. Dyes were obtained from Molecular Probes (Eugene, Ore., USA) and from Sigma.

Results

Solubilization and unloading of CO_2 as measured by gas exchange. Figure 1 shows traces of the rate of CO_2 release into CO_2-free air from a spinach leaf as recorded indirectly by the oxygen analyzer and directly by the CO_2 analyzer. Before loading, the stomata of the leaf had been opened by illuminating the leaf in air under saturating illumination. Before starting the unloading of CO_2 into CO_2-free air, the spinach leaf had been loaded with 16% CO_2 in air for 20 s in the dark. Recordings are shown at two sensitivity levels. It is apparent that the combination of oxygen recording and CO_2 recording maximized time resolution and accuracy of recording.

In Fig. 2, rates of CO_2 release from a darkened spinach leaf are plotted against the total amount of released CO_2 after the leaf had been loaded for 20 s with CO_2 ranging from 1 to 16% in air. As expected, more CO_2 had dissolved in the leaf at the higher than at the lower concentrations. In consequence, more could be released into CO_2-free air. Actually, a loading time of 4.5 s produced as much CO_2 uptake as a 20-s loading period (data not shown) whereas a loading time of 300 s with 16% CO_2 increased CO_2 solubilization as shown during unloading by a total release of about 3000 μmol m^{-2} CO_2 compared to about 2400 μmol m^{-2} CO_2 after 20 s loading with 16% CO_2. This increase in solubilization by longer loading times may be caused either by the non-catalyzed slow conversion of CO_2 into bicarbonate in cellular regions devoid of carbonic anhydrase or by pH regulation (i.e. an increase in cytoplasmic pH), or both. pH regulation countering the cytoplasmic acidification that is caused by high CO_2 in leaves has been demonstrated by photosynthesis and CO_2 solubilization experiments (Wagner et al. 1990; Heber et al. 1994; Hauser et al. 1995a,b) and by ^{31}P-NMR methodology (Bligny et al. 1997).

When related to the CO_2 solubility in water at the different CO_2 concentrations, the data of Fig. 2 appear as shown in Fig. 3. The ratio of released CO_2 to the CO_2 which had been physically dissolved in the water space of the leaves decreased as the CO_2 concentration increased. This is a consequence of the acidifying effect CO_2 has on leaf compartments during solubilization according to

$$CO_2 + H_2O \rightarrow HCO_3^- + H^+ \quad (1)$$

The Henderson/Hasselbalch equation

$$pH = pK + \log(HCO_3^-)/(CO_2) \quad (2)$$

shows that decreased pH values will lower CO_2 solubilization by reducing the concentration of HCO_3^-. At the ionic strength of cellular contents, the pK of CO_2 is about 6.1 (Yokota and Kitaoka 1985).

Figure 1 shows that most of the CO_2 desolubilized during the transition from high CO_2 to CO_2-free air is released during the first 2 s. In addition to physically dissolved CO_2, this must be CO_2 formed from bicarbonate by the action of carbonic anhydrase which is known to possess a high activity in the chloroplasts of mesophyll cells. If the volume of chloroplasts is known and only bicarbonate from there is considered together

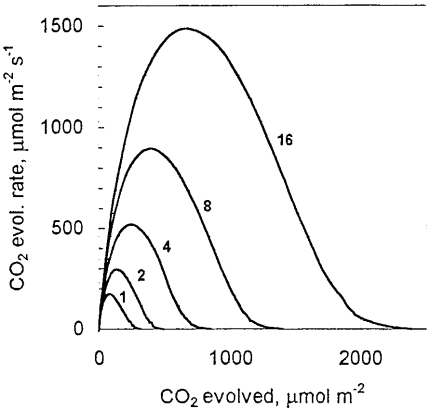

Fig. 2. Rates of CO_2 release from a spinach leaf versus amount of released CO_2 after loading the leaf with 1, 2, 4, 8 and 16% CO_2 in air for 20 s, in that order. Individual loading and unloading experiments followed one another at time intervals of about 30 min. Recordings were initially by the oxygen analyzer and, 2 s after starting the release experiment, by infrared recording of released CO_2

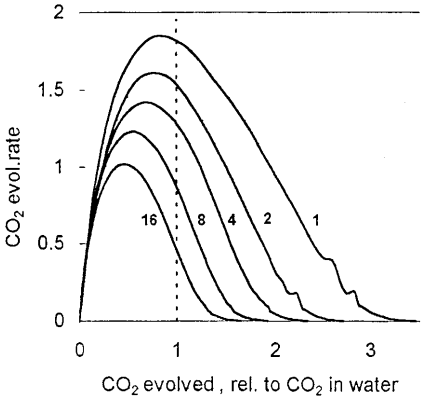

Fig. 3. Data of Fig. 2 are replotted to show on the abscissa the ratio of released CO_2 to the CO_2 which had been physically dissolved in the aqueous phase of the spinach leaf before the CO_2 was released into CO_2-free air. The *vertical line* that intercepts the abscissa at unity defines the extent to which CO_2 is dissolved physically. Values higher than unity on the abscissa define additional CO_2 which is released as CO_2 from bicarbonate. Rates of release of CO_2 are in nmol cm^{-2} s^{-1}. They are divided by the concentration of dissolved CO_2 in the water space of the leaf in nmol cm^{-2}. The correct dimension of the rates is therefore s^{-1}

with the fraction of physically dissolved CO_2 which can be attributed to the chloroplast space, the data of Fig. 3 can be used to calculate the pH of the chloroplast stroma with the help of the Henderson/Hasselbalch equation. For spinach leaves, 9.5% of the volume of mesophyll cells has been reported to be chloroplast volume (Winter et al. 1994). This percentage is lowered by a sizeable contribution of chloroplast-free tissues such as epidermis and cells of the vascular system to the total cellular volume of leaves. Moreover, the thickness of spinach leaves (i.e. the ratio of mesophyll tissue to epidermal tissue) depends on growing conditions. We have chosen greenhouse-grown spinach because of the flatness of its thinner leaves. Basic assumptions underlying the calculations shown in Fig. 4 are that the chloroplastic space is either 6 or 10% of the total aqueous space and that only bicarbonate released with time constants lower than 2 s originates from chloroplast bicarbonate. On this basis, chloroplast pH values can be calculated using Eq. 2. By extrapolation to zero CO_2, the pH of the chloroplast stroma of darkened leaves was about 7.7 (assumption: 6% of water space is chloroplast space) or 7.4 (10% of water space is chloroplast space). The pH was lowered by 1% CO_2 in air to pH 7.5 (6% assumption) or 7.3 (10% assumption) and by 16% CO_2 to pH 6.8 (6% assumption) or 6.55 (10% assumption).

When the amounts of CO_2 rapidly released from chloroplasts are related to the chloroplast space, chloroplast bicarbonate concentrations can be calculated. Figure 4 (inset) shows that calculated bicarbonate concentrations depend much on assumptions about chloroplast volumes. However, the validity of the assumptions can be tested. Changes in calculated bicarbonate concentrations (Fig. 4 inset) can be related to changes in chloroplast pH values (Fig. 4). Knowing that calculated bicarbonate concentrations correspond to equal concentrations of protons formed when carbonic acid dissociates forming HCO_3^- and H^+, buffer capacities can be obtained from the data of Fig. 4. Table 1 shows corresponding buffer capacities in mM pH^{-1}. In the spinach experiment of Figs. 2 and 3, the buffer capacities were about 20 mM pH^{-1} if the chloroplast space was assumed to be 6% of the total aqueous leaf space, and about 12 mM pH^{-1} under the assumption of a 10% contribution of the chloroplasts to the total aqueous leaf space. As chloroplast concentrations of phosphate and phosphate esters (pK values close to 7.1) are between 20 and 25 mM (calculated from Gerhard et al. 1987) and buffer concentrations, c, are related to the buffer capacity, β, close to the pK of the buffer by $\beta_{max} = 0.575$ c, chloroplast buffer capacities cannot be lower than 12 mM pH^{-1}. In that case, and with an initial stromal pH of 7.4, buffering would rest almost completely on phosphate and phosphate esters. Since this and a 10% assumption for the chloroplast space appear to be unrealistic for spinach, the buffer capacity must be higher than 12 mM pH-$unit^{-1}$. Consequently, the initial chloroplast pH in darkened spinach is also higher than pH 7.4.

Buffer capacities would be higher than 20 mM pH-$unit^{-1}$, as calculated for a 6% contribution of the chloroplasts to the aqueous leaf space in Table 1, only if the chloroplast space were below 6%. However, in that case, calculated initial pH values would increase unrealistically. They would be higher than pH 7.7 in darkened leaves as calculated for a 6% contribution of the chloroplasts to the total aqueous leaf space. For these reasons, an assumption of 6%, or a little more, for the chloroplast space is considered realistic. It yields internally consistent data for both pH and chemical buffering.

Whereas the enzymic conversion of CO_2 into HCO_3^- is very fast in leaf compartments with carbonic anhydrase, it is slow where this enzyme is absent. The same is true for the converse reaction when HCO_3^- is converted into CO_2 and OH^- during unloading. This accounts for differences in the rates and extent of CO_2 loading as well as for differences in the rates of unloading into a CO_2-free atmosphere. Slow unloading of CO_2 from HCO_3^-

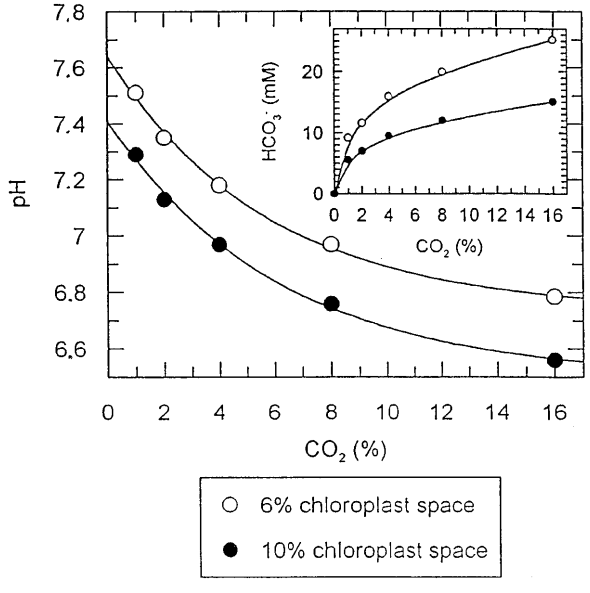

Fig. 4. Calculation of chloroplastic pH values from CO_2^- release experiments. Data are shown as a function of CO_2 loads during 20-s exposures of a spinach leaf to CO_2 concentrations between 1 and 16% (see Fig. 2). Basic assumptions for the calculation are that the chloroplastic space is either 6 or 10% of the total water space of the leaf. For explanation, see text. *Inset*: calculated chloroplast bicarbonate concentrations as a function of CO_2 concentrations in air. Data from CO_2-release experiments (see Fig. 2)

Table 1. Stromal buffer capacities calculated from the data of Fig. 4 assuming that the chloroplast space was either 6 or 10% of the total aqueous leaf space

% CO_2 in air	Buffer capacity (mM pH^{-1}) assuming 6% chloroplast space	Buffer capacity (mM pH^{-1}) assuming 10% chloroplast space
2	16.7	10
4	21.2	12.8
8	20.4	12.3
16	22.7	13.4

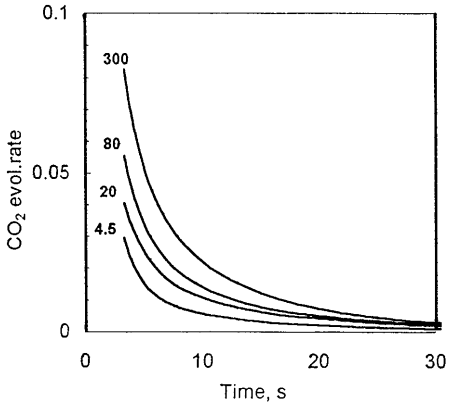

Fig. 5. Dependence of rates of CO_2 release from a spinach leaf versus time on different exposure durations to 16% CO_2. Exposure times were 4.5, 20, 80 and 300 s (recordings by the CO_2 analyzer). Rates of release of CO_2 are in nmol cm^{-2} s^{-1} divided by the concentration of dissolved CO_2 in the water space of the leaf in nmol cm^{-2}. The correct dimension of these rates is therefore s^{-1}

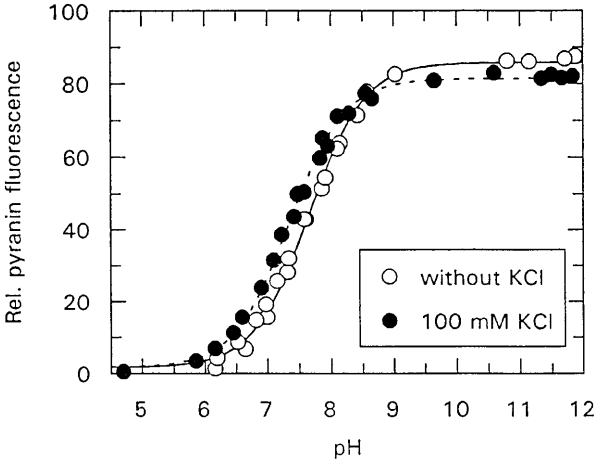

Fig. 6. Fluorescence of pyranine as a function of pH in 20 mM Hepes, with and without 100 mM KCl also present. The pK values were 7.64 without KCl and 7.34 with 100 mM KCl

after different loading times with 16% CO_2 has been recorded by the CO_2 analyzer (Fig. 5). The data are given in relation to the solubility of 16% CO_2 in water to show the magnitude of this bicarbonate release. For comparison, the ratio of bicarbonate to physically dissolved CO_2 was 32 at pH 7.6 and still 8 at pH 7 in the chloroplast stroma. It is apparent that there is a clear dependency of rates and amounts of bicarbonate that are slowly released as CO_2 on the length of loading periods with 16% CO_2. Assuming that desolubilization of CO_2 from HCO_3^- as shown in Fig. 3 follows first-order kinetics, it is possible to derive time constants of desolubilization. In addition to very fast CO_2 release with time constants lower than 2 s, three more components of release could be resolved by plotting log c against time (data not shown). Approximate time constants were 5, 15 and 150 s. Amounts of CO_2 released with time constants of 5 and 15 s are listed in Table 2 for different loading times with 16% CO_2.

According to the data published in Bligny et al. (1997), pH regulation is responsible for the very slow CO_2 release with a time constant of 150 s. With respect to the time constant of 15 s, it is tempting to speculate that this reflects liberation of CO_2 from vacuolar and apoplastic bicarbonate.

The results of titration experiments with CO_2 at concentrations between 1 and 16% with leaves of *Mercurialis perennis*, *Solanum tuberosum* and *Helianthus annuus* were very similar to those shown in Figs. 1–3 for spinach.

Table 2. Amounts of slowly released CO_2 after loading a spinach leaf for 4, 20, 80 and 300 s with 16% CO_2 in air. Temperature 20 °C

Time of exposure to 16% CO_2	Time constant 5 s (nmol cm^{-2})	Time constant 15 s (nmol cm^{-2})
4	16	16
20	22	23
80	35	31
300	57	41

Monitoring pH in the extrachloroplastic part of the cytoplasm. The fluorescent pH indicator pyranine is known to enter the cytoplasm of leaf cells rapidly when fed to the leaves through the petiole via the transpiration stream (Yin et al. 1990). Figure 6 shows fluorescence of the dye in a buffer (20 mM Hepes) as a function of pH. Fluorescence is low in the acidic and high in the alkaline range. The pK of the dye was 7.6 without KCl and 7.3 with 100 mM KCl. After feeding 1 mM pyranine to a spinach leaf for 1 h, intercostal areas of the leaf were strongly fluorescent whereas veins remained dark, indicating quenching of dye fluorescence in the vascular tissue. After feeding the dye, the petiole of the leaf was placed into water to remove excess dye from the apoplastic leaf space during transpiration. In some experiments, apoplastic pyranine was removed by infiltrating the leaf with water and subsequent centrifugation. Microscopy of leaf cross-sections failed to reveal fluorescence from the chloroplasts. Either the dye did not penetrate into the chloroplasts, or the chloroplasts absorbed most of the exciting blue light and of the fluorescence emission. Transport of pyranine into the vacuoles is slow in spinach (Yin et al. 1990). Also, dye that slowly enters the vacuoles is largely nonfluorescent at vacuolar pH values. Residual fluorescence originating from the vacuoles responds only slowly to CO_2 owing to the slow rate of the uncatalyzed conversion of CO_2 into bicarbonate. Thus, fast changes in dye fluorescence originate mainly from the extrachloroplastic cytoplasm.

The pyranine-containing leaf was exposed to 1, 2, 4, 8 and 16% CO_2 for 1 min each and fluorescence was monitored. Each exposure to CO_2 was separated from the next by a 5-min period in CO_2-free air. Figure 7 shows quenching of pyranine fluorescence as a function of CO_2 concentration. In the inset of Fig. 7, the kinetics of quenching are shown. Quenching and its reversal were much faster than expected if the cytoplasm were devoid

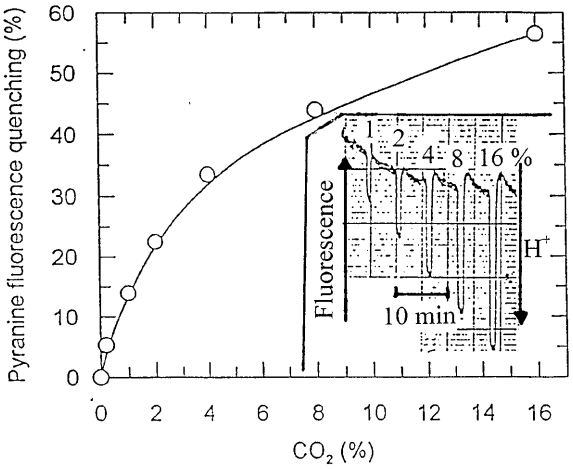

Fig. 7. Quenching of pyranine fluorescence as a function of CO_2 concentration. The stomata of the spinach leaf had been opened under illumination in CO_2-free air. Exposure times to CO_2 were 1 min. *Inset*: kinetics of the quenching of pyranine fluorescence in a darkened spinach leaf by CO_2 (1–16% in air) and restoration of fluorescence in CO_2-free air

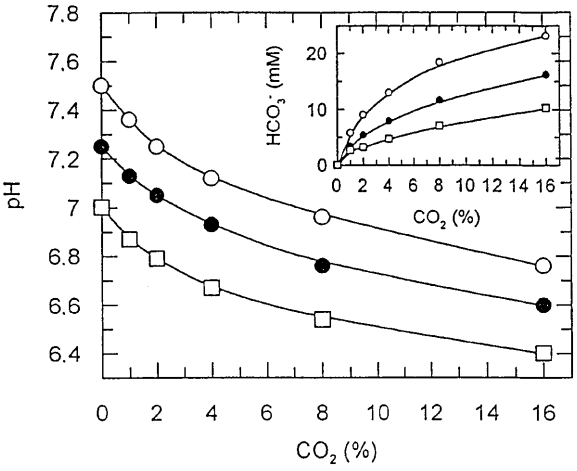

Fig. 8. Cytoplasmic pH values as a function of CO_2 concentration in a spinach leaf. Calculations are from fluorescence quenching as shown in the inset of Fig. 7 and the quenching curve of Fig. 6. The basis of the calculations was that the starting pH was either 7.5 or 7.25 or 7.0. For explanation, see text. *Inset*: calculated extrachloroplast bicarbonate concentrations as a function of CO_2 concentration in air. Data from the pH curves (same symbols)

of carbonic anhydrase. The observed fast kinetics indicate the presence of this enzyme in the cytoplasm (Hauser 1996).

The quenching curve of Fig. 7 can be used in combination with the calibration curve of Fig. 6 to estimate the effect of CO_2 on cytoplasmic pH if assumptions are made concerning initial pH values of the cytoplasm in air in the absence of high CO_2 concentrations.

Electrophysiological evidence has revealed cytosolic pH values to be between pH 7 and 7.5 in a variety of plant cells (Smith and Raven 1979; Felle 1988; Thaler et al. 1992). We have therefore calculated the acidifying effect of high CO_2 on the cytoplasmic pH, starting from initial pH values in air of either 7.5 or 7.25 or 7 and using the pH calibration of Fig. 6 and a pK for pyranine of 7.3. The pK of 7.6 measured in the absence of 100 mM KCl in the calibration experiment of Fig. 6 does not take into account the ionic strength of the cytoplasm. The results of the calculations are shown in Fig. 8. The depression of pH by high CO_2 does not depend much on the different assumptions concerning initial pH values. Application of 16% CO_2 reduced the pH by 0.5 pH units when the starting pH was 7.0, and by 0.6 pH units when it was 7.5. The corresponding CO_2-dependent pH depression in the chloroplasts (Fig. 4) was about 1 pH unit. The smaller pH depression in the extrachloroplastic part of the cytoplasm indicates a somewhat higher buffering capacity compared to that of the chloroplast stroma.

Figure 8 (inset) shows calculated cytoplasmic bicarbonate concentrations as a function of CO_2 concentration for the different pH values shown in Fig. 8, and Table 3 shows the corresponding calculations of buffer capacities from the individual titration steps. The buffer capacities were about 35 mM pH-unit^{-1} if the starting pH was considered to be 7.5. Considerably lower buffering capacities were calculated for the starting pH

Table 3. Buffer capacities of the extrachloroplastic cytosol calculated from the data of Fig. 8 and assuming that the starting pH of the extrachloroplastic cytoplasm was either 7.5 or 7.25 or 7.0

% CO_2 in air	Buffer capacity (mM pH^{-1}) calculated in relation to a starting pH of:		
	7.5	7.25	7.0
2	33.6	26.6	16.4
4	34.3	24.5	14.7
8	33.9	24.4	15.6
16	30.7	24.7	16.9

values of 7.25 and 7.0. For pH 7.25, they were similar to the buffering capacity of chloroplasts which was close to 20 mM pH unit^{-1} at a starting pH of 7.7 (Fig. 4, Table 1). Thaler et al. (1992) have reported cytoplasmic pH values between 7.2 and 7.3 for *Eremosphaera viridis*.

Monitoring pH in the apoplast. With a pK of 6.1, FITC is sufficiently sensitive to record pH changes in the apoplast of leaves (Hoffmann and Kosegarten 1995). The fluorescence of its dextran conjugate is shown as a function of pH in Fig. 9. By infiltration, a solution of the dye (0.25 or 1 mM) and of sodium vanadate (4 mM) was brought into the apoplast of a leaf. The purpose of using vanadate was to inhibit the plasmalemma ATPase. The vanadate had a buffering capacity of 0.8 mM pH-unit^{-1} between pH 6 and 7. Centrifugation at 450 *g* for 5 min removed excess infiltrate and restored effective gas exchange between the atmosphere and the leaf interior. Leaves were then titrated with CO_2 at concentrations between 1 and 16% in air. Quenching of FITC fluorescence indicated acidification by CO_2. Recordings of quenching are shown in Fig. 10 for a potato (A) and a spinach leaf (B). Apoplastic responses to CO_2 differed considerably in the two species. In spinach, where the

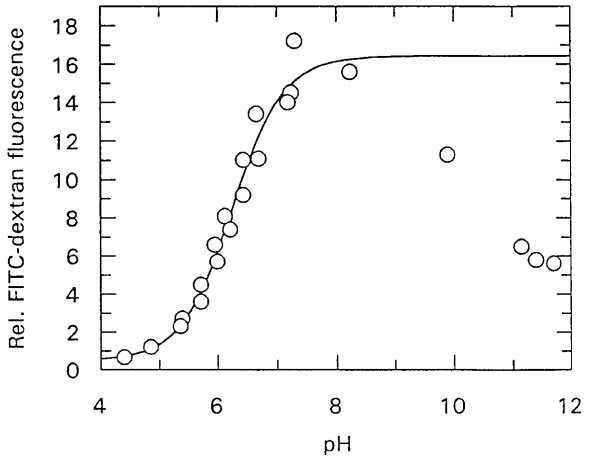

Fig. 9. Fluorescence of FITC as a function of pH in 20 mM Mes buffer with 100 mM KCl also present. pK was 6.13. (pK was 6.33 without KCl, data not shown)

vanadate had not been very effective in closing the stomata, 1% CO_2 failed to produce appreciable acidification. Instead, CO_2 actually increased FITC fluorescence, indicating apoplastic alkalinization rather than acidification. Some fast quenching of fluorescence, indicating acidification, was only observed at the higher CO_2 concentrations. Maximum quenching of FITC fluorescence by 16% CO_2 was below 10% of total FITC fluorescence in spinach. Even while high CO_2 was present, fluorescence increased after its initial fast decrease, revealing slow progressive alkalinization. Removal of the CO_2 overcompensated the initial CO_2 dependent fluorescence quenching. Thus, even at high concentrations, CO_2 initiated progressive apoplastic alkalinization in spinach, a process which was only slowly reversed after removal of high CO_2.

Similar responses were observed with potato only when vanadate was absent. When it was present, stomata closed slowly as revealed by transpiration data (data not shown). The kinetics of CO_2-dependent quenching and of the release of quenching were then slow. They were influenced by the diffusion barrier of the stomata and did not reflect the faster kinetics seen when the stomata were partially open. These faster kinetics were not monophasic. Moreover, the initial kinetics of this quenching were always faster than the initial kinetics of its reversal indicating complex reversibility of quenching (data not shown). This complexity is also apparent by the different extent of quenching and of its reversal in the recordings of Fig. 10. After the withdrawal of 16% CO_2 from the gas stream, FITC fluorescence increased more than it had decreased on addition of CO_2. This shows that during acidification by high CO_2 some of the protons released by apoplastic bicarbonate formation had not remained in the apoplast but had entered the more alkaline cytosol, not only in the spinach experiment but also in the potato leaf. In consequence, removal of the CO_2 led to a transient alkalinization of the apoplast, a process which was only slowly corrected as protons re-entered the apoplast from the cytosol where they had been sequestered. Intermediate responses were seen between the extremes of 2 and 16% CO_2.

In Fig. 11, the extent of the reversal of quenching of FITC fluorescence after CO_2 had been removed from the gas phase is plotted as a function of the CO_2 concentration in air for the potato experiment shown in Fig. 10A. Maximum quenching by 16% CO_2 was almost 60% of total FITC fluorescence. Corrections were made by extrapolation to account for the slow transmembrane

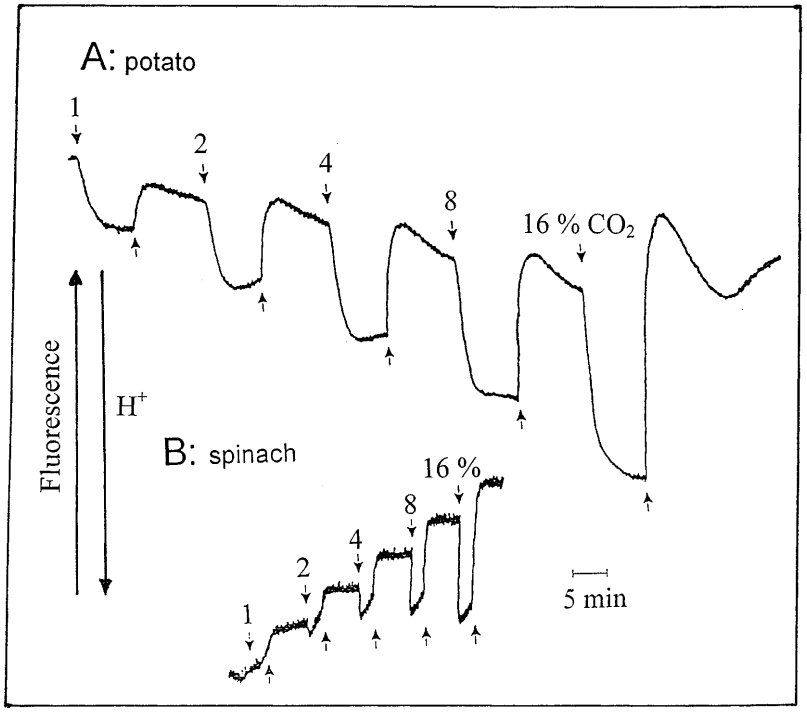

Fig. 10A,B. Recordings of quenching of FITC fluorescence in the apoplast of a potato (**A**) and a spinach (**B**) leaf by CO_2 (1–16% in air) and restoration of fluorescence in CO_2-free air. **A** The stomata of the potato leaf had been largely closed by 4 mM sodium vanadate in the infiltration medium. **B** The spinach leaf was exposed to CO_2 for 2 min. Stomata were open

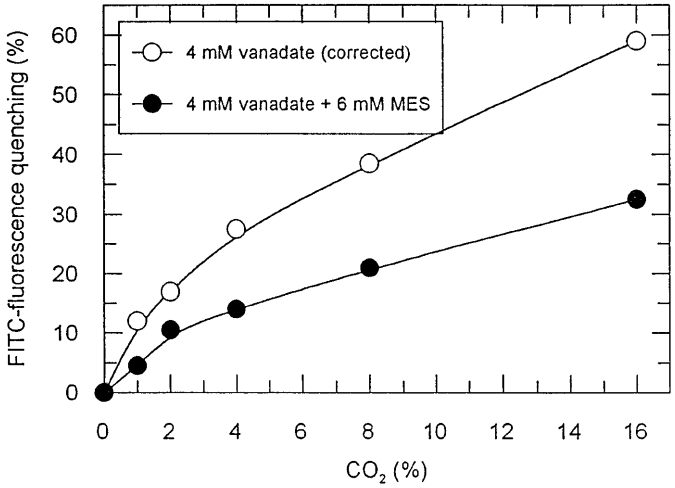

Fig. 11. Extents of the reversal of quenching of FITC fluorescence in the apoplast of a potato leaf after removal of CO_2 from the gas stream. *Open symbols* are from the recordings of Fig. 10A. They are corrected for transmembrane proton fluxes which counter apoplastic acidification by CO_2. The curve shown with *closed symbols* was obtained from an experiment in which Mes buffer (6 mM, pH 6.1) was infiltrated into the leaf together with FITC and vanadate

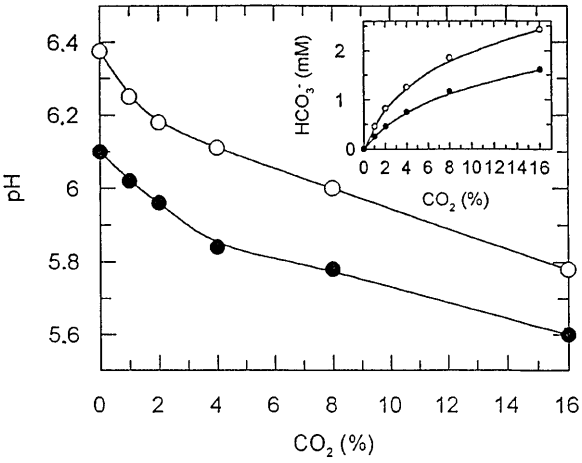

Fig. 12. Apoplastic pH values as a function of CO_2 concentration. Calculations from fluorescence quenching as shown in Fig. 10. The basis of the calculations was that the starting pH was either 6.37 or 6.1. *Inset*: calculated apoplastic bicarbonate concentrations as a function of CO_2 concentration in air. Data from the pH curves (same symbols)

proton fluxes which give rise to the complex kinetics of the reversal of quenching seen particularly after addition of 16% CO_2.

When, in a second experiment, a potato leaf was infiltrated with 1 mM FITC, 4 mM vanadate and, in addition, 6 mM Mes buffer (pK 6.1), CO_2-dependent quenching of FITC fluorescence was little more than 50% of the quenching observed in the absence of the Mes buffer. In this case, the overshoot phenomena seen particularly with 16% CO_2 in the recordings of Fig. 10 were no longer observed.

Using the pH-dependence of FITC fluorescence shown in Fig. 9, the acidification of the apoplast by CO_2 which would be observed in the absence of compensatory proton fluxes is shown in Fig. 12 for initial apoplastic pH values of either 6.37 or 6.1. In both cases the pH depression by 16% CO_2 was about 0.5 pH-units. The corresponding bicarbonate accumulation is shown in Fig. 12 (inset). A comparison with Fig. 8 (inset) for cytoplasmic bicarbonate accumulation as a function of CO_2 concentration is instructive in regard to the increased bicarbonate levels in the more alkaline cytoplasmic leaf cell compartments shown in Fig. 8.

With the aid of the pH data and the corresponding bicarbonate accumulation shown in Fig. 12, apoplasmic buffer capacities can be calculated (Table 4). Depending on assumptions regarding initial pH values, buffer capacities are either below 5 or below 4 mM pH-unit^{-1}. From these values, vanadate buffering must be subtracted so that correct buffering capacities are, depending on assumed initial pH values, about 4 or 3 mM pH-unit^{-1}. It is important to note that the buffering capacity of the infiltrate, which had been obtained by centrifugation after water infiltration of leaves, was too low to be measurable by conventional titration. It thus appears that bound ionic groups of the cell wall matrix are largely responsible for apoplastic buffering, not soluble apoplastic constituents.

Table 4. Apoplastic buffer capacities calculated from the data of Fig. 12 and assuming that the starting pH of the apoplast was either 6.37 or 6.1

% CO_2 in air	Buffer capacity (mM pH^{-1}) calculated in relation to a starting pH of:	
	6.37	6.1
2	5.1	3.6
4	4.5	3.3
8	4.5	3.4
16	4.1	3.2

When data on fluorescence quenching, by CO_2 as shown in Fig. 10 were plotted directly as extent of quenching, and not after correcting the reversal of quenching for transmembrane proton fluxes as done in Fig. 11, calculated pH depressions by higher CO_2 concentrations were lower and calculated bicarbonate accumulations higher than shown in Fig. 12. In these cases calculated buffer capacities were comparable to values shown in Table 4 only at CO_2 concentrations of 1 or 2%. In contrast to the data shown in Table 4, they increased with increasing CO_2 concentration. This is a consequence of the CO_2-dependent transmembrane proton fluxes from the more alkaline cytosol into the more acidic apoplast, which counter the apoplastic acidification by CO_2 that is shown by the overshoot phenomena in Fig. 10. These CO_2-dependent calculated buffer capacities are in no direct relation to the buffering by ionic groups of the apoplastic matrix.

Discussion

We have taken a non-invasive approach to determine pH and buffer capacities of different cellular compartments

in intact leaves. The acidifying agent used to titrate the base in the apoplastic and cytoplasmic compartments was gaseous CO_2 which entered the leaves by diffusion and left them by diffusion. The state of the stomata was monitored in all experiments. As long as they were reasonably open, we were kinetically limited only by the response time of the system used for measuring CO_2 exchange or by the gas flow system used to bring CO_2 to the leaf. In the absence of carbonic anhydrase which catalyzes the fast conversion of CO_2 to bicarbonate, acidification by CO_2 is expected to be slow. Its rate constant is 0.036 s^{-1} and the time constant t/2 about 20 s at 25 °C (Gibbons and Edsall 1963).

However, the main part of acidification and its reversal as measured by CO_2 exchange was very fast. This part is thought to monitor the pH in the chloroplast stroma (Hauser et al. 1995a). Chloroplasts are known to contain highly active carbonic anhydrase. In the present work, we have improved the method of measuring fast CO_2 exchange by keeping all leaf parts capable of rapid gas exchange inside the cuvette. This eliminated artifacts by fast intercellular CO_2 diffusion between leaf parts outside and inside the cuvette. In this way, we realized that buffer capacities measured earlier for chloroplasts in vivo (Hauser et al. 1995b) were overestimates. This does not affect the regulatory aspects of the earlier work. Calculations of buffer capacities from data obtained now with the improved method agree with the results of direct titrations of isolated intact chloroplasts (Pfanz and Heber 1986) showing that little buffer had escaped from the chloroplasts during isolation.

Quenching of the fluorescence of the cytosolic pH indicator pyranine by CO_2 was also invariably fast when stomata were sufficiently open. As chloroplasts and extracytoplasmic compartments fail to show green pyranine fluorescence in leaf cross-sections (Yin et al. 1990), this dye records mainly the pH of the cytosol and of nuclei when properly used. Acidification by CO_2 as recorded by pyranine was far faster than expected on the basis of an uncatalyzed reaction between CO_2 and bicarbonate. Fast quenching of pyranine fluorescence by CO_2 was first observed by Hauser (1996) who attributed it to the presence of carbonic anhydrase in the extrachloroplastic cytoplasm.

For the apoplast, the situation is less clear. Neither acidification nor its reversal as monitored by FITC fluorescence were monophasic processes in the apoplast. Time constants varied considerably in different experiments. When stomata were open, the fast part of acidification often had time constants which varied between 4 and 10 s. The reason for this variability is not yet clear but there is preliminary evidence that it is related to effective apoplastic pH regulation. When the complete absence of the regulatory overshoot phenomena demonstrated in Fig. 10 indicated the absence of effective apoplastic pH regulation (as it did when Mes buffer was included in the infiltration medium; data not shown), apoplastic acidification by CO_2 was as slow, as expected for the uncatalyzed reaction, even when stomata were partially open.

There are two results of the present work which merit additional discussion. (i) Even in darkness, the pH of the chloroplast stroma is likely to be higher in leaves than the pH of the extrachloroplastic cytoplasm. (ii) Cytosolic alkalinization under acid stress as an expression of active pH regulation does not involve extrusion of excess protons into the apoplast but rather into central vacuoles.

(i) The calculation of pH in chloroplasts in vivo by the measurement of CO_2 release rests on assumptions about stromal volumes. Uncertainty about volumes influences the results. However, this disadvantage is balanced by the results of calculations of the stromal buffer capacity. The higher the pH, the higher the accumulation of bicarbonate in equilibrium with CO_2. The two pH curves of Fig. 4 were calculated assuming chloroplast volumes of 6 or 10% of the total aqueous space of the leaves. Winter et al. (1994) have reported for spinach that chloroplasts accounted for 9.5% of the mesophyll space. This does not consider other tissues which are devoid of chloroplasts. Extrapolation of CO_2-exchange data yielded initial pH values of 7.7 or 7.4 for a 6 and a 10% contribution of the chloroplasts (Fig. 4). The corresponding buffer capacities were about 25 or 12 mM pH-unit^{-1} (Table 1). Values lower than 12 mM pH-unit^{-1} for an initial pH of 7.5 are unrealistic because known chloroplast concentrations of phosphate and phosphate esters alone would provide such buffering. Values much higher than 25 mM pH-unit^{-1} are unlikely because chloroplast volumes would be lower than 6% of the aqueous space and initial pH values would be higher than 7.7. Moreover, direct titration of isolated intact chloroplasts of spinach (Pfanz and Heber 1986) agree with a buffer capacity of chloroplasts close to 25 mM pH-unit^{-1}. The titration work revealed a buffer capacity of 1.1 μmol (mg chlorophyll)$^{-1}$ between pH 7 and 8. At a chloroplast volume of 40 μl (mg chlorophyll)$^{-1}$, this corresponds to 28 mM pH-unit^{-1} for intact chloroplasts. Higher buffer capacities were also reported (Hauser et al. 1995b). It is now realized that these values were in part the result of experimental overestimations and in part of active pH regulation caused by proton export from the chloroplasts.

Such proton export under acid stress is not confined to chloroplasts. The inset of Fig. 7 shows quenching of the fluorescence of the cytoplasmic pH indicator pyranine by high concentrations of CO_2. The higher the concentration of CO_2, the larger was the overshoot in fluorescence after its removal. This overshoot indicates alkalinization of the cytoplasm which occurred during exposure to high CO_2 as a result of proton removal from the cytoplasm. In the experiment of Fig. 7, exposure was only 1 min. Prolonged exposures produced larger overshoots (data not shown). Quite obviously, proton transport from one to another compartment during measurements of buffering will influence measured buffering capacities. If protons are removed, apparent buffering increases, but is then not based on locally available chemical buffers. Chemical buffering must therefore be distinguished from pH regulation by proton transport. This simple fact will be discussed in a

subsequent publication. It appears to be the reason for considerable controversy in the literature regarding widely differing buffer capacities (Frohnmeyer et al. 1998; Schönknecht and Bethmann 1998).

With minimal interference by proton transport, pH curves as a function of CO_2 concentration were for the extrachloroplastic part of the cytoplasm less steep than the chloroplast curves (Fig. 8 versus Fig. 4) suggesting a higher buffer capacity of the cytoplasm outside the chloroplasts than in the chloroplast stroma. At an initial pH of 7.5, calculated buffer capacities are about 35 mM pH-unit^{-1}, i.e. almost twice the calculated chloroplast buffer capacities (Table 1, left column). At pH 7.25, cytosolic buffer capacities are about 25 mM pH-unit^{-1} and at pH 7, about 16 mM pH-unit^{-1} (Table 3). Values for cytosolic pH values are usually between 7.2 and 7.4 in the literature (Felle 1988; Thaler et al. 1992). Thus, cytosolic buffering is likely to be around 25 mM pH-unit^{-1} in the pH range reported typical of the cytosol, i.e. somewhat higher than chloroplast values.

Simultaneously, there appeared to exist a pH gradient between the more alkaline chloroplast stroma and the surrounding cytosol. Such a gradient has not yet been seen in ^{31}P-NMR work which is sufficiently sensitive to reveal pH gradients between cytoplasm and vacuoles. However, the chloroplast envelope is known to contain an ATPase which acts as a proton pump (Berkowitz and Peters 1993). Also, it has long been noticed that the distribution 3-phosphoglycerate and of dihydroxyacetone phosphate between chloroplasts and the extrachloroplastic part of the cytoplasm is strikingly different (Urbach et al. 1965; Heber et al. 1967). About 70% of the phosphoglycerate occurred in the chloroplasts of spinach leaves both in the dark and in the light, whereas it was less than 30% for dihydroxyacetone phosphate. Both metabolites are transported in the form of their divalent anions across the chloroplast envelope by the phosphate translocator. If the chloroplast stroma is more alkaline than the cytosol, the trivalent phosphoglycerate anion will be trapped in the chloroplasts, accounting for the observed accumulation of phosphoglycerate in the chloroplasts. Thus, the differential distribution of these metabolites supports the present results of a pH gradient between chloroplasts and cytosol not only in the light (Heldt. et al. 1973) but even in the dark.

(ii) In algal cells suspended in and surrounded by large volumes of water, pH regulation by proton pumping across a membrane barrier might be expected to occur across the plasmalemma. This is not necessarily the expectation for cells of higher plants. In spinach, the apoplast of mesophyll cells occupies only 5% of the total cellular space (Winter et al. 1994) and in potato 3% (Leidreiter et al. 1995). For this reason, the report of Gout et al. (1992) was somewhat of a surprise since it showed that sycamore cells suspended in an aqueous environment regulated their pH under acid stress mainly by proton export into the medium rather than by proton transport into central vacuoles. In the present work with intact leaves, however, where the aqueous apoplast occupies just a minor fraction of the total aqueous leaf space, no proton extrusion could be observed under acid stress. It rather appeared that high levels of CO_2 opened a gate for protons into the more alkaline cytosol. Thus, in the absence of vanadate, which is usually considered to be an inhibitor of the proton-exporting plasmalemma ATPase, little apoplastic acidification was indicated by the quenching of the fluorescence of the apoplastic pH indicator FITC-dextran. Rather, and paradoxically, addition of CO_2 was often accompanied by apoplastic alkalinization rather than acidification (data not shown). Vanadate was helpful in obtaining reliable apoplastic pH titration curves with CO_2. Calculated buffer capacities were independent of CO_2 concentration only in its presence. Without vanadate, they increased with CO_2 concentration, indicating interference by fast apoplastic pH regulation which rapidly compensated for the acidification caused by CO_2. In these cases chemical buffering in the apoplast could not be reliably assessed. When measured in the presence of vanadate, apoplastic buffering was much lower than cytosolic or chloroplast buffering.

Our CO_2 titrations provided no evidence that cytosolic pH regulation under acid stress involves proton extrusion from the cytosol into the apoplast. Since ^{31}P-NMR work with pea leaves has shown that cytoplasmic alkalinization under acid stress is accompanied by vacuolar acidification (Bligny et al. 1997), it must be concluded that in acid-stressed leaves the pumping of excess protons across the tonoplast into the vacuoles is mainly responsible for fast cytoplasmic pH regulation.

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