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Light-induced alkalization of the chloroplast stroma in vivo as estimated from the CO_2 capacity of intact sunflower leaves

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Rapid CO_2 gas exchange by *Helianthus* leaves was analysed kinetically using a computer model which distinguished different components of the gas exchange by different time constants. A rapid phase of CO_2 uptake was ascribed to the solubilization of CO_2 in all leaf compartments and to the conversion of the dissolved CO_2 to HCO_3^- in the chloroplast stroma which contains carbonic anhydrase. From stromal HCO_3^-/CO_2 ratios the stroma pH of darkened leaves was estimated to be close to 7.5. Occasionally, values as high as 8 or as low as 7 were also obtained. If fast HCO_3^- formation also occurs in the cytosol, pH values may be lower by about 0.3 pH units than those calculated under the assumption that carbonic anhydrase is localized in chloroplasts only. Illumination with a light intensity close to saturation of photosynthesis caused an increase in CO_2 solubilization which indicated the alkalization of the chloroplast stroma by about 0.6 pH units. This is an underestimation, if the pH of cytosol decreases in the light liberating CO_2 by the action of carbon anhydrase. An alkalization of the stroma by 0.6 pH units indicates the export of about 450 nmol H^+/mg chlorophyll from the stroma. This forms the basis of a large transthylakoid pH gradient which drives light-dependent ATP synthesis. A pH gradient between stroma and cytosol is capable of supporting secondary gradients between these compartments in the light, such as a gradient in the ATP/ADP ratio. On darkening, the stroma alkalization was reversed. The rate of stroma acidification was much higher in the presence of CO_2 than in its absence.

Introduction

Illumination of thylakoids isolated from green leaves results in an alkalization of the suspending medium and an acidification of the intrathylakoid space [1]. The transfer of protons into the thylakoids is coupled to light-dependent electron transport, and a proton gradient is established across the thylakoid membrane which together with a membrane potential constitutes the driving force for the phosphorylation of ADP by the chloroplast coupling factor [2]. The chloroplast envelope is an effective barrier against the transport of protons between the medium and the chloroplast stroma [3,4]. Therefore, in intact chloroplasts the alkaline shift caused by proton transfer into the thylakoids remains confined to the stroma space. Its extent is a function of the number of protons transferred and of the buffer capacity of the chloroplast stroma. In experiments performed by different authors, the alkalization of the stroma ranged from about 0.25 to 1 pH unit [4–9]. It increased biphasically with light intensity,

Abbreviations: Rbu-1,5-P_2 ribulose 1,5-bisphosphate; 3-PGA, 3-phosphoglycerate.

and the larger part of the alkalization reaction was saturated at a light intensity as low as about 12 $W \cdot m^{-2}$ [5].

Although much information has been derived from experiments with isolated chloroplasts [10,11] on the consequences of pH differences between chloroplast stroma and cytosol on leaf metabolism, ver little is actually known about the stroma alkalization in vivo. Light-scattering measurements have been the source of information on thylakoid acidification in leaves as a function of illumination and composition of the gas phase surrounding the leaves [12,13]. This information has considerable comparative value. But is not quantitive and in particular does not directly pertain to stroma alkalization. The pH of the cytosol of green cells does not increase on illumination and may actually decrease [14,15].

Under steady-state conditions, CO_2 is very close to equilibrium with bicarbonate in leaf tissues [7]. The equilibrium is pH- and temperature-dependent. Using sensitive gas-exchange methodology, Laisk [16] observed CO_2 evolution from an aspen leaf during a rapid temperature decrease which exceeded the dissolved CO_2 by a factor of 2–7. With a more sophisticated technique, Oja [17] could show that about 4-times more CO_2 was taken up from a gas stream by a darkened sunflower leaf within a time span of 5–10 s than could be accounted for by the solubility of CO_2. Most of the CO_2 exchanged within 2 s, but slow exchange persisted for a much longer time. Since fast equilibration between CO_2 and HCO_3^- requires the presence of carbon anhydrase which is a chloroplast enzyme [7,18], the rapid component of the gas exchange was attributed to the solubilization of CO_2 in the chloroplast stroma. Since the volume of the stroma is known, a pH of about 7.9 was calculated for the stroma of darkened leaves from the CO_2 uptake data. In the present work, the same technique has been applied to measure pH in the stroma of chloroplasts in sunflower leaves in vivo in the light and during light/dark transients.

Material and Methods

Sunflower (*Helianthus annuus* L.) plants were grown in a growth cabinet at 10000 lux, 16/8 h, 25/18°C day/night cycle in pots filled with 4 kg of moist turfy glasshouse soil. The upper leaves of 6-week-old plants were used for the experiments. They possessed the following typical photosynthetic parameters at 22°C: mesophyll conductance for CO_2 in 1% O_2 0.7 cm/s, CO_2 and light-saturated rate of photosynthesis 4–5 nmol CO_2 per cm^2 per s, stomatal conductance 1.5–2 cm/s for CO_2 under experimental conditions (adaxial side of the leaf only).

The apparatus for the measurement of the leaf CO_2-exchange rate was described in detail in Ref. 19. In principle, it contained two open systems for measuring photosynthesis (below referred to as channels) in which the gas composition can be adjusted independently by means of gas mixers MIX (Fig. 1). A sandwich type leaf chamber LC ($4.4 \times 4.4 \times 0.3$ cm; flow rate, 20 cm^3/s) could rapidly be switched into the chain of either the first or the second channel. Laminar air flow in the leaf chamber guaranteed the transition of a I-shaped front of CO_2 over a leaf element within 0.1 s. Water vapour was condensed in thin cooled tubes before entering the gas into analysers. The volumes of the sample cuvettes were 20 cm^3. The frequency response characteristics of the amplifiers were corrected so as to obtain the shortest response time possible for the system. The func-

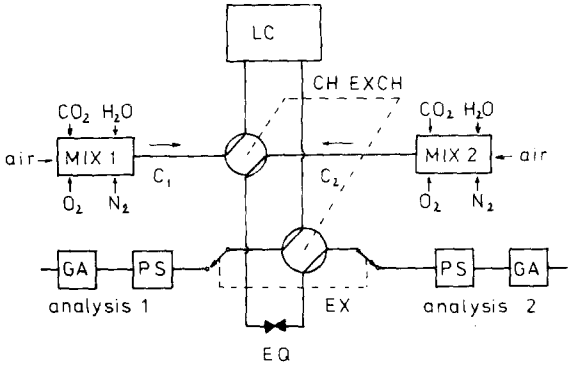

Fig. 1. Basic circuit of the two-channel leaf gas-exchange measurement system. LC, leaf chamber, MIX 1 and MIX 2, gas mixers, C_1 and C_2, different CO_2 concentrations in the gas mixtures produced by gas mixers, CH EXCH, 'channel exchange' valve for switching the leaf chamber into the circuit of either channel 1 or channel 2, EQ, an equivalent resistance to the leaf chamber, EX, exit valves for flushing the leaf chamber after channel exchanges, GA, infrared gas analysers, PS, psychrometers.

tional response of the whole system to an I-shaped CO_2-peak in the leaf chamber was a gaussian curve of 1 s at half maximum height.

After the channel exchange procedure in which the leaf chamber was switched from the 'old' to the 'new' channel, psychrometer PS and infrared CO_2 analyser GA (Infralyt IV, D.D.R.) were disconnected for 0.5 s by means of the valves EX for flushing out the 'old' gas from the leaf chamber and from the tubing. It has been shown [17] that during this time only a negligible portion of CO_2 from the interior of the leaf is exchanged through the stomata. Some of the gas filling the leaf chamber and the tubing was not flushed out. This volume I_r was about 0.02 cm^3/cm^2 surface area at 23°C. In the calculations below it will be subtracted from the total CO_2 exchanged. The pool of CO_2 which is subsequently exchanged and measured by the gas analyser is considered to be dissolved in the leaf tissue either as CO_2 or as HCO_3^-. Two different experimental procedures were used. A leaf was either pre-exposed to a known CO_2 concentration in one channel and then switched into the other where CO_2 was absent, or vice versa. In the first case, a CO_2 outburst was recorded, in the second there was a CO_2 gulp.

The main problem in these measurements was the avoidance of artifacts arising from the metabolic CO_2 exchange. Two experimental procedures minimized interference by ribulose 1,5-diphosphate (Rbu-1,5-P_2) carboxylase activity. (1) RuBP carboxylase was deactivated before the measurements. For effective deactivation sunflower leaves had to be kept in CO_2-free N_2 containing 1% O_2 for 2–3 h in the light. This resulted in an up to 10-fold decrease of the rate constant of carboxylation as derived from the kinetics of the postillumination decay of the CO_2 uptake [20]. (2) Measurements were performed at a low temperature which decreases photosynthesis and increases CO_2 solubility.

Light scattering of leaves was measured as described in Ref. 12.

Results

On admission of CO_2 to a leaf kept in a CO_2-free atmosphere, CO_2 is rapidly taken up. The magnitude of uptake depends on pH according to $CO_2 + OH^- \rightleftarrows HCO_3^-$ and on metabolic events, such as carboxylation.

Fig. 2 shows the kinetics of CO_2 solubilization (a) and desolubilization (b) for a darkened leaf. The stomata of the leaf had been opened in the light, and they remained open during the experiment. Gas streams in the channels of the gas exchange apparatus contained 2000 ppm CO_2 in 1% O_2/99% N_2 and 1% O_2/99% N_2 without CO_2. The leaf was equilibrated in one channel for several minutes, after which the channels were changed. When the leaf was preexposed to CO_2, a peak of CO_2 evolution was recorded after changing the channels (b). This rapid CO_2 liberation is attributed to CO_2 dissolved both as gas and as bicarbonate which is in contact with carbon anhydrase. Components of the slow CO_2 evolution persisting after 20 s are respiratory CO_2 production, production of CO_2 from HCO_3^- not in contact with carbonic anhydrase (vacuolar HCO_3^-) and the release of CO_2 from the activator sites of the Rbu-1,5-P_2 carboxylase [21]. In agreement with this view, the CO_2 uptake observed on switching from a CO_2-free atmosphere to a CO_2-containing atmosphere (a) is largely a mirror image of the CO_2 evolution, but the slow component is smaller than in (b), presumably because vacuolar HCO_3^--formation and the binding of CO_2 to the carboxylase still cause CO_2 uptake, while respiration produces CO_2.

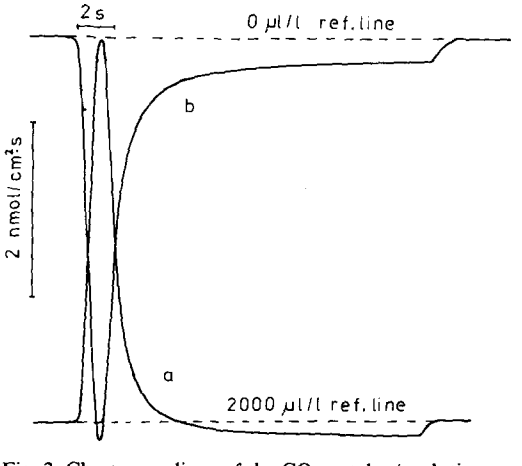

Fig. 2. Chart recordings of the CO_2 uptake/evolution processes following the transitions of the leaf from 0 to 2000 µl/l CO_2 (curve a) and from 2000 to 0 µl/l CO_2 (curve b).

Since the solubilization and desolubilization of CO_2 are not simple exponential processes, but contain rapid and slow components, it was necessary to separate components kinetically. Therefore, a mathematical model was created and realized on an Apple II computer for simulating the output signals of the gas analyzer. The structure of the model is shown in Fig. 3a. Its central point is C_w that stands for the concentration of dissolved CO_2 in the liquid phase of mesophyll cells. It can be identified by gas exchange measurements. This CO_2 is connected with the external atmosphere via the resistance R_s which is mostly stomatal. The respiratory CO_2 flux R enters into and the photosynthetic carboxylation flux F leaves from the same point. The capacity of the leaf for dissolved CO_2 species is described by three capacitors L, L_1 and L_2 which represent three components of the CO_2 solubilization process with different time constants: T_0 (= practically momentary), T_1 and T_2. Modelling the CO_2 solubilization process as a superposition of three exponents resulted in good coincidence between measured and simulated curves.

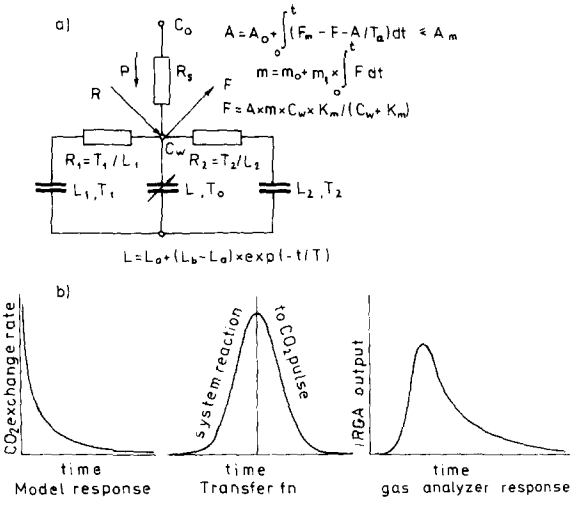

Fig. 3. Basic analog circuit of the leaf CO_2 exchange model applied for the simulation of CO_2 and light transients (a) and the scheme of filtering the model response through the transfer function of the gas exchange apparatus to obtain the gas response line. C_o, external CO_2 concentration; C_w, intracellular dissolved CO_2 concentration; R_s, gas phase resistance (mostly stomatal); R, respiration rate; F, carboxylation rate; P, gas-exchange rate; L, L_1, L_2, capacities of the leaf for CO_2; T, T_0, T_1, T_2, time constants. A, pool of CO_2 acceptor; m, rate constant for carboxylation; F_m, regeneration rate of the CO_2 acceptor; T_a, time constant for the self-decay of the acceptor pool, A_m, the maximum pool of the acceptor; K_m, Michaelis constant; m_1, activation rate constant of the carboxylase.

During light-dark transients the CO_2 solubility of the leaf changed. This phenomenon is taken into account assuming that the capacitor L changes from its initial value L_a to the final value L_b exponentially with the time constant T. Carboxylation rate F is the most complicated part of the model. In Ref. 20 it was shown that $F = mAC_wK_m/(C_w + K_m)$. A is the assimilatory power (mostly Rbu-1,5-P_2, m is the specific efficiency of carboxylation and K_m is the Michaelis constant for CO_2. In leaves with deactivated carboxylase, activation starts as CO_2 becomes available. This process was simulated as: $m = m_0 + m_1\int_0^t F\,dt$, where m_0 and m_1 are constants. The budget of the assimilatory power in each transient was taken as follows: $A = A_0 + \int_0^t(F_m - F - A/T_a)dt \leq A_m$, where A_0 is the initial pool of A, F_m is the rate of the synthesis of A, F is its rate of consumption in photosynthesis and A/T_a is the rate of self-decay of the assimilatory power. The growth of A is limited by a ceiling value A_m.

This model produces time-courses of the CO_2-exchange rate with very sharp peaks in response to changes in the external CO_2 concentration C_0 (schematically Fig. 3b to the left). The response curves of the model were filtered through the transfer function of the gas-exchange apparatus (response of the system to a very short CO_2 pulse

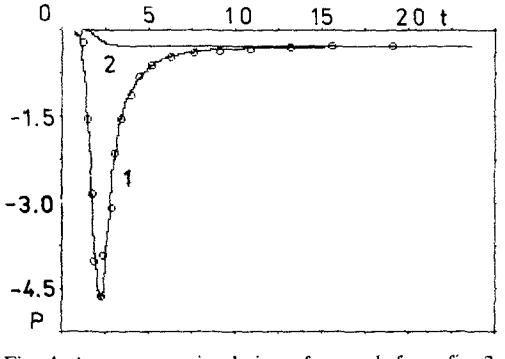

Fig. 4. A computer simulation of curve b from fig. 2. Experimental points (represented by circles) input from the Apple graphics tablet (1), and the calculated gas/analyzer response in case the CO_2 solubility is 0 (2), CO_2 exchange rate P in nmol/cm^2 per s, time t in s.

generated in the leaf chamber, a gaussian curve with 1 s half-maximum width, Fig. 3b in the centre), and finally the calculated output curves of the infra-red gas analyzer were obtained (Fig. 3b to the right). The latter were compared with the experimental curves. For each transient, the parameters of the model were varied until coincidence of the simulated curve with the experimental points was reached.

In Fig. 4, a computer curve simulating experiment (b) of Fig. 2 is recorded. Single experimental points are depicted as circles. The simulation parameters of these and of the other experiments described below are given in Table I. Different components of the leaf CO_2 capacity L_a, L_b, L_1 and L_2 are given in cm^3/cm^2 surface area (cm^2 of water layer in which the equivalent amount of gaseous CO_2 may be dissolved).

When the CO_2 concentration varied, CO_2 uptake by or evolution from the leaves also changed. There was proportionality between the peaks of gas exchange as shown in Fig. 2 and the CO_2 concentration in the gas phase indicating the absence of metabolic events such as carboxylation or decarboxylation in this part of the gas exchange. Experiments nos. 3–8 in Table I were carried out with a darkened leaf at a leaf temperature of 4.8°C. CO_2 outburst into the CO_2-free gas was measured proceeding from different concentrations. The leaf CO_2 capacities are almost equal in one series of measurements (Nos. 3–5 and 6–8). There is a difference between the average capacities in both series which is presumably caused by drifts in chloroplast pH. This result also shows that the pH of the tissue was not significantly affected by the variations of the dissolved gaseous

TABLE I

SIMULATION PARAMETERS OF TRANSIENTS ON THE CO_2 EXCHANGE OF SUNFLOWER LEAVES SHOWN IN FIGS. 2–10

Structure of the simulation model is shown in Fig. 3. Leaf CO_2 capacities L (cm^3/cm^2) and time constans T (s) correspond to those in Fig. 3. pH values in chloroplast stroma are calculated from eqn. 3. Assuming the stroma volume $L_s = 0.002$ cm^3/cm^2. $(pH)_a$ = stroma pH before a transition; $(pH)_b$ = stroma pH after a transition.

No.	Fig.	Part	L_a	L_b	T	L_1	T_1	L_2	T_2	$(pH)_a$	$(pH)_b$
1	2	b	0.087	0.087	–	0.050	1	0.02	7	8.05	8.05
2		a	0.095	0.095	–	0.045	1	0.04	12	8.06	8.05
3	–	30.5 [a]	0.047	0.047	–	0.018	1	0.019	5	7.52	7.52
4	–	62.1 [a]	0.050	0.050	–	0.015	1	0.019	5	7.52	7.52
5	–	93.7 [a]	0.048	0.048	–	0.014	1	0.019	5	7.47	7.47
6	–	14.7 [a]	0.057	0.057	–	0.020	1.5	0.015	5	7.66	7.66
7	–	46.4 [a]	0.057	0.057	–	0.018	1.5	0.015	7	7.64	7.64
8	–	77.9 [a]	0.053	0.053	–	0.018	1.5	0.015	7	7.60	7.60
9	5	a	0.135	0.047	3	0.015	1	0.035	5	8.10	7.47
10		b	0.11	0.047	3	0.015	1	0.035	5	8.00	7.47
11		c	0.09	0.047	3	0.015	1	0.035	5	7.89	7.47
12		d	0.085	0.06	3	0.015	1	0.035	5	7.85	7.64
13		e	0.05	0.05	–	0.013	1	0.07	20	7.46	7.46
14	7	a	0.13	0.09	3	0.015	1	0.02	5	8.08	7.89
15		b	0.09	0.062	3	0.015	1	0.02	5	7.89	7.66
16		c	0.062	0.048	3	0.015	1	0.02	5	7.66	7.49
17		d	0.048	0.048	–	0.015	1	0.02	5	7.49	7.49
18		e	0.050	0.050	–	0.015	1	0.02	5	7.49	7.49
19	9	a	0.14	0.14	–	0.08	1	0.035	5	8.31	8.31
20		b	0.20	0.08	2.3	0.02	1	0.035	5	8.31	7.85
21		c	0.06	0.06	–	0.02	1	0.035	5	7.69	7.69
22		d	0.06	0.14	2.8	0.02	1	0.035	5	7.69	8.14
23	11	a	0.097	0.051	4.5	0.03	1	0.025	8	8.01	7.70
24		b	0.051	0.13	8	0.03	1	0.025	8	7.70	8.14

[a] CO_2 concentration in mesophyll cells ($nmol/cm^3$) during the preexposition, leaf temperature 4.8°C in the dark.

CO_2 between O and 78 μM during the exposure time. Titration experiments have revealed a buffer capacity of the chloroplast stroma close to 30 μeqH^+/ml per pH unit not far from pH 8 [22].

From CO_2 uptake, solubility data for CO_2 and volumetric information, pH values can be calculated according to the Henderson–Hasselbach equation:

$$pH = pK +^{10} \log[HCO_3^-]/[CO_2] \quad (1)$$

The measured total CO_2 capacity of the leaf L_c is related to the bicarbonate/CO_2 equilibrium as follows:

$$[HCO_3^-]/[CO_2] = \frac{L_c - L_l}{L_l} \quad (2)$$

where L_l is the water layer of the Helianthus leaf. The fresh weight of the leaves used in the experiments was 20 ± 1 mg/cm^2 and their water content 17 mg/cm (water layer $L_l = 0.017$ cm). For calculating an average pH of the leaf blade, L_c should be taken as $L_c = L_a + L_l + L_2 - L_r$ (L_r, correction for the incomplete flushing of the leaf chamber as discussed in Materials and Methods). However, such calculations are not reasonable as leaves contain different compartments of different pH, and not all of these are in short-term CO_2/bicarbonate equilibrium. Since CO_2/HCO_3^- interconversion is a slow process in the absence of carbonic anhydrase (for rate constant of CO_2 hydration, see Ref. 23), CO_2 taken up by a leaf within the first few seconds of exposure is either dissolved gas or it is converted to HCO_3^- by carbonic anhydrase. Carbonic anhydrase is a chloroplast enzyme [7,18]. CO_2 taken up in excess of the CO_2 dissolved as gas is therefore accumulated in the chloroplast stroma. Indeed, the magnitude of CO_2 uptake by *Helianthus* leaves clearly shows that within the time span of the experiments, HCO_3^- formation in the vacuoles can be neglected. In contrast to HCO_3^- not in contact with carbon anhydrase, CO_2 dissolved as gas exchanges rapidly with the atmosphere. Estimates based on the diffusion path-length revealed that the time-constant for equilibration of a cylindrical cell of 12 μ in diameter with the intercellular atmosphere is below 0.1 s [17]. Therefore, we obtain the bicarbonate capacity of the chloroplast as $L_c = L_a + L_l - L_r$. Eqs. 1 and 2 can now be rewritten for calculating the pH of the chloroplast stroma as

$$pH =^{10} \log \frac{L_a + L_l - L_r - L_l}{L_s} + 2.987 + \frac{1000}{T} \quad (3)$$

where $2.987 + 1000/T$ stands for pK and L_s, CO_2 dissolved in the stroma space. The temperature dependence of pK has been calculated from data in Ref. 23. In microphotographs of thin sections of a *Helianthus* leaf chloroplasts occupied 10% of the cell volume. This corresponds to $L_2 = 0.002$ cm^3/cm^2 surface area or 40 μl/mg chlorophyll. This value was used for calculations, since the thylakoid space in small compared to the stroma space and can be neglected [24]. The sucrose-impermeable space of isolated chloroplasts which are usually suspended in somewhat hypertonic media has been reported to be 50 [7], 26 [4] or 20–30 [9] μl/mg chlorophyll. The results of calculations from data given in the figures are listed in Table I. Usually, the pH of the chloroplast stroma in darkened leaves was close to 7.5. The stroma of chloroplasts in illuminated leaves had a pH somewhat above 8.

Fig. 5 shows the recordings of CO_2 uptake of a sunflower leaf illuminated for 2 h in a CO_2-free

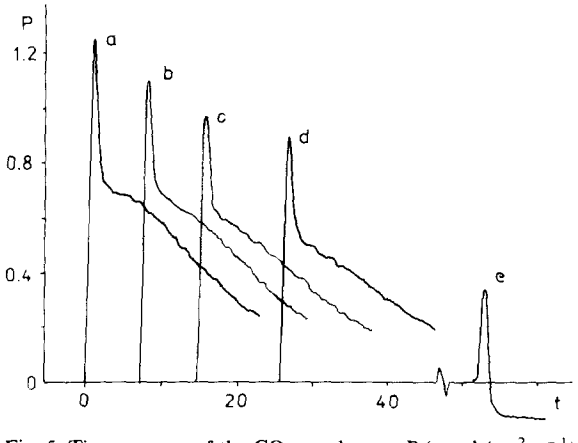

Fig. 5. Time-courses of the CO_2 uptake rate P (nmol/cm^2·s^{-1}) in a sunflower leaf with deactivated carboxylase following the transition from CO_2-free nitrogen (with 1% oxygen) to CO_2 = 230 μl/l. Light (I = 28 mW/cm^2 absorbed photosynthetically active radiation) was switched off simultaneously with CO_2-transition (a) or 7 s (b), 15 s (c) and 26 s (d) before CO_2-transition, (e) after the full self-decay of the assimilatory power. Leaf temperature, 21.8°C (dark) and 23.0°C (light). Time scale t in s.

atmosphere containing 1% O_2 to deactivate RuBP carboxylase. It was supplied with CO_2 after different periods of darkening. The first peak of CO_2 uptake corresponds to the rapid solubilization of CO_2 as shown in Fig. 2. The following shoulder is caused by carboxylation, i.e., by a metabolic event. After carboxylation had ceased, CO_2 uptake exhibited the kinetics shown already in Fig. 2b. The computer analysis of the curves as exemplified in Fig. 6 and documented in Table I (Nos. 9–13) shows that CO_2 solubilization declined with the time of darkening. This is explained by the acidification of the stroma compartment which is a consequence of the dissipation of the transthylakoid proton gradient. The data indicate that this process is slow. When the experiment was performed so that CO_2 was present during a considerable part of darkening (Fig. 7), the solubilization of CO_2 declined faster than in the absence of CO_2. The decay curves of CO_2 solubilization which reflect the time-course of stroma acidification on darkening are shown in Fig. 8.

A direct demonstration of light-dependent changes of the CO_2 capacity of a leaf is shown in Fig. 9. A photo-inhibited leaf was first illuminated in the absence of CO_2. CO_2 was then added in the light, and a rapid CO_2 uptake was observed (a). The rapid phase is mainly attributed to solubilization. When the light was turned off (b), a momentary decline in apparent CO_2 uptake is interpreted as CO_2 outburst caused by a decline in the CO_2 capacity of the leaf, i.e., by stroma acidification.

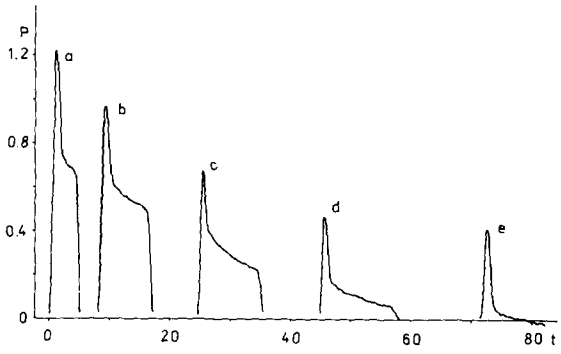

Fig. 7. Time-courses of the CO_2 uptake rate P (nmol/cm^2·s^{-1}) in a sunflower leaf with inhibited carboxylase at successive transitions from CO_2-free nitrogen (with 1% oxygen) to CO_2 = 230 μl/l in the dark. The same leaf was repeatedly switched from CO_2-free gas to CO_2-containing gas and back as indicated by the traces. Light (I = 28 mW/cm^2 absorbed photosynthetically active radiation) was turned off simultaneously with the first transition. Leaf temperature 21.8°C, time scale t in s.

This interpretation is supported by the observation that after the removal of CO_2 in the dark, readdition of CO_2 produced a solubilization peak which was much smaller than the peak observed in the light (c). Subsequent illumination increased CO_2 uptake biphasically revealing a solubilization and a carboxylation component of CO_2 uptake (d).

The simulation parameters of this experiment (Table 1, nos. 19–22) require brief comment. In the light (no. 19), the CO_2 capacity of the leaf was

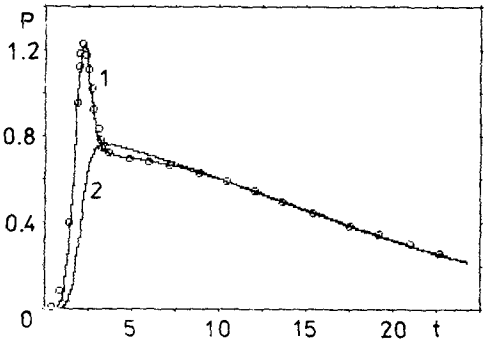

Fig. 6. A computer-simulation of the curve a from fig. 5. Experimental points (represented by circles) input from the Apple graphics tablet. Curve 1, CO_2 solubilization + photosynthetic CO_2 uptake, curve 2, photosynthetic CO_2 uptake only.

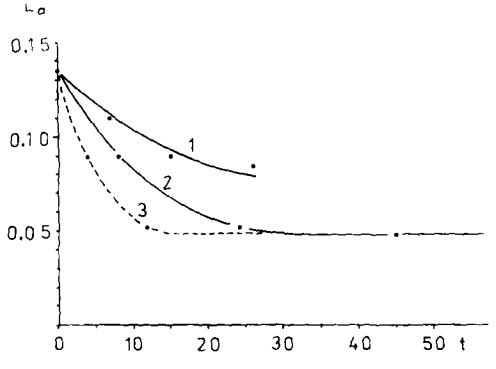

Fig. 8. Post-illumination time-courses of the rapid solubilization L_a (cm), 1, in the absence of CO_2 (data from Fig. 5); 2.3, in the presence of CO_2 (data from fig. 7), 2, natural time-scale t (s) from Fig. 7 beginning from the darkening, 3, only segments with CO_2 present included in the time-scale.

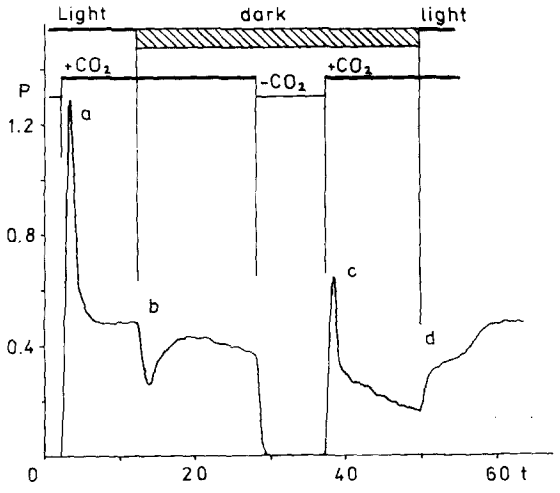

Fig. 9. Time-course of the CO_2 uptake rate P (nmol/cm^2·s^{-1}) in a sunflower leaf with inhibited carboxylase at CO_2 and light transients. Light (I = 28 mW absorbed photosynthetically active radiation) and CO_2 (C_0 = 230 µl/l) switched on and off as shown in the figure. t_1 = 21.8°C (dark) and 23.3°C (light), O_2 = 0, time scale t in s.

rather large, mainly because the 1 s component L_1 was large (0.08). For exact simulation of the light/dark transient b, the decrease in the leaf CO_2 capacity had to be taken as 0.12 cm. Such a great

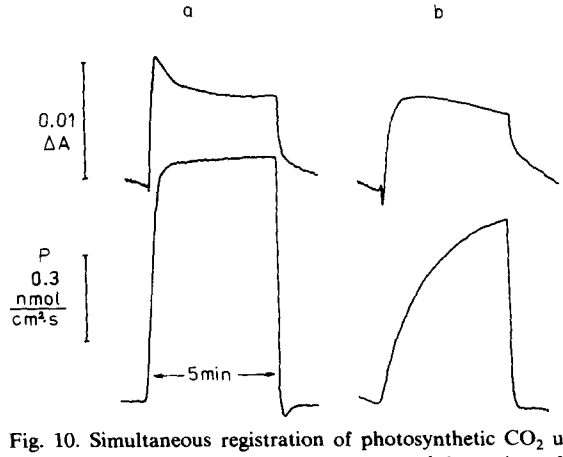

Fig. 10. Simultaneous registration of photosynthetic CO_2 uptake by a *Helianthus* leaf (lower traces) and formation of a transthylakoid protein gradient as revealed by light scattering (upper traces). (a) illumination of the leaf in air before and (b) after 40 min exposure to a CO_2-free atmosphere containing 2% O_2. After exposure, the leaf was equilibrated with air for 2 min in the dark. The intensity of red light was 270 W·m^{-2}, the maximum rate of photosynthesis in (a) 0.83 nmol/cm^2 per s (60 µmol/mg chlorophyll per h) and in (b) 0.64 nmol/cm^2 per s (46 µmol/mg chlorophyll per h).

change could not be accounted for by L_a only (its final value of 0.02 would not agree with the actually measured value of 0.06 cm in no. 21). So it was assumed that in the light/dark transient not only L_a, but also L_1 decreased. As our model (Fig. 3) did not include a variable L_1, part of the L_1 capacity was artificially ascribed to L_1. This explains why the initial value of L_a in no. 20 is taken as 0.2 cm instead of the 0.14 measured in the transient a, no. 19.

There was the question whether the illumination of leaves at a low O_2 concentration in the absence of CO_2, that was necessary for deactivating Rbu-1,5-P_2 carboxylase, influenced the extent of stroma alkalization. Fig. 10 shows photosynthetic CO_2 uptake in normal air by a *Helianthus* leaf and the formation of a transthylakoid proton gradient as revealed by light scattering before and after a 40 min illumination period in CO_2-free nitrogen containing 2% O_2. Exposure to air for 2 min in the dark brought about some reactivation of previously deactivated Rbu-1,5-P_2 carboxylase which increased during illumination resulting in the increase in photosynthesis shown in Fig. 10B. The light-scattering trace contains absorbance and scattering components. The former are fast and are seen only in the dark/light and the light/dark transients. The slow kinetics are caused by light scattering only [12]. An increase in light scattering indicates formation, while a decrease indicates decay of the transthylakoid proton gradient [13]. The formation of the gradient is accompanied by stroma alkalization. Before exposing the leaf to a

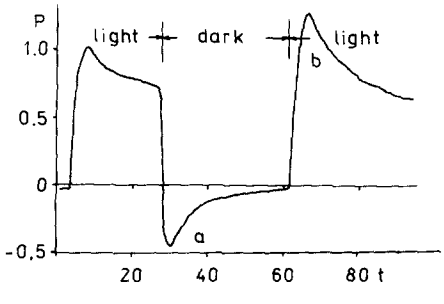

Fig. 11. CO_2 outburst and uptake peaks at light-dark transients in a sunflower leaf at 4.8°C (light) and 4.4°C (dark) induced by changing CO_2 capacity of the leaf. Photosynthesis P (nmol·cm^{-2}·s^{-1}) at 1960 µl/l CO_2 and 9.14 mW/cm^2 absorbed photosynthetically active radiation was disrupted by switching the light off (a) or on (b). Time scale t in s.

CO_2-free atmosphere, light scattering increased fast on illumination and then decreased somewhat, while photosynthesis approached the steady state. This decrease is due to the consumption of protons by increasing phosphorylation. After exposing the leaf to a CO_2-free atmosphere, light scattering increased on illumination more slowly than before exposure, and the maximum level seen in an inhibited leaf was not reached. Still, the transthylakoid proton gradient was large after the deactivation of Rbu-1,5-P_2 carboxylase indicating that the exposure of the leaf to a CO_2-free atmosphere did not interfere seriously with stroma alkalization.

The experiment shown in Fig. 11 was carried out at 4.4°C. At this low temperature, the solubility of CO_2 is high and rates of photosynthesis and respiration are low. It was therefore unnecessary to photoinhibit the leaf before the experiment. The peaks of the CO_2 burst after turning the light off, and of the uptake on turning the light on, are clearly distinguishable from the photosynthetic and respiratory CO_2 exchange processes. The dark/light increase in the CO_2 capacity exceeds somewhat the light/dark decrease (nos. 23 and 24 in Table I). This is to be expected for a leaf which had not been photoinhibited and may therefore show the induction response of the proton gradient demonstrated in Fig. 10.

Discussion

The question which pH the chloroplast stroma has in vivo in the dark and to which extent it becomes alkaline in the light is of great interest in regard to the regulation of leaf cell metabolism. It has been shown that the reductive activation of fructose bisphosphatase, an enzyme of the Calvin cycle which is inactive in the dark, requires an alkaline pH [25]. Even the light-activated enzyme is incapable of hydrolyzing fructose bisphosphate at neutral pH. Accordingly, photosynthesis of isolated chloroplasts proceeds optimally at a pH of the medium of 7.6. The pH of the stroma is actually higher. A light-dependent pH gradient is maintained between medium and stroma so that the latter is more alkaline during photosynthesis than the medium [11]. An alkaline stroma pH results in the accumulation of 3-phosphoglycerate (3-PGA) during the carboxylation of RuBP, since the chloroplast phosphate translocator does not catalyse the export of 3-PGA^{3-} and accepts only 3-PGA^{2-} as a transferable species [11]. The former accumulates at alkaline stroma pH values according to

$$3\text{-PGA}^{2-} + OH^- \rightleftharpoons 3\text{-PGA}^{3-} + H_2O$$

The accumulation of PGA in the stroma permits effective carbon reduction at relatively low phosphorylation potentials and low ratios of NADPH to NADP. Furthermore, the high levels of PGA activate adenosine diphosphoglucose pyrophosphorylase thereby opening the gate to starch synthesis [26,27]. A transenvelope pH gradient which maintains the stroma pH alkaline also stabilizes secondary gradients in the mesophyll cell. It supports a gradient between the phosphorylation potentials of chloroplasts and cytosol and a gradient in the ratios of reduced to oxidized pyridine nucleotides [10]. Thus stroma alkalization has far-reaching consequences on mesophyll metabolism.

The light-dependent alkalization of the chloroplast stroma is mainly, but not exclusively, caused by the light-dependent transfer of protons from the chloroplast stroma to the intrathylakoid space. There is also a transfer of protons from the stroma to the extrachloroplast space [4,28]. The transthylakoid proton gradient is a component of the proton-motive force which drives ATP synthesis and may be written as follows:

$$\Delta G_{H^+} = 2.3RT\Delta pH + F\Delta E$$

R and F are constants and ΔE is a light-generated membrane potential. The pH and electrical gradients can substitute one another in supporting ATP synthesis. In vitro, the pH gradient is known to be mainly responsible for thylakoid energization [35]. Isolated thylakoids may accumulate about 70 $meqH^+$ per litre of thylakoid space or 300 $neq.H^+$ per mg chlorophyll [4]. Since the buffering capacity of the stroma is about 0.65 μeq H^+ per mg chlorophyll pH unit close to pH 8 or even somewhat higher [22], this would lead to a stroma alkalization of less than 0.5 pH units in the light. However, in vivo the situation may be quite different from that observed in vitro. If in vivo the electrical gradient plays a larger role in energy

conservation than in vitro, the stroma alkalization might actually be far below 0.5 pH units. On the other hand, the stroma alkalization might be larger in vivo than predictable on the basis of the measured proton capacity of thylakoids if additional protonation reactions contribute to proton drainage from the stroma. Leaves contain significant concentrations of NH_4^+. Ammonia is formed during glycine oxidation in photorespiration. NH_4^+ ions accumulate in the light in the intrathylakoid space according to

$$\frac{[NH_4^+]_i}{[NH_4^+]_o} = \frac{[H^+]_i}{[H^+]_o}$$

i.e., an ammonium gradient comparable in magnitude to the proton gradient is formed [22]. The transport form of NH_4^+ is NH_3 which diffuses easily across biomembranes. The protons required for the intrathylakoid protonation of NH_3 are derived from the chloroplast stroma [22]. In the presence of significant concentrations of NH_4^+ the stroma alkalization might therefore be considerably larger than calculated from the capacity of thylakoids to accumulate protons.

In view of these considerations, it is highly significant that the rapid phase of bicarbonate accumulation observed in darkened leaves indicates a stroma pH of usually about 7.5 or 7.6, although higher and lower values were also occasionally observed. The pH calculation is based on

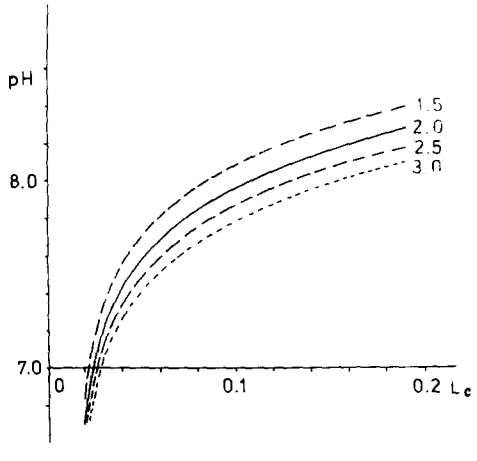

Fig. 12. Stromal pH calculated from the bicarbonate equilibrium equation (Eqn. 3) at $T = 298$ K for different possible stromal volumes per cm^2 of leaf (shown at the curves in $\mu l/cm^2$). The abscissa $L_c = L_a + L_l - L_r - L_l$ in Eqn. 3.

the assumption that rapid bicarbonate formation occurs only in the stroma where carbon anhydrase is located, and that the stroma volume is 40 μl/mg chlorophyll or 2 $\mu l/cm^2$ leaf. If the stroma volume were only 1.5 $\mu l/cm^2$, as some publications indicate [4.9], the pH would be higher, and if bicarbonate were also rapidly formed in the cytosol whose volume is about 1.5 $\mu l/cm^2$ leaf, it would be lower than 7.5. Fig. 12 shows to which extent pH values would differ from those calculated in Table I if either the stroma volume were smaller than 2 $\mu l/cm^2$ or the cytosolic volume had to be added to the stroma volume for a pH calculation. It is evident that the differences are, although significant, not very large.

The increased bicarbonate accumulation in the light is attributable to bicarbonate trapping in the stroma. It indicates considerable light-dependent stroma alkalization. Calculations such as those listed in Table I reveal an increase in the stroma pH by usually 0.6 pH units in saturating light. This is somewhat larger than expected on the basis of the proton capacity of isolated thylakoid. It may be explained by the accumulation of protonated species such as NH_4^+ in the intrathylakoid space which contribute to stroma alkalization.

Actually, it is possible that the stroma alkalization is even larger than indicated by the increased bicarbonate accumulation in the light. Fig. 10 shows that under the conditions of photoinhibition the transthylakoid proton gradient is somewhat smaller than in uninhibited leaves. The stroma alkalization should reflect this difference. If not only the chloroplast compartment, but also the cytosol contains carbon anhydrase activity [29] (carbon anhydrase is synthesized in the cytosol as indicated by inhibitor studies [30]), acidification of the cytosol as observed by Adrianov and coworkers [14,15] would decrease the CO_2 uptake due to bicarbonate accumulation in the stroma, as less CO_2 would enter into the cytosol in the light compared with the situation in the dark. Thus, the light-dependent stroma alkalization may actually be larger than calculated in Table I.

On darkening, the stroma alkalization of the leaves declined. The rate of the decline was accelerated by CO_2 (Fig. 8). Carboxylation of the pool of Rbu-1,5,P_2 by CO_2 produces PGA whose phosphorylation decreases the chloroplast ATP level

and, consequently, the proton gradient, since the chloroplast ATPase is only slowly deactivated in the dark [31]. In the absence of CO_2, ATP hydrolysis by the ATPase may actually maintain a transthylakoid proton gradient which decays by proton leakage (rate constant of leakage, about 0.14 s^{-1} [32]).

It is necessary to add a note on the variability of the data. Although usually the pH of the stroma of darkened leaves was calculated to be close to 7.6; occasionally values as high as pH 8 (Table I) or as low as 7 (not listed) were calculated. We feel that these differences cannot be attributed to experimental error. They rather appear to reflect the flexibility of leaf mesophyll metabolism. Clearly, when the pH of the stroma is low in the dark, pH modulation of enzyme activation must be more important than when it is already high. The relative contribution of the pH shift and of the formation of reductant to shortening the induction phase of photosynthesis appears to be variable. The pH modulation of the activity of photosynthetic enzymes during illumination may in vivo be less important than suggested by in vitro experiments with isolated chloroplasts [33,8]. This conclusion is supported by recent experiments which show that in leaves fructose bisphosphatase, an enzyme easily subject to pH inhibition in vitro, becomes inactive in the light only at extremely high CO_2 concentrations which shift the stroma pH towards acidity [34]. RuBP carboxylase is insensitive even to the large pH shift experienced during the light/dark transition. There was no decrease in the rate constant of the carboxylation of Rbu-1,5-P_2 (measured as described in Ref. 20) which exhibited kinetics similar to those of the stroma acidification.

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