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THE MECHANISM OF THE CONTROL OF CARBON FIXATION BY THE pH IN THE CHLOROPLAST STROMA

STUDIES WITH NITRITE-MEDIATED PROTON TRANSFER ACROSS THE ENVELOPE

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Summary

1. CO_2 fixation of intact spinach chloroplasts is inhibited by nitrite in a pH-dependent mode. At pH 7.3 in the medium 1 mM $NaNO_2$ and at pH 7.9 5 mM $NaNO_2$ were required for 50% inhibition.

2. The addition of nitrite leads to an acidification in the stroma. It appears that nitrite renders the envelope permeable for protons resulting in a breakdown of the pH gradient between the external space and the stroma.

3. In view of earlier results on the pH sensitivity of CO_2 fixation it is concluded that this pH shift in the stroma is responsible for the observed inhibition of CO_2 fixation by nitrite.

4. Octanoate and to some extent also high concentrations of bicarbonate and acetate have a similar effect as nitrite in inhibiting CO_2 fixation through an acidification in the stroma.

5. The levels of the intermediates of the CO_2 fixation cycle were measured. A strong rise of the levels of fructose- and sedoheptulose biphosphates and a concomitant decrease of the corresponding monophosphates was observed during inhibition of CO_2 fixation. It appears that the enzymatic steps of the CO_2 fixation cycle responsible for the overall inhibition of CO_2 fixation caused by lowering of the H^+ concentration in the stroma are fructose- and sedoheptulose bisphosphatase. These two enzymes have an important function in the light regulation of CO_2 fixation.

Abbreviation: CCCP, *m*-chlorocarbonylcyanide phenylhydrazone.

Introduction

On illumination of chloroplasts protons are transported across the thylakoid space. With broken chloroplasts this leads to an alkalization of the medium [1] and with intact chloroplasts to an increase of the pH in the stroma [2]. The pH in the stroma appears to be an important factor for the regulation of CO_2 fixation. Evidence has been presented that the pH changes occurring in the stroma after illumination are sufficient to switch CO_2 fixation from zero to maximal activity [3]. Experiments on the influence of valinomycin and NH_4^+ on nitrite penetration into intact chloroplasts have shown that the chloroplast envelope is permeable to undissociated HNO_2 and also to the nitrite cation [4]. A transfer of HNO_2 and NO_2^- would constitute a shuttle system for an indirect transfer of protons across the envelope, and is therefore expected to decrease the proton gradient between the external space and the stroma.

Hiller and Bassham [5] reported in their early studies on regulatory sites of CO_2 fixation in green algae that CO_2 fixation in *Chlorella pyrenoidosa* was inhibited by nitrite. The principal site of inhibition was found in the carbon cycle between fructose- and sedopheptulose bisphosphate and the corresponding monophosphates. The mechanism of this inhibition, however, was not then fully understood. Subsequently, an inhibition of CO_2 fixation by nitrite was also reported with isolated chloroplasts [6—9]. As will be shown in the following, the inhibitory effect of nitrite is due to an acidification of the stroma. Nitrite appears to be a valuable tool for manipulating the pH in the stroma in order to investigate the pH-dependent steps in the reductive CO_2 fixation cycle.

Methods

(a) Spinach (*Spinacia oleracea* L. cv. True Hybrid 102, Arthur Yates and Co., NSW, Australia) was grown in water culture according to Lilley and Walker [10].

(b) Chloroplasts with intact envelopes were prepared from fully grown leaves according to the method of Cockburn et al. [11] as modified by Heldt and Sauer [12] or by a modified Jensen and Bassham [13] procedure [14].

(c) The chloroplasts were suspended in a medium containing 0.33 M sorbitol, 50 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethane sulfonic acid (HEPES), neutralized to the pH stated in the legend with NaOH, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 0.5 mM K_2HPO_4, 5 mM $NaHCO_3$ and 2000 units/ml catalase from beef liver (Boehringer Mannheim). Other additions are indicated in the legends.

(d) Measurements of O_2 evolution were carried out with an oxygen electrode [15]. CO_2 fixation was measured from the incorporation of $[^{14}C]$bicarbonate (specific activity 0.2 Ci/mol) into material which is not volatile in the presence of 3 M acetic acid at 90°C. The pH in the stroma and the thylakoid space was measured from the uptake of 5.5-di$[^{14}C]$methyloxazolidine-2,4-dione (DMO) and $[^{14}C]$methylamine using silicone layer filtering centrifugation. For details see ref. 3.

(e) Illumination of the samples was carried out with a tungstenhalogen light source provided with a heat filter consisting of 10 cm water. The light intensity measured with a RG 630 cutoff filter (Schott, Mainz, Germany) was 120 W/m^2 if not stated otherwise. Illumination was continued during centrifugation. The labelling of phosphate-containing metabolites in the chloroplasts by $^{32}P_i$, silicone layer filtering centrifugation and the metabolite assay by ion-exchange chromatography has been described elsewhere [16].

Results and Discussion

Inhibition of CO_2 fixation by nitrite

Fig. 1 shows the inhibition of CO_2-dependent O_2 evolution by nitrite at various pH in the medium. At pH 7.3 the addition of 2 mM nitrite decreased the rate of O_2 evolution to 5%. This inhibition occurred with a lag phase of 120 s. Nitrate had no effect (data not shown). The low rate of O_2 evolution in the presence of nitrite was also observed when bicarbonate had been omitted from the medium. Apparently, this rate reflects nitrite reduction, which is known to occur in chloroplasts [8,17]. The inhibition of CO_2-dependent O_2 evolution by nitrite had a strong pH dependency, which was aslo found earlier with *chlorella* cells [5]. The inhibition was lowered when the pH of the medium was increased. Thus at pH 7.9 O_2 evolution was only slightly inhibited by nitrite. The situation is complicated by the fact, that the nitrite-dependent

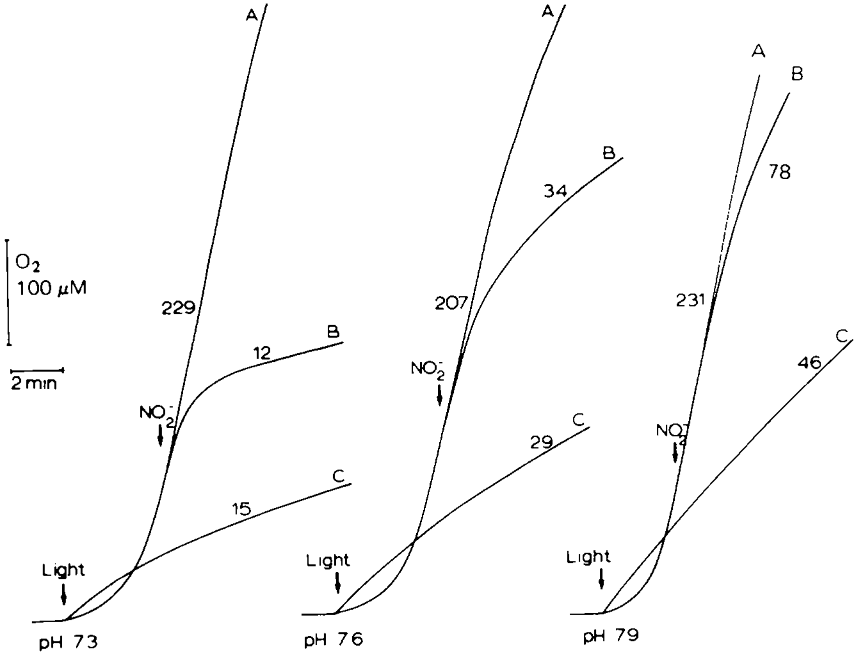

Fig. 1. Photosynthetic O_2 evolution by spinach chloroplasts (33 µg chlorophyll/ml) in the presence of $NaHCO_3$ and/or KNO_2 at different pH in the medium. A, $NaHCO_3$ (2 mM) present from beginning; B, $NaHCO_3$ (2 mM) present from beginning; addition of KNO_2 (2 mM) when indicated; C, KNO_2 (2 mM) present from beginning, $NaHCO_3$ absent. The numbers are the rates of oxygen evolution in µmol O_2/mg chlorophyll per h.

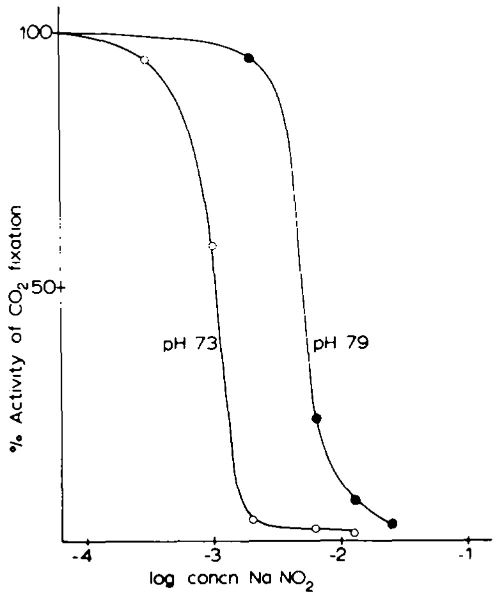

Fig. 2. Concentration dependency of the inhibition of CO_2 fixation by $NaNO_2$. Chloroplast concentration 0.025 mg chlorophyll/ml. $NaNO_2$ was added 4 min after the start of illumination, CO_2 fixation measured 7—10 min after start of illumination.

O_2 evolution in the absence of bicarbonate was increased under these conditions. For this reason, the measurment of O_2 evolution in the presence of nitrite does not fully reflect CO_2 fixation. Therefore, direct measurements of CO_2 fixation were carried out as shown in the following. Fig. 2 shows the concentration dependence of the inhibition of CO_2 fixation. Whereas at pH 7.3 50% inhibition was caused by 1 mM nitrite, 5 mM were required for 50% inhibition at pH 7.9. It should be mentioned that the nitrite concentration required for 50% inhibition varied considerably with different chloroplast preparations.

Effect of nitrite on the pH in the stroma

The strong pH dependency of the inhibition of CO_2 fixation by nitrite strengthens the afore-mentioned supposition that nitrite may act by decreasing the pH in the stroma. As shown in Table I this is indeed the case. With pH 7.6 in the medium the pH in the stroma of illuminated chloroplasts performing CO_2 fixation was 7.8 which is in accord with earlier results. The addition of 6 mM nitrite decreased the stroma pH to 7.2. As has been recently demonstrated, CO_2 fixation has a very strong dependency on the pH in the stroma [3]. Though in absolute terms this pH dependency showed some variability in different chloroplast preparations, a decrease of the stroma pH to 7.2 usually resulted in an almost complete inhibition of CO_2 fixation. Thus the inhibition by 6 mM nitrite can be fully explained as the consequence of an acidification in the stroma. With pH 7.9 in the medium, the addition of nitrite decreased the stroma pH from 7.8 to 7.6. The small inhibition of CO_2 fixation by nitrite in a medium of pH 7.9 concurs with the observation that under these conditions

TABLE I

RELATIONSHIP BETWEEN THE INHIBITION OF CO_2 FIXATION BY NITRITE AND THE pH AND METABOLITE LEVELS IN THE STROMA

Chloroplast concentration, 0.05 mg chlorophyll/ml; HCO_3^-, 5 mM. Light intensity, 100 W/m^{-2}. Time schedule after start of illumination: Addition of nitrite, 3 min; termination of samples, 5.5 min; CO_2 fixation measurement, 4—7 min after onset of illumination.

Expt.	$NaNO_2$ (mM)	pH			ΔpH (stroma —thylakoid)	CO_2 fixation (μmol/mg chlorophyll per h)	Metabolites in the stroma (nmol/mg chlorophyll)						
		Medium	Stroma	Thylakoid			FBP	SBP	HMP	RuBP	DHAP + GAP	PGA	$\frac{ATP}{ADP}$
A	—	7.60	7.81	5.39	2.42	95	3	5	47	26	3	50	0.5
	6	7.60	7.22	5.00	2.22	4	31	29	11	21	10	4	1.9
B	—	7.30	7.51	4.98	2.53	128	3	2	45	9	5	72	0.7
	2	7.30	6.89	4.64	2.25	7	25	8	17	4	13	8	1.8
	—	7.90	7.82	5.21	2.61	127	2	2	68	6	7	116	0.3
	2	7.90	7.62	5.05	2.57	109	4	3	62	6	9	77	0.3

FBP, fructose 1,6-biphosphate; SBP, sedoheptulose 1,7-biphosphate; HMP, mixture of hexose and heptose monophosphates; RuBP, ribulose 1,5-biphosphate; DHAP, dihydroxyacetone phosphate; GAP, glyceraldehyde phosphate; PGA, 3-phosphoglycerate; CCCP, *m*-chlorocarbonylcyanide phenylhydrazone.

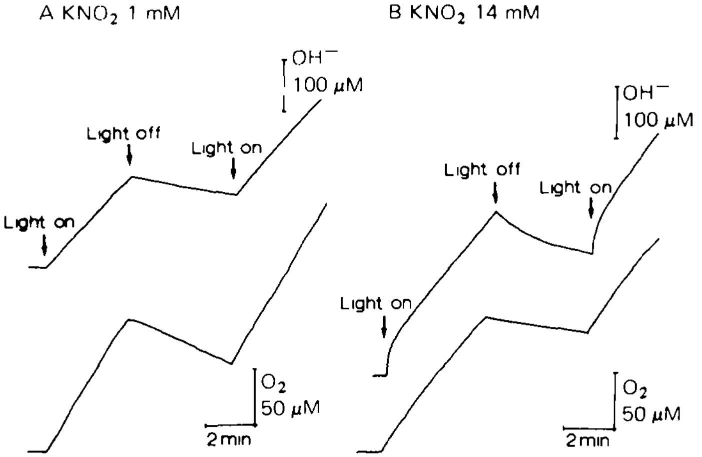

Fig. 3. Simultaneous recording of OH$^-$ formation in the medium and O_2 evolution by intact chloroplasts which were illuminated in the presence of different concentrations of KNO_2. Chlorophyll concentration 0.1 mg chlorophyll/ml. Upper trace recording of a pH electrode. Lower trace recording of an oxygen electrode.

the stroma pH was not much lowered beyond the optimal range for CO_2 fixation.

Indirect proton transfer across the envelope by HNO_2-NO_2^- shuttle

In the experiment of Fig. 3A, the nitrite facilitated proton transfer across the envelope is further demonstrated. When chloroplasts were illuminated in the presence of 1 mM KNO_2, there was a steady increase in the pH of the medium which was parallel to oxygen evolution. This is caused by OH$^-$ production during nitrite reduction [4] according to the following equation:

$$NO_2^- + 3\,H_2O \rightarrow NH_4^+ + 2\,OH^- + 1.5\,O_2 \qquad \left(\frac{OH^-}{O_2} = 1.33\right)$$

In the presence of higher nitrite concentrations (Fig. 3B), OH$^-$ production in the medium showed two phases. There was first a fast proton uptake from the medium, which was followed by a slower stationary OH$^-$ production. Only the latter was parallel to oxygen evolution. When the light was turned off, an increase in the proton concentration in the medium was observed. Thus the initial fast proton uptake in the light was reversed in the dark. This phenomenon is interpreted to reflect the light-dependent proton transport across the thylakoid membranes. Due to the low permeability of the envelope for protons the resulting light-dependent pH increase outside the thylakoid membrane is normally confined to the stroma space. However, in the presence of sufficient nitrite, this pH increase extends into the medium where it can be measured with a glass electrode. In this way it is possible to measure the amount of protons transported across the thylakoid membrane of intact chloroplasts. From the experiment of Fig. 3B it is elavated that 0.37 μequiv. H$^+$/mg chlorophyll are transported during a dark-light transient.

Since the chloroplast envelope is permeable to HNO_2 and also NO_2^- [4] the effect of nitrite is explained by allowing an indirect proton transfer across the envelope through a HNO_2—NO_2^- shuttle, which results in a collapse of the proton gradient between the stroma and the external space. In the dark or in the presence of uncoupler, the pH in the stroma is usually more acidic than in the medium. With a medium pH of 7.6, the pH in the stroma is normally found to be about 7.2 [3]. This pH gradient in the dark appears to be due to a Donnan equilibrium [18]. Illumination results in an alkalization of the stroma space to pH 7.8—8.0 [3]. For the evaluation of the effective proton gradient across the envelope in the light the observed Donnan potential has to be taken into account. Therefore, the effective proton gradient across the envelope of illuminated chloroplasts will be much higher (0.6—0.8) than the measured pH differences between the stroma and the medium (0.2—0.4). This explains why the addition of nitrite in Table I lowers the pH from 7.8 to 7.2. The maintenance of the proton gradient across the envelope required some active proton transport from the stroma into the external space in order to balance unspecific proton leakage or indirect proton transfer by substrate shuttles across the envelope. Such a light-dependent extrusion of protons from the stroma has been demonstrated recently [2,18,19] with a transport capacity of about two orders of magnitude lower than light-dependent proton transport across the thylakoid membrane. The acidification of the stroma in illuminated chloroplasts by nitrite would imply that the passive proton influx from the medium to the stroma caused by nitrous acid- nitrite shuttle is faster than the active proton extrusion.

pH dependency of the inhibition of CO_2 fixation by nitrite

A discussion of the inhibition by nitrite has to account for two partial effects: firstly the effect of nitrite on the pH in the stroma reflecting the rate of passive proton influx into the stroma and secondly the influence of the pH change in the stroma on CO_2 fixation. The latter effect is strongly pH dependent, as discussed previously. Due to the pH sensitivity of CO_2 fixation, a pH change in the stroma from 7.8 to 7.6 has only a small effect, whereas a pH change from 7.5 to 6.9 results in a dramatic inhibition of CO_2 fixation. It seems likely that the nitrite-mediated passive proton flux is limited by the HNO_2 flux across the envelope. With a p*K* of 3.4, the concentration of HNO_2 in equilibrium with 6 mM NO_2^- at pH 7.6 is as low as 0.4 μM. A pH change of 1 would alter the HNO_2 concentration by a factor of 10. Both of these factors may contribute to the strong pH dependency of the nitrite inhibition, not only observed here with isolated chloroplasts but also found earlier by Hiller and Bassham [5] with green algae. These authors required only about 50 μM nitrite for the inhibition of CO_2 fixation in *Chlorella* cells, which is two orders of magnitude lower than the corresponding concentration needed in our experiments. This concentration difference may be explained by the fact, that Hiller and Bassham [5] incubated the *Chlorella* cells in a medium of pH 5.0, and therefore the applied HNO_2 concentrations were very similar to those in our experiments.

Effect of nitrite on the proton gradient across the thylakoid membrane

It might be expected that the addition of nitrite also facilitates a passive proton efflux from the thylakoid space into the stroma, thus lowering the ΔpH across the thylakoid membrane. In fact, only a slight decrease of the ΔpH was found, as shown in Table I. As the ATP/ADP ratio observed in these chloroplasts was even elevated, the inhibition of CO_2 fixation by nitrite cannot be explained by uncoupling of photophosphorylation. Apparently, light-dependent proton transport into the thylakoid space exceeded the nitrite-mediated proton efflux. It should be mentioned, that in experiments with isolated thylakoids (not shown) 2 mM nitrite had very little effect on the magnitude of the light-dependent pH gradient. At high light intensities, no significant uncoupling of phosphorylation from electron flow was observed in the presence of 2 mM nitrite.

Effect of other salts of weak acids on CO_2 fixation

As reported earlier [3], also the uncoupler *m*-chlorocarbonylcyanide phenylhydrazone (CCCP) when applied at very low concentrations, also lowered the pH gradient across the envelope, whilst only little affecting the ΔpH across the thylakoid membrane. It also strongly inhibited CO_2 fixation when photophosphorylation was still proceeding. In this respect, CCCP appears to have a similar effect on the envelope as nitrite. The question arises whether the effect of nitrite on the proton gradient across the envelope is also produced by other salts of weak acids. In principle, some lowering of the proton gradient leading to partial inhibition of CO_2 fixation has been also observed with acetate and bicarbonate, but very large concentrations are required. In one experiment previously published [3], 20 mM bicarbonate at pH 7.6 decreased the stroma pH from 7.9 to 7.6, resulting in 44% inhibition of CO_2 fixation. Very similar effects were also observed with 20 mM acetate. It appears that in these cases the indirect proton transfer is limited by the low permeability of the envelope to acetate and bicarbonate anions [4]. Pedersen et al. [20] reported earlier that a number of fatty acids, e.g. 0.3 mM octanoic acid, inhibited CO_2 fixation in *Chl. pyrenoidosa* in a medium of pH 5, but most of this inhibition was reversed when the medium was changed to pH 7.0. Since this effect of fatty acids somewhat resembled the effect of nitrite, it was to be expected that octanoate may also affect the proton gradient across the envelope. The experiment of Table II reveals that the addition of octanoate decreases the stroma pH,

TABLE II

INHIBITION OF CO_2 FIXATION BY OCTANOATE

Chloroplast concentration 0.05 mg chlorophyll/ml; HCO_3^-, 5 mM; medium, pH 7.6. 2.5 min after the start of illumination, the additions were made and 4.5 min after onset of illumination, the measurements carried out.

Addition	O_2 evolution in the presence of HCO_3^-		pH		
	µmol/mg chlorophyll per h	Inhibition %	Stroma	Thylakoid	Δ
—	90		7.58	5.17	2.41
Octanoate (10 mM)	48	47	7.38	5.18	2.22
Octanoate (20 mM)	38	58	7.36	5.14	2.23
Nitrite (6 mM)	21	77	6.98	4.79	2.19

TABLE III

THE EFFECT OF NH_4Cl ON THE INHIBITION OF CO_2 FIXATION BY NITRITE

Chloroplast concentration, 0.05 mg chlorophyll/ml. The additions were made 5.5. min after the start of illumination. The measurements were carried out 7—9 min after onset of illumination.

Addition (mM)	O_2 evolution (μmol/mg chlorophyll per h)	CO_2 fixation	pH		
			Stroma	Thylakoid	Δ
—	75	75	7.92	5.31	2.62
$NaNO_2$ (2)	15	3	7.45	4.77	2.67
$NaNO_2$ (2) + NH_4Cl (5)	46	17	7.58	5.81	1.77
NH_4Cl (5)	46	44	7.74	5.95	1.79

resulting in an inhibition of CO_2 fixation. This inhibition shows a time dependence. The data of Table II were obtained after 4.5 min incubation with octanoate. Longer incubation times lead to much stronger inhibition. Also with octanoate, as with nitrite, the ΔpH across the thylakoid membrane is less affected than the proton gradient across the envelope.

The prevention of the inhibitory effect of nitrite by ammonia

The inhibition of O_2 evolution by nitrite may be partially released by the addition of NH_4Cl, as shown in Table III. A large portion of the oxygen evolved under these conditions seems to be due to an increase of nitrite reduction, but there was also an increased rate of CO_2 fixation observed (Table III). This effect of NH_4Cl is only seen with lower concentrations of nitrite which do not fully inhibit CO_2 fixation and is not observed in all chloroplast preparations. It appears from the pH measurements that NH_4Cl acts on CO_2 fixation by preventing the nitrite-mediated acidification in the stroma. The mechanism of this effect of NH_4Cl is not fully understood. Further experiments are required to clarify this matter.

pH-dependent steps of the reductive CO_2 fixation cycle

There is the question which enzymatic steps of the metabolic pathway of CO_2 fixation are responsible for the observed inhibition of CO_2 fixation. Since phosphoglycerate reduction, which is a partial step of the CO_2 fixation cycle, was shown to be almost insensitive to pH changes in the stroma between pH 7.0 and 8.5, it must be concluded that the pH-dependent steps were neither the generation of NADPH nor ATP by photosynthetic electron transport, but steps of the CO_2 fixation cycle between dihydroxyacetone phosphate and phosphoglycerate [3].

To further identify these pH-dependent steps, illuminated chloroplasts were first allowed to perform CO_2 fixation under optimal conditions. During illumination, the pH in the stroma was changed to a value normally observed in the dark. The inhibited steps of the CO_2 fixation cycle should then be recognized from an accumulation of the substrates and decrease of the products of these particular steps. It was desirable to obtain the pH changes in the stroma in such a way that the ΔpH across the thylakoid membrane, generally assumed to be the driving force of photophosphorylation [21], is only little altered. Such pH changes in the stroma have been produced earlier by the addition of acetate or of low concentrations of *m*-chlorocarbonylcyanide phenylhydrazone [3]. As discussed above, acetate is not very effective in this respect, and even low concentrations of CCCP have a considerable uncoupling effect. Therefore, nitrite appeared to be a much better tool for a specific manipulation of the pH in the stroma. In the experiment of Table I the intermediates of the CO_2 fixation cycle were quantitatively analyzed. Since all these intermediates contain phosphate they can be conveniently labelled when CO_2 fixation is allowed to proceed in the presence of [^{32}P]phosphate. A very rapid separation of the chloroplasts from the surrounding medium by silicone layer filtering centrifugation, followed by a quantitative determination of the labelled substances using ion-exchange chromatography permits a sensitive and accurate measurement of the metabolites in the stroma in the steady state of CO_2 fixation [16]. As shown in Table I, the inhibition of CO_2 fixation by nitrite at pH 7.6 in the medium is accompanied by dramatic changes of the amounts of hexose- and heptose phosphates. During active CO_2 fixation the amounts of fructose- and heptose biphosphates are low, and the sum of hexose- and heptose monophosphates (these compounds are usually not very well separated by our chromatographic technique and are therefore determined in total) is relatively high. The addition of nitrite causes a very strong increase of the fructose- and sedoheptulose bisphosphates and a decrease of the corresponding monophosphates. This clearly indicates that the hydrolysis of fructose- and sedoheptulose bisphosphate is blocked under these conditions. The addition of nitrite had no dramatic effect on the amount of ribulose bisphosphate in the stroma. However, that a considerable amount of ribulose bisphosphate is found even though the rate of CO_2 fixation is inhibited to about 95% may indicate that the carboxylation is inhibited under these conditions. The large decrease of the phosphoglycerate and the increase of the ATP/ADP ratio after the inhibition by nitrite is a consequence of the inhibition of the overall process of CO_2 fixation, as has been discussed elsewhere [22]. With a medium pH of 7.9, where CO_2 fixation is only little affected by nitrite, there is also no major change of the metabolite levels observed after nitrite addition. Obviously, an acidification in the stroma is a prerequisite for the inhibition of fructose and sedoheptulose bisphosphatase in the stroma. It appears therefore that the observed inhibition of CO_2 fixation caused by nitrite-mediated pH changes in the stroma is primarily due to the inhibition of the fructose- and sedoheptulose bisphosphatase. Hiller and Bassham [5] also observed with *Chlorella* cells that the addition of nitrite caused a marked increase of fructose- and sedoheptulose bisphosphates accompanied by a decrease of the corresponding monophosphates, and similar metabolite changes were also seen when CO_2 fixation in *Chlorella* was inhibited by octanoate [20].

As shown recently with intact chloroplasts, the activities of the fructose- and sedoheptulose bisphosphatase are also very effectively controlled by changes of the stromal Mg^{2+} [22]. An activation of the stromal fructose bisphosphatase by increasing pH and Mg^{2+} has been also found with the isolated enzyme [23, 24] and with reconstituted chloroplasts [25]. From previous results on the light-dependent decrease of H^+ [3] and the corresponding increase of Mg^{2+} [26,27] in the stroma, the pH and the Mg^{2+} dependency of CO_2 fixation [3, 26] and the identification of the Mg^{2+} [22] and the pH-dependent steps it is concluded that CO_2 fixation is controlled via the activities of the fructose- and sedoheptulose biphosphatase by light-dependent cation transport across the thylakoid membrane. This offers a simple explanation how CO_2 fixation is controlled by light in order to avoid wasteful CO_2 fixation [3] in the dark.

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