Biochimica et Biophysica Acta, 592 (1980) 577—591

BBA 47912

METABOLIC REGULATION BY pH GRADIENTS

INHIBITION OF PHOTOSYNTHESIS BY INDIRECT PROTON TRANSFER ACROSS THE CHLOROPLAST ENVELOPE *

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(Received November 8th, 1979)
(Revised manuscript received April 28th, 1980)

Key words: Proton transfer; Chloroplast envelope; Photosynthesis inhibition; Fructose-bisphosphatase; pH effect; Glyoxylate

Summary

Anions of several weak acids inhibited photosynthesis in isolated spinach chloroplasts. Inhibition was drastic at low pH and weak or absent at high pH. Glyoxylate was particularly effective and inhibition decreased in the order: glyoxylate, nitrite, glycerate, formate, hydroxypyruvate, glycolate, propionate, acetate, pyruvate. These anions operated as indirect proton shuttles across the chloroplast envelope. They compensated active proton fluxes into the medium, minimized gradients in proton activity across the chloroplast envelope, and so prevented light-dependent stroma alkalization. This caused inhibition of sugar bisphosphatases which are known to be pH-regulated. At concentrations that caused photosynthesis inhibition, the proton shuttles were not effective in decreasing the proton gradient across the thylakoids. Some anions also inhibited fructose-bisphosphatase directly, when present at concentrations higher than needed for photosynthesis inhibition.

Introduction

On illumination, the stroma space of intact chloroplasts becomes alkaline owing to the pumping of protons into the intrathylakoid space [1]. Leakage of protons from the medium into the alkaline stroma is compensated by an active

* Dedicated to Professor H. Ullrich, Bonn, on the ocassion of his 80th birthday.
Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid.

counter-flux of protons across the chloroplast envelope into the medium [2]. The alkalization of the stroma activates pH-sensitive enzymes of the Calvin cycle such as fructose-bisphosphatase [3,4] thus permitting photosynthesis to occur. Excessive inflow of protons from the medium into the stroma interferes with pH regulation and results in inhibition of photosynthesis. Inflow may be direct or indirect. Nitrite, which can permeate the chloroplast envelope both after protonation and in the anionic form [5], has been shown to be a powerful inhibitor of photosynthesis. Inhibition has been explained by the capability of nitrite to mediate indirect proton transport and decrease the proton gradient across the chloroplast envelope [6]. Later it became apparent that nitrite can also interfere with the reductive activation of enzymes of the photosynthetic carbon cycle [7]. Inhibition of photosynthesis by effects on the stroma pH should be non-specific for the anion used. In the following, different proton shuttle systems are described which are capable of inhibiting photosynthesis. Both non-specific and specific inhibitory effects are identified and discussed.

Materials and Methods

Intact chloroplasts capable of photoreducing CO_2 at high rates were prepared at 4°C from freshly picked green-house or field-grown spinach by a modification [8] of the procedure of Jensen and Bassham [9]. The percentage of intact chloroplasts was measured by the ferricyanide method [10]. Preparations which contained less than 75% intact chloroplasts were not used.

Illumination was provided by a halogen lamp. Light was filtered through water (8 cm), a heat absorbing filter (1 mm Calflex C from Balzers, Liechtenstein) and a cutoff filter (3 mm RG 630 from Schott, Mainz) which permitted passage of red light with a halfband width from 630 to 760 nm. Light intensity was 270 W/m^2 if not mentioned otherwise. It was measured by a YSI-Kettering radiometer model 65 A.

Oxygen evolution was recorded by a Clark type electrode in a well-stirred medium which contained 330 mM sorbitol, 40 mM Hepes, 10 mM NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 0.5 mM KH_2PO_4 and catalase, 1500 I.U./ml, pH 7.6, if not mentioned otherwise. Chlorophyll concentration was 33 to 66 μg/ml. Bicarbonate was usually added to a final concentration of 2 mM. When more was added, the medium pH was readjusted after addition to the original value. The fluorescence of 9-aminoacridine (5 μM) was excited by a weak beam of 400 nm light and was recorded through a 459 nm interference filter by a photomultiplier which was protected against actinic light by Corning filters 5-57 and 4-96. pH changes were measured in chloroplast suspensions with an Ingold electrode in the same medium as above except that buffer was omitted. The recorded proton exchange was calibrated by addition of 0.01 N HCl. Light scattering was measured as apparent absorbance at 535 nm in a Zeiss spectrophotometer. The medium contained 220 mM sorbitol, 33 mM Hepes, 1.32 mM EDTA, 0.66 mM $MnSO_4$, 0.66 mM $MgSO_4$, pH 7.6. Chlorophyll concentration was 50 to 100 μg/ml. To avoid chloroplast dilution on adding small volumes of concentrated salt solutions, the additions also contained chloroplasts.

The pH of the stroma and of the intrathylakoid space of intact chloroplasts

was calculated from the distribution of 5,5-dimethyloxazolidine-2,4-dione and methylamine according to Heldt et al. [1]. ^{14}C-labelled compounds were obtained from Amersham Buchler, Braunschweig. After photosynthesis in the presence of $^{14}CO_2$, the distribution of labelled metabolites in the chloroplast stroma was measured as described by Lilley et al. [11]. For separation of metabolites, the method of Giersch [12] was used. Fructose-bisphosphatase was measured spectrophotometrically at 340 nm according to Latzko and Gibbs [13].

Results

1. Inhibition of photosynthesis by salts of weak acids

Fig. 1 shows bicarbonate-dependent oxygen evolution of intact spinach chloroplasts at different pH of the suspending medium before and after addition of sodium glyoxylate or before and after increasing the sodium bicarbonate concentration from 2 to 30 mM at constant pH. At 2 mM bicarbonate, and without further additions, the pH optimum of photosynthesis was usually close to pH 7.6, although some variation was observed with different chloroplast

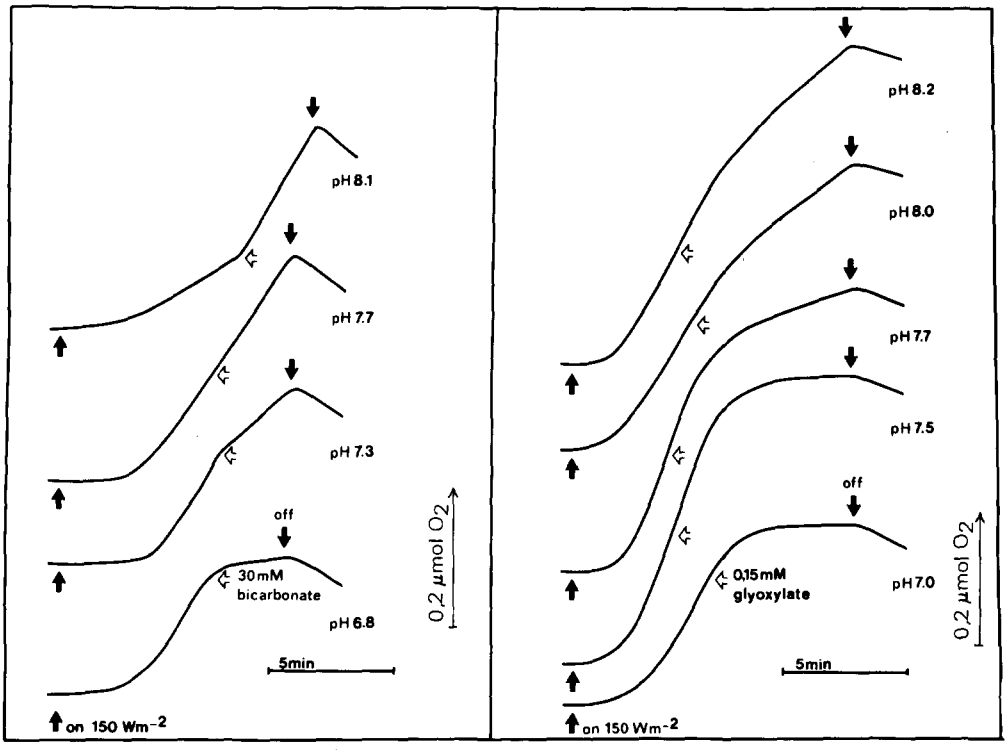


Fig. 1. Effect of 30 mM bicarbonate (left) and 0.15 mM glyoxylate (right) on CO_2-dependent oxygen evolution of intact spinach chloroplasts at different pH of the suspending medium. The oxygen concentration of the medium was recorded by a Clark electrode. Illumination with 150 W/m^2 red light. The rate of oxygen evolution was in the pH 7.5 experiment after the initial lag phase 204 µmol/mg chlorophyll per hour.

preparations. Increased concentrations of bicarbonate inhibited photosynthesis at low pH. At high pH, when photosynthesis was limited by the availability of CO_2, high bicarbonate concentrations stimulated photosynthesis by increasing the CO_2 concentration. From this, it is clear that the pH optimum of photosynthesis is shifted by bicarbonate into the alkaline range. Inhibition of photosynthesis by bicarbonate was first reported by Werdan et al. [14].

Glyoxylate also produced drastic inhibition of photosynthesis at low pH. At high pH, it was much less effective as an inhibitor. It also shifted the pH optimum of photosynthesis to higher values. Glyoxylate did not support oxygen evolution in the light and was therefore not photoreduced at significant rates. In contrast to inhibition of photosynthesis by glyoxylate, which slowly increased with time, inhibition by bicarbonate became immediately effective after addition. The different kinetics of inhibition is explained by faster penetration of CO_2 than of glyoxylic acid into the chloroplast stroma. Once photosynthesis became inhibited, it did not recover subsequently when the pH of the medium was held constant. However, titration to a higher pH restored photosynthesis. Inhibition at low pH was not specific. Table I lists concentrations of different salts that produced 50% inhibition of CO_2-dependent oxygen evolution at pH 6.9. Glyoxylate was the most and pyruvate the least effective inhibitor. Inhibition was always more pronounced at low than at high pH.

2. Permeability of the chloroplast envelope

Light scattering measurements can be used to monitor ion fluxes across the chloroplast envelope [5,15]. Fig. 2 shows the response of intact chloroplasts to the addition of different salts, to valinomycin and to ammonium sulfate as recorded by light scattering. On the stepwise addition of potassium salts, light scattering increased stepwise and then remained constant. The rapid scattering increase indicates osmotic chloroplast shrinkage and the constant level of scattering the absence of significant salt fluxes [5]. When the K^+ ionophore valinomycin was added, light scattering slowly decreased. The decrease was faster with propionate than with pyruvate. It reflects osmotic chloroplast

TABLE I

p*K* VALUES AND CONCENTRATIONS OF DIFFERENT SALTS THAT PRODUCED 50% INHIBITION OF PHOTOSYNTHESIS BY INTACT CHLOROPLASTS AT pH 6.9 (BICARBONATE CONCENTRATION 2 mM)

	Concentration (mM)	p*K*	Concentration of free acid (μM)
Glyoxylate	0.04	3.5	0.016
Nitrite	0.2	3.37	0.06
Glycerate	3	3.3	0.75
Formate	5	3.75	3.5
Hydroxypyruvate	10	—	3.5
Glycolate	15	3.83	12.7
Propionate	15	4.88	143
Acetate	20	4.75	141
Pyruvate	90	2.5	3.5

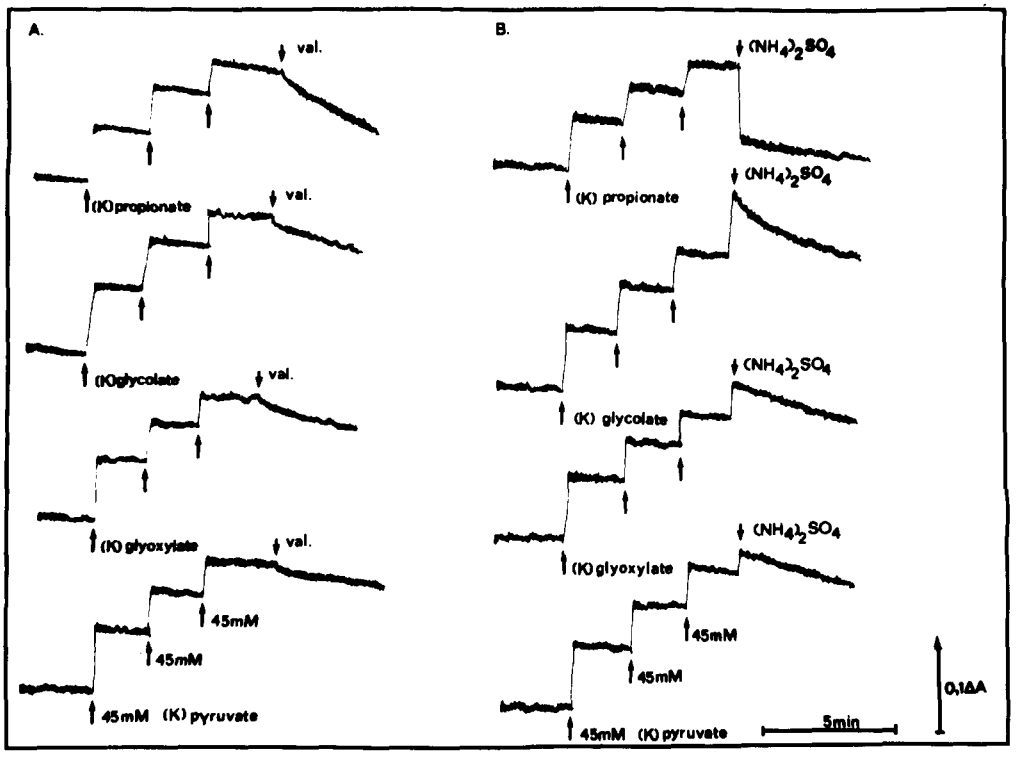


Fig. 2. Osmotic response of intact chloroplasts to the addition of different potassium salts (three additions of 45 mM each) and to 0.15 μM valinomycin or to 60 mM $(NH_4)_2SO_4$ as measured by the apparent absorption of a chloroplast suspension at 535 nm. For explanation, see text.

swelling which results from the influx of both anions and K^+. The data indicate that the chloroplast envelope permits transfer of the propionate, glycolate, glyoxylate and pyruvate anions. Pyruvate passage appeared to be very slow.

When, instead of valinomycin, ammonium sulfate was added to chloroplasts which were osmotically shrunk in a medium containing different potassium salts, an immediate scattering decrease was observed in the presence of potassium propionate. With glycolate, glyoxylate and pyruvate, there was first a transient scattering increase which was followed by a slow decrease. Since the chloroplast has only a low permeability to NH_4^+, K^+ and SO_4^{2-} [5], and since in the absence of cation fluxes net transfer even of penetrating anions is impossible, the scattering decrease indicates fluxes of NH_3 and protonated anions which are formed from the charged species according to

$$NH_4^+ + A^- \rightarrow NH_3 + AH.$$

The scattering data thus indicate that propionic acid penetrates the chloroplast envelope very rapidly, while penetration of glycolic acid, glyoxylic acid and of pyruvic acid is slower.

3. Proton gradients

Table II lists pH values of the stroma and of the intrathylakoid space of intact chloroplasts as measured by the distribution of ^{14}C-labelled 5,5-dimethyl-

TABLE II

EFFECT OF FORMATE ON THE pH GRADIENTS ACROSS THE ENVELOPE OF INTACT CHLOROPLASTS AND ACROSS THE THYLAKOID MEMBRANES WITHIN THE CHLOROPLASTS

pH of the medium 7.6. Average of 4 determinations.

	pH, stroma	Transenvelope ΔpH	pH, thylakoids	Transthylakoid ΔpH
Dark, 4 min	7.54	−0.06	5.91	1.63
Dark, 4 min + 40 mM formate	7.57	−0.03	6.05	1.52
Light, 4 min	7.90	+0.3	5.43	2.47
Light, 4 min + 5 mM formate	7.70	+0.1	5.42	2.28
Light, 4 min + 10 mM formate	7.58	−0.02	5.21	2.37
Light, 4 min + 20 mM formate	7.54	−0.06	5.00	2.54
Light, 4 min + 40 mM formate	7.52	−0.08	5.02	2.50
Light, 4 min + 60 mM formate	7.56	−0.04	5.13	2.43

oxazolidine-2,4-dione and [^{14}C]methylamine between the chloroplasts and the medium [1,16]. In the dark, the stroma pH was somewhat lower than the pH of the medium, and the pH of the intrathylakoid space was in turn lower than the pH of the medium. The pH gradients in the dark probably reflect the Donnan distribution of H^+. They were not decreased by carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone which increases the proton conductivity of biomembranes. On illumination, the pH of the intrathylakoid space decreased by about 0.5 pH units, while the pH of the stroma increased by almost 0.4 pH units. Formate added to darkened chloroplasts did not decrease the stroma pH of illuminated chloroplasts. While it abolished the ΔpH across the chloroplast envelope, it did not significantly affect the ΔpH across thylakoid membranes. Glyoxylate was more effective than formate in decreasing the stroma pH in illuminated chloroplasts (not shown).

The fluorescent dye 9-aminoacridine is an amine that distributes across biomembranes according to the H^+ distribution [17]. It forms non-fluorescent dimers when concentrated after protonation in an acidic compartment [18]. 9-Aminoacridine fluorescence quenching is often used to indicate the formation of proton gradients in biological systems. In illuminated thylakoids, it monitors the formation of a transthylakoid proton gradient. In intact chloroplasts, light-dependent 9-aminoacridine fluorescence quenching indicates formation of a gradient between the intrathylakoid space of the chloroplasts and the medium [19]. If properly calibrated, fluorescence quenching can be used for a quantitative estimation of ΔpH [20]. Fig. 3 records photosynthetic oxygen evolution and 9-aminoacridine fluorescence in a suspension of intact chloroplasts before and after addition of glyoxylate. On illumination, 9-aminoacridine fluorescence was quenched indicating the formation of a proton gradient. As photosynthetic oxygen evolution accelerated after an initial lag phase which is due to the buildup of pools of carbon cycle intermediates [21], the proton gradient decreased slightly, apparently owing to photosynthetic energy consumption [22]. On addition of glyoxylate, ΔpH increased, and there was a close correlation between the kinetics of this increase and inhibition of oxygen evolution. Fig. 4 shows the increase of the proton gradient as indicated by

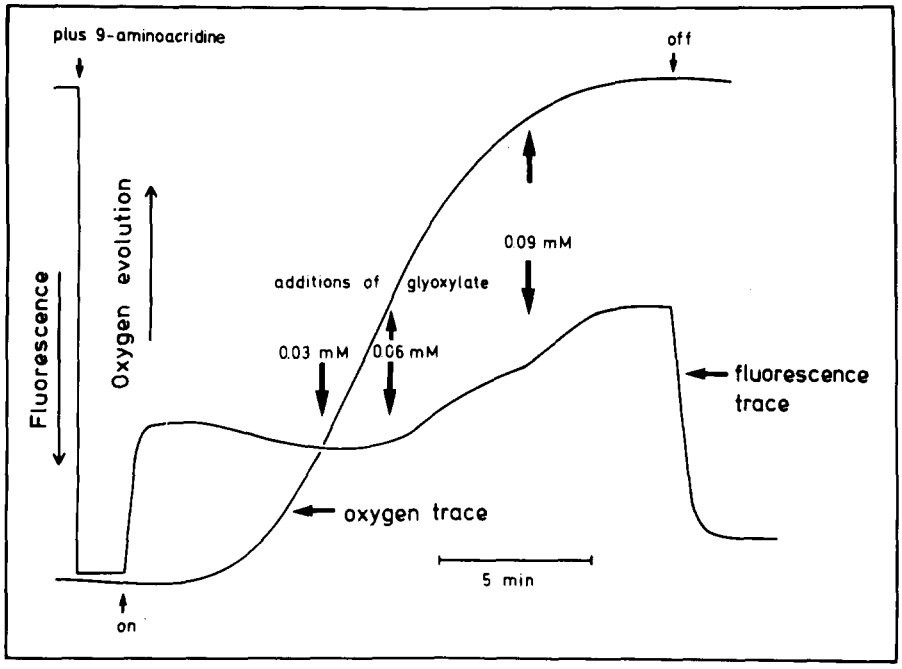


Fig. 3. Effect of glyoxylate on CO_2-dependent oxygen evolution by intact chloroplasts and on the proton gradient between the intrathylakoid space and the suspending medium. Simultaneous recording of oxygen concentration and of 9-aminoacridine fluorescence quenching which indicates formation of the proton gradient. pH 7.2. The slope of the oxygen trace after the initial lag phase corresponds to 78 μmol evolution/mg chlorophyll per hour.

9-aminoacridine fluorescence quenching and inhibition of photosynthesis as a function of the concentration of glyoxylate, nitrite and formate. Again the correlation between increase in fluorescence quenching and inhibition of photosynthesis is apparent. It appeared possible that the proton gradient increased as a consequence of increased ATP/ADP ratios, when photosynthesis became inhibited. This is ruled out by the finding that the proton gradient also increased on addition of inhibitory salt, when photosynthesis was inhibited by an excess of inorganic phosphate (not shown) or when oxaloaceatate served as electron acceptor (Fig. 4). The reduction of oxaloacetate is not accompanied by the consumption of ATP. In contrast to effects caused by glyoxylate, nitrite increased 9-aminoacridine fluorescence quenching less when oxaloacetate served as electron acceptor than in the presence of bicarbonate. Other salts capable of inhibiting photosynthesis also increased ΔpH as indicated by aminoacridine fluorescence quenching. However, the inhibitory salts failed to increase fluorescence quenching in broken chloroplasts (not shown).

Heldt et al. [1] have reported that the intrathylakoid pH decreases in illuminated chloroplasts as the stroma pH decreases. Table II also shows that a decrease in the stroma pH is accompanied by a decrease in the intrathylakoid pH. The fluorescence response of intact chloroplasts to inhibitory salts is therefore not due to an increase in the transthylakoid ΔpH, but to a decrease in the proton gradient across the chloroplast envelope. The transenvelope pH gradient decreased on addition of glyoxylate in the experiment of Fig. 3 by 0.4 pH (cal-

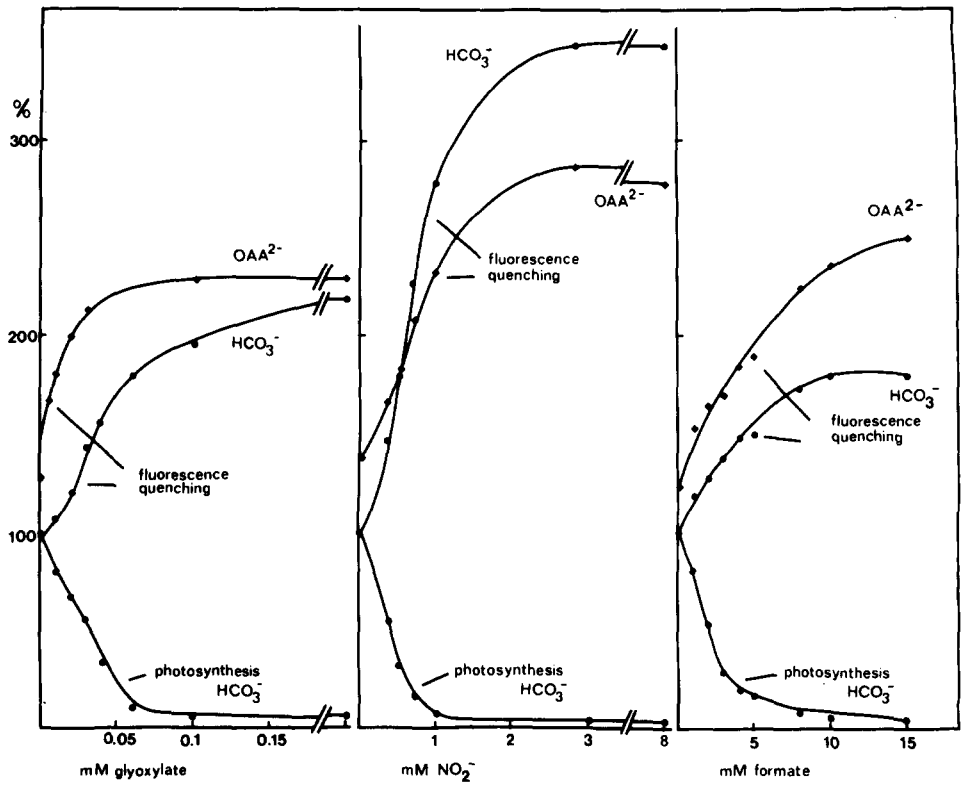


Fig. 4. Photosynthesis and quenching of 9-aminoacridine fluorescence by intact chloroplasts as a function of the concentration of glyoxylate, nitrite and formate. Note different scales on the abscissae. Photosynthesis without added salt (100%) was 153 μmol O_2 evolution/mg chlorophyll per hour in the glyoxylate experiment, 136 μmol in the nitrite experiment and 113 μmol in the formate experiment. pH of the reaction medium was 7.2, light intensity 150 W/m^2. 2 mM $NaHCO_3$ or 1 mM oxaloacetate were substrates. The extent of 9-aminoacridine fluorescence quenching in the absence of inhibitory salt and with HCO_3^- as substrate was taken as 100%.

culated from 9-aminoacridine fluorescence quenching according to de Benedetti and Garlaschi [20]). The formation of the transenvelope pH gradient in the absence of inhibitory salts needed very little light. Fig. 5 shows fluorescence quenching as a function of light intensity with and without added formate. At about 8 W/cm^2 the increase in fluorescence quenching brought about by formate was maximal. In comparison, the light saturation of photosynthesis is above 100 W/m^2. The data of Fig. 5 indicate that the alkalization of the chloroplast stroma which can be abolished by inhibitory salts is saturated at a very low light intensity. The decrease in ΔpH caused by formate was calculated to be 0.35 pH units at 8 W/m^2.

Fig. 6 shows the increase in fluorescence quenching as a function of pH in the suspending medium. Again formate was used as the inhibitory salt. There was no or only very little decrease in ΔpH between the intrathylakoid space pH of the medium the ΔpH across the chloroplast envelope is small. The increase in fluorescence quenching was maximal at low pH where photosynthesis is effectively inhibited by formate. At low pH, the pH across the chloro-

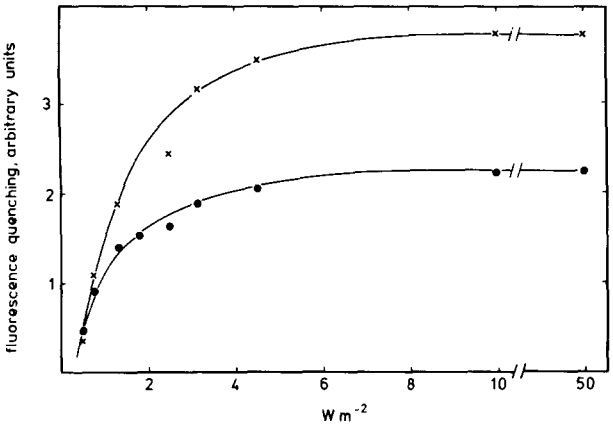


Fig. 5. 9-Aminoacridine fluorescence quenching with and without 20 mM potassium formate as a function of the intensity of red light. pH of the reaction medium, 6.7. •———•, without formate; X———X, after addition of formate.

plast envelope is considerable. Glyoxylate differed from other inhibitory salts in that it increased fluorescence quenching significantly not only at low but also at high pH.

4. Salt-induced proton uptake by intact chloroplasts

In the absence of substrates, intact chloroplasts excrete protons on illumination in a cation exchange reaction [2,23]. In the presence of inhibitory concentrations of glyoxylate, proton release was replaced by proton uptake (Fig. 7). The kinetics of the uptake reaction reflect the kinetics of light-dependent proton uptake by thylakoids. In agreement with the fluorescence data, it appears that in the presence of an inhibitory anion the chloroplast envelope ceases to be a barrier to the movement of protons. Fig. 8 shows proton

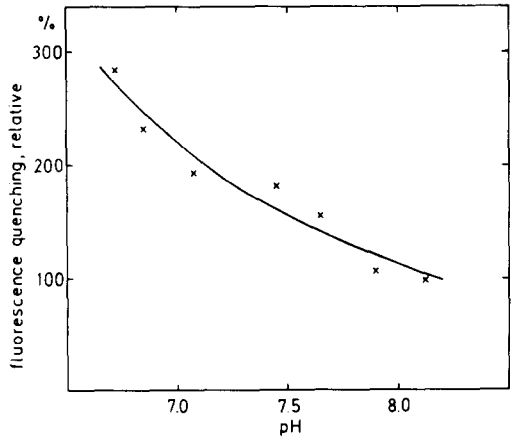


Fig. 6. Increase in 9-aminoacridine fluorescence quenching on addition of 10 mM formate to intact chloroplasts as a function of the pH of the suspending medium. 100% indicates fluorescene quenching without formate. Illumination with 100 W/m^2 red light.

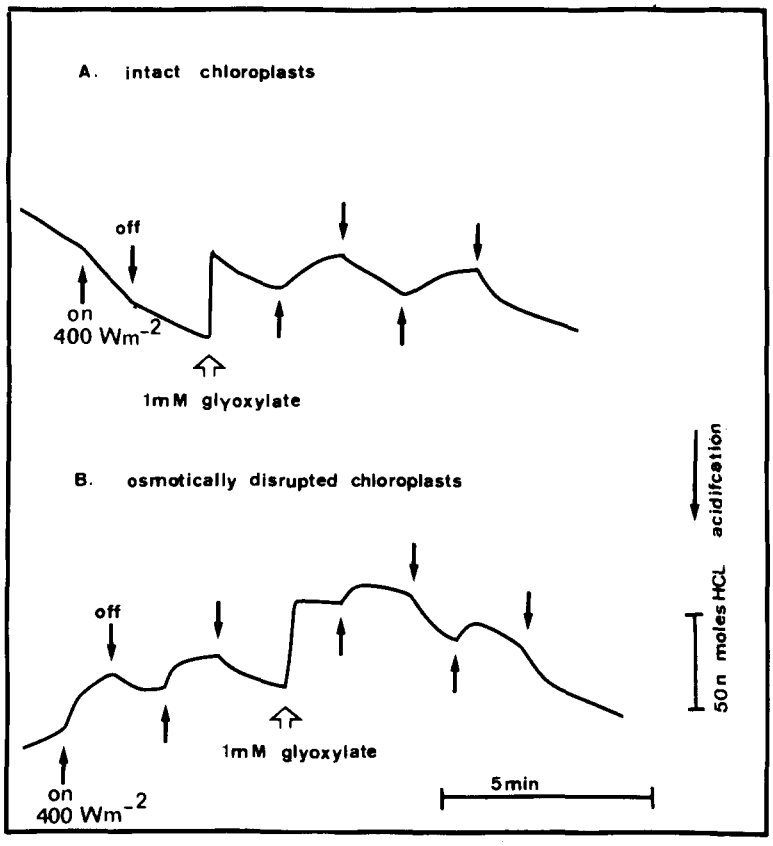


Fig. 7. Light-dependent pH changes in a slightly buffered isotonic reaction medium (pH 7.1) containing intact or broken chloroplasts (45 μg chlorophyll/ml) as influenced by glyoxylate. Light-dependent pH changes were recorded by a pH electrode.

exchange of intact chloroplasts as a function of the concentration of formate. In the absence of formate, proton release was considerable. A low concentration of formate was sufficient to suppress proton excretion. Proton uptake was maximal and amounted to 180 nmol H^+/mg chlorophyll at a concentration of about 5 mM formate. Light-dependent proton uptake by broken chloroplasts at pH 7.1 was comparable to this figure.

5. Distribution of stromal metabolites

Table III shows the distribution of metabolites labelled with ^{14}C during photosynthesis in the presence of $^{14}CO_2$ as measured after the separation of chloroplasts from the suspending medium by silicone layer centrifugation [11]. In the absence of formate, 21% of the labelled carbon was in sugar monophosphates and only 4% in fructose bisphosphate. Obviously, dephosphorylation of fructose bisphosphate was so fast as to prevent any significant accumulation of this intermediate. After inhibition of photosynthesis by formate, however, fructose bisphosphate accumulated dramatically and levels of sugar monophosphates declines. Another decline was observed in ribulose bisphosphate which

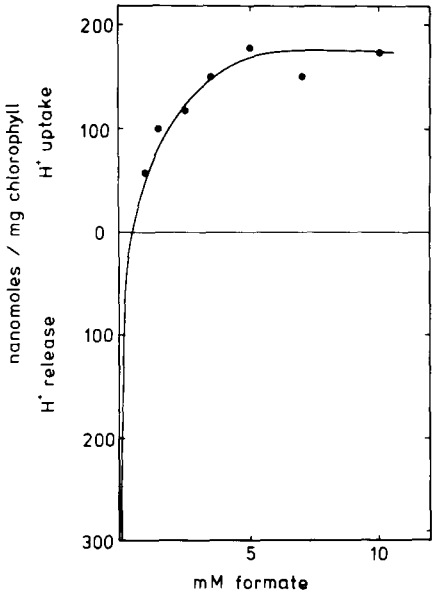


Fig. 8. Proton exchange of intact chloroplasts illuminated with saturating red light as a function of the concentration of formate. pH of the reaction medium 7.1.

is formed from the sugar monophosphates. The accumulation of sedoheptulose bisphosphate after formate inhibition of photosynthesis was much less significant than that of fructose bisphosphate. The ratio of phosphoglycerate to dihydroxyacetone phosphate was high before and low after inhibition of photosynthesis. Part of the change in the ratio is caused by the acidification of the stroma. Protons take part in the reaction

$$PGA^{3-} + ATP^{4-} + NADPH + H^+ \rightarrow DHAP^{2-} + ADP^{3-} + P_i^{2-} + NADP^+,$$

and an increase in the proton concentration decreases the ratio of phosphoglycerate (PGA) dihydroxyacetone phosphate (DHAP). Another factor in the

TABLE III

EFFECT OF 15 mM POTASSIUM FORMATE ON THE DISTRIBUTION OF LABELLED STROMAL METABOLITES AFTER 8 min PHOTOSYNTHESIS IN THE PRESENCE OF $^{14}CO_2$

pH of the medium 7.2, bicarbonate concentration 2 mM. The rate of photosynthesis was 140 μmol per mg chlorophyll per hour in the control and 11 μmol per mg chlorophyll per hour in the presence of formate. Figures are presented as percentages.

	Control	+ Formate
Total	100	100
Free sugars, glycolate and minor components	25	14
Sugar monophosphates	21	7
Ribulose bisphosphate	16	9
Fructose bisphosphate	4	49
Seduheptulose bisphosphate	7	10
Dihydroxyacetone phosohate	4	9
Phosphoglycerate	23	2

decrease of the ratio is an increase in the ratio of ATP/ADP, as has been observed in studies on the effect of nitrite on photosynthesis [6].

As the pH of the stroma decreased in the presence of formate, a higher percentage of phosphoglycerate was exported from the chloroplasts. The concentration ratio of phosphoglycerate in the chloroplasts to phosphoglycerate in the medium was 3.8 in the absence and 0.41 in the presence of formate after 8 min illumination.

Together with earlier evidence [6], the data of Table II demonstrate that inhibition of photosynthesis by formate is caused by inactivation of fructose-bisphosphatase. This enzyme is known to be active in the light under slightly alkaline conditions and to be inactive at neutral pH [3,4]. Inhibition of photosynthesis is not caused by lack of ATP, as this would have caused an increase in phosphoglycerate.

The distribution of metabolites in the stroma of intact chloroplasts in the presence of 0.2 mM glyoxylate was very similar to that observed when photosynthesis was inhibited by formate. Again dephosphorylation of fructose bisphosphate appeared to be blocked.

6. Direct enzyme inhibition

In Table I it is shown that glyoxylate is much more inhibitory than formate or propionate. This observation is difficult to reconcile with observations that show the permeability of the chloroplast envelope to propionic acid and its anion to be higher than that to glyoxylic acid and its anion (Fig. 2). Inhibition by glyoxylate may, therefore, not be solely attributable to a lowering of the stroma pH as the fluorescence and photosynthesis data of Fig. 4 suggest.

After activating fructose bisphosphatase in intact chloroplasts by illumination, the chloroplasts were lysed osmotically and fructose-bisphosphatase was measured in the lysate (Table IV). Addition of glyoxylate, nitrite or formate at concentrations that inhibited photosynthesis in intact chloroplasts did not affect enzyme activity significantly. However, progressive inhibition of fructose bisphosphatase was observed as glyoxylate or formate concentrations were

TABLE IV

ANION EFFECTS ON THE ACTIVITY OF FRUCTOSE-BISPHOSPHATASE

Fructose-bisphosphatase was activated by illuminating intact chloroplasts at pH 7.6 for 5 min. It was released by suspending chloroplasts with about 2 μg chlorophyll in a hypotonic medium containing 20 mM Tris and 5 mM $MgCl_2$, pH 8.2. Controls (100%) dephosphorylated fructose bisphosphatase at a rate of about 50 μmol/mg chlorophyll per hour. Salts were added to the enzyme assay. Figures are presented as percentages.

mM	Additions		
	Glyoxylate	Nitrite	Formate
0	100	100	100
1	90	96	100
3	70	93	94
6	40	93	84
10	27	97	65

increased. As anions are accumulated in the chloroplast stroma as long as the latter is more alkaline than the medium (see Discussion) direct enzyme inhibition might, in addition to pH effects, contribute to inhibition of photosynthesis.

Discussion

1. Proton shuttles

Weak acid anions are in or at least very close to equilibrium with the undissociated acid both inside and outside the chloroplast envelope

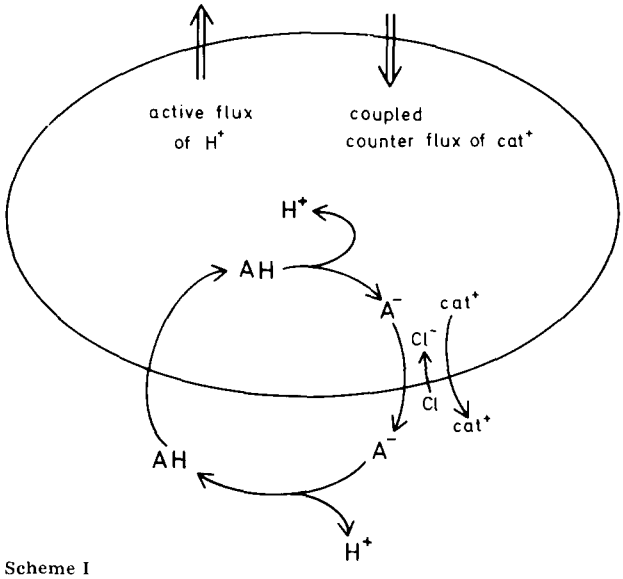
$$\frac{[A^-]_o[H^+]_o}{[AH]_o} = \frac{[A^-]_i[H^+]_i}{[AH]_i} \quad (1)$$

When a salt of a weak acid is added to a chloroplast suspension, AH will penetrate the chloroplast envelope, if the latter is permeable, and dissociate in the chloroplast stroma decreasing the stroma pH. At flux equilibrium, the activity of AH must be the same inside and outside the chloroplast envelope. It follows that

$$\frac{[A^-]_i}{[A^-]_o} = \frac{[H^+]_o}{[H^+]_i} \quad (2)$$

However, the acidification of the chloroplast stroma in the light by salts of weak acids as documented in Table II cannot simply be explained by the dissociation of an acid that has penetrated the chloroplast envelope in the undissociated form. According to Heldt et al. [1], the stroma pH rises to about 7.6 in the light, when the pH of the medium is 6.9 as in the experiment of Table I. From the Henderson/Hasselbach equation and the buffer capacity of intact chloroplasts (about 48 μequiv. H^+/ml per pH unit at pH 7.6; Kaiser, G. and Heber, U., unpublished data) it can be calculated that direct acidification by acid influx into the stroma is very different for different salts at similar photosynthesis inhibition. An acidification of less than 0.01 pH unit with glyoxylate, 0.02 unit with nitrite, 0.3 unit with formate and about 0.6 unit with acetate is indicated by Table I. In the experiment of Table II, direct acid influx in darkened chloroplasts should have decreased the stroma pH by about 0.35 pH units. However, no significant decrease was seen. Moreover, if inhibition of photosynthesis were simply caused by acid influx, it should be transient because the chloroplast can pump protons into the medium in exchange for external monovalent cations [23]. Inhibition should cease after the light-driven H^+/cation exchange reaction had reestablished the original stroma pH which permitted photosynthesis to occur before the inhibitory salt had been added. The activity of the proton pump is considerable: ion fluxes up to 24 μequivalents per mg chlorophyll have been measured in the dark/light transition [23] which is sufficient capacity to re-establish the original stroma pH within a few minutes after addition of an inhibitory salt concentration. However, no secondary pH increase in the stroma was observed and inhibition of photosynthesis was not temporary, presumably because anion permeability of the chloroplast envelope permits a shuttle transfer of protons. Scheme I shows the ion fluxes

across the envelope of illuminated chloroplasts in the presence of a proton shuttle system. Loss of A^- from the chloroplast is facilitated in part by



Scheme I

counter-transport of permeant anions such as chloride but mainly by passive co-transport of cations (cat^+) that have previously been imported in exchange for H^+. If the anions could not penetrate, they should accumulate in the chloroplast stroma according to Eqn. 2 as the stroma pH is increased by active proton pumping into the medium above the pH dictated by the Donnan distribution of ions. Penetrating anions, on the other hand, should leak back into the medium, if such leakage is permitted by appropriate cation co-transport or anion counter-transport.

2. Enzyme inactivation

Although Fig. 4 indicates that the potent inhibitors glyoxylate and nitrite are very effective in decreasing the ΔpH across the chloroplast envelope, it is possible that other effects of these compounds such as direct inhibition of fructose-bisphosphatase also contribute to inhibition (Table IV).

Interference of salts of weak acids with light-dependent enzyme activation [24] has been shown to occur [7]. In addition to this, nitrite can cause oxidative enzyme inhibition [7]. It is photo-reduced in the chloroplasts in a ferredoxin-dependent reaction. As enzyme activation by the chloroplast thioredoxin system is also ferredoxin-dependent [24], nitrite interferes not only with pH regulation but also with redox regulation.

3. Inhibition of photosynthesis

The accumulation of fructose bisphosphate and the depletion of sugar monophosphate pools in chloroplasts on addition of salts of weak acids show that fructose-bisphosphatase becomes rate determining when the stroma pH is not

optimally regulated for photosynthesis. In the absence of inhibitory salts the stroma pH is regulated so as to permit considerable enzyme activation at very low light, as the data of Fig. 5 indicate. The proton gradient across the chloroplast envelope that permits stroma alkalization is formed not only in isolated chloroplasts but exists also in vivo. Photosynthesis in algae [25] and in protoplast from leaves (Kaiser, G. and Heber, U., unpublished data) is sensitive to salts of weak acids, and the ratio of phosphoglycerate to dihydroxyacetone phosphate is higher in the chloroplasts than in the cytoplasm of leaf cells [26]. It has been shown above that this ratio is indicative of pH differences in the chloroplast stroma and the extrachloroplast space.

Acknowledgments

We are very grateful to Professor H.W. Heldt for introducing us to the secrets of the silicone layer centrifugation technique and to both Professor Heldt and Dr. E. Weis for stimulating discussions. Dr. C. Giersch was so kind as to aid us in the analytical separation of metabolites and one of the reviewers helped to improve the manuscript. This investigation was supported by the Deutsche Forschungsgemeinschaft.

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