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THE ROLE OF pH IN THE REGULATION OF CARBON FIXATION IN THE CHLOROPLAST STROMA. STUDIES ON CO_2 FIXATION IN THE LIGHT AND DARK

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SUMMARY

1. The pH in the stroma and in the thylakoid space has been measured in a number of chloroplast preparations in the dark and in the light at 20 °C. Illumination causes a decrease of the pH in the thylakoid space by 1.5 and an increase of the pH in the stroma by almost 1 pH unit.

2. CO_2 fixation is shown to be strongly dependent on the pH in the stroma. The pH optimum was 8.1, with almost zero activity below pH 7.3. Phosphoglycerate reduction, which is a partial reaction of CO_2 fixation, shows very little pH dependency.

3. Low concentrations of the uncoupler *m*-chlorocarbonylcyanide phenylhydrazone (CCCP) inhibit CO_2 fixation without affecting phosphoglycerate reduction. This inhibition of CO_2 fixation appears to be caused by reversal of light induced alkalisation in the stroma by CCCP.

4. Methylamine has a very different effect compared to CCCP. Increasing concentrations of methylamine inhibit CO_2 fixation and phosphoglycerate reduction to the same extent. The light induced alkalisation of the stroma appears not to be significantly inhibited by methylamine, but the protons in the thylakoid space are neutralized. The inhibition of CO_2 fixation by higher concentrations of methylamine is explained by an inhibition of photophosphorylation. It appears that methylamine does not abolish proton transport.

5. It is shown that intact chloroplasts are able to fix CO_2 in the dark, yielding 3-phosphoglycerate. This requires the addition of dihydroxyacetone phosphate as precursor of ribulosemonophosphate and also to supply ATP, and the addition of oxaloacetate for reoxidation of the NADPH in the stroma.

6. Dark CO_2 fixation in the presence of dihydroxyacetone phosphate and oxaloacetate has the same pH dependency as CO_2 fixation in the light. This demonstrates that CO_2 fixation in the dark is not possible, unless the pH in the medium is artificially raised to pH 8.8.

Abbreviations used in tables, figs and equations: PGA, 3-phosphoglycerate; DAP, dihydroxyacetone phosphate; FDP, fructosediphosphate; DTT, dithiothreitol.

7. It is shown that pH changes occurring in the stroma after illumination are sufficient to switch CO_2 fixation from zero to maximal activity. This offers a mechanism for light control of CO_2 fixation, avoiding wasteful CO_2 fixation in the dark.

INTRODUCTION

Illumination of chloroplasts causes a transport of protons across the thylakoid membrane [3]. We have shown recently that this light induced proton transport leads to a considerable alkalisation of the chloroplast stroma [4]. The question arose whether this change in the chloroplast stroma caused by illumination is of importance in the regulation of CO_2 fixation by light. To answer this question, we investigated the dependency of CO_2 fixation by intact chloroplasts in the light and in the dark on the pH in the stroma.

METHODS

(a) Chloroplasts with intact envelopes were prepared from spinach obtained at the local market according to the method of Cockburn et al. [5] modified by Heldt and Sauer [6]. The intactness of the chloroplasts was checked by phase contrast microscopy [7] and ferricyanide dependent O_2 evolution [8]. 80–95 % of the chloroplasts of a usual preparation appeared to be intact.

(b) The incubation was normally carried out in a medium containing 0.33 M sorbitol, 50 mM *N*-2-hydroxyethylpiperazine *N*-2-ethane sulphonic acid (HEPES), adjusted to the appropriate pH with NaOH, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 2 mM EDTA. Chlorophyll was assayed after the method of Whatley and Arnon [9].

(c) Illumination of the samples contained in the centrifuge tubes or in the vessel with the oxygen electrode was carried out with a tungsten halogen light source provided with an RG 630 cutoff filter (Schott, Mainz, Germany) and a Calflex C heat filter (Balzers, Lichtenstein). The light intensity was 80 000 ergs $s^{-1} \cdot cm^{-2}$. Illumination was continued during centrifugation.

(d) CO_2 fixation was usually carried out in a medium as described above containing 3mM bicarbonate and 0.25 mM phosphate, and phosphoglycerate reduction was done in the presence of 2 mM 3-phosphoglycerate and 3 mM phosphate. The chloroplast concentration was 0.04–0.08 mg chlorophyll/ml, and the temperature 20 °C. Both reactions were measured by the rate of O_2 evolution with an oxygen electrode [10], with 1 mM 5.5 dimethyloxazolidine-2.4-dione (DMO) and 0.03 mM methylamine present in the medium. Simultaneous assays of the pH in the stroma and in the thylakoid space were carried out in parallel samples according to the method of Heldt et al. [4]. The measuring times for assay of O_2 evolution and pH were the same (5–7 min).

(e) For enzymatic assay of 3-phosphoglycerate and dihydroxyacetone phosphate and of malate see ref. 11.

(f) CO_2 fixation in the dark was usually carried out at 20 °C in a medium as described above containing 5 mM dihydroxyacetone phosphate, 5 mM oxaloacetate, 16 mM NaH_2PO_4, 10 mM $NaH^{14}CO_3$ (specific activity 1 Ci/mol), 5 mM dithiothreitol and 50 mM *N*-tris (hydroxymethyl)-methylglycine (Tricine) instead of HEPES,

adjusted to pH 8.8. The reaction was started by addition of chloroplasts 0.1–0.4 mg chlorophyll/ml, and terminated by adding 100 μl of the suspension to 100 μl 2 M NaOH. For measurement of fixed carbon 100 μl of the soluble alkaline extract were added to 500 μl 1 M HCl, evaporated at 90 °C, redissolved in 100 μl 2 M $HClO_4$, and counted by liquid scintillation counter. From each value the control value obtained in the absence of dihydroxyacetone phosphate was subtracted. The rate of CO_2 fixation was evaluated from the difference of the radioactive carbon fixed at 4 and 6 min from the start of the reaction. Dihydroxyacetone phosphate was prepared from dihydroxyacetone phosphate dimethylketal (Boehringer, Mannheim, Germany) according to the procedure advised by the manufacturer. For further experimental details see ref. 2.

RESULTS

pH changes in the stroma caused by illumination

Measurements of pH changes in the chloroplast stroma, as mentioned in the introduction, had been carried out so far at 4 °C. Very similar results are obtained if the measurements are done at 20 °C, as shown in Table I. In the dark the difference in pH between the thylakoid space and the stroma space is almost zero. There is a pH gradient between the stroma space and the medium, which may be due to a Donnan potential, caused by negatively charged proteins in the stroma. Such a Donnan potential has been also postulated from measurements of K^+ concentrations in intact chloroplasts [12]. Illumination of the chloroplasts causes proton transport from the stroma space into the thylakoid space. Thus the pH in the stroma is increased by almost 1 pH unit which is in accordance with previously published data obtained at 4 °C [4]. The increase of pH in the stroma is accompanied by a decrease of the pH in the thylakoid space. A ΔpH of 2.5 across the thylakoid membrane is usually determined under these conditions. It may be noted that the determination of the pH in the thylakoid space is based on an estimation that the volume of the thylakoid space is 12.5 percent of the total space surrounded by the inner membrane of the envelope [4]. If the thylakoid space in the intact chloroplasts would be actually lower than this, the pH in the thylakoid space would be also lower, and the ΔpH across the thylakoid membrane

TABLE I

MEASUREMENT OF pH IN INTACT SPINACH CHLOROPLASTS

Medium pH 7.6, bicarbonate 2 mM, temperature 20 °C.

Expt No.	Stroma space		Thylakoid space		pH gradient between spaces	
	light	dark	light	dark	light	dark
	pH		pH		pH	
16	8.11	7.11	5.45	7.04	2.67	0.07
41	7.99	6.93	5.32	6.75	2.67	0.18
45	8.00	7.31	5.69	6.88	2.31	0.43
133	8.00	7.28	5.58	6.66	2.42	0.62
	∅ 8.02	∅ 7.16	∅ 5.51	∅ 6.83	∅ 2.51	∅ 0.33

higher. However, this would not significantly alter the pH values determined in the stroma space (see Table III in ref. 4).

Methods for changing the pH in the stroma during illumination

In order to investigate whether these pH changes caused by illumination play a role in the regulation of CO_2 fixation, we tried to vary the stroma pH in illuminated chloroplasts without affecting other parameters, e.g. redox state of electron carriers, which are normally also changed in the dark light transient and which may be also important for regulation of CO_2 fixation. Several methods are available for directed change of the stroma pH in illuminated chloroplasts.

(a) The pH in the stroma can be varied by changing the pH in the medium, whilst the ΔpH across the thylakoid membrane is only very little affected [4]. Fig. 1 shows the dependency of the pH in the stroma on the pH in the medium, as measured at 20 °C.

(b) The ΔpH between the medium and the stroma can be decreased by the ad-

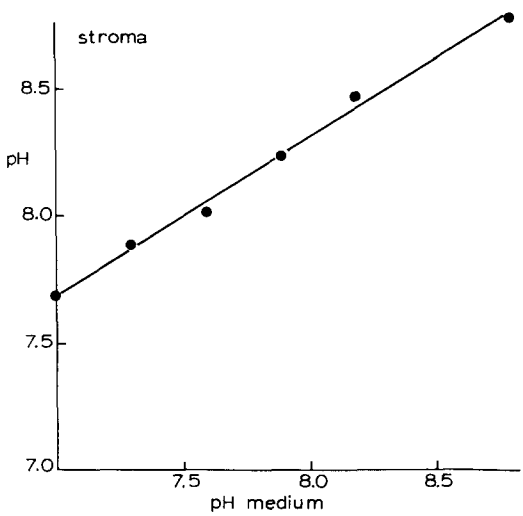

Fig. 1. Dependency of the pH in the stroma on the pH in the medium, as measured at 20 °C under conditions of CO_2 fixation. Chloroplast concentration: 0.033 mg chlorophyll/ml.

TABLE II

DEPENDENCY OF THE pH IN THE STROMA DURING ILLUMINATION ON THE CONCENTRATION OF ACETATE IN THE MEDIUM

CO_2 fixation in presence of 2 mM bicarbonate. Medium pH 7.6, temperature 20 °C.

Acetate in medium (mM)	pH stroma	ΔpH stroma–medium
0	7.97	0.37
10	7.71	0.11
20	7.64	0.04
40	7.59	−0.01

TABLE III

INFLUENCE OF THE BICARBONATE CONCENTRATION IN THE MEDIUM ON THE pH DEPENDENCY OF CO_2 FIXATION

Simultaneous measurement of the pH in the stroma and the rate of CO_2 fixation when varying the pH in the medium. Illumination. Temperature 20 °C.

Bicarbonate in medium (mM)	pH medium	pH stroma	CO_2 dependent O_2 evolution (μmol O_2/mg chlorophyll/h)
1	6.40	6.94	0
1	7.30	7.57	28.9
1	7.60	7.88	53.1
1	7.90	8.29	48.6
20	6.40	6.53	0
20	7.30	7.30	0
20	7.60	7.56	29.8
20	7.90	7.96	46.0

dition of anions of certain weak acids, e.g. bicarbonate [13], penetrating the envelope as the undissociated molecule, thus performing an indirect transfer of protons from the medium into the stroma. The same holds if both the anion and the undissociated acid move across the envelope, as has been proposed for acetic acid [14]. In this way the ΔpH between the stroma and the medium can be decreased to about zero, as shown with acetate in Table II. Similar results are obtained with high concentrations of bicarbonate (Table III) and dimethyloxazolidinedione (not shown here).

(c) The pH in the stroma and the thylakoid space is changed by the addition of *m*-chlorocarbonylcyanide phenylhydrazone (CCCP) causing a reversal of light driven proton transport (Fig. 2A). This leads to two different effects: It abolishes the pH gradient across the thylakoid membrane, which is regarded to be a driving force for photophosphorylation, and it also abolishes the alkalisation of the stroma caused by light. By using increasing concentrations of CCCP, these two effects can be distinguished from each other. Low concentrations of CCCP decrease the pH in the stroma with only little increase of pH in the thylakoid space, whereas with higher concentrations of CCCP the pH in the thylakoid space is mainly affected. This has been also observed at lower temperatures and was explained by assuming different buffer capacities with different pK in the stroma and in the thylakoid space [4].

(d) The action of methylamine appears to be entirely different from the effect of the uncoupler CCCP (Fig. 3A). Increasing concentrations of methylamine decrease the ΔpH across the thylakoid membrane without major effect on the pH in the stroma. Whereas the action of CCCP can be explained as reversing light driven proton transport, there is obviously proton transport in the presence of methylamine, since the light driven alkalisation of the stroma is not abolished by methylamine. The lowering of the ΔpH across the thylakoid membrane caused by higher concentrations of methylamine may be due mainly to neutralisation of the protons being transported into the thylakoid space by the undissociated amine.

pH dependency of CO_2 fixation in the light

In the experiment of Fig. 4 the rate of CO_2 fixation depending on the pH in the

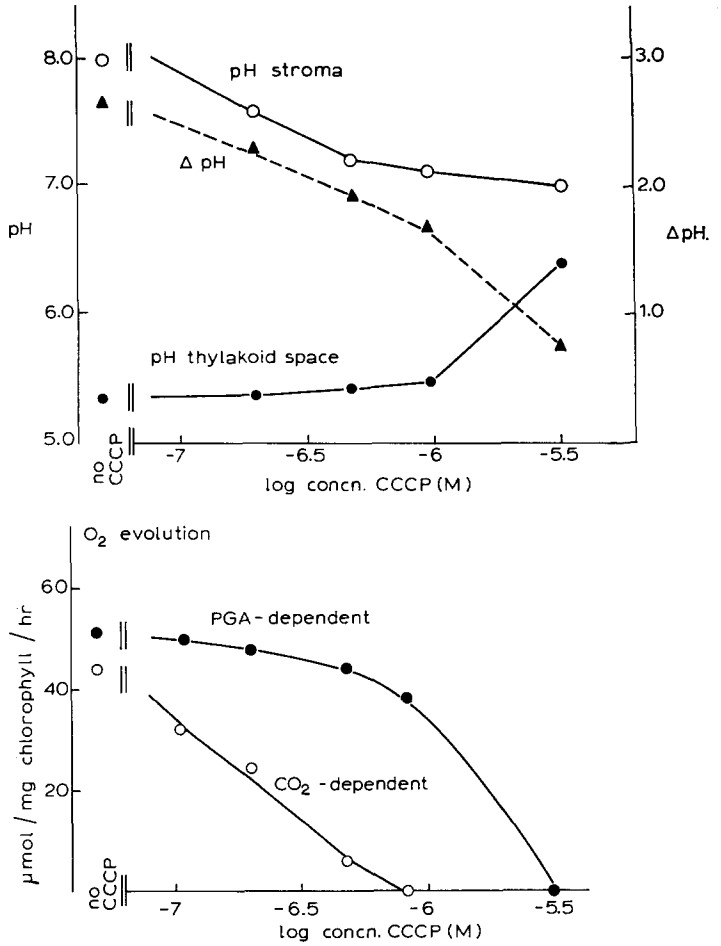

Fig. 2. The effect of CCCP on the pH in the chloroplast spaces and on CO_2 fixation and PGA reduction. The medium (pH 7.6) contained 2 mM $NaHCO_3$ plus 0.25 mM NaH_2PO_4 for CO_2 fixation and 2 mM PGA plus 3 mM phosphate for PGA reduction. Concentration of chloroplasts: 0.048 mg chlorophyll/ml, temperature 20 °C, CCCP as indicated was added after full rate of CO_2 fixation was obtained (5 min after start of illumination), the measurement of pH and of rate of CO_2 evolution was carried out 4 min after CCCP addition. (A) Measurement of the pH in the stroma and in the thylakoid space. The data were obtained under the conditions of measuring CO_2 fixation. Data obtained under experimental conditions of PGA reduction differed only slightly. (B) The rates of CO_2 fixation and of PGA reduction depending on the CCCP concentration in the medium, measured as O_2 evolution simultaneously.

medium was measured. There is a marked pH dependency observed with a pH optimum around 7.8, concurring with earlier results [15–16]. In other experiments not shown here we observed a variation of the pH optima of CO_2 fixation as related to the pH in the medium. Those chloroplasts which appeared to be more leaky and which maintained a lower pH gradient between the stroma and the medium in the light, displayed optimal rate of CO_2 fixation only if the pH in the medium was above 8.0. When the pH in the stroma is artificially lowered by addition of acetate (Fig. 4) the pH de-

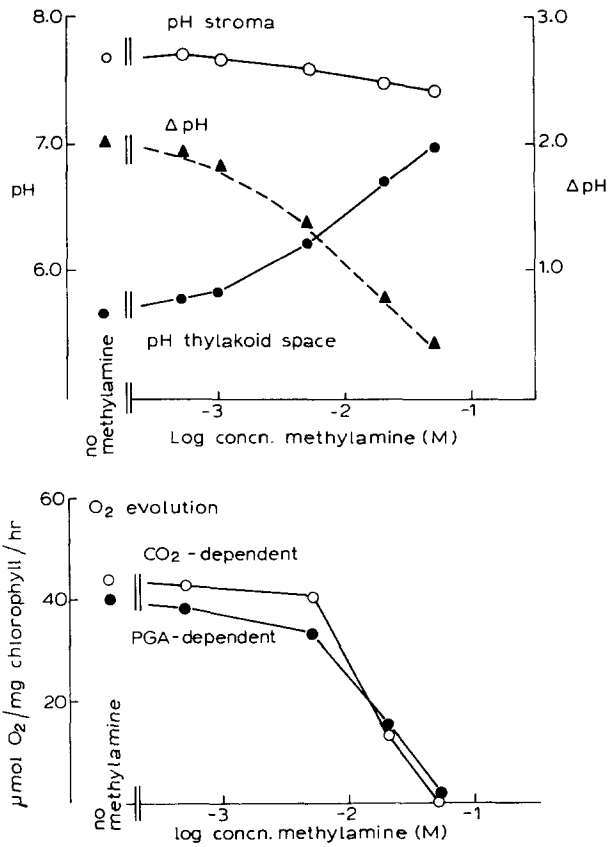

Fig. 3. The effect of methylamine on CO_2 fixation, PGA reduction and the pH in the chloroplast spaces. For experimental details see legend Fig. 2 and Methods. (A) Measurement of the pH in the stroma and in the thylakoid space. (B) The rate of CO_2 fixation and PGA reduction depending on the concentration of methylamine in the medium, measured simultaneously.

pendency curves of CO_2 fixation are shifted towards the alkaline region. A similar shift of these curves is also observed when the ΔpH between the stroma and the medium is lowered by high concentrations of bicarbonate (Table III). From these findings it is obvious that the pH optimum of CO_2 fixation, as related to the pH in the medium, is rather dependent on the chloroplast preparation and the experimental conditions.

However, if the rate of CO_2 fixation is related to the pH in the stroma, a single curve is obtained, as shown in Fig. 5 and Table III. Since the pH in the stroma has been varied in these experiments by two independent methods (variation of the pH in the medium and of the pH between the stroma and the medium) these findings demonstrate that CO_2 fixation in the light is strongly dependent on the pH in the stroma. Table IV lists the results of 5 typical experiments, in which quite similar values for optimal and half optimal pH have been obtained. It appears from these experiments that maximal rate of CO_2 fixation requires pH 8.1 in the stroma. There is virtually no CO_2 fixation occurring if the pH in the stroma is below 7.2. In sharp contrast to the

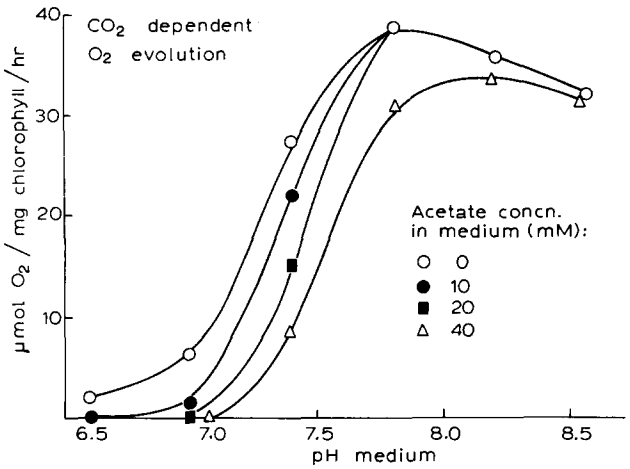

Fig. 4. Dependency of CO_2 fixation on the pH in the medium. The medium contained various concentrations of acetate, as indicated. CO_2 fixation was measured as oxygen evolution (see Methods). Bicarbonate concentration 2 mM, chloroplast concentration 0.08 mg chlorophyll/ml, temperature 20 °C.

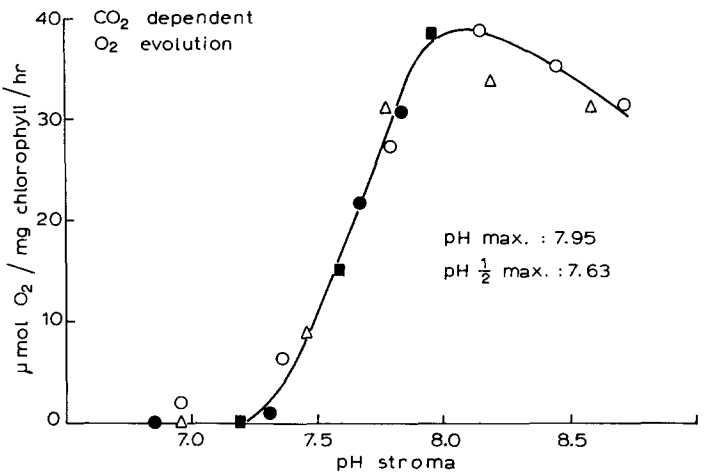

Fig. 5. The data of Fig. 4 as related to the pH in the stroma, which has been assayed simultaneously with each measurement. For symbols see Fig. 4.

pronounced pH dependency of CO_2 fixation, phosphoglycerate reduction, a partial step of CO_2 fixation, appears to be almost independent of changes of the stroma pH between 7.0 and 8.5 (Fig. 6). The reduction of phosphoglycerate by intact chloroplasts requires ATP and NADPH, which are both generated by photosynthetic electron transport. This allows the conclusion, that the pH depending step controlling CO_2 fixation is neither the generation of ATP nor of NADPH.

CO_2 fixation is inhibited by very low concentrations of CCCP, an uncoupler of photophosphorylation, as shown in the experiment of Fig. 2B. Phosphoglycerate

TABLE IV

CO_2 FIXATION DEPENDING ON THE pH IN THE STROMA

Results from different experiments in which the pH in the medium was varied and the pH in the stroma and the rate of CO_2 fixation were measured simultaneously, according to the expt. in Figs 4 and 5. Temperature 20 °C, pH max (pH 1/2): pH in the stroma at maximal (half maximal) rate of CO_2 fixation.

Experiment No.	Maximum rate of CO_2 dependent O_2 evolution (μmol O_2/mg chlorophyll/h)	pH 1/2	pH_{max}
1	54.0	7.73	8.01
2	38.9	7.63	7.95
3	74.0	7.95	8.20
4	164.7	7.76	8.10
5	72.0	7.95	8.30
		∅ 7.80	∅ 8.11

reduction appears to be much less sensitive towards inhibition by this uncoupler. With 1 μM CCCP CO_2 fixation is totally inhibited, whereas phosphoglycerate reduction still proceeds at more than 50 % of the control rate. These findings can be explained from the results of simultaneous pH measurements, as shown in Fig. 2A. It appears that the ΔpH across the thylakoid membrane can be decreased considerably without major inhibition of ATP consuming phosphoglycerate reduction. This shows very clearly, that inhibition of CO_2 fixation by low concentration of CCCP is not due to inhibition of photophosphorylation. Obviously, the inhibition is caused by lowering of the pH in the stroma. Thus a decrease of the stroma pH from pH 8.0 to 7.2 appears to inhibit CO_2 fixation completely. This again shows rather clearly the control of CO_2 fixation by the pH in the stroma.

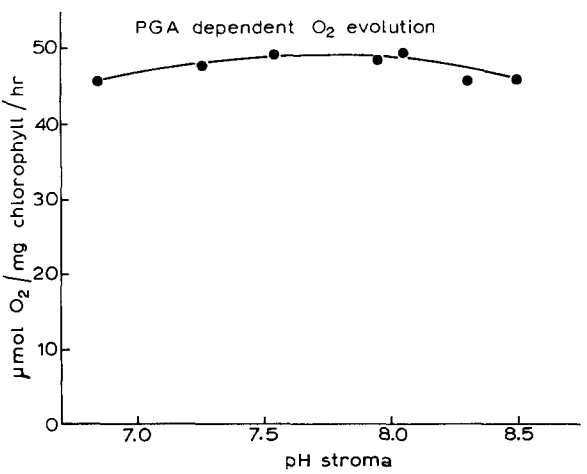

Fig. 6. Dependency of PGA reduction on the pH in the stroma. Chloroplast concentration 0.067 mg chlorophyll/ml, temperature 20 °C. For details see Methods.

Quite different results are obtained with methylamine, which is also regarded as an uncoupler of photophosphorylation. There is no selective inhibition of CO_2 fixation (Fig. 3B), which is explained by the fact that there is no marked decrease of the stroma pH even with high methylamine concentrations. With higher methylamine concentrations phosphoglycerate reduction and CO_2 fixation are reduced to the same extent, apparently because of reduced formation of ATP. It may be noted that also in this experiment the ΔpH across the thylakoid membrane is lowered considerably without decreasing the rate of CO_2 fixation or phosphoglycerate reduction. It seems that the threshold value of the ΔpH necessary for photophosphorylation in intact chloroplasts is rather low, probably lower than with broken chloroplasts [17]. However, as indicated at the beginning, there is a possibility that the size of the thylakoid space has been overestimated. In this case the pH in the thylakoid space would be lower and the ΔpH across the thylakoid membrane higher than calculated here. In some experiments we observed even an increase of the rate of CO_2 fixation after the addition of 1 mM methylamine. This has been also observed by Forti et al. [18], who speculated that this increase of rate may be due to the stimulation of enzymes of the Calvin cycle. It might have been possible that the addition of low concentrations of methylamine could have caused an additional alkalization of the stroma. We carried out experiments in which there was no significant rise of the stroma pH observed during stimulation of CO_2 fixation by 1 mM methylamine. One might conclude from these findings. that the stimulatory effect of methylamine on CO_2 fixation cannot be explained in terms of pH changes in the stroma. Further investigations will be required to clarify this matter.

CO_2 fixation in the dark

The preceding results indicate that CO_2 fixation is controlled by light. The question arises whether intact chloroplasts are able to perform CO_2 fixation also in the dark if the pH in the stroma is adjusted to the appropriate value. The fixation of CO_2 via the Calvin cycle requires ATP and NADPH for the formation of triosephosphates (dihydroxyacetone phosphate and glyceraldehyde-3-phosphate) as precursor for ribulosemonophosphate, and also ATP for the formation of ribulosediphosphate. The ATP and the NADPH are normally provided by photosynthetic electron transport. In principle, CO_2 fixation by intact chloroplasts should also be possible in the

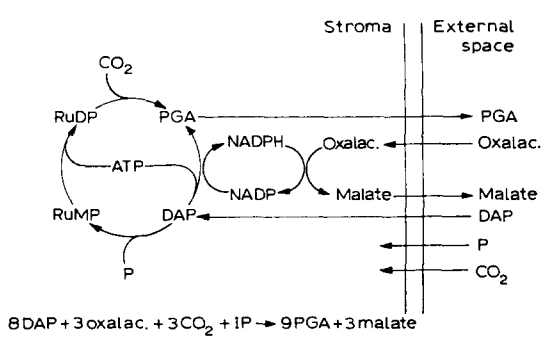

Fig. 7. Scheme for CO_2 fixation by intact chloroplasts in the dark.

dark if ATP and dihydroxyacetone phosphate are provided from external sources. Whereas dihydroxyacetone phosphate is rapidly transported into the chloroplasts by the phosphate translocator, direct transport of ATP is very slow [19]. However, by a shuttle of dihydroxyacetone phosphate and phosphoglycerate, which is mediated by the phosphate translocator [1, 20, 21], an indirect transport of ATP and NADPH is possible. Fig. 7 shows a hypothetical scheme for CO_2 fixation by intact chloroplasts occurring in the dark: dihydroxyacetone phosphate added to the medium is transported into the chloroplast stroma. There part of it is transformed to ribulosemonophosphate via aldolase and fructosediphosphatase, and the other part is oxidized to phosphoglycerate yielding ATP and NADPH. The ATP is utilized for the phosphorylation of ribulosemonophosphate to ribulosediphosphate. This scheme meets all requirements for CO_2 fixation. The balance of this hypothetical reaction would be:

$$8\ DAP + 3\ CO_2 + 1\ P_i + 3\ NADP \rightarrow 9\ PGA + 3\ NADPH \qquad (1)$$

Whereas the phosphoglycerate formed could be transported to the external space by the phosphate translocator, the NADPH also formed would be trapped in the stroma, since the inner membrane of the envelope is impermable for pyridine nucleotides. Therefore, such a CO_2 fixation in the dark could be expected to work only if the NADPH is reoxidized. Reoxidation can be achieved by adding oxaloacetate, which is

TABLE V

CO_2 FIXATION IN THE DARK

Medium pH 8.8, bicarbonate 10 mM, 0.18 mg chlorophyll/ml, 20 °C.

	Addition (mM)		Rate of CO_2 fixation evaluated between 4–6 min (μmol/mg chlorophyll/h)
Intact chloroplasts + 5 mM DTT	P_i	16	0.03
	P_i	16	
	+ Oxaloacetate	5	0.01
	P_i	16	
	+ DAP	5	0.11
	P_i	16	
	+ PGA	5	
	+ Oxaloacetate	5	0.04
	P_i	16	
	+ DAP	5	
	+ malate	5	0.13
	DAP	5	
	+ Oxaloacetate	5	0.28
	P_i	16	
	+ DAP	5	
	+ Oxaloacetate	5	3.54
Intact chloroplasts −DTT	P_i	16	
	+ DAP	5	
	+Oxaloacetate	5	1.99
Broken chloroplasts +5 mM DTT	P_i	16	
	+DAP	5	
	+Oxaloacetate	5	0.01

TABLE VI

BALANCE OF CO_2 FIXATION IN THE DARK

Data from the 4 min value in Fig. 9.

	10^{-9} mol/ mg chlorophyll	Relative amount
DAP consumption	256	≡ 8
PGA formation	277	8.7
Malate formation	145	4.5

transported by the dicarboxylate translocator into the stroma [1, 20], where malate-dehydrogenase catalyzes reduction to malate, which leaves the stroma in exchange for oxaloacetate via the dicarboxylate translocator. Thus the balance of CO_2 fixation (1) is modified:

$$8\ \text{DAP} + 3\ CO_2 + 1\ P_i + 3\ \text{oxaloacetate} \rightarrow 9\ \text{PGA} + 3\ \text{malate} \qquad (2)$$

We utilized this system for CO_2 fixation with intact chloroplasts (Table V). A considerable rate of CO_2 fixation is observed in the dark, if all components of the system (dihydroxyacetone phosphate, inorganic phosphate and oxaloacetate) are present, but there is almost no reaction if any one of these compounds is omitted. Phosphoglycerate cannot be substituted for dihydroxyacetone phosphate and malate cannot be substituted for oxaloacetate without loss of activity. The concentration ratio of inorganic phosphate/dihydroxyacetone phosphate = 3 was found to be optimal. This may be due to the fact that the K_m of the phosphate carrier for dihydroxyacetone phosphate is lower than for inorganic phosphate [21]. Dark CO_2 fixation does not occur when the chloroplasts have been broken by osmotic shock (Table V). This shows clearly, that an intact chloroplast envelope is prerequisite for dark CO_2 fixation. Apparently, CO_2 fixation by broken chloroplasts did not occur since there was no ATP added to the medium. CO_2 fixation in the dark was found to be stimulated to various degrees by the addition of the SH-donor dithiothreitol, which may be due to stimulation of ribulosemonophosphokinase [22] or of NADP specific malic dehydrogenase [23]. In the experiment of Fig. 8 the product of dark CO_2 fixation has been subjected to thin-layer electrophoresis. There is only a single spot observed, migrating the same distance as added [^{14}C]phosphoglycerate. A single spot, migrating the same distance as phosphoglycerate was also obtained with thin-layer chromatography (*n*-butanol/formic acid/water; 6 : 1 : 2 v/v/v) not shown here. This leads to the conclusion that the product of dark CO_2 fixation is indeed phosphoglycerate.

In the experiment of Fig. 9 the kinetics of dark CO_2 fixation has been followed by enzymatic assay of dihydroxyacetone phosphate consumption and of phosphoglycerate formation. For the sake of accurate measurements of dihydroxyacetone phosphate consumption, suboptimal dihydroxyacetone phosphate concentrations in the medium were employed, yielding lower rates of CO_2 fixation than in Table V. It appears from the data that CO_2 fixation does proceed until all dihydroxyacetone phosphate has been consumed. This illustrates the fact that CO_2 fixation in the dark is an exergonic process. Exergonic reaction steps in this overall reaction are hydrolysis of fructosediphosphate, phosphorylation of ribulose-monophosphate and carboxylation

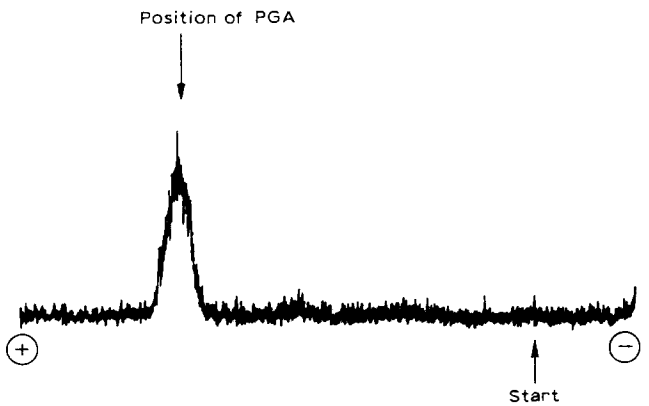

Fig. 8. Electrophoretic separation of the products of CO_2 fixation in the dark obtained after 6 min. The electrophoresis was carried out for 1 h according to Schürmann [24] on a cellulose thin-layer plate (20 cm long, DC Fertigplatten, Merck, Darmstadt, Germany) in a buffer containing 10 ml pyridine and 35 ml acetic acid in 955 ml H_2O. Radioactivity was scanned by a TLC scanner (Berthold-Friesecke, Karlsruhe, Germany). The specific activity of the $NaHCO_3$ (10 mM) was 10 Ci/mol and the chloroplast concentration 0.37 mg chlorophyll/ml. 20 μl of the neutralized $HClO_4$ extract were applied.

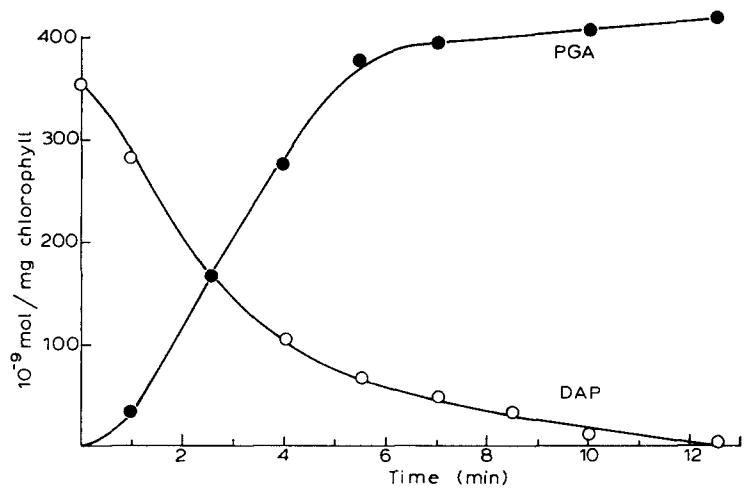

Fig. 9. Kinetics of the consumption of dihydroxyacetone phosphate (DAP) and the formation of phosphoglycerate (PGA) and malate during CO_2 fixation in the dark. The medium contained at the beginning of the reaction: DAP 1 mM, oxaloacetate 1 mM, phosphate 3 mM, dithiothreitol 10 mM. Chloroplast concentration: 0.25 mg chlorophyll/ml temperature 20 °C.

of ribulosediphosphate (see ref. 25).

The stoichiometry of the reaction was measured from the sample terminated after 4 min (Table VI). The balance obtained from enzymatic assay is rather similar to Eqn 2, but there is an excess of malate formed. This might be due to partial unspecific hydrolysis of ATP in the chloroplasts. If e.g. 33 % of the ATP generated by dihydroxyacetone phosphate oxydation in the chloroplasts would be used for other pur-

poses than phosphorylation of ribulosemonophosphate, the balance of the reaction would be:

8 DAP : 8.8 PGA : 3.8 malate

The dependency of the rate of dark CO_2 fixation on the bicarbonate concentration in the medium was investigated (Fig. 10). From the data a linear plot is obtained, yielding a K_m for bicarbonate of 2.6 mM. With pH 8.8 in the medium, this is equivalent to about 6 μM CO_2. Since the envelope is freely permeable for CO_2, the CO_2 concentration in the stroma is supposed to be equal to the concentration in the medium. The K_m of 6 μM CO_2 is very similar to the K_m of ribulosediphosphate carboxylase for CO_2 found in freshly ruptured chloroplasts [26].

Figs 11 and 12 show the dependency of the rate of dark CO_2 fixation on the pH

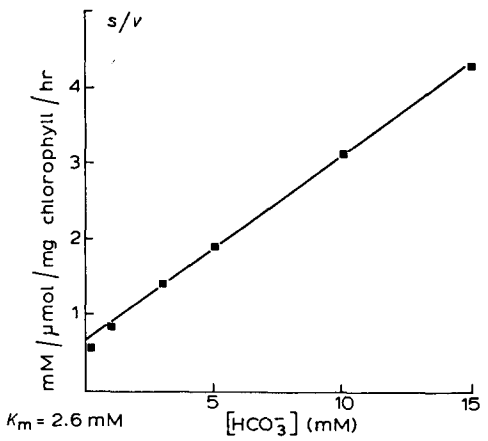

Fig. 10. Dependency of CO_2 fixation in the dark on the concentration of bicarbonate in the medium (pH 8.8). Chloroplast concentration: 0.37 mg chlorophyll/ml, temperature 20 °C.

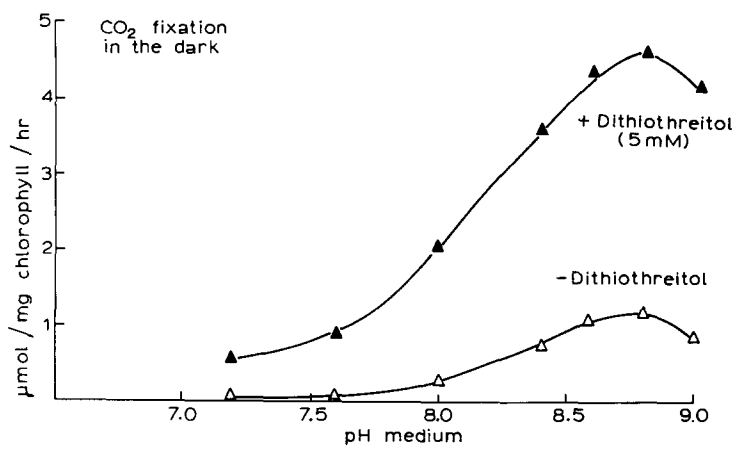

Fig. 11. Dependency of the rate of CO_2 fixation in the dark on the pH in the medium. Concentration of bicarbonate 7.2 mM, dithiothreitol if indicated 5 mM, chloroplasts 0.10 mg chlorophyll/ml.

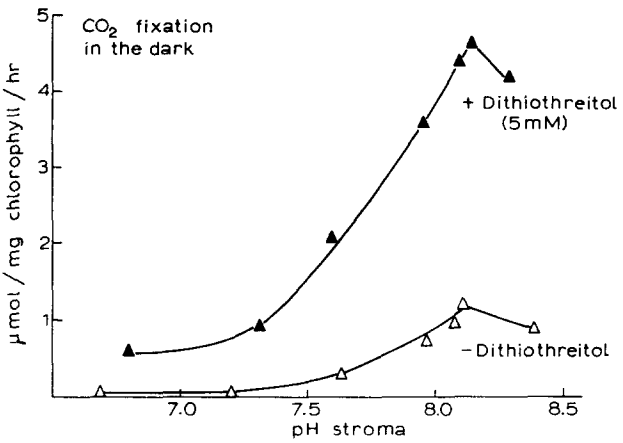

Fig. 12. Dependency of the rate of CO_2 fixation in the dark on the pH in the stroma. Data from Fig. 11 and simultaneous measurement of the pH in the stroma (see Methods).

in the medium and in the stroma. Similar to that in the light, CO_2 fixation in the dark is found to be strongly dependent on the pH in the stroma. Addition of dithiothreitol increases the rate of CO_2 fixation, but does not alter the pH dependency. Maximal activity of dark CO_2 fixation is observed with pH 8.15 in the stroma. This compares well with pH 8.1 being optimal for CO_2 fixation in the light. In order to obtain such an alkalisation of the stroma in the dark, the pH in the medium has to be 8.8. With pH 7.6 in the medium, which might resemble physiological conditions, the pH in the stroma is about 7.3, and there is hardly any dark CO_2 fixation observed under these conditions. Thus, CO_2 fixation in the dark, although possible in principle, is shown to proceed only when the stroma is alkalized to the extent normally observed when the chloroplasts are illuminated. This demonstrates very clearly that the pH in the stroma has a role in mediating the regulation of CO_2 fixation by light, in particular for preventing fixation during darkness.

DISCUSSION

The data shown are clear evidence for the occurrence of carboxylation of ribulosediphosphate by intact chloroplasts in the dark, if the necessary dihydroxyacetone phosphate is transferred from the medium into the stroma. If such a CO_2 fixation in the dark should occur in the plant, the carbohydrates of the plant would become oxidized to yield phosphoglycerate during darkness, the malate formed being oxidized in the mitochondria. It seems essential for plant metabolism that this reaction, which is favoured by thermodynamics, should be inhibited. These considerations make obvious the necessity for regulation of CO_2 fixation in the plant by light. From measurement of metabolite levels of the intermediates of the Calvin cycle during light-dark transients it has been suggested earlier that CO_2 fixation is under the control of light [25, 27, 28], and it has been speculated that pH changes in the stroma may be involved in such a control of CO_2 fixation [29].

We have shown that there are considerable pH changes occurring in the stroma

after illumination, and we have further demonstrated the effect of these pH changes on the rate of CO_2 fixation. This strongly suggests that CO_2 fixation is regulated by the pH in the stroma. However, there may be additional regulatory mechanisms acting in a complementary fashion. Evidence has been presented that light-dependent proton transport into the thylakoid space is at least in part counter balanced by a transport of magnesium ions [30–34]. These findings suggest that light-dependent alkalization of the stroma is accompanied there by an increase of the magnesium concentration. In a reconstituted chloroplast system CO_2 fixation was shown to be stimulated by increasing the magnesium concentration [35]. It seems therefore that CO_2 fixation may be also regulated by changes of the magnesium concentration in the stroma. Furthermore, light regulation of CO_2 fixation may also involve the activation of enzymes by -SH groups, which are reduced by photosynthetic electron transport (see ref. 36). Such additional mechanims for regulation of CO_2 fixation could be responsible for the comparatively low rates of CO_2 fixation in the dark, observed even with optimal pH (Table V, Fig. 12). Dithiothreitol stimulating CO_2 fixation in the dark may be a model for a regulation of CO_2 fixation by reduced -SH groups.

Without questioning the possible importance of the other regulatory mechanisms discussed here, our data clearly demonstrate that those pH changes occurring in the stroma at the light-dark transient are sufficient to switch CO_2 fixation from almost zero to maximal activity. This is apparent from the data of Fig. 5, where the pH in the stroma has been changed in a way that the ΔpH across the thylakoid membrane is not altered [4]. It is most likely that under these conditions the magnesium and potassium concentration in the stroma and the reduction state of -SH donors are also not changed. Therefore, in this case the inhibition of CO_2 fixation at low stroma pH seems to be entirely due to the proton concentration there. This well illustrates the capacity of the physiological pH changes in the stroma for regulating CO_2 fixation.

Which are the enzymatic step(s) of the Calvin cycle being controlled by pH changes? These step(s) should be in the reaction chain between dihydroxyacetone phosphate and phosphoglycerate, since phosphoglycerate reduction was shown to be almost insensitive to pH changes. The pH dependent step might be fructosediphosphatase, the activity of which has a very strong pH dependency very similar to pH dependency of CO_2 fixation [37]. In order to clarify the matter we are presently studying the levels of the intermediates of the Calvin cycle in spinach chloroplasts during CO_2 fixation. In preliminary experiments we found that the addition of 1 μM CCCP, causing inhibition of CO_2 fixation only (see Fig. 2B), resulted in a marked increase of the FDP concentration in the chloroplasts (Lilley and Heldt, unpublished). These findings indicate that fructosediphosphatase is indeed a pH dependent step regulating CO_2 fixation.

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