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ALKALIZATION OF THE CHLOROPLAST STROMA CAUSED BY LIGHT-DEPENDENT PROTON FLUX INTO THE THYLAKOID SPACE*

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SUMMARY

1. From the uptake of dimethyloxazolidinedione and of methylamine into the sucrose-impermeable space of intact spinach chloroplasts as measured by silicon-layer filtering centrifugation and from estimation of the size of the thylakoid space by planimetry of electron micrographs the pH in the stroma and in the thylakoid space is evaluated. The reliability of the method is checked.

2. Illumination causes an alkalization in the stroma and an acidification in the thylakoid space, with a ΔpH of about 2.5 between the two spaces, reflecting light-dependent proton transport across the thylakoid membrane. By addition of *m*-chlorocarbonyl cyanide phenylhydrazone or of nigericin these pH changes are reversed to the corresponding dark values.

3. Beside this there is also some light-dependent proton movement from the stroma across the inner membrane into the external space.

4. The pH in the stroma and in the thylakoid space of illuminated chloroplasts depends on the pH in the medium, whereas the ΔpH across the thylakoid membrane is almost independent from this.

5. From comparison of the pH changes in the stroma and in the thylakoid space it appears that in the thylakoid space there is a buffer with pK 5.5 and in the stroma with pK 6.8 and that the buffer concentration in the thylakoid space is about four times higher than in the stroma.

6. It is discussed that the light-dependent alkalization in the chloroplast stroma may be involved in the regulation of CO_2 fixation at the fructose diphosphatase step.

INTRODUCTION

Illumination causes a transport of protons across the thylakoid membrane[3] and leads to a decrease of pH in the thylakoid space[4–8] as was found with broken chloroplasts which had lost their envelope. It has been shown that the inner membrane of the chloroplast envelope is impermeable for protons[9,10]. Therefore, light-dependent

Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine *N'*-2-ethane sulphonic acid; DMO, 5,5-dimethyloxazolidine-2,4-dione; CCCP, *m*-chlorocarbonyl cyanide phenylhydrazone.

* Part of the results have been included in preliminary reports[1,2].

proton transport across the thylakoid membrane might increase the pH in the stroma space, depending on the unknown buffer capacity in the stroma. A light-induced change of the pH in the stroma may influence CO_2 fixation, the enzymes of which are located in the stroma space.

Recently we have observed that there is indeed alkalization in the stroma when the chloroplasts are illuminated[11]. This report is an extension of these findings and also a systematic study of the pH changes brought about by illumination in the stroma and in the thylakoid space.

METHODS

(a) Chloroplasts with intact envelope were prepared according to the method of Cockburn *et al.*[14] modified by Heldt and Sauer[15]. The integrity of the chloroplasts was checked by phase contrast microscopy. Furthermore, as a quantitative measure of intactness the ferricyanide-dependent O_2 evolution was employed according to Heber and Santarius[24]. With this criterion, 80–95% of the chloroplasts of a usual preparation appeared to be intact. If not stated otherwise the incubations were carried out in a medium containing 0.33 M sorbitol, 50 mM *N*-2-hydroxyethylpiperazine *N'*-2-ethane sulphonic acid (HEPES), pH 7.6, neutralized with NaOH, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 2 mM EDTA. Chlorophyll was assayed after the method of Whatley and Arnon[16]. For details of incubation, filtering–centrifugation and radioactivity measurement see Werdan *et al.*[11]. Illumination of the samples contained in the centrifugation tubes 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, Liechtenstein). The light intensity was 80000 ergs$\cdot s^{-1}\cdot cm^{-2}$. During centrifugation, illumination was continued. If not stated otherwise the pH in the medium was 7.6 and the time of illumination was 3 min prior to centrifugation. All experiments, except those shown in Figs 4 and 6 have been carried out at 4 °C. In all experiments except those with [^{14}C]bicarbonate the tubes used for filtering centrifugation contained 20 μl of 1 M $HClO_4$ in the bottom layer. With bicarbonate they contained 20 μl of 2.5 M NaOH. For measurement of pH in the stroma and in the thylakoid space chloroplasts (20–60 μg chlorophyll per ml) were incubated in duplicate samples with 1 mM 5,5-dimethyloxazolidine-2,4-dione (DMO) *plus* 30 μM methylamine containing either [^{14}C]DMO (New England Nuclear, Boston, Mass., U.S.A.; 0.3–1 Ci/mole) or [^{14}C]methylamine (New England Nuclear, Boston, Mass., U.S.A.; 6 Ci/mole). Other radioactive compounds employed were: $NaH^{14}CO_3$ (Amersham, Braunschweig, Germany; 0.1–1 Ci/mole), ^{3}HHO (Amersham, Braunschweig, Germany; $1\cdot10^{-4}$ Ci/mole), 10 mM [^{14}C]sucrose (New England Nuclear, Boston, Mass., U.S.A.; 0.1 Ci/mole), 20 μM *p*-nitro[^{14}C]phenol (I.C.N., Irvine, Calif., U.S.A.; 10 Ci/mole).

(b) The evaluation of the concentrations of the above-mentioned compounds in the sucrose-impermeable ^{3}HHO space of the chloroplasts (C_c) from the radioactivity measurements was carried out as follows:

$$C_c = \frac{(\mathrm{dpm_s} - \alpha\,\mathrm{dpm_m})\cdot V_m \cdot C_m}{\mathrm{dpm_m}\cdot \mathrm{chl}\cdot V_c}$$

dpm_s = decays per min in sediment fraction; dpm_m = decays per min in incubation medium; V_m = volume of incubation medium; C_m = concentration in incubation medium; chl = amount of chloroplasts (mg chlorophyll) in incubation; V_c = sucrose-impermeable ^{3}HHO space of the chloroplasts employed (μl/mg chlorophyll); α = dpm [^{14}C]sucrose in sediment/dpm [^{14}C]sucrose in medium. α is evaluated from a control experiment. This value represents the medium which has been carried through the silicone oil[15].

A considerable amount of DMO ($pK = 6.64$)[13] is present in the undissociated form under the conditions of our experiments. In order to evaluate the concentration of DMO anions in the medium $[D^-]_m$, the concentration of undissociated DMO [DH] has to be subtracted from the concentration of total DMO in the medium $[D^{tot}]_m$.

$$[D^-]_m = [D^{tot}]_m - [DH]_m$$

$$[DH]_m = [DH]_c = \frac{[D^{tot}]_m}{1 + 10^{pH_m - pK}}$$

For the evaluation of the concentration of DMO anions in the sucrose impermeable space of chloroplasts $[D^-]_i$, the concentration of undissociated DMO in the medium is subtracted from the concentration of total DMO $[D^{tot}]_c$ in the sucrose-impermeable space.

$$[D^-]_c = [D^{tot}]_c - [DH]_c$$

In an analogous way the bicarbonate ($pK = 6.2$)[17] is corrected for CO_2. With methylamine ($pK = 10.6$)[8] such a correction is not necessary since the concentration of undissociated methylamine can be neglected in our experiments. The data shown in this report are all mean values from triplicate experiments carried out simultaneously.

RESULTS

Estimation of the size of the stroma space and the thylakoid space

The space surrounded by the inner membrane, which may be functionally defined as the sucrose-impermeable ^{3}HHO space, consists of the stroma space and the thylakoid space[15] (Fig. 1). With intact chloroplasts, the size of these two spaces cannot be measured with functional methods. Therefore, an estimation of the relative

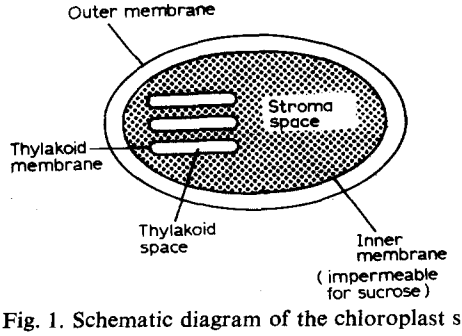


Fig. 1. Schematic diagram of the chloroplast structure.

sizes of these spaces was carried out by planimetry of electron micrographs from isolated spinach chloroplasts fixed with glutaraldehyde[15]. The planimetric estimation of the thylakoid space and the stroma space was carried out in the following way: The electron micrographs (21 000-fold magnification) were put under transparent paper, and the contours of the inner membrane and the grana were drawn. The chloroplasts were cut out along the inner membrane and the relative size of the sum of thylakoid and stroma space was calculated from weighing the paper. In the same way the area occupied by the grana was evaluated, and it was estimated, how much of this space was covered by the thylakoid space.

The results obtained with different preparations (Table I) are rather similar. The method employed for this estimation is not very exact and it may be doubtful if the minor differences of spaces observed between the incubation in the light and in the dark are real. It may be noted that we also did not find any significant changes of the size of the sucrose-impermeable ^{3}HHO space under the conditions of our experiments. Earlier investigations have shown a flattening of the chloroplast shape and a decrease of the absolute volume of the thylakoid space on illumination[18–23]. For the calculation of pH, 0.125 will be employed as mean value of the relative size of the thylakoid space as compared to the sucrose-impermeable ^{3}HHO space.

TABLE I

ESTIMATION OF THE RELATIVE SIZES OF THE THYLAKOID SPACES IN CHLOROPLASTS, AS OBTAINED BY PLANIMETRY OF ELECTRON MICROGRAPHS

For details see text.

Conditions of incubation	*Fraction of the area of the chloroplasts occupied by the space within the thylakoid membranes related to the area enclosed by the inner membrane; n = number of chloroplasts*			
	Expt 1	*Expt 2*	*Expt 3*	*Mean value*
Light	0.130, $n=21$	0.128, $n=15$	0.132, $n=6$	0.130 | 0.125
Dark	0.118, $n=26$	0.103, $n=23$	0.139, $n=6$	0.120 |

Evaluation of the pH in the stroma space and in the thylakoid space

When a dissociable compound is added to a suspension of membrane-surrounded vesicles, the membrane of which is only permeable for the uncharged molecule, the concentrations of the uncharged molecule will be equal on both sides, whereas the concentrations of the corresponding ions will depend on the concentrations of protons in both spaces.

$$\frac{[H^+]_i}{[H^+]_m} = \frac{[BH^+]_i}{[BH^+]_m} = \frac{[A^-]_m}{[A^-]_i} \qquad (1)$$

i = internal; m = external = medium.

With cationic compounds the ion concentration inside will be higher than outside if the internal space is more acidic than the external one and vice versa with

anionic substances. Measurement of the concentration of these substances in the vesicles allows the calculation of the internal pH. Compounds, suitable for such a pH measurement are DMO which has been widely used for determination of pH in tissues[12] and in mitochondria[13], and methylamine, which has been used earlier for pH measurement in the thylakoids of broken chloroplasts[8].

In the experiment shown in Table II, the uptake of [^{14}C]DMO and of [^{14}C]-methylamine was measured using silicone layer filtering centrifugation. In order to have identical conditions, two samples have been treated simultaneously, one containing [^{14}C]DMO and unlabelled methylamine and the other containing [^{14}C]-methylamine and unlabelled DMO. When the amounts of DMO and of methylamine analyzed in the sedimented chloroplasts are related to the total size of the sucrose-impermeable ^{3}HHO chloroplast space, the apparent ion concentrations of both compounds ($[DMO^-]_c$, $[MA^+]_c$) are higher than in the medium. In view of Eqn 1 it appears to be paradoxical that both these compounds are accumulated in the chloroplasts. From this it can be assumed that there are two different spaces in the sucrose-

TABLE II

pH MEASUREMENT IN THE STROMA AND IN THE THYLAKOID SPACE

For conditions of incubation *etc.* see Methods and legend of Fig. 4. Temp., 8 °C.

Specific space (µl/mg chlorophyll)			
Chloroplast (sucrose impermeable)	V_c	26.3	
Thylakoid (0.125 V_c)	V_T	3.3	
Stroma (0.875 V_c)	V_S	23.0	
Concentrations in medium (µM)			
DMO anions	$[D^-]_m$	900	
Methylammonium cations	$[M^+]_m$	30.0	
Concentrations measured in the sucrose-impermeable space (µM)			
DMO anions	$[D^-]_c$	1480	
Methylammonium cations	$[M^+]_c$	383	
Concentrations calculated (µM)*		*(1) Solution Thylakoid space more acidic than stroma*	*(2) Solution Stroma more acidic than thylakoid space (not valid, see text)*
DMO anions – stroma	$[D^-]_S$	1691	(61.8)
Methylammonium cations – stroma	$[M^+]_S$	16.0	(437)
DMO anions – thylakoid	$[D^-]_T$	9.2	(11366)
Methylammonium cations – thylakoid	$[M^+]_T$	2937	(2.4)
pH in medium		7.60	
pH calculated			
Stroma	pH_S	7.87	(6.44)
Thylakoid	pH_T	5.61	(8.70)
	ΔpH	2.26	(−2.26)

* Two solutions possible (see text).

impermeable ^{3}HHO space, one being more acidic and the other being more alkaline than the medium. It seems obvious that these two spaces are identical with the stroma space and the thylakoid space.

From the amount of [^{14}C]DMO and [^{14}C]methylamine found in the sucrose-impermeable ^{3}HHO space, the pH in the stroma and in the thylakoid space can be calculated. This calculation is based on the following three relations:

$$[D^-]_m \cdot [M^+]_m = [D^-]_S \cdot [M^+]_S = [D^-]_T \cdot [M^+]_T \quad \text{(see Eqn 1)} \tag{2}$$

$$D_c^- = D_S^- + D_T^+ = [D^-]_S \cdot V_S + [D^-]_T \cdot V_T \tag{3}$$

$$M_c^+ = M_S^+ + M_T^+ = [M^+]_S \cdot V_S + [M^+]_T \cdot V_T \tag{4}$$

D_c^-, M_c^+ = amount of DMO anion (methylamine cation) in the sucrose-impermeable ^{3}HHO space (nmoles/mg chlorophyll); M_T^+, M_S^+ = amount of methylamine cation in the thylakoid (stroma) space (nmoles/mg chlorophyll); D_T^-, D_S^- = amount of DMO anion in the thylakoid (stroma) space (nmoles/mg chlorophyll). For other symbols see Table II.

These three equations can be combined into a quadratic equation dissolved for D_S^-. $[M^+]_m$ and $[D^-]_m$ are fixed by the incubation procedure. The sum of $V_T + V_S$ is determined from the measurement of the sucrose-impermeable ^{3}HHO space by silicone layer filtering centrifugation; the relative sizes of the chloroplast are estimated in Table I.

$$D_S^- = -\frac{a}{2} \pm \sqrt{\left(\frac{a}{2}\right)^2 - b} \tag{5}$$

$$a = \frac{[M^+]_m \cdot [D^-]_m \cdot (V_T^2 - V_S^2)}{M_c^+} - D_c^- \tag{6}$$

$$b = \frac{D_S^-}{M_c^+} \cdot [M^+]_m \cdot [D^-]_m \cdot V_S^2 \tag{7}$$

$$[D^-]_S = \frac{D_S^-}{V_S} \tag{8}$$

From the distribution of DMO anions between the stroma and the medium, it is calculated how much methylamine is present in the stroma space. When this value is subtracted from the amount of methylamine analyzed in the sucrose-impermeable ^{3}HHO space, the corresponding methylamine concentration in the thylakoid space can be evaluated and from this the pH in the thylakoid space:

$$[M^+]_T = \frac{1}{V_T}\left(M_c^+ - \frac{[D^-]_m \cdot [M^+]_m \cdot V_S^2}{D_S^-}\right) \tag{9}$$

From the concentration of DMO anions in the stroma space and of methylammonium ions in the thylakoid space, the pH in these two spaces is calculated:

$$pH_S = pH_m + \log \frac{[D^-]_S}{[D^-]_m} \quad (10)$$

$$pH_T = pH_m + \log \frac{[M^+]_m}{[M^+]_T} \quad (11)$$

Due to the quadratic equation these calculations yield two different solutions. As an example, both solutions have been calculated in Table II. In the first solution the thylakoid space is more acidic than the stroma space, and the application of this solution to the experiment of Fig. 4 yields light-dependent proton transport from the stroma into the thylakoid space. This is in agreement with earlier experiments with isolated thylakoids showing light-dependent proton transport into the thylakoid space[3]. Therefore, the second solution, which would indicate a proton transport in the opposite direction is disregarded.

The pH in the stroma can also be calculated in a less complicated way[1] if one simplifies the matter by assuming that all DMO anions analyzed in the sucrose-impermeable ^{3}HHO space are located in the stroma. Since the thylakoid space is comparatively small and since it is more acidic than the stroma space, the error introduced by this assumption is low.

The calculation is based on the mean value for the size of the thylakoid space as estimated in Table I. The actual size of the thylakoid space in a preparation may be different from this mean value. Table III investigates how a deviation of the thylakoid space will influence the results of the calculation.

It was investigated whether the evaluation of pH in both compartments is influenced by the chloroplast concentration (Table IV). The experiments dealt with in this report have been carried out with chloroplast concentrations of 20–60 μg chlorophyll per ml. Within this range, the results of pH measurement are almost unaffected by changes of chloroplast concentration.

The chloroplasts which have lost the envelope remain on top of the silicone layer during the centrifugation. Only the intact chloroplasts are filtered through the silicone layer into the $HClO_4$. For this reason, the broken chloroplasts which are present in the preparation, do not affect the results of our pH measurements.

TABLE III

EVALUATION OF THE pH IN THE STROMA SPACE AND IN THE THYLAKOID SPACE FROM THE UPTAKE OF [^{14}C]DMO AND [^{14}C]METHYLAMINE

Possible errors introduced by deviations of the size of the thylakoid space. The measured value is taken from Table II.

Size of the thylakoid space as part of the sucrose-impermeable ^{3}HHO space	*pH stroma space*	*pH thylakoid space*	*ΔpH across the thylakoid membrane*	*Errors*
0.125 (measured)	7.87	5.61	2.26	
0.063 (assumed)	7.84	5.33	2.51	+0.25
0.188 (assumed)	7.91	5.78	2.13	−0.13

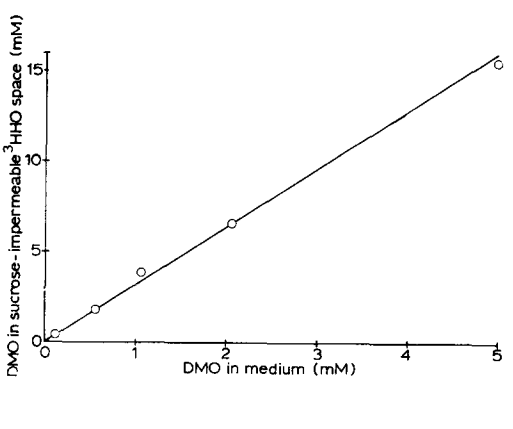
TABLE IV

CALCULATION OF THE pH IN THE STROMA SPACE AND IN THE THYLAKOID SPACE FROM THE UPTAKE OF DMO AND METHYLAMINE USING DIFFERENT CONCENTRATIONS OF CHLOROPLASTS IN THE EXPERIMENT

Chloroplast concentration (μg chlorophyll/ml)	*Sucrose-impermeable ^3HHO space (μl/mg chlorophyll)*	*DMO concentration in sucrose-impermeable ^3HHO space (mM)*	*Methylamine concentration in sucrose-impermeable ^3HHO space (mM)*	*pH stroma space*	*pH thylakoid space*	*ΔpH across the thylakoid membrane*
13.0	31.8	1.50	0.553	7.85	5.44	2.41
26.6	33.5	1.62	0.586	7.88	5.42	2.47
57.3	33.8	1.71	0.607	7.91	5.40	2.51
92.0	30.4	1.76	0.689	7.92	5.34	2.58

The result of pH measurement in the stroma is independent of the DMO concentration employed for a large concentration range. This is shown from the experiment in Fig. 2, as a linear relationship is observed between the DMO anion concentration in the medium and in the sucrose-impermeable ^3HHO space. Normally our measurements are carried out with 1 mM DMO in the medium.

With methylamine such a linearity between the concentrations is only observed when the methylamine concentration in the medium is below 60 μM (Fig. 3). With higher concentrations the uncoupling effect of methylamine becomes noticeable leading to slight changes of the pH in the stroma and in the thylakoid space (Table V).



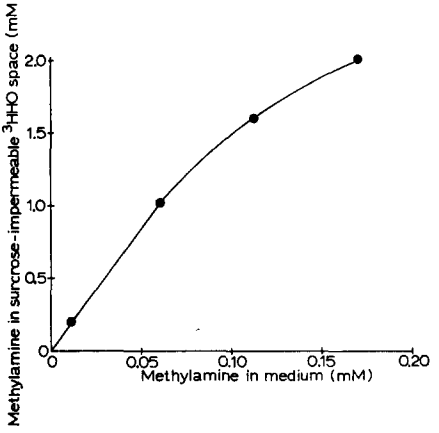


Fig. 2. DMO^- concentration in the sucrose-impermeable ^3HHO space (26.3 μl/mg chlorophyll) of spinach chloroplast (23.0 μg chlorophyll/ml) depending on the DMO^- concentration in the medium. Illumination. For details see Methods.

Fig. 3. Methylamine concentration in the sucrose-impermeable ^3HHO space (24.5 μl/mg chlorophyll) of spinach chloroplasts (23.0 μg chlorophyll/ml) depending on the methylamine concentration in the medium. Illumination. For details see Methods.

TABLE V

ΔpH ACROSS THE THYLAKOID MEMBRANE DEPENDING ON METHYLAMINE CONCENTRATION

Methylamine (μM)	*ΔpH across the thylakoid membrane*
10	2.84
60	2.75
110	2.61
1000	2.51

TABLE VI

CALCULATION OF THE pH IN THE STROMA FROM THE UPTAKE OF DMO, BICARBONATE OR *p*-NITROPHENOL INTO THE STROMA SPACE

Expt No.	*Compound used*	*Concentration in the medium* (mM)	*Concentration in the sucrose-impermeable* ^3HHO *space* (mM)	*pH in the stroma calculated*
1	DMO	10	12.70	7.77
	HCO_3^-	10	11.09	7.70
	p-Nitrophenol	0.02	0.156	8.66
2	DMO	1	0.72	7.50
	HCO_3^-	10	7.61	7.53
3	DMO	1	1.40	7.82
	HCO_3^-	3.3	5.57	7.89

The effect of higher methylamine concentrations on the pH in the thylakoid space has been reported earlier[8]. In order to avoid this, our measurements are carried out with 30 μM methylamine. With this concentration neither the pH in the stroma nor the pH in the thylakoid space are affected. No effect on CO_2 fixation or photophosphorylation is observed. This also applies to the DMO concentrations used in our experiments.

We have shown that the pH in the stroma can be calculated from the distribution of bicarbonate[11]. Table VI shows the comparison of the pH measurements with DMO and with bicarbonate. In one experiment *p*-nitrophenol was also tried. With DMO and with bicarbonate almost identical results are obtained (see also ref. 11), whereas the pH value obtained with *p*-nitrophenol is entirely different. The uptake of *p*-nitrophenol is much higher than that of DMO or CO_2, and it is not stimulated by light (experiments not shown here) as in the case of the uptake of CO_2 or DMO (Fig. 4).

The evaluation of pH based on the distribution of ions could yield erroneous results if the corresponding undissociated molecules are accumulated in the membrane

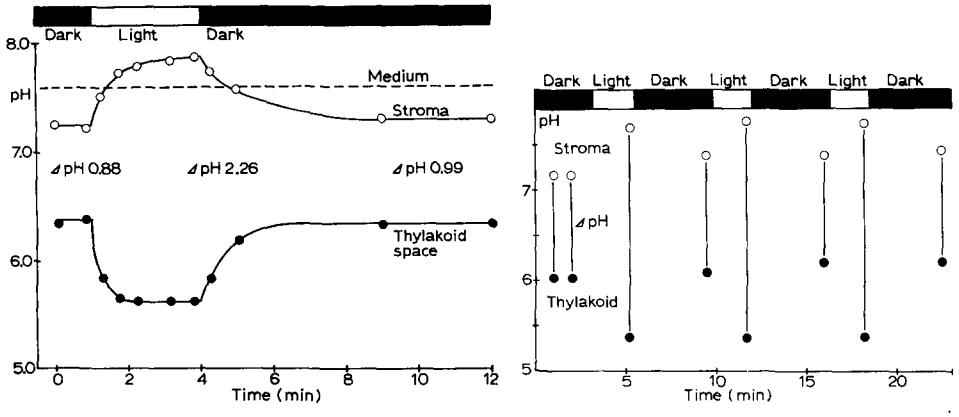


Fig. 4. Changes of the pH in the stroma and in the thylakoid space of spinach chloroplasts (45.0 μg chlorophyll/ml) caused by dark–light and light–dark transients. Temp., 8 °C. Sucrose-impermeable ^{3}HHO space, 26.3 μl/mg chlorophyll. Each sample has been incubated in the centrifugation tube and the incubation was terminated by centrifugation. For details see Methods.

Fig. 5. Change of the pH in the stroma and in the thylakoid space of spinach chloroplasts (57.0 μg chlorophyll/ml) caused by repetitive dark–light and light–dark transients. For details see Methods and legend Fig. 4. Sucrose-impermeable ^{3}HHO space: light, 21.3 μl/mg chlorophyll; dark, 21.9 μl/ml chlorophyll. ○, pH in the stroma space; ●, pH in the thylakoid space.

phase. This is probably the case with *p*-nitrophenol which seems to be unsuitable for pH measurements in the stroma, but in the case of DMO it has been demonstrated that it is not accumulated in tissue homogenates[12]. With methylamine erroneous results caused by accumulation of the undissociated base can also be ruled out. The experimental data of Table II may be taken as an example. With pH 7.6 in the medium, only $1^0/_{00}$ of the methylamine (p*K* 10.6) is in the undissociated form. Even if there would be a 100-fold accumulation of undissociated methylamine, and the membrane phase would be 5% of the chloroplast space, the amount of methylamine accumulated in this way would be less than 0.5% of the methylammonium accumulation in the thylakoid space (Table II). This would not affect the results of pH measurement in the thylakoid space.

Light-dependent pH changes in the stroma and in the thylakoid space

It has been shown recently that the pH in the stroma becomes more alkaline when the chloroplasts are illuminated, and it has been assumed that this was due to light-driven proton transport across the thylakoid membrane[10,11]. This assumption is confirmed from the experiment in Fig. 4. When the chloroplasts are kept in the dark, there is a ΔpH of about 0.9 unit across the thylakoid membrane, the thylakoid space being more acidic than the stroma. Illumination causes a rapid rise of the pH in the stroma, accompanied by a fall of the pH in the thylakoid space, reflecting light-driven proton transport. The ΔpH across the thylakoid membrane increases to 2.26 units. Similar pH gradients across the thylakoid membrane in the light have been observed earlier with broken chloroplasts devoid of the inner and the outer membrane[4–8]. It may be noted that the initial fall of the pH in the thylakoid space is more rapid than the corresponding rise of the pH in the stroma. Although the

kinetics shown here may be limited by the diffusion of DMO and of methylamine these differences of pH changes cannot be explained in these terms, since DMO was found to diffuse chloroplasts more rapidly than methylamine (Werdan, K. and Heldt, H. W., unpublished). These differences between the pH changes are probably due to different buffer capacities in the stroma and in the thylakoid space as discussed in connection with Fig. 11. When the light is turned off, the pH gradient across the thylakoid membrane collapses and a ΔpH very close to the original dark value is approached in an exponential time course. These pH changes can be repeated many times, the ΔpH in the light and in the dark does not markedly change (Fig. 5).

The same ΔpH changes are obtained when the experiment is carried out at 20 °C, but in this case the ΔpH across the thylakoid membrane decreases to almost zero when the light is turned off (Werdan, K. and Heldt, H. W., unpublished). The relatively high ΔpH observed in the dark seems to be characteristic for chloroplasts incubated at low temperatures.

Proton transport from the stroma into the external space

In addition to the proton transport from the stroma into the thylakoid space as demonstrated in the experiment of Fig. 4, there appears to be a slight light-dependent proton transport from the stroma across the inner membrane to the outer space. This has been shown by Heber and Krause[9]. It is confirmed by the experiment in Fig. 6, in which the proton concentration in the medium of a chloroplast suspension

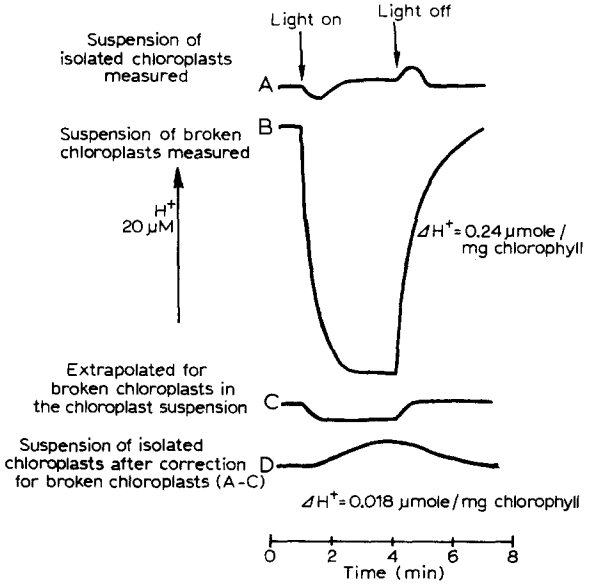


Fig. 6. Recording of light-dependent pH changes in the medium of a chloroplast suspension (0.23 mg chlorophyll/1.6 ml). The measurement was carried out with a Metrohm E 388 pH meter provided with a glass electrode (U 59 Schott, Mainz). The medium contained 0.33 M sorbitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA and 1.25 mM HEPES, pH 7.6. Temp., 20 °C. In order to prevent CO_2 fixation the media were bubbled through with nitrogen. The scale was adjusted by addition of HCl standard. For measurement of broken chloroplasts, distilled water was added to the chloroplast suspension and 1 min later an equal volume of double-concentrated medium was added. See text.

was measured with a glass electrode. When intact chloroplasts are illuminated (Trace A), the proton concentration in the medium decreases rapidly at first, and then it rises again more slowly. Both effects are reversed when the light is turned off. This indicates that there are two processes occurring: a rapid uptake of protons and a slower release. As measured with ferricyanide-dependent oxygen evolution[24], 5% of the chloroplasts in this preparation had lost the inner membrane. In Expt B, the same chloroplast suspension was osmotically shocked in order to disrupt the inner membrane. Here illumination causes a large decrease of proton concentration in the medium reflecting proton transport into the thylakoid space[3]. From this the proton uptake due to the broken chloroplasts in the suspension of undisrupted chloroplasts can be estimated to be 5% of the proton uptake by the shocked preparation (C). Obviously, the rapid uptake of protons observed with suspensions of intact chloroplasts in the light is due to broken chloroplasts being present in the preparation. There does not seem to be any light-dependent proton uptake by intact chloroplasts. When Trace C is subtracted from Trace A, it appears that in intact chloroplasts there is indeed a light-driven transport of protons into the outer space (D). However, this transport is very small as compared to the proton transport into the thylakoid space. It may be, therefore, concluded that light-dependent alkalization of the stroma is primarily due to proton transport across the thylakoid membrane.

Influence of the pH in the external space on the pH in the stroma and in the thylakoid space

When the pH in the medium is changed, there is also some change of pH in the stroma (Fig. 7). This could be mediated by proton transport across the inner membrane, it could be also due to partial leakage of the inner membrane. In the

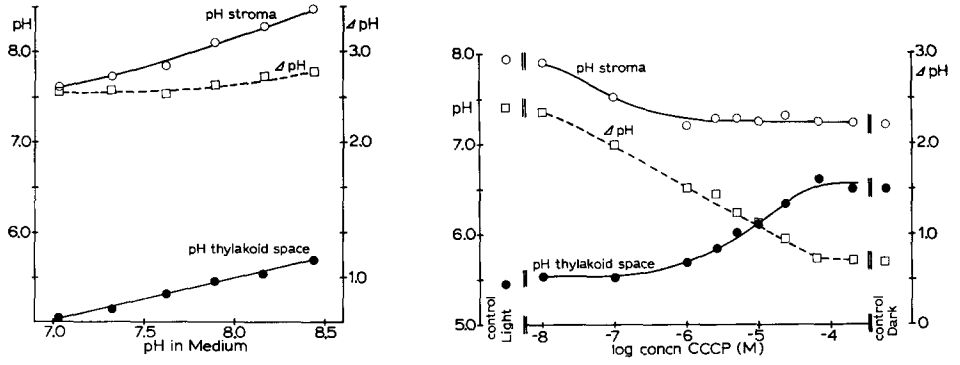


Fig. 7. The pH in the stroma and in the thylakoid space of spinach chloroplasts (45 μg chlorophyll/ml) depending on the pH in the medium. Illumination. For details see Methods. Varying the pH of the medium did not influence the sucrose-impermeable ^{3}HHO space: pH 7.03, 24.2 μl/mg chlorophyll; pH 7.32, 24.9 μl/mg chlorophyll; pH 7.62, 24.1 μl/mg chlorophyll; pH 7.89, 25.6 μl/mg chlorophyll; pH 8.16, 23.8 μl/mg chlorophyll; pH 8.43, 25.8 μl/mg chlorophyll.

Fig. 8. The effect of CCCP on the light-dependent pH changes in the stroma and in the thylakoid space of spinach chloroplasts (58 μg chlorophyll/ml). CCCP was added to the illuminated chloroplasts. Incubation time, 2 min. For details see Methods. No influence of CCCP on the sucrose-impermeable ^{3}HHO space: light, 19.3 μl/mg chlorophyll; light and CCCP (0.1 μM), 20.3 μl/mg chlorophyll; light and CCCP (1 μM), 19.4 μl/mg chlorophyll; light and CCCP (10 μM), 19.1 μl/mg chlorophyll; light and CCCP (200 μM), 19.7 μl/mg chlorophyll; dark, 19.8 μl/mg chlorophyll.

experiment shown, the pH in the stroma increased by 0.86 pH unit when increasing the pH in the medium by 1.41 units. The changes of pH in the stroma are reflected by changes of pH in the thylakoid space. It may be noted that the ΔpH between the stroma and the thylakoid space is almost unaffected by these pH changes. This result is similar to data obtained by Rottenberg *et al.*[8] with broken chloroplasts. Since the buffering capacity in the stroma and in the thylakoid space will depend on the pH, as discussed later, the ΔpH across the thylakoid membrane during illumination seems to be independent from the amount of protons transported to build up to this pH gradient.

Decrease of the ΔpH across the thylakoid membrane caused by the addition of uncoupler or by lowering of light intensity

Nigericin and *m*-chlorocarbonyl cyanide phenylhydrazone (CCCP) are known to cause the decay of the energy linked proton gradient in chloroplasts[25,26]. These substances prevent the increase of the pH gradient between the stroma and the thylakoid space in the presence of light[8].

The experiment of Fig. 8 investigates the dependence of the pH in the two chloroplast spaces on the concentration of CCCP. When the CCCP concentration is increased, the ΔpH across the thylakoid membrane is gradually decreased to the dark value. The pH changes in the stroma and in the thylakoid space do not fully correspond with each other. The addition of low concentrations of CCCP causes a decrease of the pH in the stroma with only little increase of pH in the thylakoid space. With higher concentrations of CCCP there is hardly any change of the pH in the stroma, but there is a large increase of pH in the thylakoid space. Essentially the same results are obtained when nigericin is used instead of CCCP (Fig. 9) and a very similar picture is also observed when the light intensity is varied (Fig. 10). Lowering of the light intensity causes a gradual decrease of pH across the thylakoid membrane. With higher light intensities mainly the pH in the stroma is affected, whereas in the range of low light intensities the pH in the thylakoid space is primarily influenced.

It appears from these different experiments that with increasing proton transport across the thylakoid membrane the rise of the pH gradient is at first predominantly caused by a lowering of the pH in the thylakoid space and then from a rise of the pH in the stroma. Therefore, the light-dependent alkalization of the stroma is almost abolished when the ΔpH between the stroma and the thylakoid space is only partially decreased.

On the buffering capacity in the stroma and in the thylakoid space

The differences between the corresponding pH changes could arise from different buffer capacities in both compartments. In order to test this possibility pH changes have been simulated by a simple model, consisting of two spaces (stroma and thylakoid space), the sizes of which are 7:1 (Table I) and which contain certain concentrations of buffer $[B]_S$, $[B]_T$ with a certain pK (pK_S, pK_T). From the experiment in Fig. 6B, which is in accordance with previous results[8], it can be estimated that about 70 mmoles of protons are taken up per liter of thylakoid space. Thus the buffer capacity in the thylakoid space must be in this range. From the pH values for both spaces in the dark, as taken from the experimental data of Fig. 8, the dissociation of the corresponding buffers is evaluated.

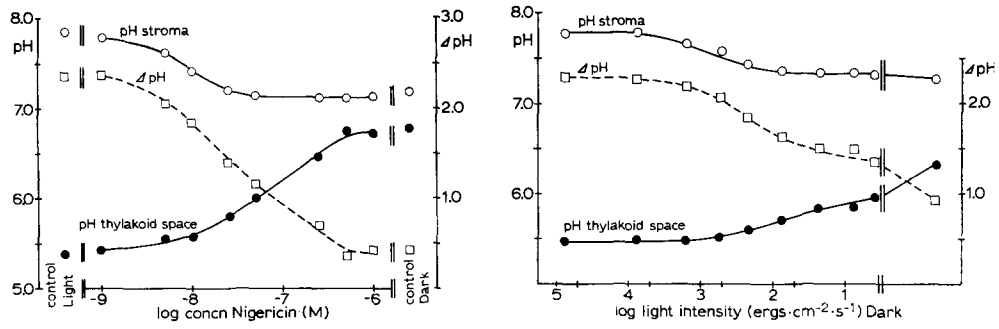


Fig. 9. The effect of nigericin on the light-dependent pH changes of spinach chloroplasts (36 μg chlorophyll/ml) in the stroma and in the thylakoid space. Nigericin was added to the illuminated chloroplasts. Incubation time, 2 min. For details see Methods. The medium did not contain K^+. The endogenous K^+ (ref. 27) of the isolated chloroplasts will be responsible for the effect of nigericin. Addition of nigericin did not significantly change the sucrose-impermeable ^{3}HHO space: light, 23.4 μl/mg chlorophyll; light and nigericin (0.01 μM), 23.9 μl/mg chlorophyll; light and nigericin (0.2 μM), 24.4 μl/mg chlorophyll; light and nigericin (1.0 μM), 24.0 μl/mg chlorophyll; dark, 23.9 μl/mg chlorophyll.

Fig. 10. The effect of light intensity on the light-dependent pH changes in the stroma and in the thylakoid space of spinach chloroplasts (38 μg chlorophyll/ml). For details of illumination and incubation see Methods. The sucrose-impermeable ^{3}HHO space is independent of the light intensity: light (80000 ergs·cm^{-2}·s^{-1}), 24.6 μl/mg chlorophyll; light (8000 ergs·cm^{-2}·s^{-1}), 24.1 μl/mg chlorophyll; light (80 ergs·cm^{-2}·s^{-1}), 23.5 μl/mg chlorophyll; dark, 24.3 μl/mg chlorophyll.

$$\frac{[B^-]_S}{[HB]_S} = 10^{pH_S - pK_S}$$

$$[B]_S = [B^-]_S + [HB]_S$$

For thylakoid space accordingly.

After successive proton movement [ΔH^+] from the stroma into the thylakoid space, the resultant pH values are calculated:

$$pH_S = pK_S + \log \frac{[B^-]_S + [\Delta H^+]}{[HB]_S - [\Delta H^+]}$$

$$pH_T = pK_T + \log \frac{[B^-]_T - 7[\Delta H^+]}{[HB]_T + 7[\Delta H^+]}$$

The model has been tried with various values for pK_S, pK_T and $[B]_S$. The best fit of the model with the experimental data of Figs 8–10 is obtained with $pK_T = 5.5$, $pK_S = 6.8$ and $B_T/B_A = 4$. This is shown in Fig. 11, where the pH values calculated from our model for the two spaces are plotted against ΔpH (solid line). Furthermore, the experimental data of Figs 8–10, which have been obtained with the same plant material, are plotted. There is a fairly good agreement between the different experimental data and the values calculated from our model.

In conclusion, the differences between the pH changes in the stroma and in the thylakoid space, as shown in the experiments of Figs 4 and 8–10, can be explained

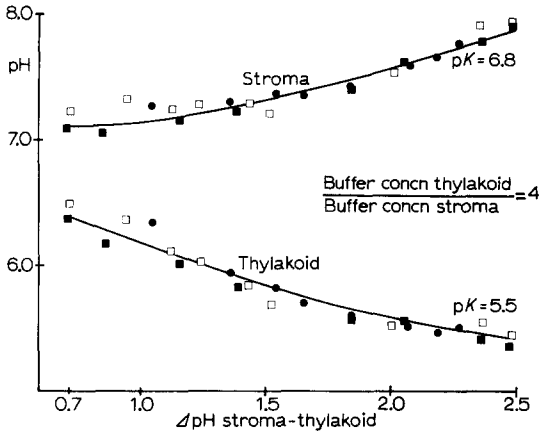


Fig. 11. pH changes in the stroma and in the thylakoid space depending on the ΔpH across the thylakoid membrane. The data of Figs 8–10 have been plotted. ■, experiment with CCCP (Fig. 8); □, experiment with nigericin (Fig. 9); ●, experiment with varied light intensity (Fig. 10). The solid lines represent the calculated pH changes in the two spaces of a model system as described in the text.

in terms that in the thylakoid space there is a buffer with a pK about 5.5 and in the stroma a buffer with a pK about 6.8 and the relationship of the buffer concentrations between the thylakoid space and the stroma is 4:1.

The pK assumed for the thylakoid space is in agreement with the results of Rottenberg *et al.*[8], who found a maximal buffer capacity around pH 5 with broken chloroplasts. The nature of this buffer is not known. It has been speculated that the buffering capacity in the thylakoid space may be due to phospholipids in the thylakoid membrane[28]. The buffer in the stroma might consist partly of inorganic phosphate and phosphate esters, which are known to be present[29,30].

DISCUSSION

On the method

The use of methylamine as a reliable tool for measuring the pH in the thylakoids has been demonstrated by Rottenberg *et al.*[8]. The suitability of DMO for measuring the pH in the stroma appears from the results of Table II and of Fig. 5 in ref. 11 where identical pH values have been obtained when using CO_2 instead of DMO.

For the calculation of the pH in the stroma and in the thylakoid space, it is necessary to know the size of these spaces. The sum of the stroma and the thylakoid space can be accurately determined in each chloroplast sample as the sucrose-impermeable ^{3}HHO space. Unfortunately the thylakoid space of intact chloroplasts cannot be assayed with functional methods. Planimetric measurements of electromicrographs, as carried out in this report can be regarded only as a rough estimation. If the actual size of the thylakoid space would be only half of our estimation, the calculation of the pH in the stroma would be hardly at all affected, but the pH in the thylakoid space would be about 0.3 unit lower than calculated (Table III). This may be important for the light-induced changes of the ΔpH across the thylakoid membrane. Our calculations are based on the assumption that the size of the thylakoid

space does not change between darkness and illumination. If there would be light-induced shrinkage of the thylakoid space, the ΔpH across the thylakoid membrane in the light might be even higher than calculated in this report. However, the differences from our results are not expected to be very large, since the ΔpH values calculated here are very similar to the values obtained with thylakoids of broken chloroplasts[8] (Werdan, K. and Heldt, H. W., unpublished) where the size of the thylakoid spaces have been accurately determined.

The pH in the stroma

It has been shown that the pH in the stroma space is dependent on the pH in the medium. The question arises what is the optimal pH in the stroma for CO_2 fixation. With intact chloroplasts maximal rates of CO_2 fixation have been observed when the pH in the medium was about 7.6 (Walker, D. A., personal communication)[31]. In experiments not shown here, the dependency of CO_2 fixation by intact chloroplasts on the pH in the stroma was measured. Depending on the growth conditions of the spinach, pH optima between 7.6 and 8.4 were found (Werdan, K. and Heldt, H. W., in preparation). Taking into account that the pH optima of enzymes may be not exactly the same in diluted solution as in the densely packed stroma, and that the pH in the stroma may be not fully homogeneous, these pH optima appear to be similar to the pH optima of several stroma enzymes (fructose diphosphatase, pH 8.5–8.8 (refs 32–34), phosphoribulokinase, pH 7.9 (ref. 35), NADP–glyceraldehyde phosphate dehydrogenase, pH 8.0 (ref. 36), α-1,4-glycosyltransferase, pH 8.5 (ref. 37), and cyclic photophosphorylation, pH 8.8 (ref. 38)). Ribulosediphosphate carboxylase (pH 7.6 in the presence of 10 mM Mg^{2+} (refs 39, 40)), appears to be different. This could be explained by a local acidification in the stroma occurring at the site of ribulose-diphosphate carboxylase[11].

Light-dependent alkalization in the stroma and regulation of CO_2 fixation

In principle, CO_2 fixation could also proceed in the dark, since the NADPH and ATP necessary for this reaction, could also be supplied from the cytoplasm *via* dihydroxyacetone phosphate phosphoglycerate shuttle[24,41,42]. However, CO_2 fixation dependent on the substrates generated by oxidative metabolism in this way would lead to a futile cycle. It may be, therefore, essential for the metabolism of the plant cell that CO_2 fixation proceeds only in the light. Besides changes of the level of Mg^{2+} (refs 43–45) or of the reduction state of redox carriers[45–50], the light-dependent alkalization of the stroma could be an important factor for the regulation of CO_2 fixation. One site for the regulation of CO_2 fixation appears to be fructose diphosphatase facilitating the initial step of the cycle[51,52]. The activity of this enzyme seems to be rate-limiting for CO_2 fixation. It is largely stimulated when the pH is increased by 0.5–0.7 unit[32–34], the absolute value of the pH optimum being shifted by the Mg^{2+} concentration and by ferredoxin or the unphysiological SH reagent dithiothreitol[34,49] (Latzko, E., personal communication). Furthermore, ribulose diphosphate carboxylase was shown to be stimulated by fructose 6-phosphate and this stimulation was reversed by fructose diphosphate[53]. Thus a stimulation of fructose diphosphatase would also increase the activity of ribulose diphosphate carboxylase. It is feasible that light-dependent alkalization of the stroma is involved in "switching on" CO_2 fixation at the fructose diphosphatase step.

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