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CO_2 REDUCTION BY INTACT CHLOROPLASTS UNDER A DIMINISHED PROTON GRADIENT

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SUMMARY

9-Aminoacridine has been used to monitor the intrathylakoid pH of photosynthetically competent intact chloroplasts. Values obtained from 9-aminoacridine accumulation in the chloroplasts must be corrected for light-dependent binding of 9-aminoacridine to the thylakoid membranes. During nitrite reduction by intact chloroplasts, the intrathylakoid proton concentration increased. It decreased somewhat during CO_2 reduction. However, low concentrations of uncoupling amines such as NH_3 or cyclohexylamine, which rapidly penetrated the chloroplast envelope and decreased the intrathylakoid proton concentration, failed to reduce, and actually stimulated, rates of CO_2-dependent oxygen evolution even under rate-limiting light. In contrast, low concentrations of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) or nigericin, which inhibited CO_2 reduction, even appeared to increase the intrathylakoid proton concentration. As indicated by measurements of the 515 nm signal of the chloroplasts, the light-induced membrane potential was not much affected by low concentrations of the uncoupling amines, but was decreased by FCCP and by high concentrations of the amines. Even in the presence of high concentrations of NH_4Cl, ATP/ADP ratios of illuminated chloroplasts remained far above the ratios observed in the dark. In contrast, low concentrations of FCCP were sufficient to reduce ATP/ADP ratios to the dark value even under high intensity illumination. The observations are difficult to explain within the framework of the chemiosmotic hypothesis as presently discussed.

INTRODUCTION

According to current concepts, photosynthetic CO_2 reduction in the Calvin cycle requires three molecules of ATP and four reducing equivalents per molecule

Abbreviations: CHA, cyclohexylamine; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; $\Delta\Psi$, electrical potential difference; P_i, inorganic phosphate; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid.

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assimilated CO_2. Two energy-conserving sites are thought to be located in the thylakoid membrane of chloroplasts and contribute to synthesis of ATP [1–6]. During light-driven transport of an electron from water to a suitable acceptor molecule two protons have been observed to be transferred across the thylakoids into the closed intrathylakoid space [1,7–9]. Under steady-state electron transport a pH difference of about 3 pH units can be measured across the membrane (for reviews see refs. 10 and 11). In addition to proton translocation, electron transport induces an electrical potential difference across the thylakoid membranes. Values as high as 100 to 135 mV have been reported [12, 13]. According to Mitchell's chemiosmotic theory [14] both the proton gradient and the membrane potential are the driving forces of phosphorylation. Jagendorf and Uribe [15] have in elegant experiments shown that thylakoids can synthesize ATP in the dark instead of in the light when a proton gradient of sufficient magnitude is set up across the membrane. Racker and Stoeckenius [16] have incorporated bacteriorhodopsin, a retinal-containing protein acting as light-dependent proton pump in the cell membranes of *Halobacterium halobium* into liposome membranes. The particles were shown to take up H^+ in the light and to generate ATP from ADP and phosphate, when a mitochondrial ATPase was also incorporated into the liposomes. Witt et al. [17] recently reported that it is possible to induce ATP formation in isolated class II chloroplasts [18] in an external electrical field even in the absence of a significant pH gradient. These observations lend solid support to Mitchell's hypothesis according to which proton translocation and charge separation are the primary acts of energy conservation. While aware of and strongly impressed by these findings, we wish to report the disturbing fact that even under rate-limiting light intact chloroplasts continue to evolve oxygen during CO_2 assimilation, when the proton gradient has been diminished by the addition of an amine, and the membrane potential appears to be unaltered or even decreased. In contrast, ATP/ADP ratios are drastically decreased by low concentrations of FCCP or nigericin, which even increase ΔpH.

MATERIALS AND METHODS

Intact chloroplasts capable of photoreducing CO_2 at high rates were prepared at 4 °C from fresh and rapidly growing spinach leaves by a modification [19] of Jensen and Bassham's procedure [20]. Chlorophyll was determined according to Arnon [21]. The percentage of intact chloroplasts in the preparations was routinely measured by the ferricyanide method [22]. Preparations which contained less than 75 % intact chloroplasts were not used. For the experiments chloroplasts with 33 μg chlorophyll/ml were suspended in an isotonic reaction medium of the following composition: 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, pH 7.6. Catalase (1500 I.U./ml) was also added. Concentrations of substrates ($NaHCO_3$, KNO_2, oxaloacetate) were 1 or 2 mM, the concentration of 9-aminoacridine 5 or 10 μM. For Δ pH calculations, the thylakoid volume of intact chloroplasts was assumed to be 3.3 μl/mg chlorophyll [23]. Oxygen evolution was recorded by a Clark type electrode, 9-aminoacridine and chlorophyll fluorescence by photomultipliers which were protected against red actinic and short wavelength blue light by proper filter combinations (filters 9782 and 5030 from Corning, 4 mm each, and interference filter 457 nm from Balzers for 9-

aminoacridine fluorescence, and 4 mm 5030 from Corning, 3 mm RG 10 from Schott, 1 mm Calflex C from Balzers and interference filter 742 nm for chlorophyll fluorescence). Red actinic light was provided by a halogen lamp and was filtered through 8 cm water, 1 mm Calflex C and 3 mm RG 630 from Schott. The latter filter was substituted by 3 mm RG 610 and a broad band interference filter K_6 from Balzers, when chlorophyll fluorescence, 9-aminoacridine fluorescence and oxygen evolution, or 515 nm absorption, were simultaneously recorded. Short wavelength blue light of low intensity (peak transmission 360 nm) served for excitation of 9-aminoacridine fluorescence. It was provided by a 450 Watt Xenon lamp. Filters used were 8 cm water, Corning filters 5874 and 5860, 4 mm each, and 4 mm of a heat absorption filter from a Leitz projector. Absorption measurements were carried out in a sensitive single beam spectrophotometer as previously described [24] or in a dual wavelength spectrophotometer (DW 2, from Aminco). The photomultipliers were protected against actinic light by suitable filters. The intensity of actinic light was recorded by a silicone photodiode calibrated against a thermopile.

Determination of adenylates. The adenylate content was determined by the luciferin-luciferase method. The chloroplasts were inactivated by $HClO_4$, final concentration 0.17 M, and after 15 min neutralized with KOH to pH 7.3. After a fast centrifugation the supernatant was used for determination of ATP and ADP.

ATP Determination: 0.1 ml of a solution containing extract from 10 mg firefly lanterns per ml (Sigma FLE 50) in 50 mM $KHAsO_4$ and 20 mM $MgSO_4$, pH 7.4, was injected into 10 ml of a solution composed of 0.4 ml of the sample and 15 mM HEPES, 100 mM $NaHAsO_4$, 100 mM $MgSO_4$, pH 7.3. The light emissions under 10 s were immediately recorded in a Packard liquid scintillation counter. Calibration was done by adding known amounts of ATP to a darkened sample treated in the same way. Luminescence was proportional to ATP in the physiological concentration range.

ADP determination: ADP was converted to ATP by adding 50 μg pyruvate kinase (200 I.U./mg) to 0.4 ml of the sample in a solution containing 15 mM HEPES, 10 mM $MgSO_4$, 1.5 mM sodium phosphoenolpyruvate, pH 7.3, 1 ml total volume. After 30 min incubation at room temperature the total ATP content was measured as described above. ADP was obtained by subtracting ATP from the total value.

RESULTS AND DISCUSSION

Penetration of 9-aminoacridine into intact chloroplasts

When a suspension of chloroplast or bacterial thylakoids containing 9-aminoacridine is illuminated, 9-aminoacridine fluorescence decreases. It increases on darkening. Light-dependent fluorescence quenching of 9-aminoacridine has been interpreted to be the consequence of protonation and retention of the amine inside the thylakoids [25, 26]. Fluorescence quenching is thus supposed to indicate and actually to measure a light-induced pH differential across thylakoid membranes. Fig. 1 compares fluorescence quenching of 9-aminoacridine by intact and by broken chloroplasts with their oxygen exchange in the absence and presence of 0.5 mM KNO_2. It is apparent that nitrite does not influence fluorescence quenching by broken chloroplasts under low intensity (15 W/m^2) illumination but produces an increase of quenching in intact chloroplasts. While in broken chloroplasts illumination results in some

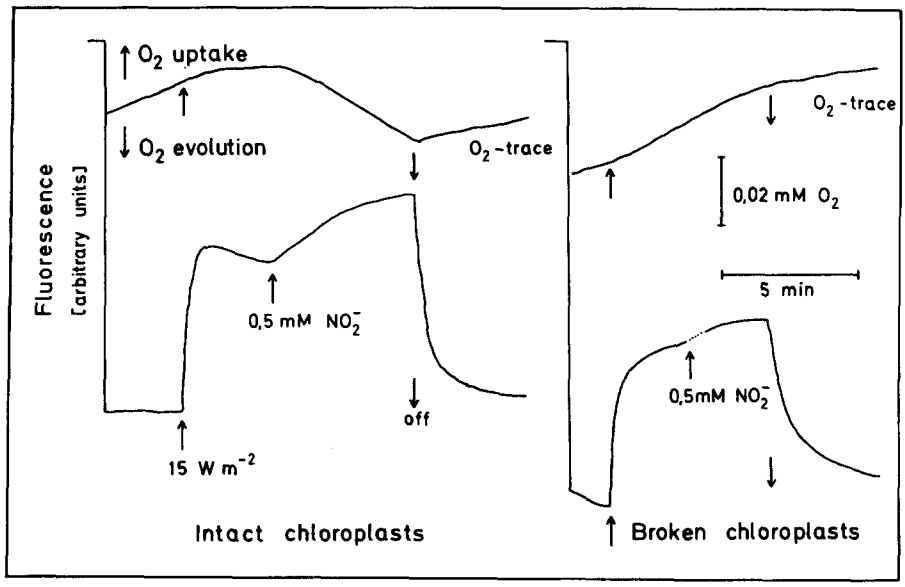

Fig. 1. Simultaneous measurement of the kinetics of oxygen exchange and fluorescence of 9-aminoacridine (10 μM) during illumination of a suspension of intact and osmotically ruptured chloroplasts. Composition of the medium was identical in both cases. Addition of KNO_2 as indicated. The rate of nitrite-dependent oxygen evolution of intact chloroplasts was 8.6 μmol/mg chlorophyll per h. There was some light-dependent oxygen uptake by broken chloroplasts which was not influenced by nitrite.

oxygen uptake due to a Mehler reaction which is not influenced by nitrite, intact chloroplasts respond to nitrite addition with oxygen evolution according to

$$NO_2^- + 3\,H_2O \rightarrow NH_4^+ + 2\,OH^- + 1\tfrac{1}{2}\,O_2$$

The decrease of 9-aminoacridine fluorescence on nitrite addition to intact, but not broken chloroplasts clearly indicates that 9-aminoacridine penetrates the envelope of intact chloroplasts and moves across the stroma into the thylakoids. It also implies that the light-induced proton gradient across the thylakoid membranes which leads to accumulation of 9-aminoacridine inside the thylakoid vesicles is increased in intact chloroplasts during electron transport to nitrite, whose reduction does not require ATP.

Decrease in the intrathylakoid proton concentration of intact chloroplasts by uncoupling amines without inhibition of CO_2 assimilation

Fig. 2 shows the effect of additions of cyclohexylamine and NH_4Cl on the quenching on 9-aminoacridine fluorescence by chloroplasts illuminated with saturating red light and the simultaneous effect on CO_2-dependent oxygen evolution. During the lag phase of photosynthesis [27] fluorescence quenching was maximal. In accordance with Mitchell's [14] concept of energy conservation, it decreased when oxygen evolution started. As is predictable, addition of uncouplers such as cyclohexylamine or NH_4Cl decreased fluorescence quenching drastically. Higher concentrations practically abolished it. Surprisingly, at low concentrations, the uncouplers failed to

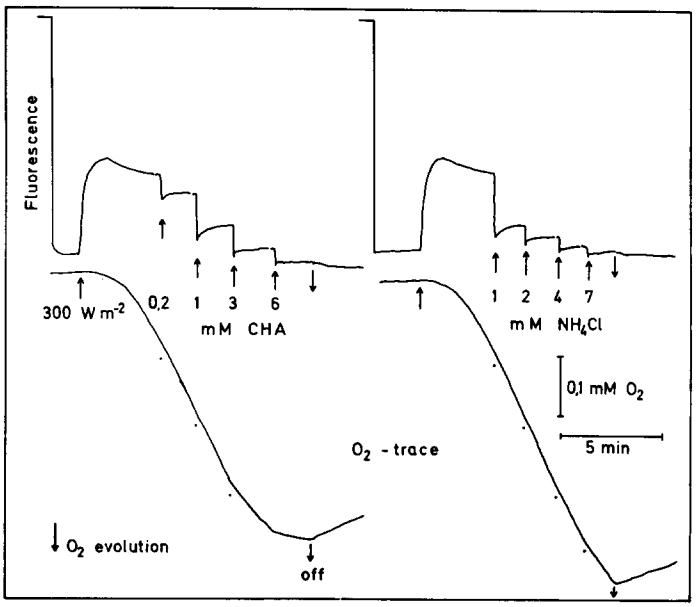

Fig. 2. Effect of cyclohexylamine (CHA) and NH_4Cl on the time course of oxygen exchange and fluorescence of 9-aminoacridine during illumination of intact chloroplasts in the presence of 2 mM HCO_3^- with 300 W/m^{-2} red light (half bandwith from 630 to 760 nm). Addition of amine as indicated. Rates of O_2 evolution, in μmol/mg chlorophyll per h: without NH_4Cl, 156; with 4 mM NH_4Cl, 176; with 7 mM NH_4Cl, 135. Without cyclohexylamine, 143; with 0.2 mM cyclohexylamine, 183; with 3 mM cyclohexylamine, 108.

decrease CO_2-dependent oxygen evolution. They actually stimulated photosynthesis (see legend to Fig. 2). This stimulation is due to pH regulation of stroma enzymes, particular of fructose bisphosphatase, which can be stimulated by a very small increase in the stroma pH. Stimulation should be absent when the stroma pH is optimal initially. Indeed, when the medium pH is raised to 8.0 there is no stimulation by NH_4Cl. Otherwise, there is no difference to the results with the lower pH. Stimulation of CO_2-dependent oxygen evolution has already been reported by Forti et al. [28] but ascribed to another regulating step of the carbon cycle. More than 20 mM NH_4Cl were usually required by intact chloroplasts to inhibit CO_2 reduction extensively. There was some variability in the sensitivity of intact chloroplasts from different leaf material against NH_4Cl uncoupling, but in all instances fluorescence quenching was lowered by NH_4Cl long before CO_2 reduction was affected. It appeared possible that at the high light intensity used in the experiment of Fig. 2 the proton motive force

$$\Delta G_{H^+}/F = 2.3\, RT/F \cdot \Delta\mathrm{pH} + \Delta\Psi$$

(where F is the Faraday constant, R the gas constant and $\Delta\Psi$ a light-generated membrane potential) which, according to Mitchell [14] drives ATP synthesis, was excessive and actually higher than required for the ATP production needed in CO_2 assimilation. The experiment was therefore repeated under a light intensity which was strictly rate limiting for CO_2 reduction (Fig. 3). It has previously been established [19] that under these conditions not NADPH but ATP limits CO_2 reduction. Fig. 3 shows that even

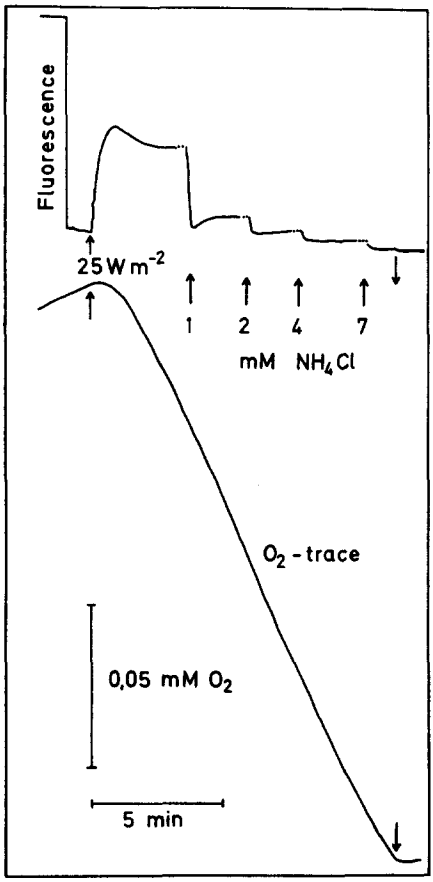

Fig. 3. Effect of NH_4Cl on oxygen exchange and fluorescence of 9-aminoacridine (10 μM) during illumination with low light intensity. Light quality as for Fig. 2. Addition of NH_4Cl as indicated. Rates of O_2 evolution in μmol/mg chlorophyll per h: without NH_4Cl, 40; with 1 mM NH_4Cl, 47; with 4 mM NH_4Cl, 41; with 7 mM NH_4Cl, 39.

under rate-limiting light oxygen evolution was not decreased but actually stimulated by 1 mM NH_4Cl. Inhibition of CO_2 reduction was not yet significant even in the presence of 7 mM NH_4Cl. Fluorescence quenching was drastically lowered by 1 mM and almost abolished by 7 mM NH_4Cl.

Very similar results were obtained when 9-aminoacridine was substituted by the same concentration of *N*-(naphthyl)-1-ethylenediamine as a pH indicating fluorescent dye.

The observations, if taken at face value, appear to indicate that the proton gradient is much more sensitive to an uncoupler than the ATP-requiring CO_2 assimilation itself. However, interpretation of fluorescence quenching as an indicator of ΔpH is not unequivocal. Fluorescence quenching may also be caused by binding of the amino group of 9-aminoacridine to negative charges of the thylakoid membrane which become exposed to the dye during illumination [29].

To investigate this, the percentage of light-induced quenching was measured as

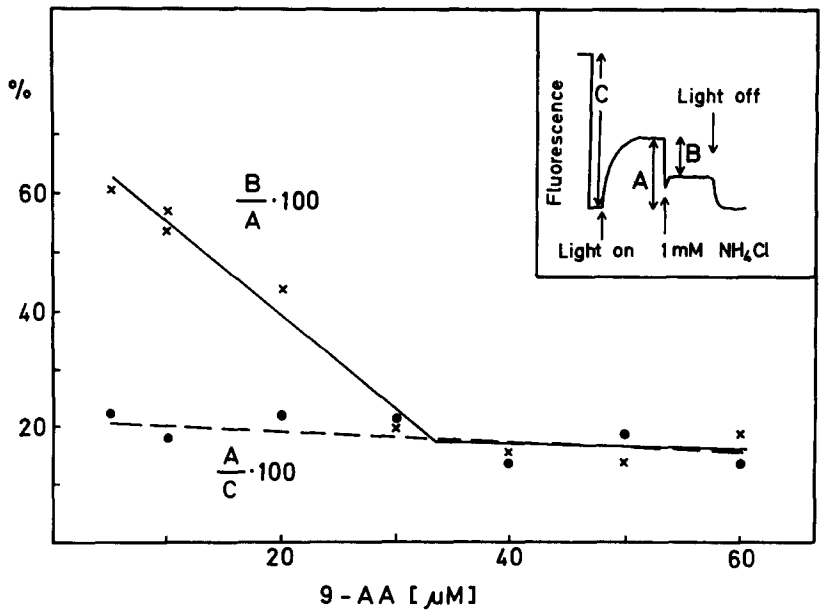

Fig. 4. Light-induced quenching of 9-aminoacridine (9-AA) (– – –) and decrease of quenching caused by addition of 1 mM NH_4Cl (—). See insert for explanation of the ordinate. Intensity of short wavelength red light 50 W/m^{-2}.

a function of the 9-aminoacridine concentration. The percentage of quenching due to binding alone should decrease with increasing concentration of 9-aminoacridine as a consequence of saturation of the binding sites. The uncoupling effect of 9-aminoacridine as seen by inhibition of CO_2-dependent O_2 evolution should also be correlated with a decreased ΔpH which is seen as a decreased quenching. As the percentage of light-induced quenching is not much decreased (Fig. 4) it is concluded that the percentage of bound amine cannot be very high. Its magnitude can roughly be estimated from competition studies. If there is binding of 9-aminoacridine to the membrane, the relative fluorescence increase caused by NH_4Cl should according to the law of mass action be higher at low than at high concentrations of the fluorescent amine. This was indeed observed for concentrations of 9-aminoacridine from 5 to 30 μM (Fig. 4). A further increase in the concentration of the fluorescent amine did not cause a significant increase in relative fluorescence after addition of NH_4Cl. With 10 μM 9-aminoacridine the light-induced 9-aminoacridine fluorescence quenching was reduced by about 55 % by 1 mM NH_4Cl (Fig. 4). In contrast, at concentrations of 9-aminoacridine higher than 30 μM, reduction of quenching by 1 mM NH_4Cl was only about 20 % of the total signal. Obviously, at the higher 9-aminoacridine concentrations, release of 9-aminoacridine from the membranes, where it was bound, became insignificant in relation to the increased release of dye from the intrathylakoid space, where protonation of NH_3 increased the pH. Thus at higher concentrations of 9-aminoacridine it may be assumed that the fluorescence response to NH_4Cl reflects largely the pH change in the intrathylakoid compartment.

Schuldiner and coworkers [25] calculated ΔpH according to

$$\Delta\text{pH} = \log\frac{Q}{1-Q} + \log\frac{V}{v} \quad (1)$$

(Q percentage of fluorescence quenching, V total volume of medium, v volume of osmotically active space). In this expression the Q term has to be corrected for binding. Since under our experimental conditions (light intensity 50 W/m^{-2}, 10 μM 9-aminoacridine, 33 μg chlorophyll/ml) 35 % of the total fluorescence change (55 % minus 20 % = 35 %) are caused by amine binding and 65 % by pH-dependent distribution of 9-aminoacridine between an acid chloroplast compartment and the outside medium, the correction factor is equal to 1−0.35 = 0.65. Schuldiner et al. [25] get an overestimation of ΔpH in the order of

$$\Delta(\Delta\text{pH}) = \log\frac{Q}{1-Q} - \log\frac{0.65Q}{1-0.65Q} \approx 0.3 \text{ pH units},$$

depending somewhat on the Q value.

From these data it is possible to calculate the percentage of the fluorescent amine bound to the membrane. As the quenching is caused either by binding or retention the Q term in Eqn. 1 has to be replaced by $Q - A_B/A_0$, where A_B/A_0 is the ratio of 9-aminoacridine bound to the membranes to the total concentration of 9-aminoacridine. Comparing this corrected expression

$$\Delta\text{pH} = \log\frac{1}{1-Q}(Q - A_B/A_0) + \log\frac{V}{v} \quad (2)$$

with Eqn. 1 in which the Q term is replaced by $Q' = 0.65Q$, it can be calculated that about 10 % of the fluorescent amine is bound to the membrane under our conditions. In conclusion, binding does occur, but the portion of bound amine is small. Calculating ΔpH according to Eqn. 1 thus results in some overestimation of ΔpH, but the value is by no means very unrealistic. For illustration, calculation of ΔpH was done from the data of Fig. 2 according to Eqn. 1 but with $Q' = 0.65Q$ instead of Q. For comparison, values calculated according to the original Eqn. 1 are given in parentheses: 3.3(3.6) pH units without NH_4Cl, 2.8(3.0) and 2.6(2.8) pH units with 1 and 2 mM NH_4Cl, respectively. For bacterial chromatophores, Casadio et al. [26] have found that 9-aminoacridine behaves as an ideal amine and that binding to the membranes does not have to be considered.

The kinetics of the response of 9-aminoacridine fluorescence to addition of NH_4Cl is also consistent with the conclusion that the uncoupling amine increases the intrathylakoid pH. On addition of NH_4Cl there was a rapid increase in fluorescence (Figs. 2 and 3) reflecting fast penetration of the undissociated amine into the thylakoid interior, where it neutralized protons thus decreasing the proton concentration. The kinetics of this increase is very different from that observed on addition of the uncoupler FCCP (see Fig. 8) which induces leakage of protons from the thylakoid interior. It shows that it cannot be the result of unbinding of 9-aminoacridine from the membranes by NH_4Cl which kinetically should resemble the FCCP effect. Continued electron transport and accompanying proton pumping were necessary after NH_4Cl addition to raise the inner proton concentration to a new steady state. From the chemiosmotic hypothesis it should be expected that during the filling of the proton sink produced inside the thylakoid by amine penetration phosphorylation

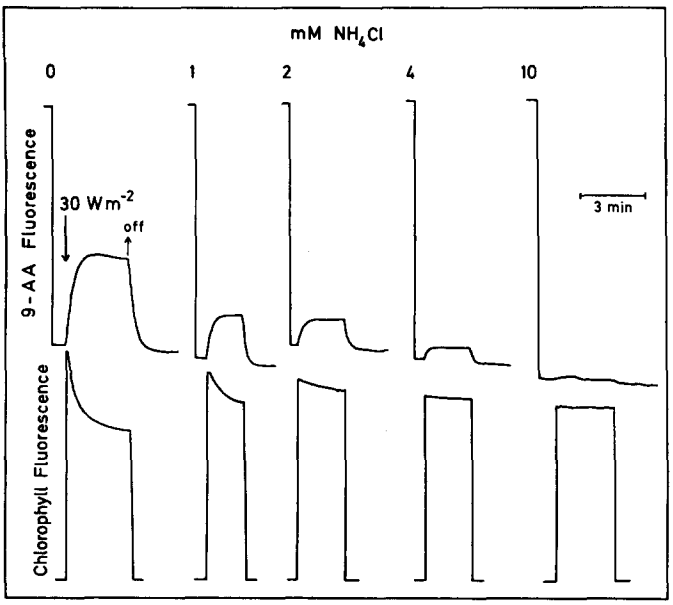

Fig. 5. Simultaneous recordings of 9-aminoacridine (9-AA) fluorescence (top) and chlorophyll fluorescence at 742 nm (bottom of the figure) in a suspension of intact chloroplasts. 9-Aminoacridine concentration 10 μM. Intensity of short wavelength red light 30 W/m^{-2}.

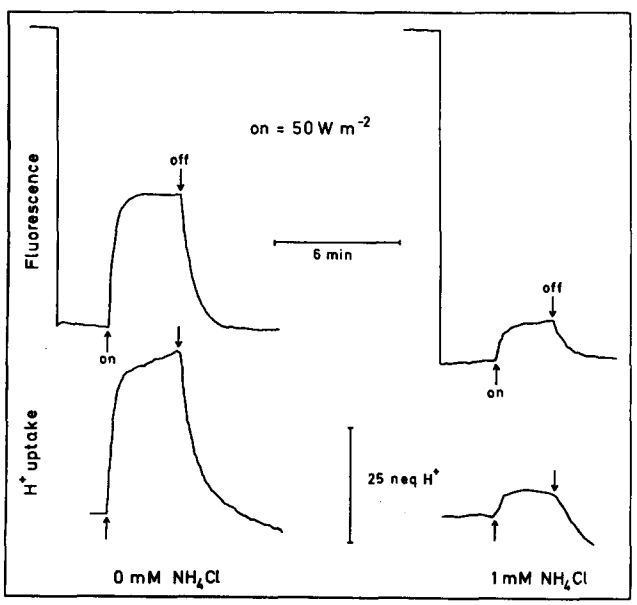

Fig. 6. Simultaneous measurements of 9-aminoacridine fluorescence and medium pH in a suspension of osmotically shocked chloroplasts. The pH was followed by a glass electrode. Chloroplasts containing 100 μg chlorophyll were ruptured in 1.7 ml 5 mM $MgCl_2$. The same volume of double strength reaction medium, but without HEPES and P_i, was added. The pH was adjusted to 7.2 with KOH. Light quality as for Fig. 2.

should decrease resulting in an at least temporarily decreased rate of CO_2-dependent oxygen evolution. No such effect has been observed (see Fig. 3).

There are other observations confirming that the decrease in 9-aminoacridine fluorescence quenching brought about by additions of NH_4Cl or another uncoupling amine which do not inhibit CO_2 reduction reflects a decrease in the intrathylakoid proton concentration. Fig. 5 shows simultaneous recordings of 9-aminoacridine fluorescence quenching and chlorophyll fluorescence as influenced by NH_4Cl.

Without NH_4Cl, illuminated chloroplasts showed the Kautsky phenomenon, i.e. a secondary decrease in the fluorescence yield of chlorophyll (Fig. 5, lower traces, left), which is attributed to Mg^{2+} efflux from the thylakoids into the stroma compartment of intact chloroplasts [30, 31].

This efflux serves largely to compensate electrically for the influx of protons. In the presence of increasing concentrations of NH_4Cl the Kautsky effect was diminished and finally abolished, while the steady-state fluorescence yield of chlorophyll increased, showing that Mg^{2+} efflux decreased. A decrease in Mg^{2+} efflux is most likely the consequence of a lowered proton gradient.

Thus the kinetics of chlorophyll fluorescence also indicate in accordance with the 9-aminoacridine fluorescence measurements that NH_4Cl decreases the proton concentration in the intrathylakoid space of intact chloroplasts.

While this cannot be directly shown because of the impermeability of the chloroplast envelope to protons, it is confirmed by experiments with broken chloroplasts. Under a low light intensity, proton uptake as measured directly by a glass electrode was large in the absence of NH_4Cl and much smaller in its presence (Fig. 6). The extent of proton uptake was related to the extent of 9-aminoacridine fluorescence quenching again confirming that this is a reliable, though owing to binding of 9-aminoacridine not entirely quantitative, indicator of the transmembrane proton gradient.

Effect of uncoupling amines on the light-induced membrane potential of intact chloroplasts

The puzzling observation of an increased CO_2 reduction by intact chloroplasts seen under rate-limiting light when the intrathylakoid proton concentration was decreased by NH_4Cl (Fig. 3) could find a simple explanation, if in the presence of NH_4Cl the steady-state electrical potential of the thylakoid membranes is increased. According to Mitchell [14], the proton motive force, which is supposed to drive ATP synthesis, has the two components ΔpH and $\Delta\Psi$. Clearly an increase in $\Delta\Psi$ could substitute for a loss in ΔpH. The electrochromic shift at 515 nm has been used to measure the light-induced membrane potential of thylakoid membranes [32]. Fig. 7 shows the light-induced 515 nm absorption change and 9-aminoacridine fluorescence quenching of intact chloroplasts at different NH_4Cl concentrations, as measured by a single beam spectrophotometer. Slow changes in 515 nm absorption are caused by light scattering [24, 32]. They indicate membrane transport processes and were inhibited by NH_4Cl. Fast field-indicating absorption changes were less affected. There was no indication from the 515 nm signal that the membrane potential was increased by NH_4Cl. Rather it was somewhat decreased (Fig. 7, Table I). To differentiate between the 515 nm signal and light scattering, measurements of the light-induced membrane potential were also performed in a dual wavelength spectrophotometer

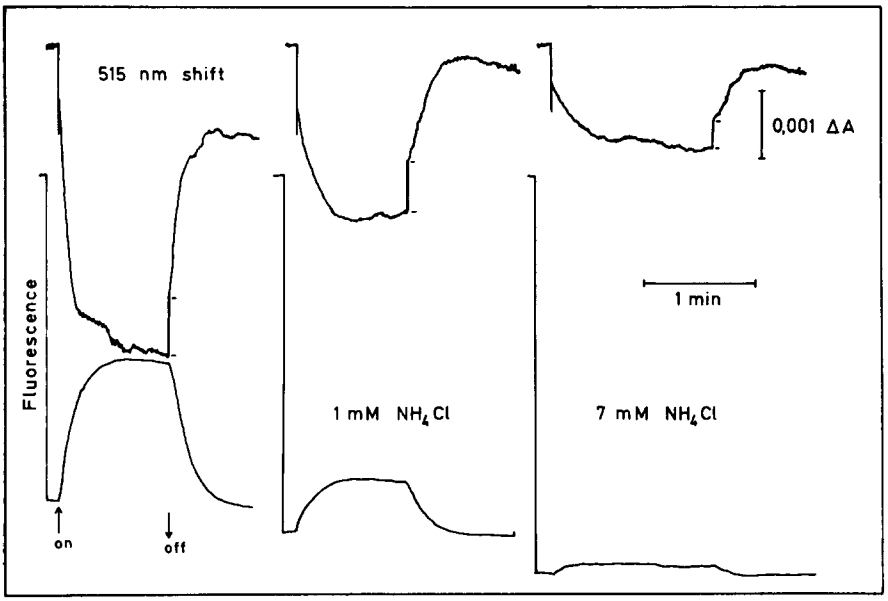

Fig. 7. Simultaneous recordings of 9-aminoacridine fluorescence and of absorbance changes at 515 nm in a suspension of intact chloroplasts (light path 0.5 cm) in the absence and presence of NH_4Cl. Field-indicating absorbance changes at 515 nm are fast. Slow changes in apparent absorbance are caused by light-dependent changes in the scattering of 515 nm light by the chloroplasts. Illumination with 300 W/m^{-2} red light. 91 % of the chloroplasts in the suspension had intact envelopes. CO_2 reduction by the preparation was 141 μmol/mg chlorophyll per h in the absence of NH_4Cl.

with the measuring beam set at 515 nm and the reference beam at 539 nm (not shown). This largely eliminated light scattering. Again NH_4Cl did not increase the 515 nm absorption change seen when the light was turned on. The steady-state signal was progressively decreased with increasing NH_4Cl concentration.

As should be expected, the 515 nm signal was more than 40 % inhibited by 0.5 μM FCCP plus 0.5 μM valinomycin. FCCP alone was less inhibitory (Table I).

Effects on ΔpH and CO_2 reduction by FCCP and nigericin

FCCP, which like NH_4Cl is a noted uncoupler of phosphorylation and is supposed to increase the proton conductivity of biomembranes, actually increased 9-aminoacridine fluorescence quenching at low concentrations which, however, were

TABLE I

INHIBITION OF THE 515 nm SIGNAL BY NH_4Cl AND FCCP

The values are taken from the fast absorption change seen when the light was turned on.

	NH_4Cl (mM)				FCCP (μM)			
	0	1	2	4	0	1	2	4
Percent inhibition of 515 nm absorption	0	6	6	22	0	47	59	68

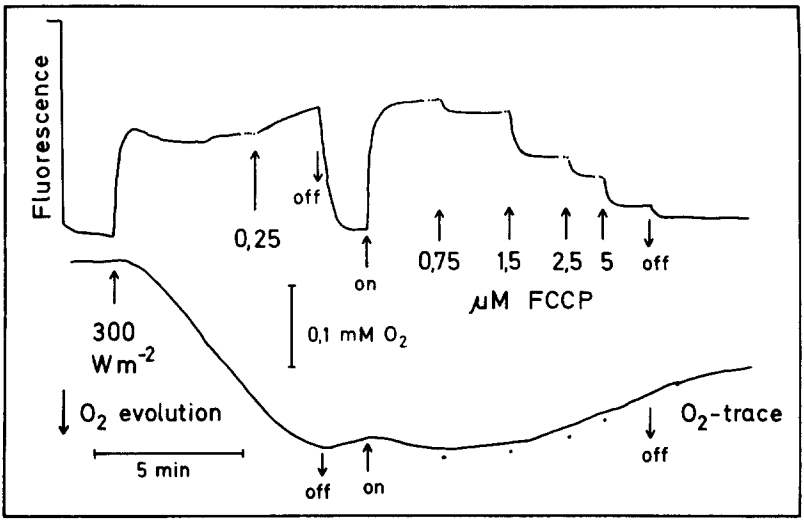

Fig. 8. Effect of FCCP on oxygen exchange and fluorescence of 9-aminoacridine (10 μM) during illumination with 300 W/m^{-2} red light. Light quality as for Fig. 2. Addition of uncoupler as indicated. Rates of O_2 evolution in μmol/mg chlorophyll per h: without FCCP, 111; 0.25 μM FCCP, 43; 5 μM FCCP, 0.

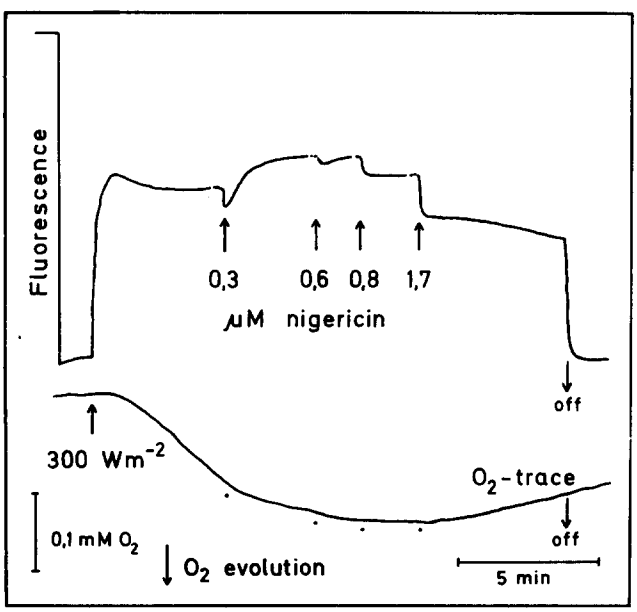

Fig. 9. Effect of nigericin on oxygen exchange and fluorescence of 9-aminoacridine (10 μM) during illumination with 300 W/m^{-2} red light. Light quality as for Fig. 2. Addition of uncoupler as indicated. Rates of O_2 evolution in μmol/mg chlorophyll per h: without nigericin, 89; 0.3 μM nigericin, 21; 0.8 μM nigericin, 9.

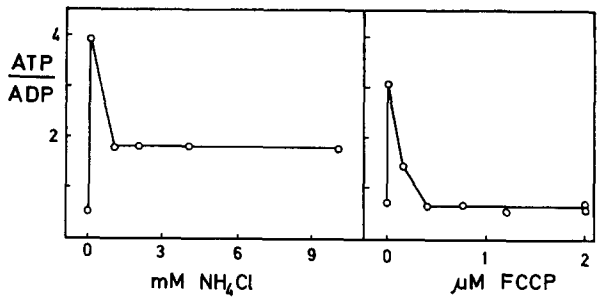

Fig. 10. Effects of NH_4Cl and FCCP on ATP/ADP ratios in intact chloroplasts. Chlorophyll concentration, light quality and intensity as for Fig. 2.

sufficient to inhibit CO_2 reduction significantly (Fig. 8). This is in striking contrast to the decrease of fluorescence quenching by NH_4Cl without simultaneous inhibition of CO_2 reduction. In contrast to low concentrations, high concentrations of FCCP decreased 9-aminoacridine fluorescence quenching. Nigericin, another well known uncoupler, was similar to FCCP in its effect on 9-aminoacridine fluorescence and CO_2 reduction (Fig. 9).

Adenylate determinations

On illumination with high intensity light, the intrachloroplast ratio of ATP to ADP rose from less than 1 to more than 3 or close to 4 in the absence of uncouplers (Fig. 10). With 1 mM NH_4Cl present, the ratio decreased to somewhat less than 2. This was still quite sufficient to support fast CO_2 reduction (see Figs. 2 and 3). A ratio close to 2 was maintained even at higher NH_4Cl concentrations, which progressively decreased ΔpH and $\Delta\Psi$. In contrast to NH_4Cl, 0.5 μM FCCP was sufficient to bring the intrachloroplast ATP/ADP ratio back to its dark value. This contrasts to the effect of FCCP on 9-aminoacridine fluorescence quenching, which suggests a higher ΔpH in the presence of 0.5 μM FCCP than in its absence. CO_2 reduction was largely inhibited by 0.5 μM FCCP.

CONCLUSIONS

The situation encountered by intact chloroplasts suspended in an isotonic medium containing an uncoupling amine is somewhat different from that encountered by thylakoids. The latter are directly exposed to both the added amine and its protonation product whose ratio can be predicted from the Henderson-Hasselbalch equation. In contrast, intact chloroplasts possess an envelope which is a barrier to the penetration of the protonated amine. In consequence, only the base can easily penetrate (Fig. 11). It will be protonated in the stroma thereby raising its pH. The extent of the stromal pH rise is a function of the amine concentration and the stromal buffering capacity. On illumination proton transfer from the stromal compartment to the intrathylakoid space will give rise to the trapping of amine in the thylakoid compartment inducing a flux of amine from the outside medium into the thylakoids. This brings about a reversible acidification of the outside medium (Fig. 11) which is not observed on illumination of thylakoids in the presence of an amine. The 9-amino-

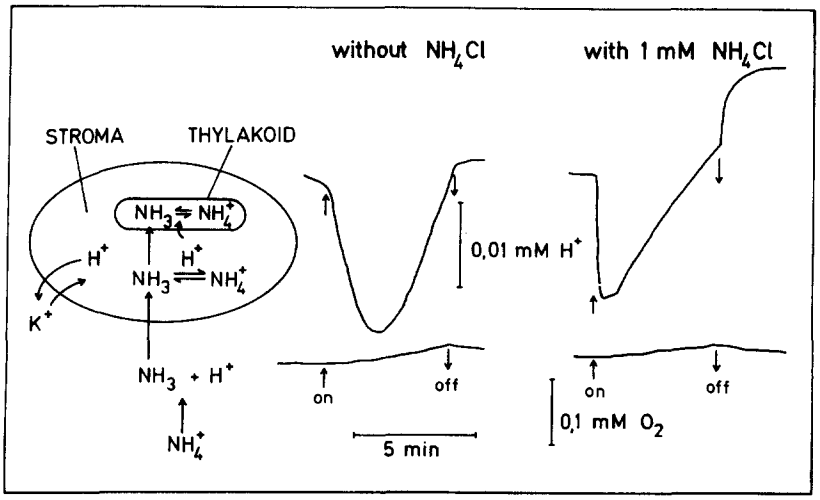

Fig. 11. Simultaneous recordings of oxygen exchange and H^+ formation in the medium on illumination of intact chloroplasts. The reaction medium, from which the buffer was omitted, contained some residual CO_2. Chlorophyll concentration was 55 μg/ml. Upper traces are recordings from a pH electrode inserted into the medium, lower traces recordings from an oxygen electrode. Downward deflection of upper traces shows increase of H^+ concentration in the medium, upward deflection of lower traces oxygen production. The slow decrease in H^+ seen after the initial increase is caused by uptake and reduction of CO_2. Arrows indicate illumination (300 W/m^{-2}) or darkening. Scheme at left explains light-dependent increase of H^+ concentration in the medium. Slow H^+ excretion in the absence of NH_4Cl is caused by K^+/H^+ exchange [46], fast increase of H^+ in the presence of NH_4Cl is caused by NH_3 uptake.

acridine fluorescence method as applied above measures the pH difference between the outside medium and the thylakoid compartment, not the bioenergetically more relevant ΔpH across the thylakoid membrane. Theoretically, a decrease in the ΔpH between the outside medium and the thylakoid compartment as observed in the presence of NH_4Cl or other uncoupling amines (Figs. 2 and 3) might be compensated by an increase in the ΔpH between the outside medium and the stromal compartment. This might leave the ΔpH between the stromal and the thylakoid compartments unaffected thus explaining the surprising resistance of CO_2 reduction to uncoupling conditions.

However, this possibility does not apply. The protein concentration in the stroma is very high (more than 20 % by fresh weight, under isotonic conditions). In addition to buffering by proteins, there is buffering by phosphate and phosphate esters whose stromal concentration is approx. 18 mM [33]. Indeed, Werdan et al. [34] did not find a significant effect of low concentrations of uncoupling amines on the stromal pH, while the intrathylakoid pH was increased. Thus, the resistance of CO_2 reduction during a lowering of the ΔpH between medium and intrathylakoid space cannot be attributed to an increased pH in the stroma which would leave the transthylakoid ΔpH unaffected.

In contrast to amines which do not much affect the ΔpH across the chloroplast envelope, FCCP was shown by Werdan et al. [34] to collapse this gradient. These workers suggested that the resulting decrease in the stromal pH inactivates carbon

cycle enzymes and is thereby responsible for inhibition of CO_2 reduction. At first sight, the increase in ΔpH between medium and intrathylakoid space seen on addition of 0.25 μM FCCP (Fig. 8) appears to support this suggestion. Also, our observation that by simply raising the pH of the outside medium from 7.6 to 7.9 or 8.2 it is possible to partially restore CO_2-dependent oxygen evolution (not shown) indicates that pH regulation of stroma enzymes is indeed a factor in the observed inhibition of photosynthesis by low concentrations of FCCP. Another factor, however, is loss of ATP synthesis. Fig. 10 shows that even very low concentrations of FCCP which increase the intrathylakoid proton concentration, decrease the stromal ATP/ADP ratio drastically. They also decrease the extent of the 515 nm shift. It is likely that the decay of the light-induced membrane potential which is signaled by the decreased 515 nm shift is the cause of the increased ΔpH. Still, phosphorylation is inhibited as seen by a decrease in the reduction of 3-phosphoglycerate and by the drop in the ATP/ADP ratio. While amines also decrease somewhat the steady-state ATP/ADP ratio, the high rate of CO_2 reduction is witness that rates of phosphorylation are not decreased but actually increased by low amine concentrations even though the intrathylakoid pH is increased and the proton gradient lowered.

Under rate-limiting light, photosynthetic CO_2 reduction is obviously limited by the availability of either ATP or NADPH. Both are generated by light. Which of them is rate limiting, depends on the coupling ratio ATP/2*e*. The ATP/2*e* requirement of photosynthesis is 1.5. There is still controversy as to the coupling ratio of the thylakoid membrane (see ref. 35 for review). Different groups have reported ATP/2*e* ratios of 1, 1.33 and 2 [36–40]. Only the latter would make NADPH rate limiting in photosynthetic CO_2 reduction. Measurements of the quantum yield of photoreactions of intact chloroplasts have suggested that the coupling ratio (ATP/2*e* ratio) during CO_2 reduction is above 1, but below 1.5 [41]. If this is correct, extra ATP must be supplied to photosynthesis by either non-cyclic electron transport to oxygen [42] or by cyclic electron transport [43]. Indeed, there is evidence that NADPH is not rate limiting in CO_2 reduction of isolated chloroplasts. This is indicated by the observation that the NADPH/NADP ratio does not increase on uncoupling by antimycin A or low concentrations of amines [44]. In contrast to ATP, it does not decrease when CO_2 reduction decreases owing to a reduction in light intensity [19]. In view of this, it is difficult to understand within the framework of the chemiosmotic hypotheses, why CO_2 reduction is not decreased by concentrations of uncoupling amines which reduce ΔpH without increasing $\Delta\Psi$, while it is decreased owing to loss of ATP synthesis by concentrations of FCCP which increase ΔpH while decreasing $\Delta\Psi$. To make confusion complete, $\Delta\Psi$ has been reported by Larkum and Boardman [45] not to contribute significantly to energy conservation of intact chloroplasts.

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