

Photophosphorylation by Chloroplasts: Effects of Low Concentrations of Ammonia and Methylamine

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Intact chloroplasts exposed to hypotonic assay conditions are capable of photophosphorylating exogenous ADP. The rate of phosphorylation by these unbroken plastids is increased by 10–50% upon the addition of low concentrations (< mM) of NH_3 or CH_3NH_2 . Stimulation of phosphorylation is abolished by washing chloroplasts with MgCl_2 . Evidence is presented that washing removes a factor responsible for amine-induced increase of ATP production and that this factor is associated with the thylakoid membrane. Addition of CH_3NH_2 increased the proton permeability of the thylakoid membrane of unbroken and washed chloroplasts during the light/dark transition. Hence, differences of the membrane permeability for protons between the two preparations seem not to be responsible for an increase of ATP production upon the addition of amines. Stimulation of photophosphorylation by methylamine is observed even at light intensities which do not saturate the proton motive force, which in turn is reduced upon the addition of the uncoupler. Apparently, phosphorylation can be stimulated, although the limiting driving force is diminished. It is concluded that phosphorylation by unbroken chloroplasts under low light illumination is limited kinetically, not energetically. Consequences of these findings for observation made with intact chloroplasts are discussed.

Introduction

The chemiosmotic concept holds the transmembrane electrochemical proton gradient to be the driving force of photosynthetic energy conversion [1]. However, the relationship between the rate of ATP production and the magnitude of the driving force is not predicted by this concept. Quantitative description of the relationship can be formulated by irreversible thermodynamics, provided the force-flow relations of energy conversion have been experimentally characterized [2, 3]. Therefore, observations concerning the dependence of the rate of phosphorylation on the pmf should aid not only an understanding of the molecular mechanism of energy conversion but will also be relevant for the formulation of force-flow relations.

Recently, it has been shown that intact chloroplasts in hypotonic assay conditions exhibit a de-

creased pmf in the presence of low amine* concentrations although the rate of steady-state light-dependent ATP production is increased [4]. This observation is not readily accommodated within the framework of the chemiosmotic concept of energy conversion [1]. Additional work with isolated intact chloroplasts provides further, although indirect, evidence for increased phosphorylation in the presence of a diminished proton gradient [5]. Sorgato et al. showed that the rate of ATP synthesis in submitochondrial particles can decrease at a constant pmf [6]. At the moment it seems unclear whether these observations demonstrate that ATP production is at least partially independent of the pmf or whether they can be explained as having resulted from artificial, non-physiological conditions. Thus, it is the aim of this contribution (i) to report observations which are relevant for understanding the mechanism of stimulated ATP production at a decreased pmf, (ii) to show that phosphorylation by unbroken chloroplasts is limited kinetically, not energetically, not only under saturating illumination [4] but also under limiting light, and (iii) to propose that such a kinetic limitation should be considered

Abbreviations: Ap_5A , P^i, P^s -di(adenosine-5')-pentaphosphate; BSA, bovine serum albumin; Chl, chlorophyll; DCCD, dicyclohexyl-carbodiimide; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid; pmf, proton motive force; 9-AA, 9-aminoacridine.

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* In this article, "amine" is frequently understood to include ammonia and its cation, NH_4^+ .

by thermodynamic descriptions of energy conversion.

Materials and Methods

Intact chloroplasts were isolated from spinach leaves of greenhouse plants by a modification [7] of Jensen and Bassham's procedure [8]. BSA (0.3%, w/v) was added to the grinding medium instead of ascorbate. The same concentration of BSA was added to the storage medium. Thylakoids were obtained by washing intact chloroplasts twice in MgCl_2 . The washing procedure was as follows: intact plastids suspended in the storage medium were diluted 20 fold with 5 mM MgCl_2 ; after centrifugation for 5 min at $3900 \times g$, the pellet was resuspended in the storage medium. Chlorophyll was determined according to Arnon [9]. Chlorophyll concentration in the assay was 25–33 $\mu\text{g/ml}$. pH changes of weakly buffered chloroplast suspensions were recorded by a glass electrode (M3, LOT-405, Ingold) connected to a Knick-53 pH meter. Oxygen evolution was measured using a Clark-type electrode; the electron acceptor was 1 mM ferricyanide if not indicated otherwise. Quenching of 9-AA fluorescence and absorption changes of chloroplast suspensions were measured as described previously [5]. Actinic light was provided by passing a beam of white light through a heat protection filter, 1 mm Calflex C (Balzer), and a 2 mm RG 630 (Schott) glass filter. Light intensities were determined by a radiometer (Yellow Springs Instruments Co., model 65A). Photophosphorylation was measured by enzymic determination of ATP, by determination of P_i , or incorporation of $^{32}\text{P}_i$. For the enzymic ATP-test [10], corrections were made for activity of adenylate kinase in the assay (E.C. 2.7.4.3), or activity of this enzyme was inhibited by addition of 0.15 mM Ap_5A [11] which does not affect the rate of phosphorylation as measured by incorporation of $^{32}\text{P}_i$. Unlabelled phosphate was determined according to Fiske and Subbarow [12]. For measurement of incorporation of labelled phosphate, P_i was replaced by $\text{KH}_2^{32}\text{PO}_4$ (5 mCi/mmol). The label recovered in phosphate and in ATP was determined by high performance liquid chromatography as described in [4]. The rate of phosphorylation was calculated from the percentage of total label recovered in the ATP peak, the amount of added P_i , and the chlorophyll concentration.

Results

1. Electron transport and phosphorylation by unbroken chloroplasts

Unbroken chloroplasts (type B, ref. 13) were obtained by suspending isolated intact chloroplasts in an assay medium containing 0.1 M sorbitol as osmoticum. These type B plastids are reported to be morphologically intact [13], but possess an envelope which is permeable to smaller molecules like ferricyanide or adenine nucleotides. They are capable of phosphorylating exogenous ADP at rates up to 180 $\mu\text{mol/mg Chl} \cdot \text{h}$. Recently, it has been demonstrated that the rate of phosphorylation by these chloroplasts is increased (rather than decreased) upon the addition of low (< 1 mM) concentrations of amines [4]. Fig. 1a shows the dependence on the concentrations of NH_4Cl and methylamine of electron transport and phosphorylation.

As reported before [4], methylamine is more effective in stimulating phosphorylation. The concentration of uncoupler causing maximum stimulation is higher for methylamine than for NH_4Cl . The rate of electron transport from H_2O to ferricyanide is slightly stimulated upon the addition of methylamine, whereas concentrations of NH_4Cl exceeding 0.6 mM drastically increase the rate, indicating that ammonia is a more potent uncoupler than methylamine. Cyclohexylamine, another uncoupling amine, causes practically no stimulation of ATP production (maximum rate in the presence of 0.1 mM cyclohexylamine was 103% of the control) and drastically increases electron transport. Since cyclohexylamine has about the same pK -value as methylamine ($\text{pK} = 10.6$) it is clear that the differences observed between the three uncouplers cannot be due to different pK -values. Results comparable to those shown in Fig. 1 were obtained when ferricyanide was replaced by methylviologen in the presence of KCN (data not shown).

As expected, the ATP/e_2 ratio is decreased in the presence of cyclohexylamine (not shown) or NH_4Cl (Fig. 1b). It is not affected or even slightly increased (Fig. 1b) with low concentrations of methylamine; higher concentrations lead to a decrease which, however, is less dramatic than with ammonia. Uncouplers like simple amines or ammonia are known to stimulate electron transport, probably by increasing the permeability of the thylakoid membrane for

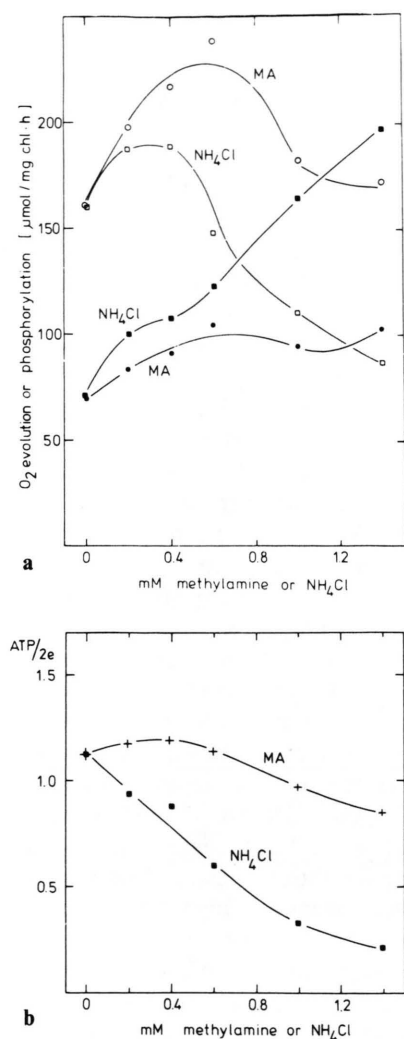


Fig. 1. (a) Dependence of oxygen evolution (closed symbols) and photophosphorylation (open symbols) by unbroken chloroplasts on uncoupler concentration. Isolated intact chloroplasts were suspended in 2 ml assay medium containing 0.1 M sorbitol, 5 mM MgCl₂, 10 mM NaCl, 1 mM KH₂PO₄ and 40 mM HEPES, pH adjusted to 7.5 with NaOH. The electron acceptor was 1 mM ferricyanide. Phosphorylation was started by addition of 2 mM ADP 30 s after the onset of illumination by 200 W/m² red light and stopped after 90 s by HClO₄, 0.67 N final concentration. The ATP content of the neutralized samples were determined enzymatically. (b) ATP/e₂ ratio calculated from the data shown in Fig. 1a.

protons via an H⁺/NH₄⁺ shuttle [14]. As the extent of stimulation of ATP production by methylamine is significantly higher than that by ammonium chloride, the former uncoupler was chosen for most of the following experiments.

2. Effects of chloroplast pretreatment

The envelope of intact isolated chloroplasts exposed to 0.1 M sorbitol is likely not to be disrupted [13, 15]. Fig. 2 shows the effect of methylamine on phosphorylation with chloroplasts exposed to isotonic conditions after brief osmotic shock in 5 mM MgCl₂. This procedure is known to disrupt the envelope totally and to produce type D chloroplasts [13]. Stimulation upon the addition of methylamine is observed also under this assay condition and may even exceed that measured under permanent osmotic stress in 0.1 M sorbitol (Fig. 2, H).

Intact chloroplasts were washed twice in MgCl₂ (*cf.* Materials and Methods) to produce a preparation of thylakoids. Phosphorylation in the absence of uncoupler was increased compared to unbroken chloroplasts with some preparations and inhibited upon the addition of 0.5 mM methylamine with all preparations tested (Table I). Thus, stimulation of phosphorylation by 0.5 mM methylamine can be abolished by washing chloroplast twice in 5 mM MgCl₂. Even methylamine concentrations as low as 0.05 mM did not stimulate phosphorylation. Phosphorylation by chloroplasts which were washed only once was stimulated on the addition of methyl-

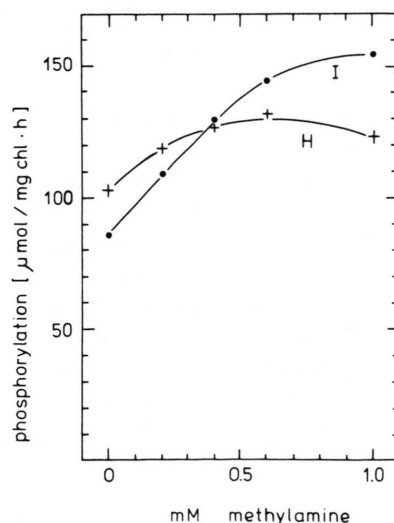


Fig. 2. Methylamine-induced stimulation of phosphorylation under isotonic (I) or hypotonic (H) assay conditions. Experimental conditions for (H) as in Fig. 1. For assay of phosphorylation under isotonic conditions, intact chloroplasts were suspended in 1 ml 5 mM MgCl₂. After 30 s, 1 ml medium was added to give an end concentration of 0.33 M sorbitol and other compounds as in (H). The ATP content of the samples was determined enzymatically.

Table I. Effect of 0.5 mM methylamine (MA) on photophosphorylation with unbroken chloroplasts and thylakoids. One portion of a preparation of intact chloroplasts was washed twice (*cf.* Materials and Methods) to yield thylakoids. The other part was used without further pretreatment. Experiments 1, 2: conditions for assay of phosphorylation were as for Fig. 1, except that 0.15 mM Ap_3A was added. Experiments 3 and 4: 1.6 μCi $^{32}\text{P}_i$ (carrier free) was added to the assay medium given in Fig. 1.

Experiment	photophosphorylation [$\mu\text{mol}/\text{mg Chl} \cdot \text{h}$]			
	1	2	3	4
intact chloroplasts				
control	157.1	169.5	121.6	146.1
+ 0.5 mM MA	181.1	185.2	157.7	180
% stimulation by MA	15.3	9.3	29.7	23.2
washed thylakoids				
control	156.7	209.2	140.1	66.5
+ 0.5 mM MA	146	189.7	122.3	56.5
% inhibition by MA	6.8	9.3	12.7	15
% stimulation by washing	—	23.4	15.2	—

amine with some preparations and inhibited with others (data not shown).

This finding raised the question of whether the observed stimulation is indeed caused by an increase in photophosphorylation or whether it is an artifact caused by the presence of the chloroplast stroma and envelope. The stroma may contain ATP-hydrolyzing enzymes. ATPase activity is also known to be associated with the chloroplast envelope [16]. An apparent increase in phosphorylation can be due a decrease in an ATP consuming reaction. To test whether methylamine inhibits ATP consumption, ATP was added to illuminated chloroplasts in the presence or absence of 0.5 mM methylamine (Table II). The Mg^{2+} -ATPase activity of the coupling factor CF_1/CF_0 was inhibited by DCCD. There was no detectable hydrolysis of ATP either in the absence or presence of the uncoupler; rather, the ATP recovery seemed to be slightly increased with time (Table II). Even if this increase is not considered as experimental scattering, it cannot be related to stimulation of phosphorylation by methylamine as it is observed both in the presence of 0.5 mM methylamine and in the control. Photophosphorylation of this preparation was stimulated by 20% upon the addition of 0.5 mM methylamine. Basically the same results were obtained with chloroplasts kept in the dark in the

absence of DCCD under otherwise identical assay conditions (data not shown). Thus, it can be excluded that methylamine affects the activity of an ATP consuming reaction associated with the chloroplast stroma.

Moreover, stimulation by methylamine depends on chloroplast energization. Fig. 3 shows the dependence on light intensity of ATP production by unbroken chloroplasts in the presence of 0.5 mM methylamine compared to the control in the absence of the uncoupler. Stimulation is light-saturated at about the same light intensity as ATP production

Table II. Recovery of ATP added to intact chloroplasts suspended in a hypotonic assay medium. Intact chloroplasts corresponding to 79 μg Chl were preincubated for 15 min in 0.6 ml assay medium (see legend of Fig. 1) in the presence of 0.15 mM DCCD at 0 °C. 0.5 ml of this suspension was exposed to conditions identical to those given in Fig. 1. ATP (10.06 $\mu\text{mol}/\text{mg Chl}$) was added 30 s after the onset of illumination. The time interval between the addition of ATP and perchloric acid, t_a , was varied between 5 and 90 s. The ATP content of the neutralized samples was determined enzymatically. In the absence of DCCD, photophosphorylation of this preparation was 154 and 184 $\mu\text{mol}/\text{mg Chl} \cdot \text{h}$ in the absence and presence of 0.5 mM methylamine, respectively.

t_a [s]	ATP recovered [$\mu\text{mol}/\text{mg Chl}$]			
	5	30	60	90
methylamine (mM)				
0	9.36	9.78	10.06	9.91
0.25	—	—	—	10.03
0.5	9.66	9.53	10.16	9.89
1.0	—	—	—	9.74

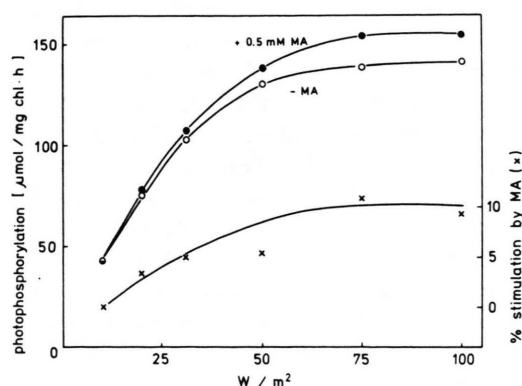


Fig. 3. Dependence of photophosphorylation with unbroken chloroplasts on light intensity in the absence and presence of 0.5 mM methylamine. Experimental conditions as for Fig. 1, except that 0.15 mM Ap_3A was added.

suggesting that this reaction is modified by the uncoupler. Moreover, the observed stimulation corresponds to ATP production of up to $36 \mu\text{mol/mg Chl} \cdot \text{h}$ (Table I) or even more (unpublished observations). It is unlikely that a light-driven ATP generating reaction of such rates, which is different from photophosphorylation, exists in chloroplasts. It is concluded that amines indeed increase photophosphorylation.

The results suggest that stimulation of photophosphorylation by amines is due to a modification of the energy converting process *per se*, not to secondary ATP turnover. Since the response of unbroken chloroplasts to uncoupling amines (Fig. 1) is different from that of thylakoids (Table I), phosphorylation is probably affected by the presence of the chloroplast stroma. Stimulation by methylamine is observed also under conditions in which the chloroplast envelope is disrupted by osmotic shock (Fig. 2) and sometimes with chloroplasts which have been washed but not with thylakoids (Table I), suggesting that the factor responsible for stimulation is associated with the thylakoid membrane.

3. Effect of methylamine on thylakoid membrane permeability to protons

Proton permeability of the thylakoid membrane can be estimated from the kinetics of increase in 9-AA fluorescence seen when a suspension of illuminated unbroken chloroplasts is darkened (Fig. 4a). About 10–15 s after switching off the light, an exponential phase of decay of the fluorescence starts (Fig. 4b).

In the absence of methylamine, the initial, non-exponential decay of the 9-AA signal is slower and apparently reflects buffering by thylakoid vesicles, not H^+ permeability of the membrane: 9-AA fluorescence remains unaffected for 2–3 s after light/dark transient (Fig. 4a), whereas proton extrusion as measured by a glass electrode is known to start simultaneously with darkening ([17], see also below, Fig. 6). This suggests that protons appearing in the medium have dissociated from buffering groups without changing the pH in the thylakoid lumen. Moreover, the initial, non-exponential phase is prolonged when the external pH is decreased to 7.0. At pH 8.0, it is nearly totally abolished. As the pK of thylakoids is about 5.5 [18], this again suggests that buffering rather than ion permeability is

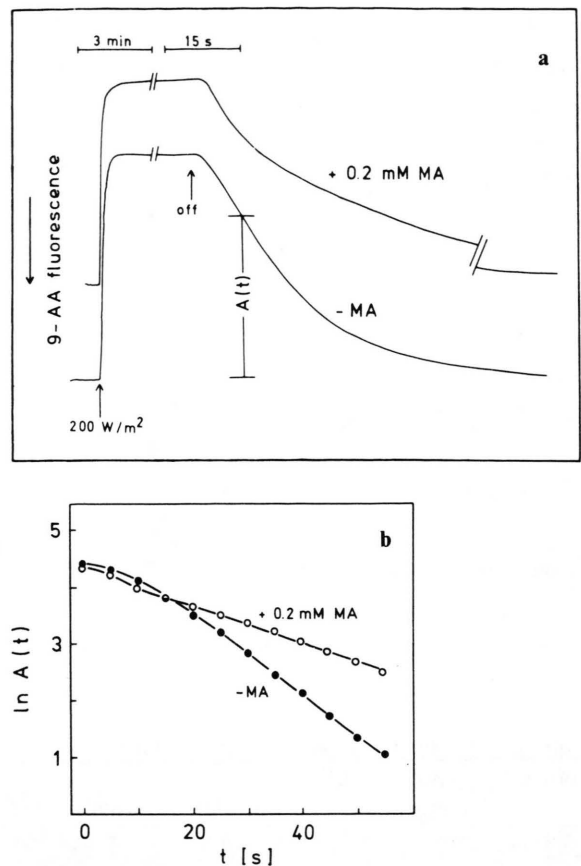


Fig. 4. (a) Quenching of 9-AA fluorescence upon illuminating and recovery upon darkening a sample of unbroken chloroplasts in the absence or presence of 0.2 mM methylamine (MA). Experimental conditions as for Fig. 1, except that ferricyanide was replaced by $25 \mu\text{M}$ methylviologen and that no ADP was added. The concentration of 9-AA was $5 \mu\text{M}$. (b) Semilogarithmic plot of fluorescence recovery curves shown in (a).

responsible for the slow initial part of the decay of 9-AA fluorescence quenching.

From the exponential part of the decay curve the half time, $T_{0.5}$, of the fluorescence signal was estimated. With unbroken chloroplasts, $T_{0.5}$ was 8–12 s; for a suspension of thylakoids it was reduced to 4–6 s. This indicates that washing increases the proton permeability of the thylakoid membrane.

In the presence of methylamine, three components contributing to the decay kinetics can be distinguished: after the slow initial phase, decline of the 9-AA signal becomes transiently faster than in the third, exponential part (Fig. 4b). With increasing methylamine concentrations the decline during the second

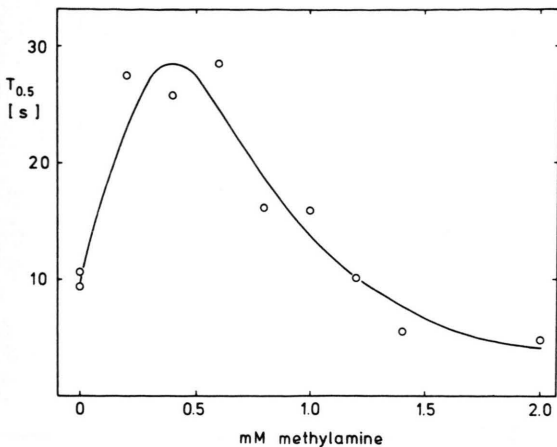


Fig. 5. Effect of methylamine on the half time of 9-AA fluorescence recovery ($T_{0.5}$) observed after light-dark transition of a suspension of unbroken chloroplasts. Experimental conditions as for Fig. 4.

phase becomes steeper (not shown). The half time of the second, transient phase is always shorter than that during the following exponential part. It is concluded that in the presence of methylamine the half time of the third, exponential part does not monitor H^+ permeability during the light/dark transition but rather reflects limitation by other processes.

The above experiments were repeated in the presence of 0.1 and 0.3 μM valinomycin. The lower concentration abolishes the field indicating 518 nm signal but does not affect the steady-state ΔpH as estimated from 9-AA fluorescence quenching, whereas 0.3 μM additionally reduces the ΔpH (not shown). However, in the presence of both of these valinomycin concentrations and 1, 30 and 150 mM KCl, the

decline observed during the exponential phase was slower than that in the second phase, indicating that the exponential part does not reflect limitation by diffusion potentials.

Fig. 5 shows the dependence of half life time $T_{0.5}$ of the 9-AA signal on the concentration of methylamine, as estimated from the exponential part of the decay curve (see Fig. 4b). $T_{0.5}$ was increased with low concentrations of methylamine and showed a maximum near 0.5 mM. With higher concentrations of the uncoupler, $T_{0.5}$ was decreased. It should be noted that $T_{0.5}$ (Fig. 5) and the rate of phosphorylation (Fig. 1a) show a striking similarity in their dependence on the uncoupler concentration.

Another indicator of membrane permeability is the rate of acidification of the medium observed upon darkening a weakly buffered suspension of chloroplasts. Fig. 6 shows a pH trace recorded by a glass electrode. The initial rate of proton extrusion is frequently used to monitor proton permeability [17]. With unbroken chloroplasts, the initial rate was increased by 30% upon the addition of 0.2 mM methylamine, indicating that the thylakoid membrane becomes more leaky due to the uncoupler. The decay kinetics is nearly linear in the absence of the uncoupler. Upon the addition of methylamine the kinetics changed in a manner similar to that of 9-AA fluorescence: a rapid acidification was followed by a slower $[H^+]$ increase; the half time of the slow phase was prolonged by methylamine (not shown). In conclusion, washing of unbroken chloroplasts increases the proton permeability of the thylakoid membrane. Addition of methylamine increases the initial rate of proton extrusion which monitors the membrane per-

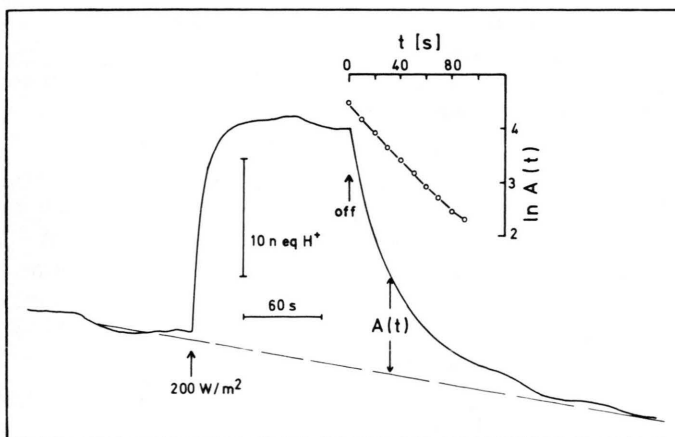


Fig. 6. Light-induced pH changes of a suspension of unbroken chloroplasts, and semilogarithmic plot of the signal height, $A(t)$, versus time. Experimental conditions as for Fig. 4, except that 9-AA and HEPES were omitted from the assay medium.

meability. The rate of proton fluxes at a later, exponential stage (starting at 10–15 s after the light is switched off) shows an optimum curve, whose dependence on methylamine concentration is comparable to that of the rate of phosphorylation. So far, it is not known which process is responsible for the exponential phase. This point is presently under investigation.

4. Effect of methylamine on photophosphorylation under low light illumination

Photophosphorylation under saturating illumination has been suggested to be limited kinetically [4]. NH_4Cl or methylamine were proposed to remove a kinetic barrier, thereby stimulating phosphorylation, provided the pmf was sufficient to drive this process. Under low illumination, the effect of amines had not yet been studied.

In Fig. 3, the dependence of the phosphorylation rate on light intensity was shown. Phosphorylation with unbroken chloroplasts is light-saturated at about 75 W/m^2 red light. This is considerably lower than the value for CO_2 fixation with intact chloroplasts which is more than 300 W/m^2 [19]. Light intensities below about 30 W/m^2 are strictly limiting for phosphorylation, both in the presence and absence of uncoupler. That energization of chloroplasts is not saturated at this low light intensity is demonstrated in Fig. 7. This figure shows a simultaneous recording of light-induced quenching of 9-AA fluorescence and the amount of proton uptake by unbroken chloroplasts. Both processes are saturated at about 50 W/m^2 ; below about 30 W/m^2 , proton transport strongly depends on light intensity. This suggests that the decrease in the rate of phosphorylation upon lowering the light intensity (Fig. 3) is caused by a reduced proton gradient.

However, phosphorylation is stimulated by methylamine also at low, limiting light intensities (Fig. 3), although this stimulation is less than at higher intensities. As mentioned above, stimulation by methylamine shows about the same dependence on light intensity as the rate of photophosphorylation itself. With the preparation shown in Fig. 3, maximum stimulation is about 10%; with other preparations, stimulation up to 40% was observed in accordance with the reported variability of the extent of stimulation by amines [4].

It should be noted that the proton motive force is reduced by the addition of methylamine not only un-

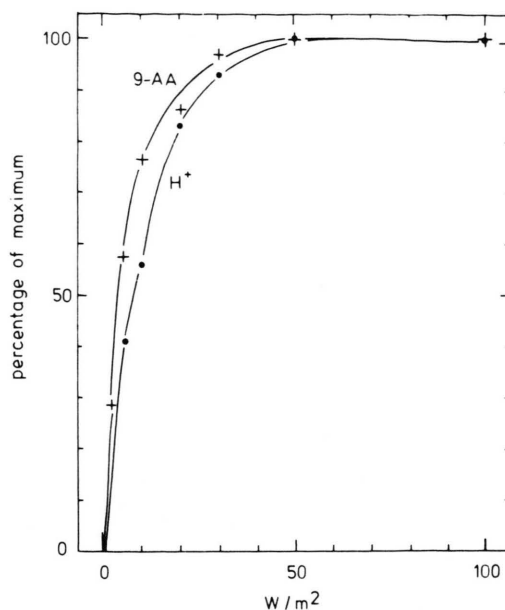


Fig. 7. Simultaneous recording of 9-AA fluorescence quenching (9-AA) and the amount of proton uptake (H^+) by a suspension of unbroken chloroplasts. Experimental conditions were as for Fig. 5, except that $5 \mu\text{M}$ 9-AA was added. 100% corresponds to quenching of 9-AA fluorescence by 75% of total fluorescence and to an H^+ uptake of $0.205 \mu\text{val/mg Chl}$, respectively.

der saturating illumination [4] but also in the presence of low light: at 15 W/m^2 , the percentage of 9-AA fluorescence quenching was 38% in the control and 37; 35; 30; 25% in the presence of 0.1; 0.2; 0.4; 0.8 mM methylamine, respectively. Under low light intensities the transmembrane potential as estimated from the 518 nm signal is hardly affected by the presence of methylamine (Fig. 8), whereas at higher light intensities it is increased as the methylamine concentration is raised. The small increase of ΔA_{518} under low light illumination is energetically insignificant. The increase with 200 W/m^2 illumination is somewhat higher than reported before [4].

Fig. 3, 7 and 8 demonstrate that the rate of phosphorylation by unbroken chloroplasts can be increased in the presence of a reduced proton motive force under light intensities which are insufficient to saturate the proton gradient. Taken at face value, this seems to exclude the pmf as the sole candidate for the driving force of phosphorylation. It should be kept in mind, however, that the rate phosphorylation depends both on the energetic and kinetic competence of the chloroplasts (see below).

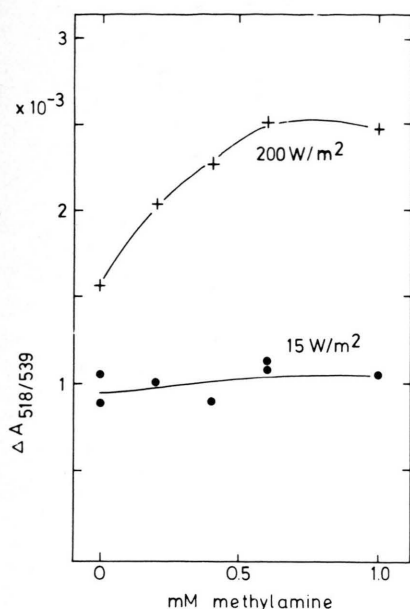


Fig. 8. Dependence on methylamine concentration of the light induced absorption change at 518 nm of a suspension of unbroken chloroplasts under high and low light illumination. Experimental conditions as for Fig. 1, except that 0.5 mM ADP was added initially. Reference wavelength was 539 nm. Data shown in the figure correspond to the absorption change observed when the light was switched off after illumination for 30 s.

Discussion

1. Electron transport and phosphorylation

It is evident that uncouplers like ammonia or methylamine have two effects on unbroken chloroplasts: first, they stimulate ATP production by an unknown mechanism; second, they increase proton permeability of the thylakoid membrane and, consequently [20], stimulate electron transport. Increased electron transport and reduced ΔpH are observed also at those low concentrations that stimulate ATP production (Fig. 1a and ref. 4). The observation that the ATP/e_2 ratio is increased with methylamine but decreased with ammonia (Fig. 1b) can be explained by the fact that the latter compound is a more potent uncoupler than the former one. This explanation implies that the mechanism responsible for increased ATP production simultaneously increases the ATP/e_2 ratio; this increase is partially masked by the uncoupling action of amines. An increased ATP/e_2 ratio can be due to increased participation of cyclic electron flow. Indeed, Slovacek *et al.* [21] reported that NH_4Cl increases cyclic electron

flow in intact chloroplasts. However, from energetic considerations it is evident that an increased ATP/e_2 ratio can only be a concomitant phenomenon, not the reason for increased ATP production.

Methylamine increases ATP production only with unbroken chloroplasts, not with washed thylakoids. The thylakoid membrane of unbroken plastids is less permeable for protons (see above). This suggests that increased phosphorylation is possible only in the presence of a relatively tight membrane. However, the rate of phosphorylation was stimulated by washing in some experiments (Table I), showing that ATP production can be increased in spite of a decreased membrane tightness. Increased membrane permeability is probably only a side effect of removal of the factor responsible for amine-induced stimulation of phosphorylation.

Amine-induced increase of ATP production is observed also under limiting light (Fig. 3). As for comparable experiments with saturating light intensities [4], this can be explained by assuming that phosphorylation is kinetically limited. Limitation is removed by amines and therefore phosphorylation is increased. This interpretation implies that phosphorylation is not energetically limited at low light intensities which do not saturate the proton gradient, *i.e.*, that also under limiting energization only part of the proposed driving force is utilized to drive ATP production.

Regarding thermodynamic descriptions of energy conversion the rate of phosphorylation is apparently not a unique function of the pmf: when the pmf is reduced by reducing light intensity (Fig. 7) the rate of phosphorylation decreases (Fig. 3); when it is decreased by addition of methylamine, phosphorylation increases. Moreover, Sorgato *et al.* demonstrated that the rate of ATP synthesis by sub-mitochondrial particles can be decreased in the presence of a constant pmf [6]. The rate of phosphorylation apparently depends on a number of factors other than the pmf and the phosphorylation potential. To the author's knowledge, no attempts were made to explain by a thermodynamic model how the rate of phosphorylation can be increased under a diminished pmf. It is proposed to consider kinetic limitation in thermodynamic descriptions of phosphorylation in order to decide whether the observed dependence of ATP production on the pmf is compatible with the suggested role of the pmf as the driving force of phosphorylation.

2. Significance of amine induced stimulation of photophosphorylation for observations made with intact chloroplasts.

The rate of CO₂ fixation by intact isolated chloroplasts is known not to be affected or even increased upon the addition of NH₄Cl [3, 20]. Likewise, the ATP/ADP ratio of intact CO₂ fixing chloroplasts was increased on the addition of methylamine [22]. Stimulation of CO₂ fixation (which is an ATP consuming process) by uncoupling amines has been explained by amine induced alkalization of the chloroplast stroma; this alkalization increases the activities of fructose and sedoheptulose biphosphatase whose turnover is thought to limit the rate of CO₂ fixation [23]. Alternatively, stimulation by low concentrations of ammonia was considered by Slovacek and Hind [24] to reflect imbalances between ATP generating and consuming processes which were overcome by partial uncoupling. The observation that phosphorylation by unbroken chloroplasts is stimulated by low concentrations of NH₄Cl or methylamine suggests

that stimulation of ATP production can be responsible for increased rates of CO₂ fixation. This possibility was not considered by the authors mentioned above. It implies that CO₂ fixation by intact chloroplasts is limited by the availability of ATP. However, concentrations of ammonia which stimulate phosphorylation with unbroken chloroplasts are considerably lower than those which may stimulate CO₂ fixation; in the latter case, 4 and even 7 mM NH₄Cl only marginally lower the rate of CO₂ dependent oxygen evolution, whereas 1.4 mM ammonia reduces phosphorylation in unbroken chloroplasts to 54% of the control (Fig. 1a).

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