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SYNTHESIS AND HYDROLYSIS OF ATP BY INTACT CHLOROPLASTS UNDER FLASH ILLUMINATION AND IN DARKNESS

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Summary

ATP concentrations were measured in isolated intact spinach chloroplasts under various light and dark conditions. The following results were obtained: (1) Even in darkened chloroplasts and in the absence of exogenous substrates, ATP levels in the chloroplast stroma were significant. They decreased on addition of glycerate, phosphoglycerate or dihydroxyacetone phosphate. When dihydroxyacetone phosphate and oxaloacetate were added together, ATP levels increased in darkened chloroplasts owing to substrate level phosphorylation. (2) Under illumination with saturating single turnover flashes, oxygen evolution in the presence of phosphoglycerate, whose reduction requires ATP, was no lower on a unit flash basis at the low flash frequency of 2 Hz than at higher frequencies. Quenching of 9-aminoacridine fluorescence, which indicates the formation of a proton gradient in intact chloroplasts, decreased with decreasing flash frequencies, until there was no significant fluorescence quenching at a flash frequency of about 2 Hz. In contrast to intact chloroplasts, broken chloroplasts did not phosphorylate much ADP at the low flash frequency of 2 Hz. (3) Flashing at extremely low frequencies (0.2 Hz) caused ATP hydrolysis rather than ATP synthesis in intact chloroplasts. At higher flash frequencies, synthesis replaced hydrolysis. Still, even at high frequencies (10 Hz), the first flashes of a series of flashes given after a long dark time always decreased chloroplast ATP levels.

From these results, it is concluded that the enzyme, which mediates ATP synthesis in the light, is inactive in darkened intact chloroplasts. Its light activation can be separated from the formation of the high energy condition,

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Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; CCCP, carbonylcyanide *m*-chlorophenyl hydrazone.

which results in ATP synthesis. After its activation, the enzyme catalyzes a reversible reaction.

Introduction

The chloroplast thylakoid system is known to synthesize ATP in the light. The responsible enzyme, which is incorporated into the thylakoid membrane, has been termed coupling factor [1]. It has a complex structure and contains several subunits [2]. When detached from the membrane, it requires special treatment such as partial digestion by trypsin to catalyze the reverse reaction, ATP hydrolysis [3]. In isolated thylakoids, ATP hydrolysis can be induced by illumination in the presence of thiols [4,5].

In intact chloroplasts, ATP synthesis has been reported to be a reversible reaction [6]. Since chloroplasts contain in vivo high levels of ATP even in the dark [7], this would pose problems, as significant ATP hydrolysis in the dark by an enzyme geared to synthesis in the light would appear to be a wasteful process.

In the light, ATP synthesis is believed to be driven by a trans-thylakoid proton gradient and a light-generated membrane potential [8—10]. Biomembranes such as thylakoids are not impermeable to ions. Under widely spaced flash illumination, therefore, the gradient energy accumulated across thylakoid membranes on short flash illumination are expected to dissipate by ion leakage during long dark intervals, before significant phosphorylation can take place. It was therefore of interest to measure the efficiency of phosphorylation under flash illumination. Such information is available for thylakoids subjected to osmotic stress during isolation [11]. It is unavailable for intact chloroplasts capable of reducing CO_2 at high rates. The membrane systems of such chloroplasts appear to be well preserved.

In the following we report data pertaining to the stability of ATP in intact chloroplasts in the dark and to ATP synthesis in the dark and under widely spaced short flashes.

Experimental

(1) Chloroplast preparation

Intact chloroplasts retaining their envelopes were isolated from freshly harvested spinach leaves (*Spinacia oleracea* L.) according to the method described by Jensen and Bassham [12] as modified by Heber [13]. They were stored in darkness at 0° C before use. Storage times were usually between 1 and 10 h. Broken chloroplasts were isolated as described by Yamashita et al. [14].

(2) ATP and ADP determination

Chloroplasts (100 μg chlorophyll) were suspended in 2 ml of a reaction mixture containing 0.33 M sorbitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 10 mM NaCl, 40 mM HEPES/NaOH buffer (pH 7.6) and substrates, incubated in a small vessel (1.5 and 5 cm in diameter and height, respectively), and then illuminated from the side with short saturating flashes (4 μs duration, $2 \cdot 10^2$

ergs/cm^2 per flash) from an Xe stroboscope (Sugawara Co., model MSK-1A) repeated at desired frequencies, or with continuous light at a saturating intensity (about 60 mW/cm^2) from a 300 W tungsten lamp after passage through heat absorbing filters. Substrate concentrations were 2 mM for nitrite (NO_2^-), bicarbonate (HCO_3^-), 3-phosphoglycerate and glycerate, and 1 mM for oxaloacetate and dihydroxyacetone phosphate. 10 mM of inorganic phosphate was added to block photosynthesis in some experiments [26].

The reaction was stopped by injection of 100 µl of 2.5 M perchloric acid. In order to ensure rapid termination of phosphorylation, all procedures were carried out under rapid stirring. After more than 20 s in darkness at room temperature, the acidified reaction mixture was neutralized with a stoicheiometric amount of aqueous KOH, and an aliquot (100 µl) of the neutralized extract was used for the determination of ATP by the luciferin-luciferase assay [15]. Another 100 µl aliquot of the same extract was incubated with phospho*enol*pyruvate and pyruvate kinase in order to convert ADP to ATP. The ADP concentration was calculated from the difference in the concentrations of ATP in the sample with and without this conversion procedure. In some experiments with broken chloroplasts, the rate of ATP formation under flashing light was estimated from the rate of OH^- production accompanying phosphorylation with a Hitachi pH meter model F-7_{ss}, according to the method described by Nishimura [16].

(3) Proton gradient and oxygen-evolution measurements

The quenching of 9-aminoacridine fluorescence was used as indicator of the trans-thylakoid proton gradient [17,18]. Oxygen evolution from intact chloroplasts was measured with a Clark-type oxygen electrode in a 1 ml vessel. The substrate concentrations in the reaction mixture were the same as those used in the experiments designed to measure adenylate levels.

Results and Discussion

(1) ATP in darkened chloroplasts and inactivity of coupling factor in darkness

Isolated chloroplasts show significant levels of ATP in darkness. The levels measured at 20 or 25°C after dark incubation of the chloroplasts for several hours at 0°C were usually not far from 15 nmol ATP/mg chlorophyll. This corresponds to an ATP concentration in the stroma of about 0.6 mM, which is comparable to the concentration found in chloroplasts in vivo in dark-adapted leaves [7]. Dark levels of ATP were 30—60% of the maximum ATP levels found in saturating light.

As shown in Table I, the ATP level in darkened chloroplasts was not much affected by the addition of such substrates as nitrite, oxaloacetate or bicarbonate, but was markedly influenced by the addition of phosphoglycerate or glycerate. The ATP levels measured after a 2 min incubation with phosphoglycerate or glycerate were 50 and 25%, respectively, of the initial level. This is attributed to the consumption of ATP by the reactions catalyzed by phosphoglycerate kinase and glycerate kinase. These enzymes occur in the chloroplast stroma [19]. The ATP decline seen on addition of glycerate to darkened chloroplasts was fast. After only 3 s, the final low ATP level was reached

TABLE I

ATP LEVELS IN DARKENED INTACT CHLOROPLASTS IN THE PRESENCE OF VARIOUS SUBSTRATES

Substrate	Concentration	Incubation	ATP content (nmoles/mg chlorophyll	
			—CCCP	+CCCP ***
No addition	—	dark *	11.2	—
Nitrite	2 mM	dark	12.7	13.0
Oxaloacetate	1 mM	dark	13.0	13.0
Bicarbonate	2 mM	dark	11.1	—
3-Phosphoglycerate	2 mM	dark	6.4	—
Glycerate	2 mM	dark	3.9	—
Nitrite	2 mM	light **	31.2	14.6

* 2 min incubation at room temperature.
** 2 min illumination at saturating intensity.
*** 5 μM CCCP for dark experiments and 10 μM for light experiment.

(kinetics not shown). The equilibrium constant of the reaction catalyzed by glycerate kinase favours the formation of phosphoglycerate from glycerate at the expense to ATP. Since, at equilibrium, the concentration of free ATP in the chloroplasts is close to zero, the ATP remaining in the system in the presence of glycerate cannot be thermodynamically active. It must be bound to chloroplast constituents. In the experiment of Table I, the bound ATP was about 4 nmol/mg chlorophyll, which amounted to almost 40% of the total ATP present in the dark. This value was not much different from the amount of ATP bound to coupling factor (1.0—3.5 nmol/mg chlorophyll) reported by Harris and Slater [20]. In contrast to the situation with glycerate, the equilibrium of the phosphoglycerate kinase reaction is reached before ATP is exhausted. This explains why the decline in ATP seen on addition of phosphoglycerate was much smaller than the decline observed, when glycerate was added.

Table II shows the effect of dihydroxyacetone phosphate on the ATP level in darkened chloroplasts. Whe dihydroxyacetone phosphate and oxaloacetate were both added to the chloroplasts, the ATP level gradually increased in the dark during the first minute, and reached saturation at a level 50% higher than the original level. The presence of oxaloacetate was essential for the increase of ATP/ADP ratio. Since NADP-dependent malic dehydrogenase is inactive in darkness [21], the ATP generation is attributed to the following reactions:

$$\text{Dihydroxyacetone phosphate}^{2-} + \text{ADP}^{3-} + \text{NAD}^{+} \rightarrow \text{phosphoglycerate}^{3-} + \text{ATP}^{4-} + \\ + \text{NADH} + \text{H}^{+} \quad (1)$$

$$\text{NADH} + \text{H}^{+} + \text{oxaloacetate}^{-} \rightarrow \text{malate}^{2-} + \text{NAD}^{+} \quad (2)$$

The NAD-dependent malic dehydrogenase will shift the equilibrium of reaction (1) toward synthesis by consuming NADH. As should be expected from Eqn. 1, phosphate increased the steady-state ATP level. In the absence of

TABLE II

EFFECT OF DIHYDROXYACETONE PHOSPHATE (1 mM), OXALOACETATE (1 mM) AND INORGANIC PHOSPHATE (P_i, 10 mM) ON THE ATP CONTENT AND THE ATP/ADP RATIO OF ISOLATED INTACT CHLOROPLASTS

Incubation mixture	Incubation condition	ATP content (μmol/mg chlorophyll)	ATP/ADP
Control			
—Dihydroxyacetone phosphate, —oxaloacetate, P_i (0.5 mM)	2 min dark	14	0.6
—Dihydroxyacetone phosphate, +oxaloacetate, P_i (0.5 mM)	2 min light	25	2.5
Complete (+Dihydroxyacetone phosphate, +oxaloacetate, 10 mM P_i)			
	3 min dark	22	1.5
	1 min dark	21	—
	20 s dark	18	—
	10 s dark	17	—
	0 s dark	14	0.6
Omission test			
—Dihydroxyacetone phosphate, +oxaloacetate, P_i (10 mM)	2 min dark	16	0.8
+Dihydroxyacetone phosphate, —oxaloacetate, P_i (0.5 mM)	2 min dark	10	0.4
+Dihydroxyacetone phosphate, +oxaloacetate, P_i (0.5 mM)	2 min dark	18	0.9

oxaloacetate, elevated dihydroxyacetone phosphate levels caused some decline in chloroplast ATP, presumably because ATP consumption in reactions such as the phosphorylation of ribulose-5-phosphate or phosphoglycerate, which are formed from dihydroxyacetone phosphate, exceeded synthesis of ATP during dihydroxyacetone phosphate oxidation. ATP synthesis in chloroplasts in the dark is not a novel observation. Werdan et al. [22] have previously reported that isolated chloroplasts fix CO_2 in darkness when supplied with dihydroxyacetone phosphate and oxaloacetate. In the absence of exogenous substrate, dihydroxyacetone phosphate is formed in the dark from starch [23,24]. Starch degradation in isolated chloroplasts is a slow process. The rate of phosphoglycerate formation during starch degradation, which yields ATP, was reported to be in the order of 0.1 μmol/mg chlorophyll per h [24]. In view of these very slow rates, the presence of significant ATP levels in darkened chloroplasts suggests that chloroplast ATP is stable in the dark and not subject to significant hydrolysis. However, Kraayenhof et al. [6] have reported that class I chloroplasts, which were presumed to be intact, showed considerable ATPase activity in the dark. If the enzyme, which forms ATP in the light from the energy of trans-thylakoid proton gradient, catalyzes a reversible reaction, ATP hydrolysis in the dark should lead to the formation of a proton gradient. Its dissipation by proton leakage would be expected to control the rate of ATP hydrolysis. The latter should be increased, and dark levels of ATP should decrease, if the rate of proton leakage is increased. However, addition of carbonylcyanide *m*-chlorophenyl hydrazone (CCCP), which increases the proton conductivity of thylakoids, was without effect on ATP levels in darkened chloroplasts (Table I). In contrast, it caused a dramatic drop in ATP in illuminated chloroplasts

(Table I). Attempts to demonstrate the dissipation of a proton gradient by measuring the fluorescence from 9-aminoacridine [17] in darkened chloroplasts on addition of glycerate whose phosphorylation consumes free ATP, were unsuccessful. Also, we did not succeed in demonstrating the increase of a proton gradient when the dark level of ATP in chloroplasts was increased by adding dihydroxyacetone phosphate and oxaloacetate.

If we assume the dark levels of chloroplast ATP are close to thermodynamic equilibrium with a proton gradient, the ΔpH value expected in darkened chloroplasts can be estimated. The dark phosphorylation potential in our intact chloroplasts was roughly (ATP)/(ADP) · (P_i) = 50. Based on this value, the dark value of $\Delta G'_{ATP}$ can be calculated as follows:

$$\Delta G'_{ATP} = \Delta G'_{0ATP} + 2.3\,RT \cdot \log(\mathrm{ATP})/(\mathrm{ADP}) \cdot (P_i)$$

Since $\Delta G'_{0ATP}$ is 8.5 kcal/mol, $\Delta G'_{ATP}$ is calculated to be 10.8 kcal/mol. The energy stored in a proton gradient is expressed as follows (F, Faraday constant):

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

In darkness, the membrane potential $\Delta\psi$ may be assumed to be zero. If 3 protons are used to synthesize an ATP molecule, 3 protons should be pumped into the intra-thylakoid space during the hydrolysis of an ATP molecule. It follows at equilibrium:

$$\Delta G'_{ATP} = 3 \times \Delta G_{H^+} = 3 \times 2.3\,RT \cdot \Delta\mathrm{pH}$$

$$\Delta\mathrm{pH} = 2.65$$

If only 2 protons are pumped, the ΔpH should be 3.97, and if 4 protons are pumped, it should be 1.99. According to Rumberg [25], thylakoid membrane potential in darkness owing to surface charges amounts to −32 mV assuming the concentration of surrounding electrolytes to be 10 mM. When we take this value into account, the $\Delta G'_{ATP}$ should be corrected by +0.74 kcal/mol to be 11.54 kcal/mol, and the expected ΔpH values are calculated, respectively, to be 2.83, 4.25 and 2.12 for the differently assumed numbers of protons expected to be pumped into thylakoids. These expected ΔpH values are much larger as compared with the measured value of 0.5 unit in darkened intact chloroplasts by Werdan et al. [22]. This indicates that the ATP found in darkened intact chloroplasts is not in equilibrium with the proton gradient.

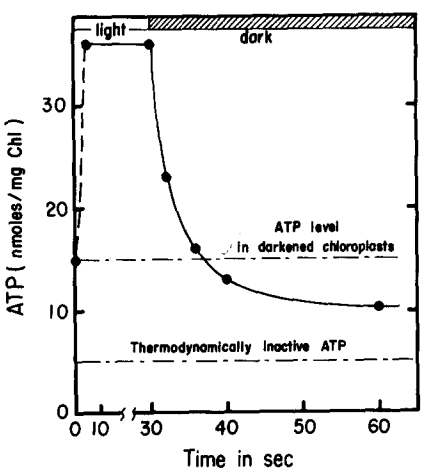
We conclude, from these results and considerations, that ATP is formed in darkened intact chloroplasts by substrate phosphorylation and that the enzyme responsible for ATP formation in the light is inactive in the dark and cannot catalyze ATP hydrolysis.

Stability of flash-generated ATP

Fig. 1 shows the stability of light-generated ATP in a subsequent dark period. 10 mM phosphate was added to the reaction mixture to inhibit photosynthesis [26] and prevent the formation of substrates which can be phosphorylated. On illumination, the ATP level rose from 15 to 35 nmol/mg chlorophyll within the first 3 s and was subsequently kept at this high level.

On turning off the light, ATP first declined very rapidly and then more slowly to reach a constant low level after 30 s. The initial rate of the ATP decrease was as high as 20 μmol/mg chlorophyll per h. Since the Calvin cycle was blocked by phosphate, the rapid decrease in ATP cannot be attributed to the phosphorylation of substrates; it must be caused by ATPase action. The final ATP level after 30 s in the dark was somewhat lower than the initial level before illumination, but was clearly higher than the level of thermodynamically inactive ATP, as determined by the addition of glycerate in the dark. From this it is concluded that the enzyme, which had catalyzed fast ATP hydrolysis immediately after the light was turned off, was rapidly inactivated in the dark. The half time of inactivation, as determined from the time course of the ATP decline, was about 2 s.

ATP formation as a function of the frequency of saturating microsecond flashes is shown in Fig. 2. Three different chloroplast preparations were used. As shown in curve C, broken chloroplasts prepared conventionally by a long period of homogenation and centrifugation did not produce ATP below a flash frequency of about 2 Hz. Above this limit, the yield of ATP per flash increased with increasing frequency. When broken chloroplasts were prepared by a brief osmotic shock of rapidly isolated intact chloroplasts which had retained a high capacity for CO_2 fixation [13], an appreciable yield of ATP



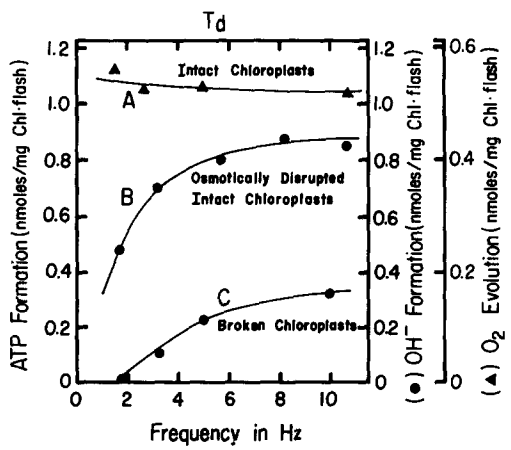


Fig. 1. Stability of light-generated ATP in a subsequent dark period. Dark-adapted chloroplasts were illuminated with saturating white light for 30 s and then kept in the dark as indicated. Electron acceptor was oxaloacetate (1 mM) and 10 mM phosphate was added to block the consumption of ATP by phosphorylation of endogenous substrates. Chl, chlorophyll.

Fig. 2. ATP formation in three chloroplast preparations as a function of flash frequency. Flash illumination was started at 10 Hz and was moved to lower frequencies. Measurement at a fixed frequency was continued for 1 min with 1-min dark interval in between. The rate of ATP formation was determined from the slope of the curve during the 1-min measurement after correction for dark drift. (A) ATP yield per flash in rapidly isolated intact chloroplasts as estimated from oxygen evolution with phosphoglycerate (2 mM) as electron acceptor. See text for details. (B) ATP yield per flash of the same chloroplast preparation used for Expt. A after osmotic rupture. ATP was determined by pH measurements [16], with methylviologen as electron acceptor. (C) ATP yield per flash of broken chloroplasts sedimented during a 15-min centrifugation period from a spinach homogenate. Experiment procedures were the same as those for Expt. B. Chl, chlorophyll.

was observed even at the low flash frequency of 2 Hz, and the yield increased with increasing frequency up to about 1 nmol ATP/mg chlorophyll per flash (curve B), which corresponds to about 1 molecule of ATP per 2 Photosystem I reaction centers. These results indicate that the quality of the chloroplast preparation is important for the yield of ATP under flash illumination.

The decrease of the ATP yield at low flash frequencies is attributed to the decay of the high energy state required for ATP synthesis during the long dark interval between flashes. The difference in ATP yields found between curves B and C, therefore, implies that the decay of the high energy state is much slower in osmotically disrupted chloroplasts than in broken chloroplasts. It was interesting to measure the stability of the high energy condition in intact chloroplasts. Unfortunately, the flash yield of ATP cannot be directly determined in intact chloroplasts, since the chloroplast envelope has a low permeability to adenylates [27]. Phosphoglycerate-dependent oxygen evolution under flashing light was therefore used to indicate the flash yield of ATP. Phosphoglycerate reduction requires 2 mol of ATP per mol oxygen evolved. Curve A of Fig. 2 shows that the flash yield of ATP, as indicated by oxygen evolution, did not decline as the flash frequency was reduced from 10 to less than 2 Hz. At still lower frequencies, accurate measurements proved technically difficult. Controls showed that oxygen evolution supported by unidentified endogenous electron acceptors was occasionally significant. In the experiment of Fig. 2, curve A, the oxygen evolution dependent on endogenous substrates was about 20% of the activity seen in the presence of phosphoglycerate. Both activities were sensitive to uncouplers. The maximum yield of ATP in intact chloroplasts was calculated to be close to 1.2 nmol ATP/mg chlorophyll per flash.

Fig. 3 shows the dependency on flash frequency of the quenching of 9-aminoacridine fluorescence with nitrite, phosphoglycerate or glycerate as electron acceptors. 9-Aminoacridine, which can penetrate the chloroplast envelope [18], is trapped on illumination in the acidified intra-thylakoid space. Trapped dye does not contribute to fluorescence. From the extent of fluorescence quenching, the gradient in H^+ between the external medium and the intra-thylakoid space can be calculated [17], if the volume of the intra-thylakoid space is known. This volume is considered to be 4 μl/mg chlorophyll in isotonic intact chloroplasts [28]. Calculated ΔpH values are overestimates, as the method of calculation does not consider light-induced binding of 9-aminoacridine to thylakoids [18,29]. Fluorescence quenching was highest in the presence of nitrite, whose reduction does not require ATP. Phosphoglycerate reduction needs 2 and glycerate reduction 4 ATP molecules per molecule oxygen evolved. Fluorescence quenching was lowest in the presence of glycerate, which had the highest ATP requirement for reduction.

Fluorescence quenching was markedly dependent on flash frequency. Below 2 Hz, there was no detectable quenching. In contrast, ATP formation, as indicated by phosphoglycerate-dependent oxygen evolution, was within the range measured independent of flash frequency (upper curve with open circles). A fluorescence quenching of 12% in the nitrite experiment of Fig. 3 indicates according to Schuldiner et al. [17] a ΔpH of about 3, a fluorescence quenching of 1% a ΔpH of 1.8. Although both values are overestimates, the failure to

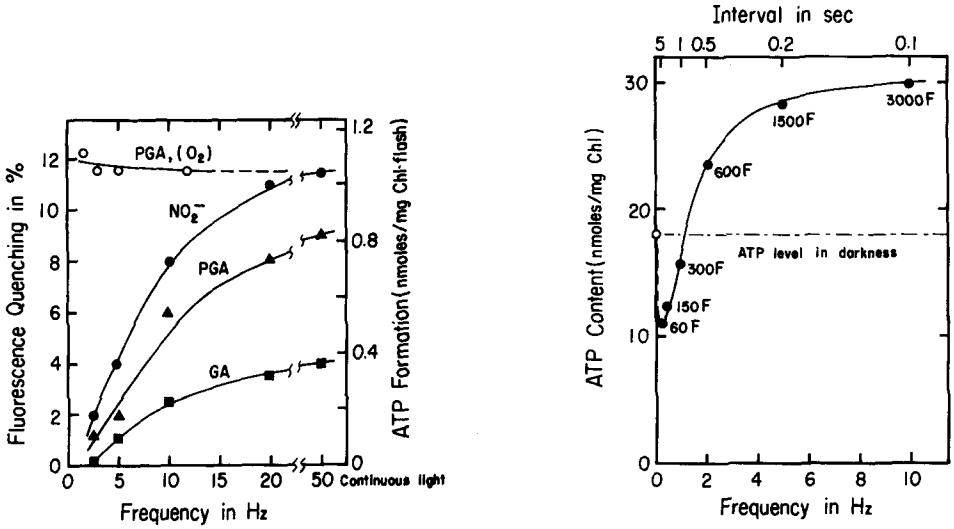


Fig. 3. Quenching of 9-aminoacridine fluorescence (solid symbols) by intact chloroplasts as a function of flash frequency. The extent of quenching was expressed on a percent basis by comparing with 9-aminoacridine fluorescence in darkness. The open circles show the ATP yield per flash as indicated by phosphoglycerate-dependent oxygen evolution in Fig. 2 experiment reproduced for comparison. PGA, phosphoglycerate; GA, glycerate; Chl, chlorophyll.

Fig. 4. Effect of flash frequency on the ATP content in isolated intact chloroplasts. Intact chloroplasts were illuminated with flashes at various frequencies for a constant period of 5 min. The total number of flashes (F) given during the 5 min is indicated by the numbers accompanying the curve. The reaction mixture contained 1 mM oxaloacetate as electron acceptor.

detect quenching below 2 Hz may indicate that the proton gradient is not higher than 1.8 unit under these conditions, since the quenching of 1% is the limit sensitivity of this method. From the view point that ATP synthesis is mainly supported by a proton gradient [8,9], the observation of a high flash yield of phosphoglycerate-dependent oxygen evolution under conditions which appear to limit the formation of a proton gradient was remarkable. It was desirable to measure intra-chloroplast ATP directly rather than through phosphoglycerate-dependent oxygen evolution.

Fig. 4 shows the level of chloroplast ATP as a function of flash frequency. Oxaloacetate, whose reduction does not consume ATP, served as electron acceptor. The chloroplasts were illuminated with flashes repeated at uniform frequencies, and the frequency was varied between 0.2 and 10 Hz. The illumination time was kept constant to 5 min regardless of flash frequency, so that the total flash number was different at different frequencies. It is indicated by the numbers accompanying the curve shown in Fig. 4. At the high frequency of 10 Hz, the ATP level reached the maximal value of 30 nmol/mg chlorophyll. Continuous illumination produced the same concentration of ATP (not shown). At 2 Hz, ATP was still increased as compared with the dark situation. However, at 0.2 Hz, the ATP level was much lower than the initial ATP content in darkened chloroplasts. The decrease in ATP is attributed to the action of an enzyme which is inactive in the dark and is activated by light to hydrolyze the ATP which has accumulated in darkened chloroplasts.

The conditions which either lead to light-dependent ATP synthesis or hydrolysis are more clearly apparent from the experiment shown in Fig. 5. Chloroplast ATP levels were measured as a function of flash number at 3 different flash frequencies. At 10 Hz, there was a small decrease in ATP during the first flashes. It was followed by a steep increase, until the maximal ATP level was reached after a total of less than 50 flashes (curve A). At 5 Hz, the initial loss of ATP was more pronounced and the subsequent rise slower than at 10 Hz. 150 flashes were not sufficient to raise the ATP level to its maximal value (curve B). At the very low frequency of 0.2 Hz, the ATP level decreased steeply during the first 10 flashes and then more gradually until it stabilized at about half the initial dark level (curve C). It should be noted that the final level attained during flashing at 0.2 Hz was higher than the level of thermodynamically inactive, bound ATP (see Table I and Fig. 1).

These data are most easily explained by assuming that the enzyme activated by light and responsible for ATP hydrolysis at the beginning of flashing and during low frequency flash illumination is the same enzyme which synthesizes ATP under high frequency flashing. After its activation, it is assumed to catalyze a reversible reaction, which equilibrates on the side of hydrolysis at low flash frequencies and on the side of synthesis at high frequencies. This interpretation is consistent with the idea on the reversibility of ATPase extensively documented for broken chloroplasts by Bakker-Grundwald and van Dam [30].

Even at the very low frequency of 0.2 Hz, significant free ATP coexists with ADP and phosphate. Its concentration is sufficient for some phosphorylation of substrates such as glycerate and phosphoglycerate, as can be seen from a comparison of Fig. 5, curve C, with the data of Table I. At 2 Hz intact chloroplasts are, in contrast to broken chloroplasts, sufficiently energized to produce their maximal ATP yield of about 1.2 nmol ATP/mg chlorophyll per flash when an efficient ATP sink is present, as has been shown in Fig. 2. The proton gradient formed at the same frequency appears to be well below a ΔpH of 2. As has been mentioned, this appears to be a rather small gradient for the syn-

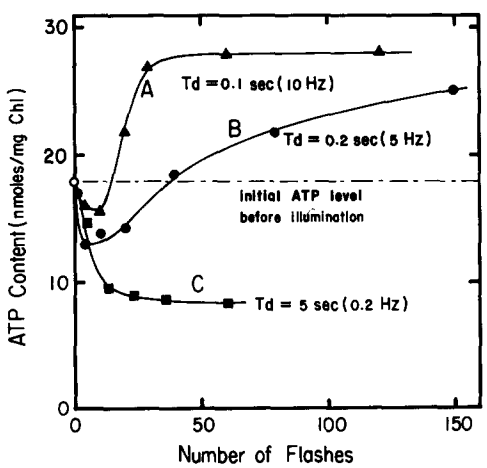


Fig. 5. Change of ATP content in isolated intact chloroplasts during illumination with flashes repeated at different intervals (Td) of 0.1 s (10 Hz, curve A), 0.2 s (5 Hz, curve B) and 5 s (0.2 Hz, curve C). The ATP content was plotted as a function of the number of flashes given.

thesis of ATP of the required phosphorylation potential. For technical reasons, we were unable to measure a flash-induced membrane potential, which might contribute energy to ATP synthesis. Larkum and Boardman [31] have reported evidence against a significant contribution of the membrane potential to energy conservation by intact chloroplasts under continuous illumination. It is worthwhile to mention in the context of this investigation, that Tillberg et al. [18] have failed to observe inhibition of CO_2 reduction by intact chloroplasts even under rate-limiting light, when the proton gradient was decreased by the addition of uncoupling amines.

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