INACTIVATION BY LIGHT OF THE PHOSPHORYLATIVE ACTIVITY OF CHLOROPLASTS*

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SUMMARY

The inactivation by light of the HILL reaction and the phosphorylating capacity of isolated chloroplasts has been studied. The results show that the ATP forming system is more sensitive than the electron transport system to light inactivation; conditions can be established under which a light pre-treatment decreases only 5 % the ability of chloroplasts to reduce TPN, while the ATP formation is decreased by 50 %. The light inactivation can be prevented to a large extent by the cofactors of photophosphorylation, vitamin K being the most efficient. ADP prevents the protection, and phosphate or arsenate restore it. The possible significance of these results is discussed.

INTRODUCTION

The finding that photosynthetic phosphorylation can be coupled to a HILL reaction[1] makes it possible to measure electron transport and ATP formation coupled to it independently of each other in isolated chloroplasts[2]. Such a possibility may be utilized in studying the mechanism of ATP formation in chloroplasts[2]. This report deals with a selectively greater inactivation by light of the ATP-forming system than of the HILL reaction.

METHODS

The chloroplasts were prepared from spinach leaves as described by JAGENDORF AND AVRON[3]. Chlorophyll was determined according to ARNON[4]. ATP formation was measured according to LINDBERG AND ERNSTER[5], as the incorporation of ^{32}P from P_i into ATP. PPNR was prepared as described by SAN PIETRO AND LANG[6]. TPN reduction was measured as the increase of O.D. at 340 mμ of a suspension of chloroplasts containing TPN and PPNR, against a blank to which no TPN was added. Ferricyanide reduction was measured by the decrease of O.D. at 420 mμ as described by AVRON *et al.*[7]. The chloroplast suspensions were illuminated in flasks agitated in an

Abbreviations: ATP, ADP, adenosinetriphosphate, adenosinediphosphate; TPN, triphosphopyridinenucleotide; PPNR, photosynthetic pyridine nucleotide reductase; FMN, flavinmononucleotide; PMS, phenazinemethosulphate; P_i, inorganic orthophosphate.

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illuminated water bath, the light intensity being 4000 foot candles at the level of the flasks. The light was provided by incandescent lamps.

EXPERIMENTAL RESULTS

In the course of studies on photosynthetic phosphorylation, it was observed that ATP formation was linear only for a short period, no matter what cofactor was added to the reaction. These results are summarized in Fig. 1. It can be seen that with PMS, FMN or vitamin K_5 as catalysts, all added at saturating concentrations, the rate of ATP formation started to decrease after 10 min of illumination and was nearly suppressed after 30 min. The subsequent addition of any of the cofactors indicated above could not reactivate the system.

It was found further that pre-illumination of chloroplasts under nitrogen for periods of 15 min or more without the reagents needed for phosphorylation led to a

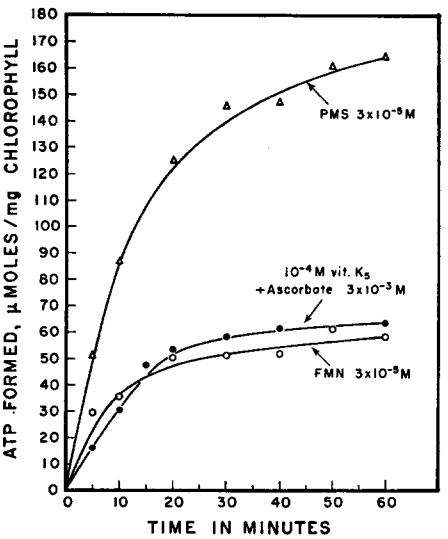

Fig. 1. Reaction mixture: NaCl 0.03 *M*; Tris 0.02 *M*, pH 8.0; $MgCl_2$ 0.003 *M*; ADP 0.005 *M*; Pi 0.005 *M*, labeled with ^{32}P. Final volume, 9 ml: N_2 atmosphere. Temperature 15°. Chloroplasts contained 0.108 mg of chlorophyll in the FMN and PMS experiment, and 0.126 mg in the vitamin K_5 experiment.

TABLE I

EFFECT OF PRE-ILLUMINATION OF CHLOROPLASTS ON THEIR CAPACITY TO COUPLE ATP FORMATION TO THE REDUCTION OF TPN

The chloroplast suspension was preincubated with KCl 0.03 *M*; Tris 0.02 *M*, pH 7.8; $MgCl_2$ 0.0033 *M*; ADP 0.0033 *M*, at 13°, under N_2 atmosphere. At the end of the time indicated, the following were added: TPN 1.6 μmoles, PPNR 20 units and 10 μmoles of phosphate, labeled with ^{32}P. Final volume 3 ml, pH 7.8. The chlorophyll content was 216 and 97 μg in Expts. 1 and 2. Reaction time, 10 min.

Experiment No.	*Pre-treatment conditions*	*TPNH formed μmoles*	*ATP formed μmoles*	*P/2e*
1	Dark 30 min	1.115	1.43	1.27
	Light 30 min	0.270	0.04	0.15
2	Dark 20 min	0.911	1.12	1.23
	Light 20 min	0.870	0.52	0.60

loss in their subsequent ability to carry out phosphorylation. The loss of activity due to pre-illumination was studied further in a system in which ATP formation is coupled to the reduction of TPN or of ferricyanide. Ih the former case, one has to deal with a "loosely coupled" phosphorylation, the rate of TPN reduction being unaffected by ADP, phosphate and Mg^{++}; in the latter, the phosphorylation is of the "tightly coupled" type, the rate of $[Fe(CN)_6]^{---}$ reduction being stimulated about 200% by the addition of the phosphorylating reagents[7]. It can be seen in Table I that as a consequence of illumination of chloroplasts their capacity to form ATP is severely decreased while the reduction of TPN is little affected (Expt. 2). With a longer time of illumination the TPN reducing activity is also decreased about 75%, but then the phosphorylation coupled to it is completely suppressed: the P/2e ratio is reduced from 1.27 to 0.15 (Expt. 1). It has to be observed that the light inactivation of the added PPNR, reported by GIOVANELLI AND SAN PIETRO[8], has no part in the phenomenon here described since PPNR was added after the pre-illumination.

In the HILL reaction with ferricyanide ATP formation is also inactivated by light at a faster rate than is ferricyanide reduction (Table II). Here again it can be seen that the P/2e ratio decreases as the time of illumination increases. The possibility that a stable, diffusible inhibitor of phosphorylation is formed during illumination of chloroplasts has been ruled out by experiments in which fresh chloroplasts were added to light inactivated ones (Table III). It can be seen that no inhibition is de-

TABLE II

EFFECT OF PRE-ILLUMINATION OF CHLOROPLASTS ON THEIR CAPACITY TO COUPLE ATP FORMATION TO $[Fe(CN)_6]^{---}$ REDUCTION

Preincubation: NaCl 0.03 *M*; Tris 0.02 *M*, pH 8.0; ADP 0.001 *M*; $MgCl_2$ 0.002 *M*; final volume 9 ml; chloroplasts containing 743 μg of chlorophyll. Atmosphere, N_2; temperature, 12°. Reaction mixture: same as preincubation, with 41 μg of chlorophyll in 3 ml; phosphate labeled with ^{32}P was added where indicated.

Pre-illumination time (min)	*$Fe(CN)_6^{---}$ reduced, in μmoles/2 min*		*ATP formed μmoles*	*P/2e*
	Without phosphate	*With phosphate 0.007 M*		
0	0.345	0.961	0.421	0.88
15	0.212	0.539	0.265	0.98
25	0.200	0.353	0.127	0.72
35	0.162	0.200	0.038	0.38

TABLE III

LACK OF FORMATION OF AN INHIBITOR OF PHOTOPHOSPHORYLATION DURING THE ILLUMINATION OF CHLOROPLASTS

Preincubation (in light or dark) as in Table I, with 15.4 μg of chlorophyll in 1.0 ml. After 30 min, 2.5 μmoles of [^{32}P]phosphate are added; FMN and non-preincubated chloroplasts where indicated. The reaction is carried on for 10 min, under N_2, at 15°.

Pre-illuminated chloroplasts	*Non-preincubated chloroplasts*	*ATP formed, μmoles/mg Chl. h.*	
		— FMN	*+ FMN $3 \cdot 10^{-5} M$*
+	—	0.4	3.1
+	+	11.0	133.5
—	+	10.5	138.5

tected, either in phosphorylation catalyzed by FMN or in the "endogenous" phosphorylation (that is, without any cofactor added). It is concluded from these experiments that the ATP-forming system of chloroplasts is inactivated by light at a faster rate than is their electron transport system.

Various reagents were added during the pre-illumination period in attempts to protect the chloroplasts from light inactivation (Table IV). No protection was afforded by ascorbate, cysteine or catalase and ethanol. Slight protection was afforded by high osmotic pressure. Of more interest is the protection afforded by PMS, pyocyanine, and vitamin K_5, all cofactors of cyclic photophosphorylation (Table IV). As can be seen, vitamin K_5 is the most effective of these cofactors in protecting the chloroplasts. Furthermore, its protection is just as efficient in red as in white light, while PMS protection is more efficient in red light than in white. Ferricyanide gives only a slight though reproducible protection.

The protection by the cofactors indicated in Table IV is suppressed or strongly reduced by the presence of ADP, while ATP has no effect (Table V). Separate experiments have shown that the presence of ADP alone during pre-incubation is harmful only when the chloroplasts are illuminated.

TABLE IV

PROTECTION OF CHLOROPLASTS FROM LIGHT INACTIVATION

The chloroplasts were preincubated for 30 min, at 14°, in N_2 atmosphere, in red light, in flasks containing NaCl 0.01 *M*; Tris 0.02 *M*, pH 8.0 and $MgCl_2$ 0.005 *M*. The suspension was then centrifuged at 18,000 × *g* for 10 min, the supernatant discarded, and the pellet washed with sucrose 0.4 *M*, containing Tris 0.02 *M*, pH 8.0, and NaCl 0.01 *M*, and finally resuspended in the same medium. Photo-phosphorylation was then measured in a system containing the same components as in Fig. 1 (PMS experiment), in a final volume of 1.5 ml. The reaction was allowed to proceed for 5 min.

Additions during preincubation	*ATP formed, μmoles/mg Chl. h.*
None	31
PMS 10^{-4} *M*	95
Pyocyanine 10^{-4} *M*	99
Vitamin K_5 10^{-4} *M*	149
Ascorbate 0.01 *M*	31
Catalase* + ethanol 0.01 *M*	32
None, preincubation in the dark	135

* The catalase added was enough to decompose 10 μmoles of H_2O_2/min.

TABLE V

EFFECT OF ADP ON THE PROTECTION FROM LIGHT INACTIVATION BY PMS

Preincubation, testing conditions and procedure as in Table IV, except for the absence of $MgCl_2$ during preincubation.

Additions during preincubation	*ATP formed, μmoles/mg Chl. h.*
None	4.3
PMS 10^{-4} *M*	68.4
PMS 10^{-4} *M* + ADP 0.003 *M* + $MgCl_2$ 0.005 *M*	15.5
PMS 10^{-4} *M* + ATP 0.005 *M* + $MgCl_2$ 0.005 *M*	73.6
None, preincubation in the dark	135.0

TABLE VI

EFFECTS OF ADP, ARSENATE AND PHOSPHATE ON THE PROTECTION FROM LIGHT INACTIVATION

Experiment No.	*Additions during preincubation**	*ATP formed moles/mg Chl. · hour*	*Increase over the control moles ATP/mg Chl. h.*
1	None	32.3	—
	Vitamin K_5	106.0	74
	Vitamin K_5 + ADP	75.0	43
	Vitamin K_5 + Pi	204.0	172
	Vitamin K_5 + ADP + Pi	117.0	85
	None, preincubation in the dark	157.5	125
2	None	59.0	—
	Vitamin K_5	212.0	153
	Vitamin K_5 + ADP	147.0	88
	Vitamin K_5 + Arsenate	254.0	195
	Vitamin K_5 + ADP + Arsenate	248.0	189
	None, preincubation in the dark	250.0	191

* Preincubation conditions: Vitamin K_5 10^{-4} *M*, and, where indicated, ADP 0.005 *M*; Pi 0.01 *M* and arsenate 0.001 *M*. Other conditions as in Table IV.

TABLE VII

EFFECT OF ADP CONCENTRATION ON K_5 PROTECTION FROM LIGHT INACTIVATION

*Additions during preincubation**		*ATP formed*	*Increase over the control*
Vitamin K_5	*ADP concentration mM*	*μmoles/mg Chl. · hour*	*μmoles ATP/mg Chl. h.*
—	0.00	80.0	—
+	0.00	156.0	76
+	0.10	168.0	88
+	0.50	143.0	63
+	1.00	118.0	38
+	5.00	89.0	9

* Preincubation for 30 min, in white light. Vitamin K_5, where indicated, 10^{-4} *M*; ADP as indicated. Other conditions as in Table IV.

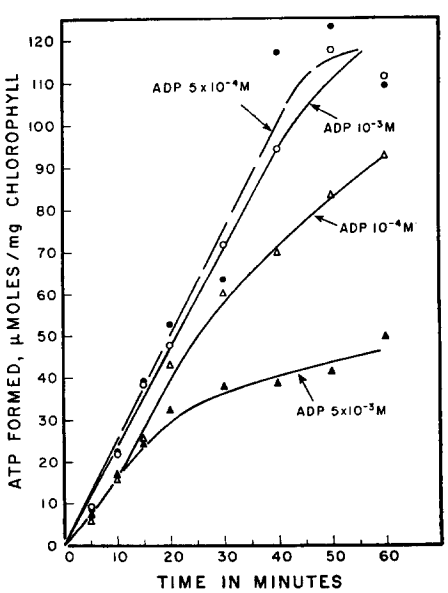

Fig. 2. Reaction mixture: NaCl 0.02 *M*; Tris 0.01 *M*; $MgCl_2$ 0.006 *M*; phosphate 0.005 *M* containing 300,000 counts/min of ^{32}P; hexokinase in large excess and glucose 0.02 *M*; vitamin K_5 10^{-4} *M*, ADP as indicated; pH 8.0. Final volume 8 ml; atmosphere: N_2·Chloroplasts containing 0.162 mg of chlorophyll.

The effect of ADP can be reversed by arsenate (Table VI). Phosphate seems also to be effective (Table VI), but the interpretation of its effect is complicated by the fact that phosphate alone, added during the light pre-incubation, provides considerable protection.

The concentration of ADP needed to reverse protection by cofactors shows a considerable variability with different chloroplasts preparations (compare Tables VI and VII); usually, however, the ADP effect is very strong or almost complete at $5 \cdot 10^{-3}$ *M*. The rate of phosphorylation, on the other hand, is saturated with about $5 \cdot 10^{-4}$ *M* ADP (Fig. 2). This suggested that the observed decline in rate of photophosphorylation with time (Fig. 1) might be due to a light inactivation facilitated by high concentrations of ADP, and that the use of lower concentrations of ADP might be able to prolong the linear part of the time course. Fig. 2 indicates that a linear time course can be achieved when the ADP concentration is not higher than $1 \cdot 10^{-3}$ *M*, and kept constant by means of the hexokinase–glucose system.

DISCUSSION

The data presented here demonstrate that light inactivates isolated chloroplasts and that the part of the system responsible for forming ATP is more sensitive than are the electron transport reactions *per se*. In fact, under the proper conditions one can observe a 50 % decrease of the ATP formation coupled to TPN reduction, while TPN reduction itself is decreased by only 5 % (Table I). An analogous phenomenon was observed with ferricyanide reduction.

The light inactivation here described is not due to the inactivation of PPNR reported by GIOVANELLI AND SAN PIETRO[8]. In fact, in our TPN reduction experiments PPNR was added after the pre-illumination, and therefore could not have been affected by the pre-treatment. Furthermore, the light inactivation of phosphorylation was observed also with ferricyanide reduction, and with cofactors such as PMS, FMN and vitamin K_1, while it is well known[9] that under such conditions photophosphorylation is independent of PPNR.

The mechanisms for light inactivation, for protection by redox cofactors (Table IV), for ADP reversal of the protection (Table V), and for phosphate or arsenate reversal of the ADP effect (Table VI) are all obscure. Some possibilities as to the nature of light inactivation can be ruled out, however. It has to be observed, first of all, that the phenomenon described here differs from the inactivation of PPNR by chloroplasts in the light[8] in two main respects: (a) PPNR inactivation is an aerobic effect, while our experiments were performed under nitrogen, and (b) PPNR photo-inactivation was prevented by ascorbate and cysteine, which had no effect here. It seems possible to rule out H_2O_2 as the damaging agent in the present case because of these two findings, and also because catalase failed to give any protection (Table IV). H_2O_2 formed in a "Mehler reaction"[10] was proposed by GIOVANELLI AND SAN PIETRO as responsible for PPNR inactivation[8].

Light inactivation might possibly be due to the photo-oxidant produced early in the HILL reaction; in this case the redox cofactors of cyclic electron transport might be able to reduce the photo-oxidant and so afford protection. This seems unlikely, however, because there is no reason why small amounts of the "photo-oxidant" could not be discharged by the evolution of oxygen. It seems reasonable to infer

that the cofactors do afford protection by permitting electron flow to occur, whatever the mechanism of light-inactivation could be. For instance, light could be inactivating directly some chloroplast component, the inactivation occurring when the component is in one redox state but not the other. In that case, a steady electron flow caused by the cofactors could change the oxidation state of this component and "poise" it in the resistant form.

As to the effect of ADP and its reversal by arsenate or phosphate (Tables V and VI), one is tempted to suggest that the protection from light inactivation afforded by the redox cofactors cannot occur whenever ADP is bound to the "activating" system[2], whereas any conditions which will decompose the ADP–catalyst complex (as the presence of arsenate or phosphate according to the scheme proposed by AVRON AND JAGENDORF[2]) will restore the protecting effect. However, the ADP effect could also be due to the depletion of the internal pool of phosphate within the chloroplasts which occurs when the chloroplasts are incubated in the light with ADP and vitamin K_5 or PMS.

It seems unlikely that light-inactivation of the phosphorylation mechanism would occur *in vivo*, at least under the usual conditions of a sustained photosynthetic rate. Indeed, protection *in vitro* is afforded by cofactors for cyclic phosphorylation provided the ADP concentration is not abnormally high. Phosphate also protects from the ADP effect. These could be models for *in vivo* protection of plastids.

The possibility of inactivating selectively the ATP forming system of chloroplasts with little damage to the electron transport system could become a useful technique to study the phosphorylation mechanism itself.

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