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THE INITIAL STAGES OF PHOTOPHOSPHORYLATION

STUDIES USING EXCITATION BY SATURATING, SHORT FLASHES OF LIGHT

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Summary

1. Photophosphorylation was studied in spinach chloroplasts on illumination, from the dark state, with saturating short ("single turnover") flashes of light.

2. At rapid flash rates (100 Hz), phosphorylation began within the first five flashes. The ATPase inhibitor protein appeared to be displaced from its inhibitory site on the ATPase also within five flashes, as deduced from the flash-induced ATPase activity.

3. At slower flash rates, or if the rate of electron transfer were reduced with 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU), phosphorylation began only after a larger number (50—60) of flashes. The displacement of the ATPase inhibitor protein was similarly delayed.

4. Partial displacement of the inhibitor protein from its inhibitory site on the ATPase (by pretreatment with dithioerythritol) allowed phosphorylation to proceed without a perceptible lag, even in the presence of DCMU. It was concluded that the ATPase inhibitor protein must be displaced on the ATPase before phosphorylation can begin, and that this process is energy dependent.

5. During the flash regime used, release of inhibitor from its inhibitory site seemed to be governed largely by the membrane potential. The light-induced pH gradient seemed to have little effect under these conditions. Our results are not compatible with a direct conformational interaction between the electron transfer chain and the ATPase causing displacement of the inhibitor.

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethyl urea; Tricine, *N*-tris(hydroxymethyl)-methylglycine.

6. The maximal rate of photophosphorylation induced by less than 200 flashes was 0.12—0.15 mol ATP made/mol ATPase per flash. This rate seemed to be limited not by the supply of energy to the ATPase molecules, nor by the maximal turnover capacity of the ATP synthesising system, but by the number of ATPase molecules which were active in synthesis, i.e., which lacked the inhibitor protein.

7. The bound nucleotides of the coupling ATPase exchanged with added nucleotides during single turnover flashes. At high flash rates, exchange began within 5 flashes. The average amount of nucleotide exchanged per flash over 100 flashes was about one tenth the amount of ATP synthesised in each flash.

8. It is concluded that, during phosphorylation, a steady state level of active coupling ATPases is set up. The energy-dependent displacement of the inhibitor protein, and its (energy-independent) relaxation back on to the inhibitory site are the two opposing factors involved in this steady state.

Introduction

It has been shown by several workers that photophosphorylation in chloroplasts is preceded by a lag phase. This is true for phosphorylation induced by light flashes [1], an acid-base transition [2] or continuous light [3,4]. This lag is about 5 ms under conditions capable of producing high rates of phosphorylation [2,4]. On a simple model of phosphorylation where ATP synthesis and hydrolysis are at equilibrium, and only the position of equilibrium is altered by energy input, no lag would be expected (see ref. 5).

Schröder et al. [6], and Gräber and Witt [7] have shown that a lag in the onset of phosphorylation would be expected if two pathways exist for dissipation of the energy "current"; one via the ATP synthase mechanism and one via a "leak" pathway, provided that the synthase pathway is of higher order in the current carrier than is the "leak". For example, if the current carrier were protons, the leakage rate might be proportional to $(H^+)^n$ and the synthase rate to $(H^+)^{n+1}$. Subsequently, however, Ort et al. [8] have shown that if the energy current is carried by protons, then the lag in phosphorylation in chloroplasts cannot be explained by this treatment or, conversely, if this treatment holds, then the high energy state is not a proton gradient.

We have considered the possibility that the lag in photophosphorylation might be due to a regulatory process. The ATPase of chloroplasts and mitochondria bears a small subunit, the ATPase inhibitor protein, which inhibits ATPase activity [9,10] but does not appear to inhibit phosphorylation. It is generally assumed that ATP synthesis and hydrolysis are at equilibrium during phosphorylation via the ATPase site, and it would therefore be surprising if this protein did not inhibit phosphorylation. It has been shown, however, that under the conditions for steady state phosphorylation, the inhibitor protein shifts from its inhibitory site to a non-inhibitory site on, or close to the ATPase [11,12]. Whether this shift was sufficiently fast to precede phosphorylation remained undetermined.

The data presented here show that inhibitor displacement from the ATPase is sufficiently fast to precede phosphorylation, and that the bound inhibitor

protein does inhibit phosphorylation. During phosphorylation induced by multiple flashes, the rate of ATP synthesis is limited by the number of uninhibited ATPase molecules rather than by energy input or the rate of ATPase turnover.

Because the inhibitor protein must be displaced from its inhibitory site on the ATPase before phosphorylation can begin, and because this displacement is energy dependent, several flashes were required to initiate phosphorylation. The number of flashes required is higher at low rates of energy input. The energy-dependent lag seen before phosphorylation in continuous light [3,4], and the lag which precedes acid-base-induced phosphorylation [2] may well be expressions of the same phenomenon.

In agreement with Gräber and Witt [7], we find that multiple flashes rapidly induce a membrane potential in chloroplasts, while a pH gradient does not develop significantly for many flashes. It appears, therefore, that release of the inhibitor preceding flash-induced phosphorylation is dependent largely on the membrane potential. This is in agreement with the conclusions of Good and co-workers [4,8] who observed that, during phosphorylation induced by continuous light, the lag was dependent on membrane potential rather than on the pH gradient.

The bound nucleotides of the chloroplast ATPase [13] exchange against added nucleotides during energisation by flashes. No lag (less than 5 flashes) was observed in the exchange at high energy input rates, and the exchange proceeded to more than one nucleotide exchanged per ATPase molecule. An interpretation of this finding, with regard to the involvement of the bound nucleotides in phosphorylation, is given.

Methods

Class II chloroplasts (lacking an outer membrane) were prepared in dim light from spinach, and washed to remove the pool of free nucleotide. The procedure used was that described previously (Mg^{2+}-containing medium) [13], except that KCl was omitted from the buffers. Chloroplasts prepared by this method were shown to be low in adenylate kinase activity [14]. The steady-state of phosphorylation by these chloroplasts, measured using $^{32}P_i$ incorporation [13], was typically 1.5—2 μmol · min^{-1} · mg chlorophyll^{-1}, about 33—50% of that observed in chloroplasts made in buffers containing KCl. The decay of the flash-induced absorption change at 515 nm (see below) was also somewhat (4—5×) faster in the chloroplasts prepared in media free of K^+.

ATPase activity was measured using a recording pH meter in a buffer containing 50 mM NaCl/4 mM dithioerythritol/2 mM Tricine/2 mM Mg-ATP/1 mM $MgCl_2$/0.1 mM EDTA brought to pH 7.8 with NaOH [11]. H^+ uptake was measured similarly, except in 100 mM NaCl/1 mM $MgCl_2$ (pH 6.6—6.8) equilibrated with air. The concentration of chlorophyll in these experiments was 20—50 μg/ml and illumination was provided by a flashlamp external to the transparent measuring cuvette. The absorbance change of chloroplasts at 515 nm was measured as described previously [15].

Flash-induced phosphorylation was measured as follows. Chloroplasts (200—300 μg chlorophyll/ml) were mixed with reagents (ADP, P_i, DCMU, etc.) in the dark and drawn into a concentric cylindrical cell (2.5 cm internal diameter)

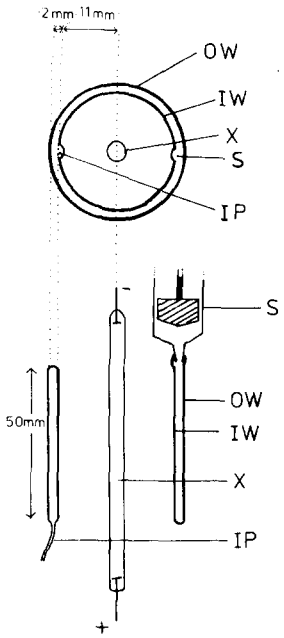

Fig. 1. Apparatus used for flash-induced phosphorylation, a, as viewed from above; b, as viewed from side (cross section). X, Xenon flash tube (Wingent, Cambridge, 200 W linear); IW, inner wall of glass cylindrical cell; OW, outer wall of glass cylindrical cell; IP, inlet and outlet port for reaction mixture; S, syringe.

with a 2 mm gap between the walls and a total volume of about 8 ml (Fig. 1). The suspension was held in place by suction from the syringe (S). Illumination was provided by brief (about 10 μs at half height) flashes from a xenon flash tube mounted centrally along the long axis of the cylinder (Fig. 1). With this arrangement, saturating flashes could be given to a large amount of chloroplasts at one time, thus eliminating the need found by other workers [4,7,8] to apply repeated flash groups separated by long dark periods for measurable incorporation of label.

Immediately after illumination, the chloroplasts were expelled from the cell through the inlet/outlet tube by downward pressure on the syringe, into either perchloric acid (final concentration 4%) for studies on $^{32}P_i$ incorporation, or ammonium chloride solution (pH 7.8) (final concentration 10 mM) for studies on the bound nucleotides. ^{32}P-Label incorporation into total nucleotide, and incorporation into the bound nucleotide were measured as previously [13].

The time between mixing the reagents and quenching the reaction mixture was about 15 s. During this time, the incorporation of $^{32}P_i$ into ATP in the dark was less than 0.5 mol/mol ATPase (less than 0.1 mol/mol ATPase in most preparations). This is corrected for in the data presented.

Chloroplasts, with part of the inhibitor protein stably displaced from its inhibitory site, were prepared by illumination with dithioerythritol in the absence of Mg^{2+} [11]. This gave a preparation with an enhanced membrane ATPase activity which persisted for several hours after illumination.

Results

Flash-induced ATPase activity

The ATPase activity of class II chloroplasts is normally very low or undetectable unless the chloroplasts are activated by pretreatment with trypsin, or light in the presence of a dithiol reagent [16,17]. These procedures act by displacing or removing the ATPase inhibitor from the ATPase [9,11].

It is shown in Figs. 2 and 3 that the ATPase activity of chloroplasts was induced by very short periods of flash illumination. At 100 Hz, the activity was stimulated in a very small number of flashes (<3). At lower flash rates (Fig. 2) or in the presence of DCMU (Fig. 3) a considerable number of flashes was required (20—30) before much stimulation occurred.

Table I shows that the light-induced ATPase was stimulated by an uncoupler (here Na^+-nigericin) as previously shown by Crofts [18] and Carmeli [19]. At low levels of activation of the ATPase, little stimulation was observed, while at higher levels, the stimulation was as much as 10-fold. This is presumably because, at low levels of activation, ATPase activity was limited by the number of active ATPase molecules present, while at higher levels by the dissipation of the energy produced during hydrolysis. Only the dissipation would be speeded up by uncouplers.

At low levels of activation of the ATPase at least, therefore, measurement of the light-induced ATPase activity gives us information about the fraction of active ATPase molecules present, i.e., about the relative number of ATPase molecules with their inhibitor protein displaced to a non-inhibitory site. Figs. 2 and 3 thus show that, at low energy input rates, a considerable number of

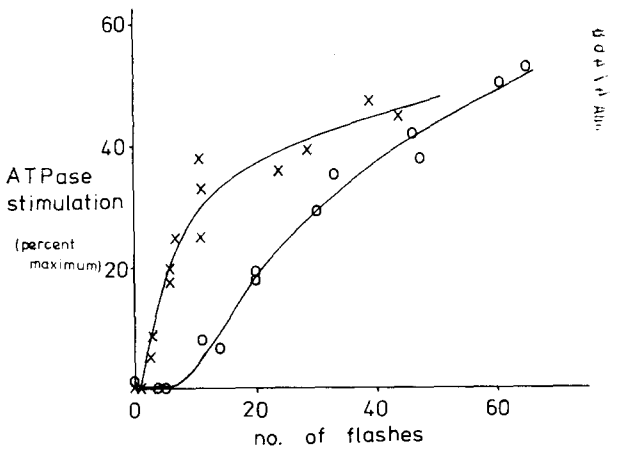

Fig. 2. ATPase induction by multiple flashes; effect of flash rate. Chloroplasts (20 μg chlorophyll) were incubated in 0.89 ml of solution containing 50 mM NaCl/2 mM Tricine/1 mM $MgCl_2$/0.1 mM EDTA/4 mM dithioerythritol/4 mM Mg-ATP/10 μg pyocyanine brought to pH 7.8 with NaOH. The ATPase activity was monitored, before and after a given number of flashes, by monitoring H^+ release with a recording pH meter. A xenon flash lamp (Osram XE1200) with an orange cut-off filter was used to provide the flashes, each of which discharged 4 J with a width at half height of 15 μs. Saturation by the flash was checked by varying the chloroplast concentration (in this and all subsequent experiments). X———X, 100 Hz; o———o, 12.5 Hz. The results are expressed as % stimulation obtained with continuous illumination.

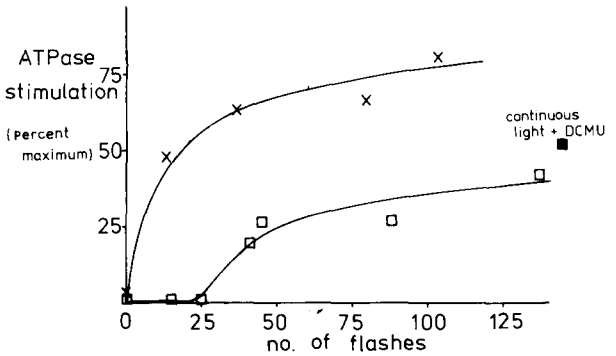

Fig. 3. ATPase induction by multiple flashes; effect of DCMU. Flash-induced ATPase activity was measured as in Fig. 2, except that 5 μM DCMU was present where indicated. The flash rate was 100 Hz. X———X, No addition; □———□, + 5 μM DCMU.

flashes was required before the inhibitor protein is displaced from its inhibitory site on the ATPase. It is noteworthy that the inhibitor displacement depended on the extent and frequency of turnover of the photosynthetic apparatus rather than simply on the number of electrons transferred or on the total exposure to light. It is also seen (Figs. 2 and 3 and Table I) that repeated flashes do not induce as high an ATPase as continuous light and, thus, that less inhibitor protein can be displaced by repeated flashes than by continuous light.

Flash-induced phosphorylation

Fig. 4 shows that, at rapid flash rates (100 Hz) phosphorylation began within the first few flashes. Omission of ADP from the reaction medium abolished phosphorylation, even though 1 mol ADP is tightly bound to the chloroplasts per mol ATPase. This is in agreement with the results of Smith et al. [2], who showed that, in phosphorylation induced by an acid-base jump, added ADP is necessary before bound ADP can be phosphorylated. During longer periods of continuous illumination (>3 s), however, it has been shown that phosphorylation of bound ADP by $^{32}P_i$ can occur in the absence of added ADP [13,20].

TABLE I

EFFECT OF UNCOUPLER ON THE FLASH-INDUCED ATPase

The ATPase was induced by light flashes in the presence of dithioerythritol and ATP, and measured as described in Fig. 2. After each group of flashes, ATP hydrolysis was allowed to proceed for about 30 s and 10 μl 2 mg/ml nigericin added as an uncoupler. The initial rate of hydrolysis after the addition of uncoupler was then measured (since in the presence of uncoupler, the activity falls in time).

No. of flashes	ATP hydrolysis	Uncoupled ATP hydrolysis	Stimulation by uncoupler
	(nmol/min per mg chlorophyll)		
0	35	65	1.9
3	40	89	2.3
7	60	178	3.0
11	64	190	2.9
39	85	480	5.7
Continuous light	154	1200	8.5

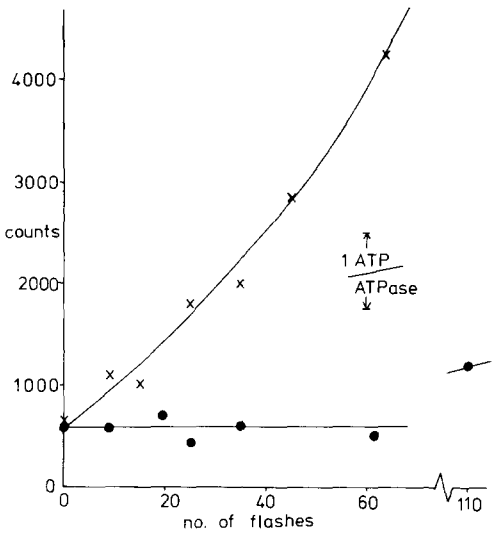

Fig. 4. Requirement of added ADP for photophosphorylation. Washed, class II chloroplasts were illuminated by saturating light flashes of 10 μs (half height) duration in the cell described in Methods. The chlorophyll concentration was approx. 300 μg/ml, and the flash frequency 100 Hz, each flash discharging 4 J. The suspension medium comprised 5 ml of 50 mM NaCl/2 mM Tricine/1 mM $MgCl_2$/10 μM pyocyanine/ 2 mM $^{32}P_i$ (2500 cpm/nmol) brought to pH 7.8 with NaOH. 200 μM ADP was added as indicated. After a number of flashes, the chloroplasts were denatured with 4% $HClO_4$ and organic ^{32}P-label (bound + free labelled nucleotide) was determined. (Inclusion of glucose (20 mM) and hexokinase (5 U/ml) did not affect the phosphorylation observed (not shown) and was omitted in subsequent measurements.) ●——●, No ADP added; X——X, 200 μM ADP added.

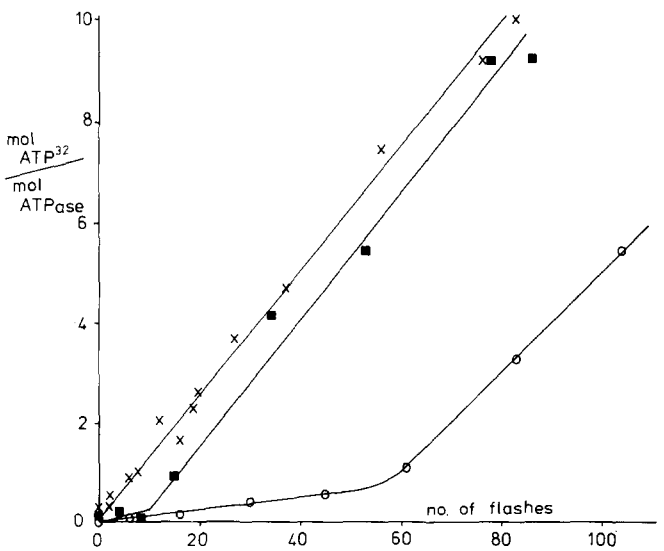

Fig. 5. Effect of flash spacing on photophosphorylation. Flash-induced phosphorylation was measured as in Fig. 4, except that 200 μM ADP was present in each case. The dark level of non-extractable phosphate (see Fig. 4) was subtracted from the readings obtained to express the values as light-induced phosphorylation, and the values expressed as mol ATP labelled per mol ATPase using a value of 1.3 nmol ATPase/mg chlorophyll [21] in this and subsequent figures. X——X, Flash rate 100 Hz; ■——■, flash rate 25 Hz; ○——○, flash rate 12.5 Hz.

Fig. 5 shows that, as the flash rate was decreased, phosphorylation did not begin until after several flashes had occurred. This delay in the onset of phosphorylation is hereafter referred to as a lag. At 50 Hz (not shown) the time course of phosphorylation was indistinguishable from that at 100 Hz, while at 25 Hz a short lag (about 8 flashes) seemed to occur, and at 12.5 Hz this lag was pronounced. This was not associated with a lag in electron transfer, as was shown by measurement of the absorption change at 515 nm (see below).

Under these conditions the electron transfer mediator, pyocyanine, mediates the turnover of Photosystem II in addition to Photosystem I since it is to some extent auto-oxidisable. Fig. 6 (lowest curve) shows that decreasing the energy input per flash, by blocking Photosystem II with DCMU, produced a lag in phosphorylation comparable (in number of flashes) to that occurring when the flash frequency was decreased.

The lags seen in phosphorylation at lower rates of energy input are reminiscent of those observed in displacement of the ATPase inhibitor protein at low

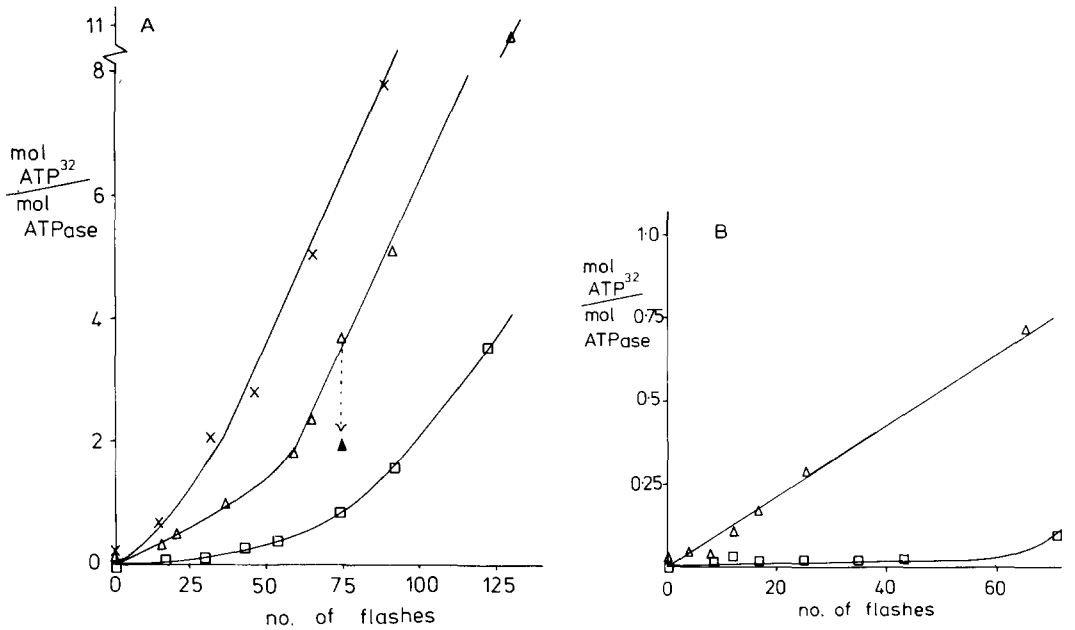

Fig. 6. Phosphorylation in chloroplasts pretreated by illumination in the presence of dithioerythritol. Results of two experiments using a small (B) or larger (A) number of flashes. Chloroplasts with a stable increased ATPase activity were prepared as in ref. 11 except that the chloroplasts were illuminated at 100—200 μg chlorophyll/ml using a 1000 W quartz-halogen lamp at a distance of 25 cm. The chloroplasts were maintained at 10°C and stirred during the 5 min illumination period. They were then spun down and washed to remove dithioerythritol by centrifugation through a medium containing 50 mM NaCl/2 mM Tricine/0.5 mM EDTA which had been brought to pH 7.8 with NaOH, and kept in this buffer at 0°C in the dark. The control particles were treated in the same manner, except that illumination was omitted and dithioerythritol and pyocyanine were not added. Photophosphorylation was measured in the presence and in the absence of DCMU (5 μM) as described (flash rate 100 Hz), except that the reaction mixture was made up in 50 mM NaCl/2 mM Tricine/0.5 mM EDTA (pH 7.8) and 1 mM free Mg^{2+} added (as $MgCl_2$) only immediately prior to flashing. This minimises "decay" of the chloroplasts back to the inhibited state. Where indicated (the point ▲) $MgCl_2$ was added to the buffer + chloroplasts 10 min prior to the addition of the other reagents and illumination. □———□, Control chloroplasts illuminated with 5 μM DCMU; △———△, pretreated chloroplasts illuminated with 5 μM DCMU; X———X, control chloroplasts illuminated without DCMU.

rates of energy input. (Figs. 2 and 3). It therefore seemed that the cause of the lag in phosphorylation might be the slow displacement of the inhibitor protein under conditions of low energy input. (That the lengths of the lags were not identical is attributed to the different conditions in the two incubations. In particular, the presence of dithiols in the ATPase assay would tend to stimulate release of the inhibitor protein even in the absence of energy input.)

To test this hypothesis, chloroplasts were prepared in which part of the inhibitor protein had been displaced from its inhibitory site. Chloroplasts were illuminated in the presence of an electron transfer mediator and dithioerythritol, and the preparation freed of dithioerythritol by repeated centrifugation in the dark. These chloroplasts had a stimulated ATPase activity stable for several hours in the absence of Mg^{2+} [11].

It is shown in Fig. 6 that chloroplasts thus treated synthesised significant amounts of ATP at low rates of energy input without the requirement for multiple flashes seen with untreated chloroplasts. The results of two experiments, involving a small (Fig. 6B) or larger (Fig. 6A) number of flashes, are given. It can also be seen that pretreatment had little effect on chloroplasts illuminated in the absence of DCMU, where no lag occurred even without preincubation. The abolition of the lag was not complete, probably because displacement of the inhibitor protein was only partial, and also because some decay to the inhibited state is unavoidable prior to phosphorylation. When this decay was accelerated, by the addition of Mg^{2+} [11], phosphorylation fell towards the level seen in untreated chloroplasts (Fig. 6A and B). We conclude, therefore, that the lag in flash-induced phosphorylation observed at low rates of energy input is a consequence of interaction between the ATPase and its inhibitor.

In all these experiments, rates of phosphorylation were less than 0.15 mol ATP/mol ATPase per flash. Table II shows the maximum rates observed over a series of experiments of the type shown in Figs. 5 and 6. In every case, a low efficiency of phosphorylation was observed; if there were about 2 mol ATPase per Photosystems (I + II) [21], and one site for energy conservation per photosystem [22], saturating single turnover light flashes might be expected to yield 2 mol ATP/mol ATPase per flash. This low rate of ATP formation was not due to the turnover capacity of the assembly of active ATPases, since, during steady

TABLE II

MAXIMUM PHOSPHORYLATION RATES INDUCED BY SINGLE TURNOVER FLASHES

Phosphorylation, measured as in Figs. 5 and 6, is expressed as average number of mol ATP/mol ATPase per flash (i.e., slopes of the lines in Figs. 5 and 6) over the range of flash numbers indicated. These numbers represent the maximum rates obtained over several preparations of chloroplasts.

Incubation conditions	Phosphorylation rate (mol ATP/mol ATPase per flash)	
	Flash nos. 5—50	Flash nos. 80—140
100 Hz	0.135	0.15
12.5 Hz	0.01	0.10
100 Hz, 5 μM DCMU	0.002	0.06
100 Hz, inhibitor-depleted chloroplasts	0.12	0.12
100 Hz, 5 μM DCMU, inhibitor-depleted chloroplasts	0.025	0.12

TABLE III

EFFECT OF K^+-VALINOMYCIN ON THE TIME COURSE OF PHOTOPHOSPHORYLATION

Flash-induced phosphorylation was measured as described in Fig. 4, in the presence of 200 μM ADP. Other additions were as indicated. The results are expressed as mol ATP labelled/mol ATPase during the total number of flashes indicated. The flash rate was 100 Hz.

Addition	ATP made up to flash number (mol/mol ATPase)		
	25	50	125
None	2.70	5.68	not determined
5 μM DCMU	0.22	0.18	2.24
40 μM valinomycin + 20 mM KCl	0.0	0.0	4.98

state phosphorylation, these chloroplasts showed ATP synthesis rates of 2 μmol/min per mg chlorophyll (see Methods), i.e., about 30 mol ATP synthesised/mol ATPase per s, which is twice as fast as the rate observed even at 100 Hz. Further, the turnover capacity of the ATP synthases is almost certainly faster than the rate of phosphorylation in continuous light [2].

Thus, during repeated flashes, something other than energy input or ATPase turnover capacity must limit photophosphorylation. This conclusion is supported by the finding that "activated" rates of phosphorylation in the presence or absence of DCMU were almost identical (in spite of the differences in energy input rate) (Table II). In view of the experiments shown in Fig. 6, it seems probable that the rate of phosphorylation was, in general, limited by the number of ATPase molecules available for turnover, i.e., those in which the inhibitor had been displaced.

As is shown in Table III, K^+-valinomycin caused a lag to occur in flash-induced phosphorylation rather as did DCMU, although the combination does not inhibit steady-state phosphorylation at these concentrations. Ort et al. [8]

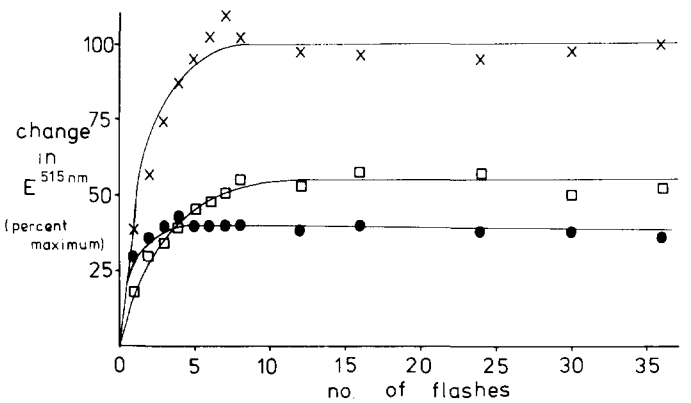

Fig. 7, Induction of membrane potential by multiple flashes. The change in absorbance at 515 nm of a suspension of chloroplasts (20 μg chlorophyll/ml) was measured during short flashes from a xenon flash lamp (Wingent, Cambridge, 200 W "snaked" tube to special design) as described previously [15]. Each flash discharged 3.5 J. The chloroplasts were suspended in a buffer containing 50 mM NaCl/2 mM Tricine/1 mM $MgCl_2$/10 μM pyocyanine (pH 7.8 with NaOH) and DCMU (5 μM) was present as indicated. The peak height after a given number of flashes is shown. X———X, 100 Hz; •———•, 12.5 Hz; □———□, 100 Hz + 5 μM DCMU.

have shown that K^+-valinomycin also increases the lag seen in phosphorylation induced by continuous light. They interpreted their results as showing a dependence of the lag period on the membrane potential.

Flash-induced energisation of the membrane

Fig. 7 shows that the change in absorbance at 515 nm (the "carotenoid" or "electrochromic" shift) measured in these chloroplasts reaches a maximum after about 5 flashes. The height of the maximum depended on the frequency of the flashes (since the half time for the decay of the shift was comparable with the time between flashes), and upon whether one or both Photosystems were operating (±DCMU).

The absorbance change at 515 nm reflects the potential across the thylakoid membrane ($\Delta\psi$) [15]. These results thus indicate that the requirement for multiple flashes in phosphorylation, at low rates of energy input, did not reflect a failure in electron transport or in membrane integrity. The different steady-state levels of membrane potential reached, however, might provide a rationale for the different lags in phosphorylation. This is discussed further below.

Fig. 8 shows that the extent of H^+ uptake by thylakoids increases only slowly during repeated flashes relative to the steady-state level seen in continuous light (cf. ref. 7). Only 20% of the steady state level was reached after 100 flashes. Lowering the flash rate from 100 Hz to 12.5 Hz had no effect on the flash yield of the H^+ uptake, presumably because of the relatively slow decay of the pH gradient. We have seen above, however, that such a change had a large effect on the lag in phosphorylation (Fig. 4) making it unlikely that, under

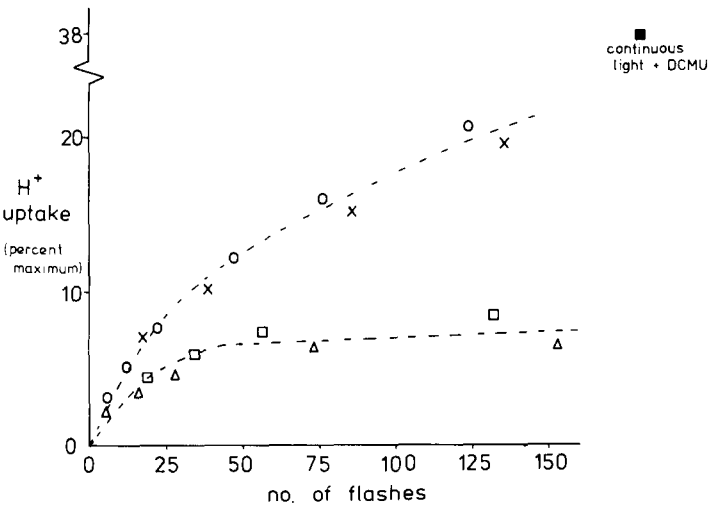

Fig. 8. Proton uptake induced by multiple flashes. Chloroplasts (50 μg chlorophyll/ml) were suspended in 150 mM NaCl/1 mM $MgCl_2$/10 μM pyocyanine equilibrated with air (pH 6.6—6.8 *). The pH of the solution was measured during flash illumination (as in Fig. 2), and the uptake of H^+ due to the flash group was estimated from the pH change induced by a group of flashes. DCMU was present as indicated. X———X, 100 Hz; ○———○, 12.5 Hz; □———□, 100 Hz + 5 μM DCMU; △———△, 12.5 Hz + 5 μM DCMU. * Similar results were obtained using a solution lightly buffered at pH 7.8, using cresol red as an indicator of the pH change [27]. These experiments were performed in collaboration with Dr. S. Saphon.

these conditions, the pH gradient is involved in the processes responsible for the lag. DCMU lowered both the flash yield of H^+ uptake, and the level of uptake seen in continuous light (Fig. 8).

Flash-induced labelling of nucleotides.

It was shown above (Figs. 4—6) that flashes of light induce chloroplasts to catalyze the incorporation of $^{32}P_i$ into organically-bound ^{32}P-label. The distribution of this label is shown in Fig. 9. Virtually all the organic label was present in adenine nucleotides, and the γ-position of ATP was labelled much faster than the β-positions of ADP and ATP. This is consistent with the view that ADP is the initial phosphate acceptor in phosphorylation but does not prove this point (see below). These findings are in agreement with the results of Aflafo and Shavit [20] using continuous light, and with Smith et al. [2] studying phosphorylation induced by an acid-base transition, but not with the results of Boyer et al. [23], using continuous light, where a rapid labelling of the β-position of ATP was observed. The reason for this discrepancy is uncertain.

The chloroplast ATPase, in its isolated and membrane-bound forms, bears tightly-bound ATP and ADP, a total of 2 mol ATP and 1 mol ADP per mol ATPase [13]. Fig. 10 shows the exchange of these nucleotides against added ATP and ADP induced by single turnover flashes. As with phosphorylation, at a flash rate of 100 Hz, the exchange started within the first few flashes and proceeded to more than 1 mol nucleotide exchanged per mol ATPase. Owing to instrumental limitations it was not possible to increase the number of flashes sufficiently to see if all the nucleotides could eventually exchange. Addition of P_i did not affect the rate of exchange against ADP (not shown).

Fig. 10 also shows that the amount of bound nucleotide exchange per flash

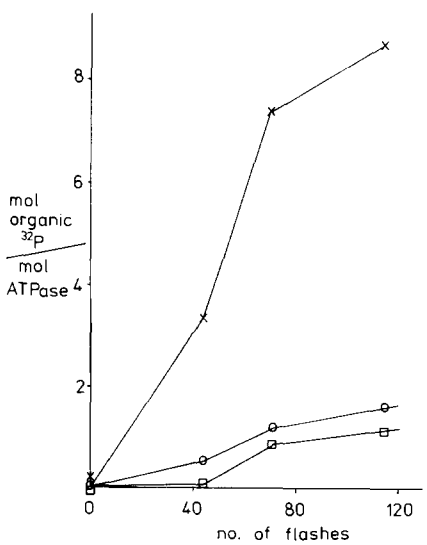

Fig. 9. Distribution of label during flash-induced phosphorylation. Phosphorylation was carried out as in Fig. 2 (100 Hz), except that $^{32}P_i$ was present at a specific activity of 16 000 cpm/nmol and the total volume was 8 ml. Distribution of label among the nucleotides was determined as previously [11]. X——X, γ-^{32}P in ATP; o——o, β-^{32}P in ADP; □——□, β-^{32}P in ATP.

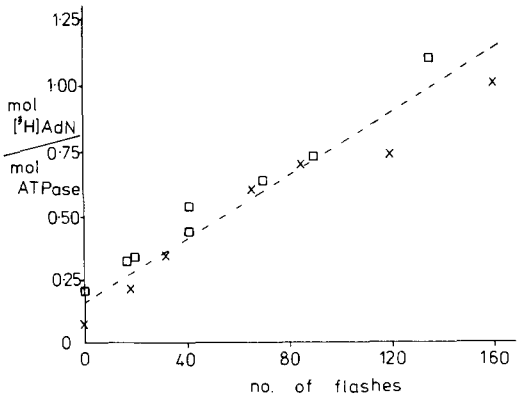

Fig. 10. Exchange of bound nucleotide with added nucleotide induced by light flashes. Chloroplasts were incubated as in Fig. 5, except that the total volume was 8 ml, $^{32}P_i$ was omitted and ADP replaced by [^{3}H]ATP or [^{3}H]ADP (7500 cpm/nmol). After illumination, the chloroplasts were expelled from the cell into a solution of NH_4Cl (pH 7.8, final concentration 10 mM) to stop further exchange, and incorporation of label into the bound nucleotides measured as described [13]. In the case of exchange against ATP, 1 mM phosphoenol pyruvate and 8 U (μmol/min) ammonia-free pyruvate kinase were also present. The flash rate was 100 Hz. □———□, Exchange against [^{3}H]ATP; X———X, exchange against [^{3}H]ADP.

is less than the phosphorylation per flash (Table II) (0.007 mol exchanged/flash as compared to 0.12 mol ATP made/flash) over about 100 flashes. This is seen more clearly from Table IV, where phosphorylation and exchange were measured on the same preparation of chloroplasts. This is discussed further below, in the light of proposals that the bound nucleotides are directly involved in the phosphorylation mechanism [13].

Discussion

It is shown above that release of the inhibitor protein from its inhibitory site on the ATPase (as indicated by induction of ATPase activity) is rapid enough to precede phosphorylation, both coming within 3 flashes at 100 Hz (Figs. 2–6). These data also show that, if the displacement of the inhibitor protein is delayed, by lowering the rate of energy input, (Figs. 2 and 3), the onset of

TABLE IV

PHOSPHORYLATION AND EXCHANGE OF BOUND NUCLEOTIDES INDUCED BY LIGHT FLASHES

Chloroplasts were incubated as in Fig. 10 except that 2 mM $^{32}P_i$ (2500 cpm/nmol) was present in addition to [^{3}H]ADP. Total phosphorylation, and [^{3}H]adenine incorporation into the bound nucleotides, after flash groups of varying length were measured as described.

No. of flashes	Total ^{32}P esterified	[^{3}H]Adenine bound
	(mol/mol ATPase)	
0	1.60	0.164
44	4.76	0.690
71	7.05	0.839
115	7.95	1.03
Mean rate	0.06/flash	0.008/flash

phosphorylation is similarly delayed (Figs. 5 and 6), thus implicating the displacement of inhibitor as a necessary initial step in phosphorylation.

Pretreatment, by illumination in the presence of dithioerythritol (followed by dark adaptation), leads to chloroplasts in which the initial "lag" in phosphorylation at low rates of energy input is partially abolished (Fig. 6). This finding can only be explained if the lag is governed by some control element, and not if the lag is due solely to the energy state of the chloroplasts at the time of measurement, as is assumed in the treatment of Gräber and Witt [7]. Since this pretreatment is known to displace the ATPase inhibitor protein [11], it is concluded that this control element is the inhibitor protein.

These conclusions are consistent with the view that the ATPase site of the coupling ATPase catalyses both the forward (phosphorylation) and reverse (ATPase) reactions in coupled chloroplasts, and that the inhibitor protein inhibits both processes. Under the conditions of the experiments above, it seems likely that the number of activated ATPase molecules limits the rate of phosphorylation, certainly at low rates of energy input (where up to 50 flashes may give no phosphorylation at all). Even at the highest flash rates (100 Hz), phosphorylation rates are less than half the maximum turnover of the assembly of ATPase molecules present, probably because a considerable proportion of the ATPase molecules are in the inhibited state.

The establishment of the ATPase inhibitor as the limiting factor in flash-induced phosphorylation opens the question as to whether it might also limit phosphorylation in continuous light. Ort and Dilley [4] have recently shown that an approximately 4 ms lag is seen prior to phosphorylation induced by continuous light in chloroplasts. It is shown above (Figs. 4—6) that at high flash frequencies less than 50 μs of light is needed to initiate phosphorylation, if this light is spaced over 20—40 ms. It appears therefore that some dark process is limiting phosphorylation during this lag phase. The relatively low rate of this process, in the ms time range, is compatible with a protein conformational change such as the displacement of the inhibitor protein.

The displacement of the inhibitor protein is "energy-dependent", i.e., it requires electron transfer in coupled chloroplast membranes. It is not triggered by a single flash, since it is affected by the spacing between flashes (Figs. 2 and 5), suggesting that a direct conformational transfer of energy is unlikely to be involved. Neither does the displacement appear, under these conditions, to depend on the pH gradient across the membrane, since the gradient is not affected by the spacing between flashes (Fig. 8).

The most obvious candidate to govern the displacement of inhibitor is the membrane potential (ψ), as mirrored by the absorbance change at 515 nm [15]. This does differ (in mean value attained) with the spacing between flashes (Fig. 7) and could thus account for the different lags seen in phosphorylation, (i.e., different lengths of time taken for the displacement of the inhibitor) at different energy input rates. Under the conditions used here, the lag appeared to depend on the membrane potential raised to a fairly high power (ψ^4 or ψ^5) since at lower energy input rates a relatively small change in the mean value of the potential (going from 100 Hz + DCMU to 12.5 Hz without DCMU) (Fig. 7) leads to a large change in the lag period (750 ms to 4800 ms) (Figs. 5 and 6).

Further evidence that the membrane potential is of major importance in the

displacement of the inhibitor protein in chloroplasts comes from the induction of a long lag preceding phosphorylation by K^+-valinomycin, both under flash conditions (Table III, above) and in continuous light [8].

It should be emphasised that the lag is not the time taken to build up the membrane potential, which is short (Fig. 7), but is due to a process whose rate depends on the steady state membrane potential attained. We might visualise the (charged) inhibitor protein moving electrophoretically in the field set up. In chloroplasts, the inhibitor is not released into free solution [16] because it is insoluble in water [9] and simply moves from one site on the ATPase to another. Possibly the electric field in the region of the ATPase is distorted to allow this to occur.

The experiments described in the last part of the experimental section above were designed to investigate the role of tightly-bound nucleotides [13] and β-labelling of ADP [24] on the coupling ATPase in the phosphorylation mechanism. Both β-labelling and exchange of bound nucleotides began with no apparent lag at a flash frequency of 100 Hz (Figs. 9 and 10), as does phosphorylation. Even at overall levels of less than one mol of label incorporated per mol ATPase however, the flash yield of bound nucleotide exchange and of β-labelling was considerably lower than phosphorylation (Figs. 9 and 10 and Table IV). It might appear at first sight, therefore, that neither energy-dependent release of nucleotides nor β-labelling could be an obligatory step in phosphorylation.

It has been demonstrated above, however, that the phosphorylation rate induced by repeated flashes is limited by the number of ATPase molecules from which the inhibitor protein is displaced. This means that the thylakoid membranes bear a heterogeneous population of ATPase molecules, some inhibited and some phosphorylating. Under these conditions, such a simple analysis of these final experiments is not possible since, if a given activated ATPase molecule turned over several times while an inhibited molecule did not turn over at all, the rate of phosphorylation observed (1 ATP made/turnover) would exceed the rate of labelling at the ATPase itself (one/active ATPase, independent of number of turnovers) even at levels of label less than one/ATPase molecule. These results are thus compatible with either of the processes involved in labelling the bound nucleotides being on the main pathway, or on a side reaction of phosphorylation.

In submitochondrial particles, it has been shown that exchange of the nucleotides bound to the coupling ATPase occurs only on ATPase molecules from which the inhibitor protein has been displaced [25,26]. If this is true also for chloroplasts, then, independent of the mechanism of this exchange, the data of Fig. 10 do provide information as to the dynamics of the ATPase-inhibitor interaction. The fact that the exchange increases, approximately linearly, up to more than 100 flashes can be explained only if a dynamic equilibrium exists between inhibited and activated ATPase molecules, and not if a fixed small section of the population of ATPase molecules is most susceptible to energisation (and thus continually active here). Any given ATPase molecule, once activated, is able to turn over several times (here about ten), but will eventually collapse back into the inhibited state, while other, inhibited ATPase molecules become activated (under the influence of the membrane potential). Re-inhibition of ATPase molecules is presumably independent of energy, since it continues

when the light is switched off, rapidly inhibiting the ATPase activity of chloroplasts in the dark.

In summary, therefore, we may represent the ATPase-inhibitor system in the form

$$\underset{\text{inhibited enzyme}}{E-I} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E^*$$

with E^* the form of the ATPase capable of ATP synthesis and hydrolysis. k_{-1} is independent of energy, while k_1, at least under the conditions used here is a function of ψ^n ($n \approx 5$). Phosphorylating thylakoid membranes in general bear an assembly of inhibited ($E-I$) and activated (E^*) ATPase molecules in a dynamic equilibrium, the ratio $E^*/E-I$ increasing with energisation of the membrane and, under some conditions at least, limiting to the rate of phosphorylation.

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