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PHOTOPHOSPHORYLATION AS A FUNCTION OF ILLUMINATION TIME

I. EFFECTS OF PERMEANT CATIONS AND PERMEANT ANIONS

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SUMMARY

(1) Very brief periods of illumination do not initiate photophosphorylation in isolated chloroplast lamellae. The time of illumination required before any phosphorylation can be detected is inversely proportional to the light intensity. At very high intensities, phosphorylation is initiated after illumination for about 4 ms.

(2) There is no similar delay in the initiation of electron transport. The rate of electron transport is very high at first but declines at about the time the capacity for ATP synthesis develops. When the chloroplasts are uncoupled with gramicidin the high initial rate persists.

(3) Various ions which permeate the thylakoid membrane (K^+ or Rb^+ in the presence of valinomycin, SCN^-, I^-, or ClO_4^-) markedly increase the time of illumination required to initiate phosphorylation. Potassium ions in the presence of valinomycin increase the delay to a maximum of about 50 ms whereas thiocyanate ions increase the delay to a maximum of about 25 ms. The effects of K^+ with valinomycin and the effect of SCN^- are not additive. Permeant ions and combinations of permeant ions have little or no effect on phosphorylation during continuous illumination.

(4) The reason for the threshold in the light requirement and the reason for the effect of permeant ions thereon are both obscure. However, it could be argued that the energy for phosphorylation initially resides in an electric potential gradient which is abolished by migration of ions in the field, leaving a more slowly developing proton concentration gradient as the main driving force for phosphorylation during continuous illumination. If so, the threshold in the presence of permeant ions should depend on internal hydrogen ion buffering.

INTRODUCTION

There are a great many reasons for believing that a transmembrane proton activity gradient is somehow involved as the driving force for photophosphorylation

Abbreviations: HEPES, *N*-2-hydroxymethylpiperazine-*N*-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; tricine, *N*-tris(hydroxymethyl)methylglycine.

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(for reviews, see refs. 1, 2). Mitchell [3] has proposed that this gradient results from the oxidation of hydrogen donors inside the thylakoid; water oxidation being one site of hydrogen ion production and the oxidation of plastohydroquinone by cytochrome *f* the other. Thus, as a result of the transfer of two electrons outward across the membrane, two protons are left inside and the thylakoid is positively charged inside. Mitchell has further proposed that the transmembrane electrochemical potential gradient is coupled to ATP synthesis by the hydrogen ion-conducting coupling factor which is located on the photosynthetic membrane, using mechanisms as yet undefined.

This hypothesis is supported by the following observations: (a) there are indeed two coupling sites along the chloroplast electron-transport chain that are correlated with proton-releasing electron transport steps [4, 5]; (b) the oxidation of hydrogen donors such as water and catechol by Photosystem II [6] and the oxidation of hydrogen donors such as diaminodurene by Photosystem I [7] both support phosphorylation whereas the oxidation of electron donors such as ferrocyanide and iodide by Photosystem II [8] and the oxidation of electron donors such as *N,N,N',N'*-tetramethyl-*p*-phenylenediamine by Photosystem I do not [9]; (c) electron transport does cause the acidification of the inside of the thylakoid [10, 11] and the efflux of the accumulated protons can cause ATP formation [12, 13]. Furthermore, the ATP yield from pre-illuminated chloroplast membranes can be enhanced by agents which increase the inside-positive potential (e.g. by valinomycin plus KCl added in the dark stage) [14, 15]; (d) many substances, which promote the transport of protons across the membrane and allow accumulated protons to escape, uncouple electron transport from phosphorylation [16].

From the evidence itemized above, it is clear that the mechanism proposed by Mitchell is capable of phosphorylation. Certainly most of the components of his chemiosmotic model are operative and many of the predictions of the model have been confirmed. But questions remain to be answered and particulars of many of the reactions have yet to be described. We do not know how the coupling factor works, we do not know exactly where the gradients are in the lamellar system, and we do not know the nature of the gradients at any particular stage in the initiation and maintenance of the phosphorylation process. For that matter, we cannot yet be sure that the chemiosmotic mechanism is actually responsible for steady-state phosphorylation. We can only conclude, on the basis of our current information, that it could be responsible.

In this paper and its sequel [37], we report on investigations of some predictions of this chemiosmotic model of phosphorylation which had not been adequately tested before. The model requires that a critical proton activity gradient be formed across the thylakoid membrane before ATP can be synthesized. This activity gradient could consist of either a proton concentration gradient, a transmembrane electric potential difference, an intramembrane electric field*, or any combination of the

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* In this paper we have distinguished between "transmembrane" potentials and "intramembrane" potentials. A transmembrane potential is a property of the membrane as a whole and describes the electromotive force acting between the aqueous suspending medium and the aqueous inside of the thylakoid vesicle. It can best be measured by its effects on the equilibrium distribution of ions on the two sides of the membrane. An intramembrane potential describes an electric field across some region within the membrane. It can be measured if pigment molecules such as carotenoids lie in the field since such pigments then undergo a change of absorption spectrum. Transmembrane potentials are,

three. There have been many attempts to measure the potential across the membrane (for reviews, see refs. 17, 18) and the pH of the inner aqueous space (e.g. 11, 19). However, there have been surprisingly few attempts to correlate the onset of phosphorylation with the development of membrane potentials and pH gradients immediately after the light has been turned on. Our papers deal with new attempts to determine the amount of electron transport required to initiate phosphorylation. We have determined phosphorylation directly by measuring the incorporation of $^{32}P_i$ into ATP, using illumination times as brief as 3 ms. In this paper, we have tried to evaluate the role of membrane potential in the early events by investigating the effects of permeant ions. These permeant ions should migrate in an electric field and, if their mobilities and concentrations are sufficient, they should dissipate any transmembrane potential. In the second paper [37], we report on the effects of permeant hydrogen ion buffers. Internal buffers must hinder acidification of the inside of the thylakoid to an extent which can be calculated from the amount inside, and therefore they must delay the development of pH gradients for a predictable length of time.

Preliminary reports of some of these experiments have appeared elsewhere [20, 21].

MATERIALS AND METHODS

Chloroplasts. Chloroplasts (intact, naked lamellae) were isolated from fresh market spinach (*Spinacia oleracea* L.) as described elsewhere [6].

Actinic light. Light from a 24 V-150 W tungsten-halogen lamp (approximately 1200 Kergs $\cdot$ cm^{-2} $\cdot$ s^{-1} of heat-filtered white light) was focused on the aperture of a Uniblitz model 26 electronic shutter. The shutter was controlled by two timing circuits (based on Signetics 555 timers) connected in series to regulate both the time open and time closed. Using an EG & G Lite-Mike in conjunction with a storage oscilloscope, it was determined that the opening and closing times of the shutter were each about 0.7 ms. Calibration of the timing circuits was accomplished using a Heathkit model SM 102-A programmable timer.

Determination of ATP formation. ATP formation was measured from the residual radioactivity after two extractions of the remaining ^{32}P-labeled orthophosphate as phosphomolybdic acid in butanol/toluene [22]. Further extraction with butanol/toluene did not remove any additional radioactivity from the labeled ATP-containing aqueous phase. $^{32}P_i$ was obtained from ICN because their product was found to have the lowest content of unextractable pyro- and polyphosphates. Careful controls were run to correct for the radioactivity attributable to these contaminants. Spinach chloroplasts are known to have a membrane-bound alkaline pyrophosphatase [23]. The activity of the enzyme made the background level of radioactivity a

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of course, the resultant of all the local intramembrane potentials and the distinction would be purely formal if the thylakoids were homogeneous with uniform properties and uniformly distributed gradients. But the thylakoids are not homogeneous and therefore transmembrane potentials as measured by ion distributions and intramembrane potentials as measured by electrochromic effects do not necessarily coincide, either in magnitude or in time-course of development. The same distinction should be made between transmembrane and intramembrane proton concentration gradients but, unfortunately, there is no known way of measuring local proton concentration differences.

time-dependent function and this was allowed for. Radioactivity was determined by Cerenkov radiation in a Beckman DPM-100 scintillation counter.

In a number of experiments ATP was trapped as glucose 6-phosphate by adding hexokinase (1 μg/2 ml) and glucose (10 mM). This procedure was not used regularly since it made no difference in the results. However, it is clear from the absence of any effect of trapping that our data were not affected by ATPase activity.

Determination of ferricyanide reduction. The photochemical reduction of ferricyanide by illuminated chloroplasts was measured in an Aminco DW-2 dual wavelength spectrophotometer. Reduction was measured as the decrease in absorbance at 420 nm with a reference wavelength of 450 nm. This wavelength selection alleviated complications due to light-scattering changes. (The absence of light-scattering effects was confirmed by making similar measurements using methylviologen as electron acceptor, in which case no change in absorbance at 420 nm was expected and none was observed). The absorbance change resulting from the complete reduction of 0.200 μmol of ferricyanide was used to calculate electron fluxes. Changes in absorbance resulting from a number of light periods, separated by 15 s of dark, were summed in order to increase accuracy.

Similar results were obtained when the ferrocyanide produced was measured by the method of Jagendorf and Hind [24].

Measurement of pH change. Changes in the hydrogen ion concentration of the reaction mixture were detected optically employing the pH-indicating dye cresol red as described by Junge and Auslander [25]. The optical changes of cresol red due to changing pH were followed at 574 nm with the Aminco DW-2 spectrophotometer, using 540 nm as the reference wavelength. For improved time resolution, the signal from the spectrophotometer was fed into a Nicolet model 1072 instrument computer before replotting on an X-Y recorder. At the end of each experiment, the pH changes registered on the chart paper were translated into H^+ equivalents by titrating the reaction mixture in the light with a known amount of 0.001 M HCl. The reaction vessel was thermostated at 19 °C. When cumulative effects were being looked for, a conventional glass electrode and pH meter were also used. In no instance were the dye and electrode measurements of pH changes significantly different.

Determination of internal chloroplast volume. The technique employed was very similar to that used by Gaensslen and McCarty [11]; 0.1 ml of a 10 % sucrose, 0.5 % Triton X-100 solution was added to a 0.4-ml polyethylene centrifuge tube. Silicone fluid (0.1 ml) was layered on top of the sucrose solution followed by 0.1 ml of chloroplast suspension containing 80–130 μg chlorophyll in the appropriate reaction mixture. Details of reaction mixtures and silicone fluids used are given in the legends of the figures and tables. Prepared tubes were centrifuged in a Beckman 152 Microfuge.

Internal chloroplast volume was calculated from the amount of ^3H_2O found in the bottom aqueous phase after centrifugation. [^{14}C]inulin (M_r = 5000) was used to determine the percentage of the ^3H_2O which was simply trapped by the chloroplasts and not part of the internal aqueous space (see ref. 11 for details). After centrifugation, the tubes were frozen in solid CO_2 and sliced to separate the aqueous phases. Chlorophyll determinations were made on each aqueous sample after centrifugation.

The bottom phase, which routinely contained 92–96 % of the chlorophyll, was placed in a scintillation vial containing 1 ml of 30 % H_2O_2 and 5 % sodium lauryl sulfate. The vials were tightly capped and placed in an oven (45 °C) for 24 h. Radio-

activity was then determined by liquid scintillation counting using external standard ratios to monitor quenching. The scintillation fluid used was Tritosol [26].

RESULTS

Electron transport threshold of photophosphorylation

We examined the relationship between the amount of ATP formed and the amount of electron transport over a range of illumination times from 3 ms to several thousand ms. Accurate determination of the very small amounts of ATP synthesized as a consequence of the shorter illumination times was difficult. Therefore it was useful to sum the ATP yields from a number of light periods. Routinely, each light period was separated from the next by a 15 s dark period, which was a sufficient amount of time for the light-induced reversible pH change to relax. Occasionally, much longer dark periods were used and did not in any way change the results. The techniques of using orthophosphate highly labeled with $^{32}P_i$ (approximately 0.05 mCi

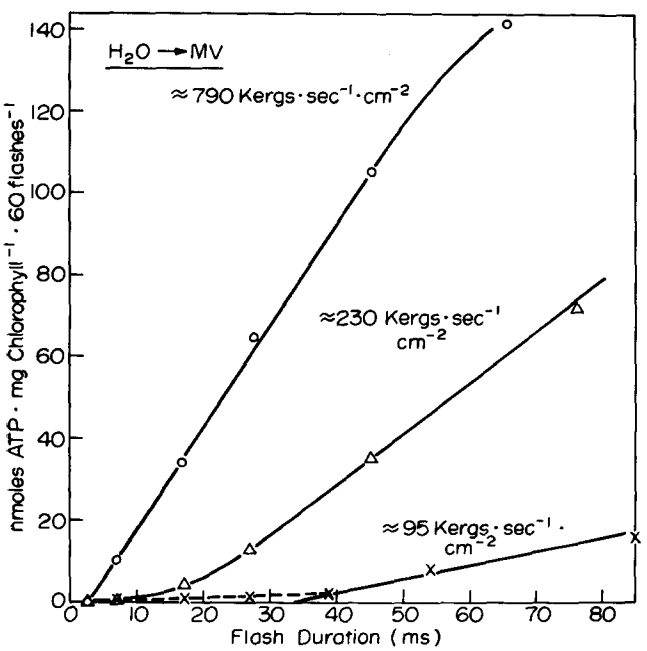

Fig. 1. ATP formation resulting from brief periods of light and the dependence of this ATP formation on light intensity. The 2-ml stirred reaction mixture contained: 0.1 M sucrose, 2 mM $MgCl_2$, 50 mM Tricine-NaOH buffer (pH 8.0), 1 mM ADP, 5 mM $Na_2H^{32}PO_4$, 100 μM methylviologen and chloroplasts containing 60 μg chlorophyll. The intensity of the heat-filtered (Corning CS 1-69) white light was measured with a YSI radiometer. Light intensity determinations had to be made outside of the reaction vessel since the probe was too large to fit inside. Intensity of the actinic beam was varied with a series of neutral-density filters. The reaction temperature was 14 °C. ATP formation was determined from a series of repetitive flashes each separated by a 15-s dark period. 0.5 ml of perchloric acid (1 M) was added to all tubes immediately after the last flash. The samples were then rapidly frozen in ethanol/solid CO_2. Results were almost exactly the same when ferricyanide replaced methylviologen as electron acceptor.

per 2 ml reaction mixture) proved to be a sensitive and reproducible assay for the amounts of ATP with which we were dealing. Most phosphorylation reactions were carried out at 12 or 14 °C, at which temperatures phosphorylation activities of these chloroplast preparations did not decrease appreciably during the 8 to 12 min required to complete a series of flashes.

Our experiments revealed that the onset of phosphorylation is not coincident with the beginning of illumination. That is to say, very brief flashes of light do not initiate ATP formation. However, the illumination time required to initiate phosphorylation is nearly three orders of magnitude shorter than some of the times previously reported [27, 28]. The relationship between the time of illumination and the amount of ATP formed is shown in Fig. 1. At high light intensities, approximately 4 ms of illumination are required before any ATP synthesis occurs; further increase in light intensity did not further reduce the time of illumination before ATP formation began nor increase the rate once it did begin. As the intensity of the actinic light is reduced, the time of illumination required to initiate phosphorylation becomes longer

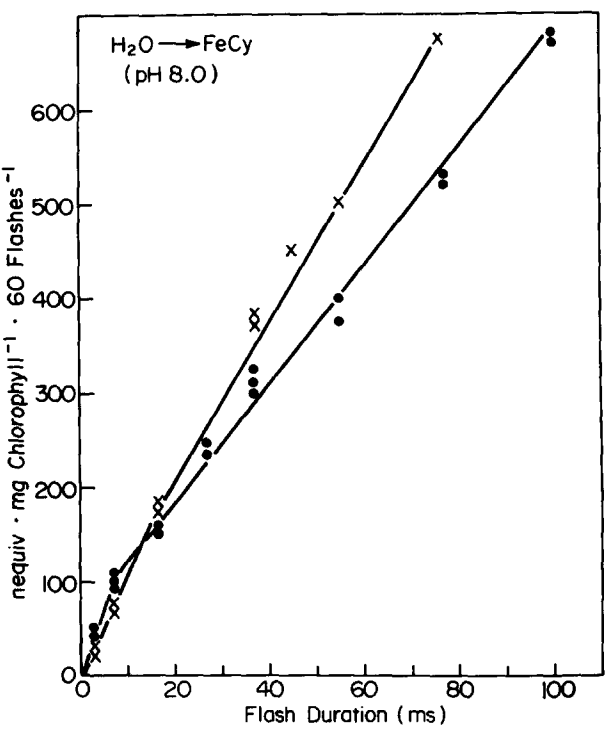

Fig. 2. Initial kinetics of the transport of electrons from water to ferricyanide under phosphorylating and uncoupled conditions. The 2.0-ml reaction mixture contained: 0.2 M sucrose, 2 mM $MgCl_2$, 10 mM KCl, 20 mM Tricine-NaOH (pH 8.0), 0.1 mM ferricyanide, 1 mM ADP, 5 mM Na_2HPO_4 and chloroplasts containing 30 μg chlorophyll ($T = 19$ °C) (● — ●). Uncoupling was obtained by adding 2 μg gramicidin D per ml (× — ×). The absorbance change (420–450 nm) resulting from the photochemical reduction of ferricyanide was determined from a series of light flashes each separated by a 15 s dark period. The greatest number of flashes used was 60 for flash time $\leq$ 20 ms. Smaller numbers of flashes were used for longer illumination times.

and over a considerable range of light intensities the duration of the lag in the initiation of phosphorylation seems to be inversely proportional to the intensity.

Fig. 1 illustrates experiments in which methylviologen was used as the electron acceptor, but the results were always the same when ferricyanide was used as the acceptor.

Electron transport does not show the lag observed in ATP formation. Rather, there is an initial phase of rapid transport which lasts about the same length of time as the lag in ATP formation (Fig. 2). Kelly and Sauer [29] have observed a similar burst in the ferricyanide reduction rate. The effect of the uncoupling ionophore gramicidin D on the time-course of electron transport is of interest, since it suggests that the attenuation of the initially high rate under phosphorylating conditions is due to a rate-limitation imposed by the phosphorylation-coupled reactions; the implication is that the initial rate of electron transport is high because the transport is initially uncoupled, even in the absence of uncouplers, and the rate remains high in the presence of the uncoupler gramicidin because the transport remains uncoupled.

The effects of permeant ions on the time of initiation of phosphorylation

The addition of valinomycin and KCl increases tenfold the length of the illumination period required before phosphorylation can begin (Fig. 3). Raising the concentration of the KCl from 10 to 100 mM does not further increase the light requirement, nor does raising the concentration of valinomycin from 0.1 to 0.5 μM. Valinomycin without KCl has very little of this effect, especially in chloroplasts which have had their endogenous potassium depleted (see legend to Fig. 3 for details). However rubidium can replace potassium.

In spite of the fact that valinomycin and KCl almost completely inhibit phosphorylation for the first 45–50 ms, subsequent phosphorylation is hardly affected at all (Table I and Fig. 3).

Gramicidin S is reported to be selective for alkali metals over hydrogen ions at low concentrations [30]. Gramicidin S (10 nM) has effects qualitatively similar to the effects of valinomycin, except that the potassium dependence is not as complete (data not shown).

The implication of these observations is that potassium and rubidium ions freely cross the thylakoid membrane in the presence of valinomycin, abolish the membrane potential and, in so doing, somehow extend the lag in the onset of phosphorylation. It is of some importance, therefore, to know if these ions do in fact equilibrate across the membrane under the conditions of our experiments. It would seem that they do. In the presence of 0.5 μM valinomycin and either 10 or 50 mM RbCl, the uptake of $^{86}Rb^{+}$ increases linearly with the internal chloroplast volume. The concentration of rubidium inside the thylakoid can be calculated from the data of Fig. 4. Such calculations reveal that the rubidium concentrations on both sides of the membrane are virtually identical. The equilibration is complete within 30 s, the shortest time in which the measurement could be made by our technique.

Inward anion movements across the thylakoid membrane should have the same effect on the membrane potential as the outward movements of cations. Thiocyanate ions readily permeate chromatophore membranes [31] and therefore were tested in our chloroplast system. The effect of 5 mM SCN^{-} on the time-course of ATP formation is similar to the effect of valinomycin and KCl, except that the maximum

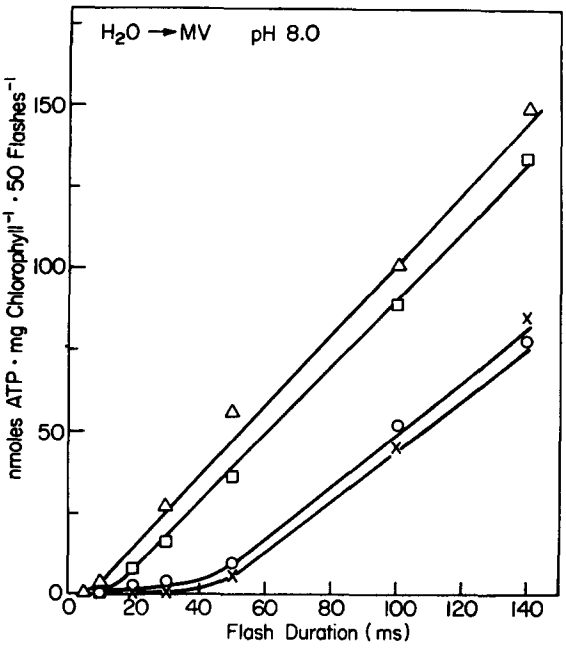

Fig. 3. Effects of valinomycin and KCl on the photophosphorylation resulting from short illumination periods. Conditions were as in Fig. 2 except that the reaction temperature was 12 °C. Concentration of valinomycin was 0.1 μM and the concentration of KCl was as follows (in mM): △—△, 0; □—□, 0.05; ○—○, 10; ×—×, 100. The light intensity measured outside the reaction vessel was approximately 450 Kergs · s^{-1} · cm^{-2} of heat-filtered white light. Chloroplasts used in this experiment were washed in a hypotonic medium to deplete them of residual potassium. The procedure for K^+ depletion was as follows. Chloroplasts were illuminated for 30 s with red light (400 Kergs · cm^{-2} · s^{-1}) in a medium consisting of: 33 mM sucrose, 5 mM $MgCl_2$ and 8 mM Tricine-NaOH (pH 8.0). The chloroplasts were then washed twice by centrifugation in the same medium. This procedure reduced potassium content from 0.7 μmol K^+/mg chlorophyll to 0.1 μmol K^+/mg chlorophyll.

TABLE I

EFFECTS OF PERMEANT IONS ON THE ATP FORMATION DURING CONTINUOUS ILLUMINATION

Reaction conditions are described for Fig. 1. The illumination time was 10 s. Reaction temperature was 15 °C. Additions were as indicated.

Addition	μmol ATP · mg chl^{-1} · h^{-1} ·	Inhibition (%)
None	170	0
0.1 μM valinomycin	148	13
0.1 μM valinomycin plus 10 mM KCl	142	17
20 mM KI	163	4
50 mM KI	139	18
100 mM NaCl	174	0
10 mM $NaClO_4$	137	19
5 mM NaSCN	144	15
0.1 μM valinomycin plus 10 mM KCl and 5 mM NaSCN	122	28

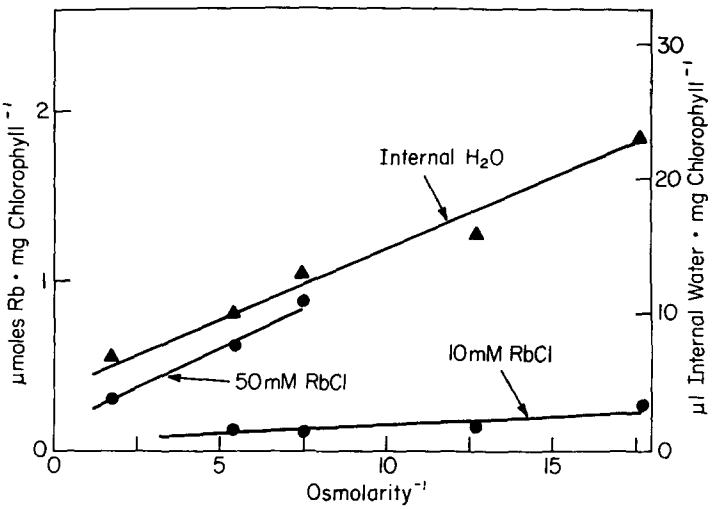

Fig. 4. Equilibration of rubidium across the thylakoid membrane in the dark. Rubidium content was measured after centrifugation of chloroplasts through silicone fluids as described in Materials and Methods. The 0.1 ml samples consisted of: 20 mM Tricine-NaOH (pH 8.0), 2 mM $MgCl_2$, 0.5 μM valinomycin, chloroplasts containing 80–120 μg chlorophyll and the indicated concentration of RbCl labeled with 0.5 μCi of ^{86}Rb. Osmolarity was varied by the addition of sucrose. The internal volume was determined from uptake of ^3H_2O in a reaction mixture identical to that used above, replacing ^{86}Rb with 1.0 μCi of ^3H_2O. The amount of ^3H_2O "trapped" by the membranes and not part of the internal volume was assumed to be same as "trapped" [^{14}C]-inulin (0.25 μCi/0.1 ml) solution. The silicone fluids used were mixtures of Versilube F-50 and SF-96 (50). Mixtures ranging from 2 : 1 (for least dense suspensions) to 4.75 : 0.25 (for most dense suspensions) by weight were employed. From this figure it is evident that the amount of rubidium inside the thylakoids depends on the internal volume and that the concentration of rubidium inside closely approximates the concentration in the suspending medium.

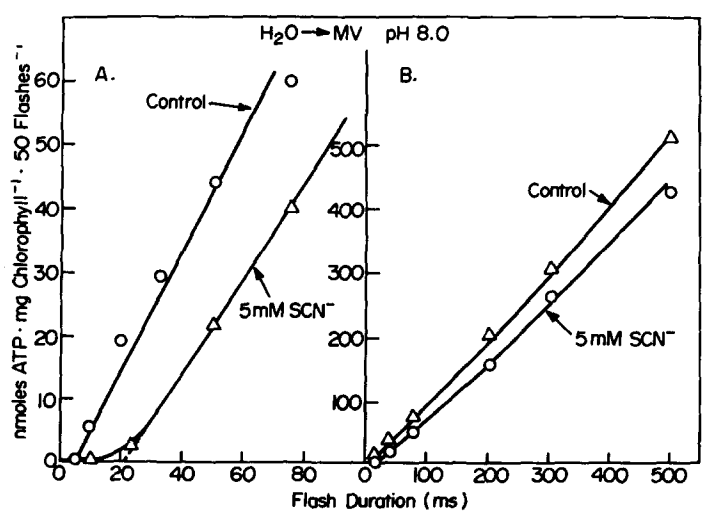

Fig. 5. Effect of NaSCN on the phosphorylation resulting from short illumination periods. Conditions were as in Fig. 3.

TABLE II

EFFECTS OF SCN^- AND VALINOMYCIN PLUS K^+ ON ATP FORMATION FROM SHORT FLASHES OF LIGHT

Reaction conditions are described in legend of Fig. 5. This Table shows that the inhibitions of ATP synthesis from short flashes caused by SCN^- and valinomycin plus K^+ are not additive.

Addition	Flash duration (ms)	nmol ATP · mg chl^{-1} · 50 $flashes^{-1}$	Inhibition (%)
None	20	21	0
	50	93	0
0.1 μM valinomycin	20	2	91
plus 10 mM KCl	50	36	61
5.0 mM NaSCN	20	10	48
	50	53	43
0.1 μM valinomycin	20	4	81
plus 10 mM KCl, 5.0 mM NaSCN	50	30	68

delay caused by SCN^- is only about half of the maximum delay caused by valinomycin with potassium or rubidium ions (Fig. 5). This implies that there is some qualitative difference in the mechanism, related perhaps to the fact that the anion is being accumulated rather than extruded in the light. Alternatively, the difference might be related to a difference in the numbers of sites accessible to anions and cations at some critical location. However, the maximum delay caused by valinomycin and KCl is not increased by SCN^-, and in this sense the effects of permeant anions and permeant cations are not additive (Table II).

Several other anions were tested. Schuldiner and Avron [32] have reported that chloroplast membranes show relatively high permeability to iodide, probably because of the formation of charge-delocalizing polyiodides [33]. Iodide ions (50 mM) completely inhibit ATP formation from a 30 ms flash of light (Table III), whereas the same concentration of iodide inhibits continuous-light phosphorylation by only 18 % (Table I and ref. 8). Chloride ions (100 mM) and perchlorate ions (10 mM) also

TABLE III

EFFECTS OF I^-, Cl^- and ClO_4^- on ATP FORMATION FROM 30 ms FLASHES OF LIGHT

Reaction conditions as in Fig. 5. Although permeant ions have little effect on steady-state photophosphorylation, they inhibit the photophosphorylation which is due to illumination periods of 30 ms or less.

Addition	nmol ATP · mg chl^{-1} · 50 $flashes^{-1}$	Inhibition (%)
None	31	0
100 mM NaCl	23	26
20 mM KI	18	42
50 mM KI	0	100
10 mM $NaClO_4$	11	65

severely inhibit the ATP synthesis from short illumination periods (c.f. Tables I and III), but not during continuous illumination.

DISCUSSION

Data presented in this paper confirm that very short periods of illumination do not cause any photophosphorylation. At least 4 ms of rapid electron transport are required before ATP synthesis is initiated (Figs. 1, 2). The time of illumination required before ATP synthesis can be detected is inversely proportional to the light intensity over a considerable range of intensities, as might be expected if a pool had to be filled by the photochemical processes. It would seem that some critical amount of electron transport is needed in order to produce the energetic state of the membrane which drives phosphorylation and, until this state has been achieved, the electron transport remains rapid.

The nature of this energetic state is not at all obvious. Observed absorbance changes at 515 nm [17] provide strong evidence that a sizeable intramembrane potential forms in a fraction of a microsecond. Yet, as we have seen, initiation of ATP formation takes several milliseconds of illumination. Since decay of the electrochromic absorption change is clearly related to phosphorylation, it is not unreasonable to suppose that the indicated electric field contributes to the energetic state by helping to push the protons out through the coupling factor, that is by adding to the protonmotive force. However, it appears from the delay in the onset of phosphorylation that this component of the protonmotive force cannot by itself drive phosphorylation and, indeed, a protonmotive force would be of little use unless protons were available. Thus, it is possible that the delay in phosphorylation reflects the need for hydrogen ions to serve as substrates for ATP formation. That is to say, an appreciable supply of protons may have to accumulate somewhere before they can be driven through the coupling factor, no matter how great the electric field propelling them. There are other reasons for espousing this concept. The fact that proton production per se, rather than electron transport and charge separation, is required for phosphorylation [8, 9] points in the same direction. The protons which are always present in water seem not to be available and, since the supply of protons in the inner aqueous phase of the thylakoid can scarcely be doubted, some question as to the role of this phase in phosphorylation arises.

A further conclusion to be drawn from our experiments is that the phosphorylation which ultimately starts in the presence of permeant ions must be independent of transmembrane potentials (as distinct from intramembrane potentials). Permeant ions do increase the lag in the onset of phosphorylation considerably, but the extent of the lag is independent of ion concentrations. The lag is not much shorter with 10 mM KCl than with 100 mM KCl (Fig. 3) nor is the lag further extended by permeant anions (Table II). Therefore the increased delay in the initiation of phosphorylation cannot represent the time required for a redistribution of ions and the consequent restoration of a transmembrane potential. This argument can be put in more quantitative terms: in the presence of valinomycin, an external concentration of 10 mM KCl would provide 8 times more internal K^+ ions than the number of H^+ ions produced by electron transport during the extended lag period. Thus, if one K^+ moved out of the thylakoid for every H^+ produced inside, there would be a

decrease of only 12 % in the internal potassium during the lag. But to restore a potential of only 60 mV would require the extrusion of 90 % of the potassium.

We must now consider the possibility that photophosphorylation requires a transmembrane potential as part of the protonmotive force for the first 50 ms, but that thereafter the transmembrane proton gradient component alone suffices. Such a situation is not difficult to envision. After a few milliseconds, the initially rapid rates of electron transport and proton accumulation begin to slow and at the same time protons presumably begin to move out of the thylakoid driven by the rising protonmotive force. Thus as the protonmotive force approaches a steady-state level, an electrically neutral proton exchange should develop, one proton being extruded for every proton produced inside. This should allow a lagging, charge-compensating movement of other ions to catch-up and nullify charge differences, leaving the pH gradient as the main component of the driving force. Such proton concentration-dependent phosphorylation should exhibit no further sensitivity to permeant ions such as K^+ in the presence of valinomycin. Furthermore the lag in the onset of phosphorylation in the presence of permeant ions would then represent the time required to acidify the inner aqueous phase of the thylakoid.

In justification of these speculations regarding the initial role of a transmembrane potential, it should be noted that Graber and Witt [37] have concluded that a membrane potential is required for photophosphorylation only when the transmembrane proton concentration gradient is sub-optimal.

Speculations aside, our observations point to the fact that photophosphorylation due to short illumination periods (3–50 ms) is dependent on some sort of electric field in or across the thylakoid membrane. There is probably no other way to explain the brief inhibition by so many kinds of permeant ions. This conclusion is consistent with the correlation between ATP formation and flash-induced 515 nm absorbance changes, changes which almost certainly measure electric potential gradients across pigment molecules [18]. Our observation that phosphorylation due to larger illumination periods seems to be independent of transmembrane electric potential differences is also consistent with the ion distribution found in continuous light [38, 19]. However, if the proton concentration gradient between the inside and the outside of the thylakoid is to provide the main driving force for ATP synthesis, the internal pH must fall below 5 in the 50 ms which elapse before phosphorylation begins. That is to say, the protons produced inside the thylakoid must overcome internal buffering in the range of pH 8 to pH 5 before ATP synthesis can occur. In the second paper of this series [37], we have explored the effects of internal buffering on the time of illumination required to initiate phosphorylation in an attempt to assess the plausibility of the above interpretations of the early events leading to phosphorylation.

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