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FACTORS AFFECTING THE DEVELOPMENT OF THE CAPACITY FOR ATP FORMATION IN ISOLATED CHLOROPLASTS

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Full development of the capacity for ATP formation in isolated thylakoid membranes coincides with the beginning of illumination. Indeed, the yield of ATP per ms of illumination is about twice as great during the first 15 ms of high-intensity illumination as it is thereafter. The presence of valinomycin and K^+ prevents the formation of a membrane potential (as indicated by the obliteration of most of the change in absorbance at 518 nm) and at the same time delays the formation of the capacity for ATP synthesis for many milliseconds. Presumably, phosphorylation is initially dependent on a rapidly formed membrane potential, whereas after a delay a ΔpH sufficient to drive ATP formation forms. The actual duration of this delay depends on the phosphoryl group transfer potential (i.e., ΔG_{ATP}) of the ATP-synthesizing reaction. If the delay in the presence of valinomycin and K^+ represents the time required to develop a ΔpH capable of driving phosphorylation by itself, then the effect of ΔG_{ATP} on the duration of the delay suggests that the onset of phosphorylation is determined by the magnitude of the electrochemical potential of protons and not by factors affecting the activation of the coupling factor enzyme. The initial ATP formation, which is almost entirely dependent on the electrical potential, should not be affected by the electrically neutral exchange of cations catalyzed by nigericin. When the external pH is 7.0 this seems to be true, since the ATP synthesis which is initially sensitive to valinomycin and K^+ is largely insensitive to nigericin and K^+. However, when the external pH is 8.0 the response to nigericin is exactly the opposite and the ATP formation which is sensitive to valinomycin is also abolished by nigericin. These data suggest that there may be either an energetic requirement for both a ΔpH and membrane potential at alkaline pH or a non-energetic requirement for a minimum proton activity in the initiation of phosphorylation.

Introduction

A portion of the free energy available from photosynthetic electron-transport reactions in chloroplasts can be captured by the membrane in the form of an electrochemical potential difference of protons. The electrical and concentration components of the potential difference are thermodynamically equivalent [1,2] and may sometimes be interconvertible [3,4]. ATP formation cannot occur until the electrochemical potential of the accumulating protons surpasses an energetic threshold for the phosphorylation of ADP. The lower limit of this energetic requirement is established by the phosphoryl group transfer potential, ΔG_{ATP} ($\Delta G_{ATP} = \Delta G^{\circ} + 1.36 \log[ATP]/[ADP]\text{-}[P_i]$), and the number of protons which move through the coupling enzyme complex for each molecule of ATP formed. The electrical capacitance of the lamellar membrane is small in comparison to its H^+

Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Tricine, *N*-tris(hydroxymethyl)methylglycine; Mops, 4-morpholinepropanesulfonic acid; Chl, chlorophyll.

buffering capacity. Consequently, the two components of the electrochemical potential develop at different rates so that the accumulation of vanishingly few protons can create an electric potential large enough to phosphorylate ADP without any significant pH difference [5,6].

Although an adequate electrochemical potential is a thermodynamic requirement for ATP formation it is not necessarily a sufficient condition. For instance, numerous transformations within the coupling factor enzyme complex seem to be necessary before the enzyme can catalyze ATP formation [7–9]. Our observations indicate that a large electrical potential alone may not be sufficient for the onset of ATP formation. Instead, there may be a non-energetic requirement for a minimum proton activity in the initiation of ATP formation which can be fulfilled by the acidity of the surrounding environment at neutral pH and below, but may require light-induced proton translocation at alkaline pH values. After this condition is met, our investigations into the effect of ΔG_{ATP} on the early events leading to ATP formation show that an energetically adequate potential difference is a sufficient condition for the onset of ATP formation and that delays in the development of the capacity for ATP synthesis probably represent delays in the development of this requisite potential difference.

Materials and Methods

Chloroplasts (intact, naked lamellae) were isolated from commercial spinach (*Spinacia oleracea* L.) Approx. 40 g of spinach were homogenized with a Waring Blendor for 5 s in 90 ml of a medium consisting of 0.3 M NaCl, 30 mM Tricine-NaOH (pH 7.8), 3 mM $MgCl_2$, and 0.5 mM Na_4EDTA. The homogenate was squeezed through 16 layers of cheesecloth and the chloroplast sedimented at 2 000 × *g* for 2 min. The chloroplast pellets were pooled and resuspended in a total volume of 40 ml of a medium containing 0.2 M sorbitol, 5 mM Hepes-NaOH (pH 7.5), 2 mM $MgCl_2$, and 0.5 mg/ml bovine serum albumin. After a 20 s centrifugation at 2 000 × *g* and filtration through a Kimwipe tissue to remove cell debris the chloroplasts were pelleted (2 000 × *g*, 4 min). The chloroplast pellet was again resuspended in 40 ml of the sorbitol medium, recentrifuged (2 000 × *g*, 4 min) and finally resuspended in a few milliliters of the sorbitol medium.

The photophosphorylation reactions were carried out in a water-jacketed chamber regulated at 25 ± 0.2°C. Actinic light was provided by a 24 V/150 W tungsten-halogen lamp and the intensity of the heat-filtered white light was about 10^6 erg · cm^{-2} · s^{-1}. The reaction was terminated 1 s after the end of the illumination period by the addition of 1 ml of a 2 M acid solution ($HClO_4$ or trichloroacetic acid) containing 10 mM EDTA. Further details of reaction conditions are given in the legends of the figures and tables.

Incorporation of radioactive orthophosphate into ATP was measured using a procedure developed in the laboratory of Boyer [10]. In order to determine the recovery of ATP from each reaction, 1.5 μmol of unlabeled ATP were added to each acid-quenched reaction mixture. To each reaction mixture 0.4 ml of a solution containing orthophosphate and pyrophosphate (0.5 M NaH_2PO_4, 0.125 M $Na_4P_2O_7$) and 0.7 ml of an AMP-treated charcoal suspension [10] (100 mg/ml) were added. The mixture was allowed to stand for 5 min. The charcoal with adsorbed nucleotides was then collected on filter paper. The charcoal was washed with 10 ml of a solution of 25 mM $Na_4P_2O_7$, 0.1 M H_3PO_4 and 0.3 M $HClO_4$ and then with three 10-ml portions of distilled water. The adsorbed nucleotides were eluted from the charcoal by placing the charcoal and filter paper in a 125 ml flask with 14 ml of 0.6 M NH_4OH in 40% ethanol and shaking for 60 min. The charcoal was removed by filtration through a 0.2 μm Millipore filter. The flask and collected charcoal were washed with an additional 3.5 ml of the ammonia/ethanol solution. The filtrate was applied to a 2.0 ml column of a quaternary amine resin (Dowex AG1-X4, 200–400 mesh) which had been previously washed with 1.0 M HCl followed by distilled water until the column eluate was neutral. After the filtrate had passed through, the column was washed with 2.0 ml of distilled water followed by 5.0 ml of 0.2 M Tris-HCl (pH 8.0). AMP and ADP were removed from the column by extensive washing with 0.060 M HCl (50 ml). The ATP was eluted from the column in 15 ml of 1.0 M HCl. Absorbance at 257.5 nm was measured for each sample in order to determine the recovery of ATP, which routinely varied between 60 and 70%. Using

this procedure the background level of ^{32}P in the ATP fraction when no [^{32}P]ATP was formed (i.e., no chloroplasts added to the reaction or $HClO_4$ added to the chloroplasts before the addition of $^{32}P_i$) was always less than 0.01 nmol per reaction. That is, less than 50 cpm compared to the $50 \cdot 10^6$ cpm originally introduced into each reaction as $^{32}P_i$. Radioactivity was determined by measuring Cerenkov radiation in a Searle Delta 300 scintillation counter *.

The fluorescent nucleotides 1-N^6-ethanoadenosine diphosphate and 1-N^6-ethenoadenosine triphosphate were synthesized by the method of Secrist et al. [11]. Final purification was by ion-exchange chromatography on a DEAE-cellulose column (3 X 40 cm, bicarbonate form). The sample was applied to the column and eluted with a 4 l linear gradient of triethylammonium bicarbonate, pH 8.0 (50–300 mM). The appropriate fractions were combined and evaporated to dryness. The triethylammonium salt was dissolved in a few milliliters of methanol and added dropwise to 1% $NaClO_4$ in acetone to form the sodium salt. The precipitate was collected and washed once in cold acetone. Thin-layer chromatography on Eastman Chromagram cellulose sheets developed with isobutyric acid/NH_4OH/H_2O (75 : 1 : 24, v/v/v) showed the final products to be free from starting material and hydrolysis products.

The electrochromic absorption band shift (518–540 nm) was measured in a 10 X 10 mm cuvette thermostatically maintained at 18°C. The actinic light source was the same as that used for the photophosphorylation reactions except that it passed through a Corning 2-58 filter. The photomultiplier (Hamamatsu R268) was protected from actinic light by a Corning 4-96 filter and a Fish-Shurman 546 interference filter. The amplified signal was improved by averaging eight flashes (0.066 Hz) using a Biomation 805 transient recorder and a Nicolet 1172 signal averager.

In some experiments ATP synthesis was assayed by the firefly method. The 2.0 ml chloroplast sample was quenched with 1.0 ml of 1.0 M trichloroacetic acid containing 10 mM EDTA and then placed in an ice bucket. After 30 min the samples were brought to room temperature and extracted with diethyl ether (three portions of 5 ml each) to removed the trichloroacetic acid [12]. The samples were diluted 100-fold in 0.1 M Tris-CH_3COOH (pH 7.75), 2 mM EDTA. For ATP analysis, each reaction contained 0.8 ml of the diluted sample and 0.2 ml of a luciferin/luciferase reagent purchased from LKB-Wallac which provided a constant light emission directly proportional to the ATP concentration. More than 98% of the ATP originally in the sample was carried through the procedure. Each experiment was calibrated with an internal standard. The intensity of the bioluminescence was measured as the current from a photomultiplier (Hamamatsu R268) operated at 600 V. Current was measured with a Keithley 610C electrometer.

Results

The data in Fig. 1 show the dependence of ATP formation on illumination time. Precise measurement of the ATP produced from a single brief flash was accomplished by adsorbing the nucleotides on charcoal and finally eluting the mono-, di- and triphosphate adenine nucleotides sequentially by acid treatment of an ion-exchange column according to the procedure of Smith et al. [10].

The yield of ATP per ms of illumination was significantly greater during the initial 15 ms or so of illumination than it was subsequently. The medium pH (from 7 to 8) had little effect on this initial yield displaying the sensitivity to pH similar to the steady-state ATP/$2e^-$ ratio rather than the steady-state rate of ATP synthesis [13].

Fig. 2 shows the results of an experiment analogous to that of Fig. 1 except that the ATP was measured by monitoring the luminescence from the reaction of ATP and luciferin catalyzed by firefly luciferase. It is apparent that the initial 'burst' in ATP formation measured by $^{32}P_i$ incorporation is also observed when ATP is assayed by luciferin/luciferase. Thus, there can be little doubt that the light-induced increase in [^{32}P]ATP (Fig. 1) is indeed a result of ATP synthesis and not the result of any light-induced ATP-$^{32}P_i$ exchange activity. We do note, that even under identical reaction conditions, greater apparent yields of ATP were observed by the luciferin/lucifer-

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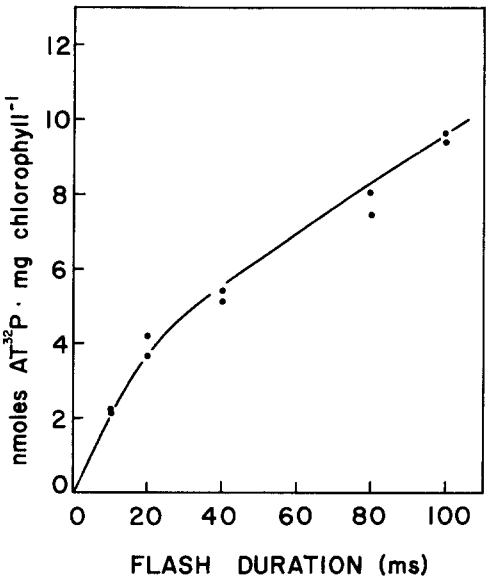

Fig. 1. The dependence of [^{32}P]ATP yield on the duration of single flashes of light. Chloroplasts containing 30 μg Chl were suspended in 2 ml of reaction mixture containing: 50 mM sorbitol, 50 mM Tricine-KOH (pH 8.0), 5 mM $MgCl_2$, 0.1 mM methyl viologen, 0.5 mM $^{32}P_i$ (15 μC_i), 0.1 mM ADP and 1.0 μM P^1,P^5-diadenosine-5′-pentaphosphate. The reaction was continuously stirred and thermostatically maintained at 25°C. Chloroplasts were added to temperature equilibrate 60 s prior to the flash. The ADP and $^{32}P_i$ were added 15 s prior to the flash. 1 s after the flash, the chloroplasts were denaturated by the addition of 1 ml of 2 M $HClO_4$.

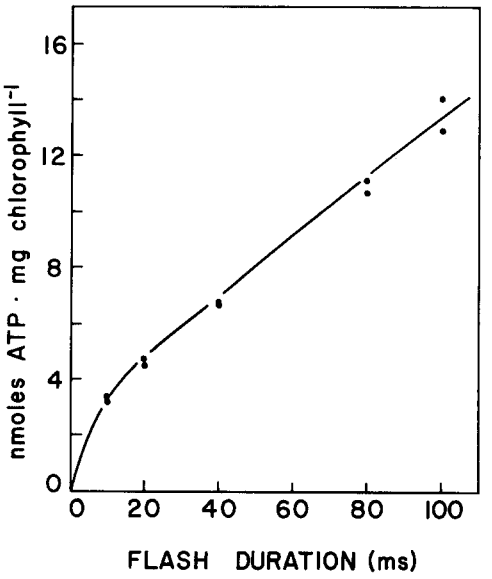

Fig. 2. Measurement of ATP resulting from single flashes by bioluminescence from the luciferase-catalyzed reaction between luciferin and ATP. Conditions were identical to those of Fig. 1 except that unlabeled phosphate replaced radioactively labeled phosphate and that trichloroacetic acid replaced $HClO_4$.

TABLE I

COMPARISON OF THE YIELD OF ATP FROM A 1 s FLASH ASSAYED BY DIFFERENT TECHNIQUES

Reaction conditions are given in Figs. 1 and 2.

Method of assay for ATP formation	nmol ATP/mg Chl
[^{32}P]ATP isolated by ion exchange	111
	115
[^{14}C]ATP isolated by ion exchange	97
	105
[^{32}P]ATP by extraction of [^{32}P]phosphomolybdate	110
	108
ATP by luciferin/luciferase	143
	148

ase assay than by $^{32}P_i$ incorporation. In fact, as Table I demonstrates, the bioluminescence assay indicates the formation of about 30% more ATP from a 1 s light exposure than do three other procedures which all agree well with one another. This unexplaned discrepancy of the firefly assay is one of the reasons that $^{32}P_i$ incoporation was used to assay ATP formation in this study.

Valinomycin-facilitated movement of K^+ through the lamellar membrane acts to oppose the formation of the electric component of the electrochemical potential of the accumulated protons. Indeed, so long as the internal K^+ reservoir remains adequate, valinomycin should prevent the formation of any delocalized electric potential altogether. The inset to Fig. 3 shows the effect of valinomycin and K^+ on the electrochromic absorption change resulting from a relatively long flash of 60 ms. The residual flash-induced absorption change observed in the presence of valinomycin was also insensitive to gramicidin and so probably was not electrochromic in origin [14]. The presence of valinomycin plus K^+ delayed the development of ATP formation capacity for 30–40 ms at

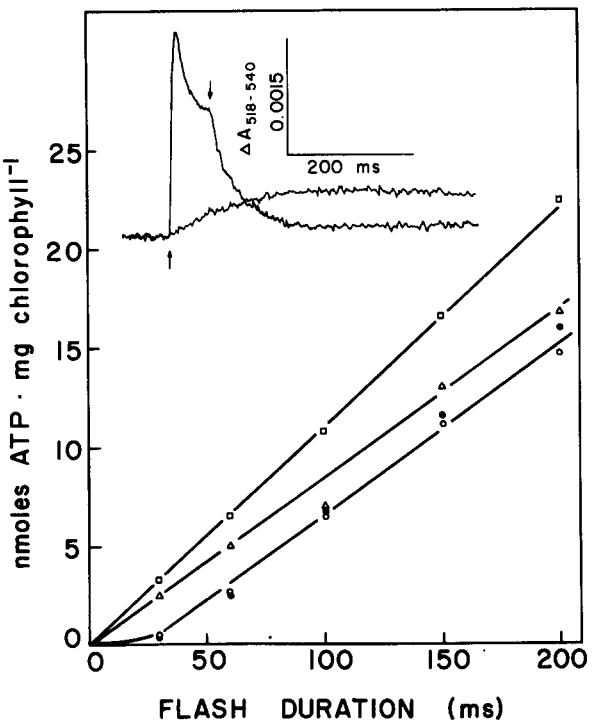

Fig. 3. The uncoupling of photophosphorylation by valinomycin at pH 8.0 as a function of flash duration. The reaction conditions were similar to those given for Fig. 1 with Hepes replacing Tricine as the buffer and the P^1,P^5-diadenosine-5′-pentaphosphate omitted. The Hepes was titrated with either NaOH or KOH depending upon which cation was desired. (□-□) 40 mM Na^+; (△-△) 40 mM Na^+ plus 0.25 μM valinomycin; (○-○) 20 mM K^+, 20 mM Na^+ plus 0.25 μM valinomycin; (●-●) 40 mM K^+ plus 0.25 μM valinomycin. The inset shows the effect of 0.25 μM valinomycin plus 20 mM K^+ on the electrochromic absorption band shift (518–540 nm) from a 60 ms actinic flash.

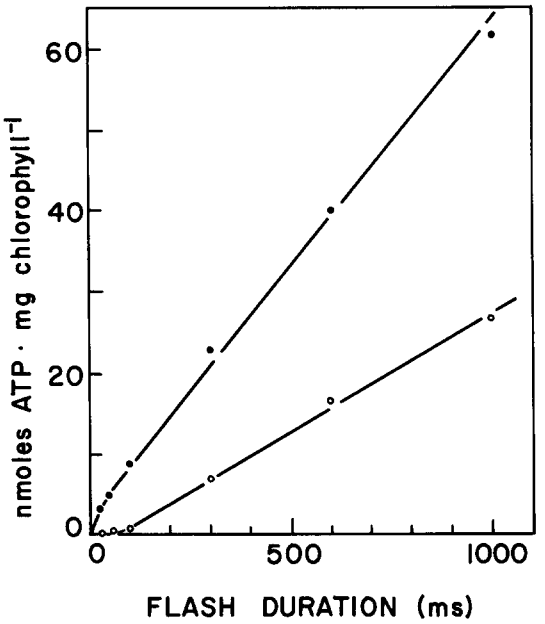

Fig. 4. The uncoupling of photophosphorylation by valinomycin at pH 7.0 as a function of the flash duration. Mops-KOH buffer replaced the Hepes buffer of Fig. 3, otherwise the reaction conditions were the same. (○-○) Control, (●-●) 0.25 μM valinomycin.

pH 8.0 (Fig. 3) and for 100 ms at pH 7.0 (Fig. 4). Fig. 3 shows that the delay was dependent on K^+ and also that there was no difference in the delay of the development of phosphorylation capacity when the concentration of K^+ was increased from 20 to 40 mM. These data demonstrate that the internal pool of K^+ is adequate in the presence of valinomycin to prevent the formation of an electric potential difference for a period of illumination longer than that needed to bring about ATP formation. This conclusion is corroborated by the lack of recovery of the electrochromic absorption change in the presence of valinomycin and K^+ during a 60 ms flash (inset Fig. 3).

Thus, under our conditions at least, there seems to be no basis for the suggestion that valinomycin and K^+ have little effect on the electric potential during millisecond illumination periods [15]. Valinomycin itself (i.e., in the absence of K^+) diminished the yield of ATP per ms of illumination by approx. 20% without inducing any delay in the onset of phosphorylation. This reduction in yield probably was a result of inhibition of electron transport by valinomycin [16]. An initial burst in ATP yield is not apparent in Fig. 3 but may be obscured by the expanded time scale (the first data point is at 50 ms while the burst is over within 15 ms).

The results in Fig. 5 show that under conditions where the electric potential was allowed to form, an increase in ΔG_{ATP} of 16.6 kJ/mol diminished the yield of ATP per ms of illumination by about 50% but did not induce a detectable lag in the onset of phosphorylation. After the electric potential was abolished by valinomycin and K^+, an increase in ΔG_{ATP} requiring an increase in the threshold ΔpH of 1 unit (assuming $H^+/ATP = 3$) delayed the onset of ATP

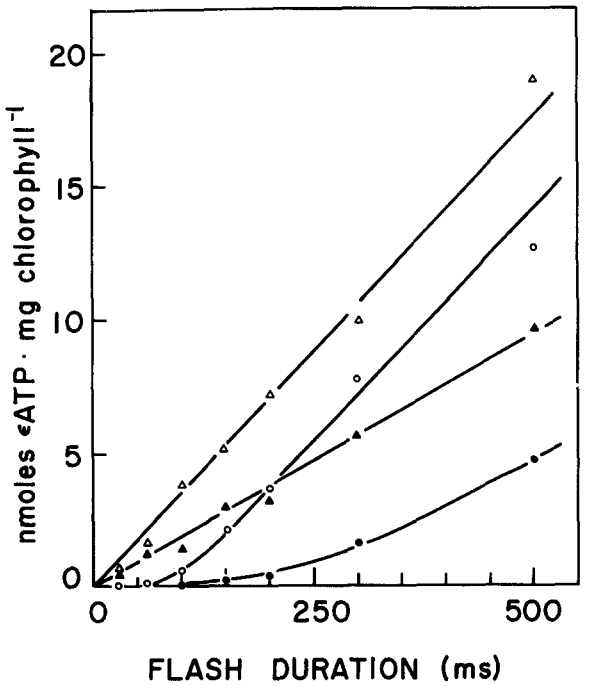

Fig. 5. The effects of $\Delta G_{\epsilon ATP}$ on initial phosphorylation. The magnitude of $\Delta G_{\epsilon ATP}$ was established by the 1-N^6-etheno ATP concentration which was either 1 μM or 1 mM. The 1-N^6-ethano ADP concentration was 0.1 mM and the $Na_2H^{32}PO_4$ concentration was 1 mM in all samples. The reaction mixture was buffered with 50 mM Hepes-KOH (pH 8.0); reaction conditions were otherwise identical to those in Fig. 3. Assuming that the 1-N^6-etheno substituted nucleotide derivatives have the same G^o as unsubstituted adenine nucleotides, the ΔG_{ATP} was poised at 38.3 and 55.4 kJ/mol. (△) $\Delta G_{\epsilon ATP}$ = 38.3 kJ/mol, (▲) $\Delta G_{\epsilon ATP}$ = 55.4 kJ/mol, (■) $\Delta G_{\epsilon ATP}$ = 38.3 kJ/mol in the presence of 0.25 μM valinomycin, (○) $\Delta G_{\epsilon ATP}$ = 55.4 kJ/mol in the presence of 0.26 μM valinomycin.

formation by an additional 100 ms or so. Moreover, the yield of ATP per ms of illumination was again depressed by about 50%. ΔG_{ATP} of the reaction was manipulated using the 1-N^6-etheno analogs of ADP and ATP (ϵADP and ϵATP) because attempts to raise the ΔG_{ATP} value with large amounts of ATP gave high levels of $^{32}P_i$ incorporation into ATP in the dark. The nature of the exchange activity leading to the dark incorporation of $^{32}P_i$ is not well characterized but, as Shahak et al. [17] showed, ϵATP does not participate significantly in the reaction(s). The effect of $\Delta G_{\epsilon ATP}$ on the yield of ϵATP per ms of illumination differed among chloroplast preparations.

As little as 20% and as much as 50% reduction in yield of ϵATP per ms of illumination were observed in experiments identical to the experiment of Fig. 5. Providing that the 1-N^6-etheno substituted derivatives have the same ΔG^o as the unsubstituted adenine nucleotides, the calculated $\Delta G_{\epsilon ATP}$ values in Fig. 5 are 38.3 and 55.4 kJ/mol. The lower of these values, 38.3 kJ/mol, is very close to the ΔG_{ATP} of the experiment shown in Fig. 3. Yet in Fig. 3 the illumination time necessary to develop a phosphorylation capacity in the absence of an electrical field was much less than when ϵADP was present. In addition, once phosphorylation has begun, the yield of ϵATP per ms of illumination was only about half the yield of ATP per ms of illumination. These differences in the phosphorylation of ϵADP and ADP could be accounted for by a difference in the ΔG^o of the reac-

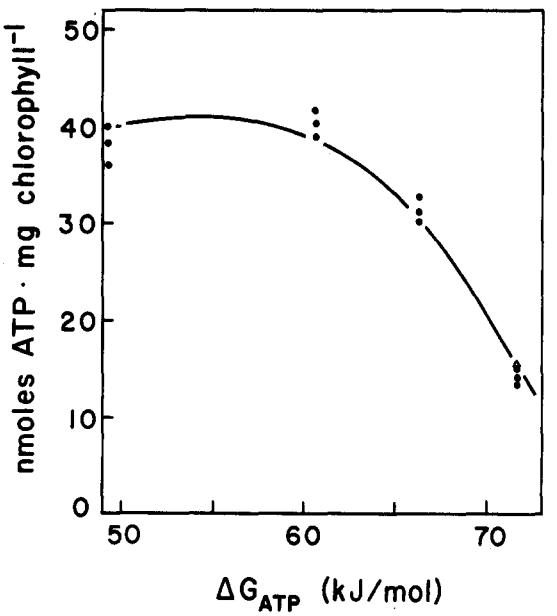

Fig. 6. The effects of ΔG_{ATP} on the rate of steady-state phosphorylation. The magnitude of ΔG_{ATP} was varied with ATP (0.3 μM to 3.0 mM) while the ADP and P_i concentrations were held constant at 30 and 100 μM, resepctively. In addition, the 2 ml reaction mixture contained 50 mM sorbitol, 50 mM bistrispropane-HCl (pH 9.0), 10 mM $MgCl_2$, 0.1 mM methyl viologen, 25 mM KCl and chloroplasts equivalent to 17 μg Chl. The illumination period was 2 s and the reaction temperature 25°C. The symbol △ indicates results obtained from duplicate experiments in the presence of 0.1 μM valinomycin.

tions. Even so, a value for ΔG° of the etheno derivatives is not necessary to determine the absolute difference in $\Delta G_{\epsilon ATP}$ values of Fig. 5.

Although high dark incorporation of $^{32}P_i$ into ATP prevented us from imposing high ΔG_{ATP} values in experiments using short flashes (e.g., Fig. 5), ATP could be used when the illumination periods were extended. The apparent effects of ΔG_{ATP} on steady-state phosphorylation are shown in Fig. 6. There appears to be no effect of ΔG_{ATP} until very high values are used (greater than about 60 kJ/mol). However, it is possible that the effects of ΔG_{ATP} are masked by the stimulation of $^{32}P_i$-ATP exchange that occurs during illumination [18]. For instance, we found that in the presence of 1 mM purified ATP the rate of exchange of $^{32}P_i$ into ATP was stimulated more than 100-fold after 1 s illumination, accounting for more than 20 nmol of [^{32}P]ATP per mg Chl. Indeed, for synthesis of ATP to occur at a ΔG_{ATP} of 71.2 kJ/mol a Δp equivalent to 270 mV would be required (assuming $H^+/ATP = 3$). Since valinomycin and K^+ did not diminish the [^{32}P]ATP formed, a ΔpH of nearly 4.5 units would be necessary. The opposite side of the issue is how to differentiate between the effects of high ATP concentrations and high ΔG_{ATP} values. An ATP concentration of 0.3 mM was used to attain a ΔG_{ATP} of 66.5 kJ/mol in Fig. 6. This concentration had no effect on the rate of ATP formation at lower ΔG_{ATP} values (established by elevating the ADP concentration). The highest ΔG_{ATP} value of Fig. 6 required 3 mM ATP and this level of ATP did diminish ATP synthesis at lower ΔG_{ATP} values.

Nigericin is a potent uncoupler of steady-state phosphorylation in chloroplasts [19] and is thought to uncouple by catalyzing an electrically neutral exchange of the accumulating H^+ for K^+ or certain other alkali metal ions. Since steady-state photophosphorylation in chloroplasts seems not to rely on any significant electrical component of accumulated protons [20–22], the H^+-K^+ exchange serves to collapse the pH difference and prevent ATP formation. However, if ATP formation occurring in the initial period of illumination depends solely on transmembrane

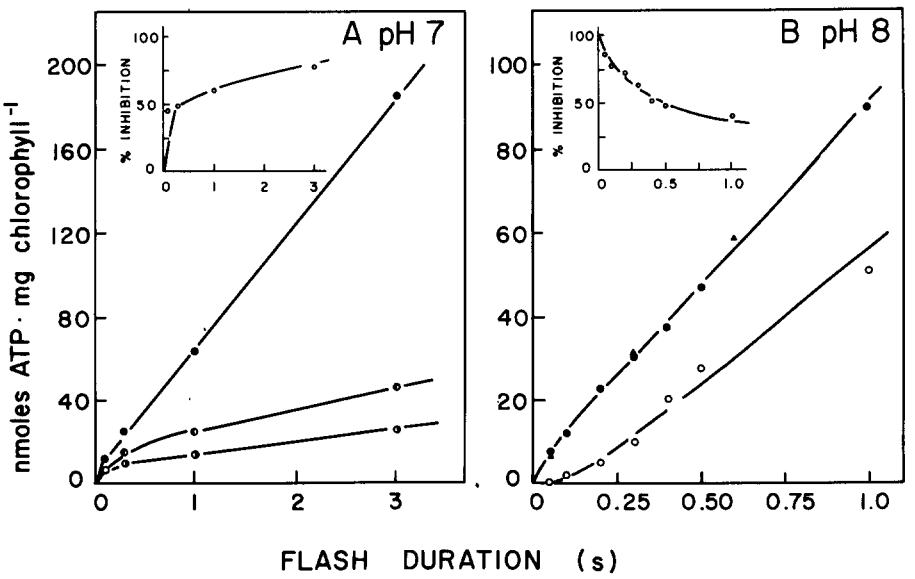

Fig. 7. The uncoupling of photophosphorylation by nigericin as a function of flash duration. (A) The effect of nigericin at an external pH of 7.0. Reaction conditions were the same as for Fig. 4. (●-●) Control, (⊙-⊙) 100 nM nigericin, (⊙-⊙) 200 nm nigericin In the inset the percent inhibition of ATP formation by 100 nM nigericin as a function of flash duration is shown. (B) The effect of nigericin at an external pH of 8.0. Reaction conditions were the same as for A with Tricine-KOH replacing Mops-KOH. (●-●) Control, (○-○) 100 nM nigericin. The inset shows the percent inhibition calculated from the data points. △ shows that 100 nm nigericin has no effect on ATP formation when K^+ was excluded from the reaction by using LiOH to tritate the buffer, the Tris salt of ADP and the bistrispropane salt of phosphate.

electrical potential differences and the action of nigericin is to exchange one cation for another, then ATP synthesis should be unaffected by nigericin and K^+. At pH 7.0 or below nigericin behaved in this manner (Fig. 7A). Uncoupling by nigericin was significantly less at the beginning of illumination than it was thereafter (inset Fig. 7A). These data are quite similar to the observations of Vinkler et al. [23]. However, when chloroplasts were suspended at more alkaline pH the effect of nigericin was markedly different (Fig. 7B). At pH 8.0, 100 nM nigericin uncoupled steady-state phosphorylation by approx. 50%. The ATP formation occurring immediately after the beginning of illumination, rather than being less sensitive to nigericin as it was at pH 7.0, was instead much more sensitive (inset Fig. 7B). That is, the ATP formation which was prevented by valinomycin-facilitated K^+ movement was also abolished by nigericin-catalyzed H^+-K^+ exchange.

It is clearly important for the interpretation of these experiments to establish that nigericin was acting in an electrically neutral manner. Either a net movement of the nigericin anion or a transient imbalance in the H^+-K^+ exchange ratio could lead to the results shown in Fig. 7B. The curve with Δ symbols in Fig. 7B shows the strict K^+ dependence of nigericin uncoupling and thus it is unlikely that the movement of the nigericin anion was involved. Furthermore, in Fig. 8 it is shown that nigericin and K^+ did not cause any acceleration in the dark decay of the flash-induced electrochromic absorption change measured at 518–540 nm. The decay of the absorption change was accelerated by phosphorylation (trace B) as is well known [24–26] but nigericin and K^+ caused no further increase in the decay rate (trace C). A partial reversal of the phosphorylation-induced acceleration was evident at pH 7.0. At an external pH of 8.0, nigericin caused a complete reversal of the acceleration brought on by phosphorylation (cf. traces A and C and the computer substraction labeled A–C). These effects of nigericin on the electrochromic absorption change are entirely consistent with the ATP formation data shown in Fig. 7, since ATP formation from a 21 ms flash is entirely abolished by nigericin at pH 8.0 and only somewhat diminished at pH 7.0.

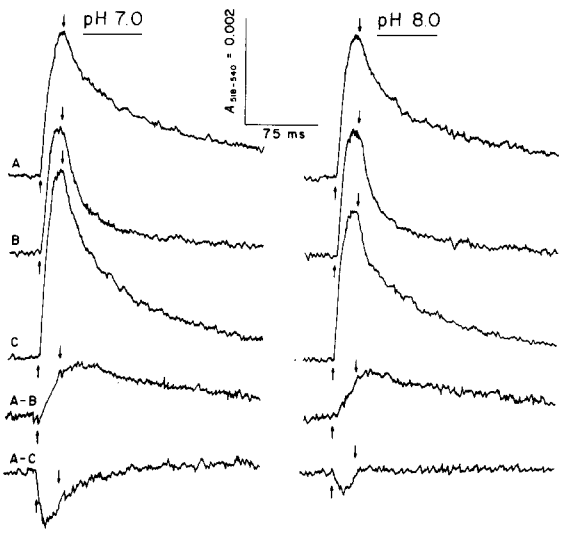

Fig. 8. The effect of phosphorylation and nigericin-catalyzed H^+-K^+ exchange on the electrochromic absorption band shift (518–540 nm) measured at pH values of 7.0 and 8.0. The conditions for these measurements were the same as those in Fig. 6A and B except that the reaction temperature was 18°C. The signal-to-noise ratio was improved by averaging eight flashes, 21 ms long, separated from one another by 15 s. Trace A, plus ADP; trace B, plus ADP and P_i; trace C, plus ADP, P_i and 100 nM nigericin. The arrows indicate the duration of the actinic flash.

Discussion

It is generally held that ATP synthesis represents the reversal of the ATP hydrolysis activity of the chloroplast coupling factor. The chloroplast ATPase activity is normally very low, requiring removal or displacement of the ATPase inhibitor polypeptide before full expression of ATP hydrolysis is observed [7,8,27,28]. If ATP synthesis and ATP hydrolysis are indeed expressions of the same catalytic site, then one would expect removal or displacement of the ATPase inhibitor to be a necessary event preceding the synthesis of ATP. Even though the data in Figs. 1 and 2 show no detectable lag in the development of the phosphorylation capacity (lag much less than 5 ms) it is well to bear in mind that we actually measured the yield of ATP resulting from a given flash followed by a 1 s dark period. Consequently, we have little knowledge of the precise time at which ATP synthesis occurred. It is conceivable that activation of the coupling factor might occur in the dark after a short flash in which case the resulting ATP synthesis would occur further still into the dark period. No

reliable kinetics of the activation of the coupling factor can be obtained without rapid quenching of the reaction following the end of the illumination period. Thus, ATP synthesis requiring about five single turnover flashes delivered at 100 Hz reported by Harris and Crofts [9] is not a demonstration of the time required for the ATPase inhibitor protein to be displaced from its inhibitory site, since both activation and ATP formation could have occurred in the dark period prior to acid denaturation of the chloroplasts.

The effects of $\Delta G_{\epsilon ATP}$ on the early events of photophosphorylation (Fig. 5) suggest that coupling factor activation may not play a limiting role in ATP formation in chloroplasts. As pointed out earlier, the yield of ATP per ms of illumination is lower and the delay in the onset of phosphorylation longer (in the absence of an electric potential difference) when $\Delta G_{\epsilon ATP}$ is poised at high values. Thus, the development of the capacity for phosphorylation as well as the yield of ATP from short illumination periods are governed principally by the energetics. These data indicate that is is not possible for the activation state of the coupling factor enzyme complex to play any intervening role, since neither the rate of activation nor the number of enzyme complexes activated should be affected by $\Delta G_{\epsilon ATP}$ (unless activation and the formation of the capacity for ATP formation have identical requirements). Melandri et al. [29] demonstrated a similar effect of ΔG_{ATP} on the onset of phosphorylation in chromatophores from *Rhodopseudomonas capsulata*. In their experiments an increasing number of single turnover flashes (delivered at 6.25 Hz) were required to initiate phosphorylation as ΔG_{ATP} was raised. A significant difference between the results with chromatophores and our results with chloroplasts is that the ATP yield per flash, once ATP formation was initiated in chromatophores, was not influenced by ΔG_{ATP}.

The initial burst in ATP formation has not been reported previously, in fact lags generally have been reported [6,23,30]. The good agreement observed when ATP was measured by two unrelated techniques (Figs. 1 and 2) shows that the incorporation of $^{32}P_i$ into ATP is really a result of net synthesis of high-energy bonds and not some kind of ATP-$^{32}P_i$ exchange. Since we have little knowledge of when the ATP formation occurs, we can make no conclusions about the rates of ATP synthesis during and after the initial burst. However, the burst cannot be explained simply on the basis of when the ATP is made relative to the flash duration. In order to account for the burst during this period it is necessary to postulate that either more protons are transported per ms of illumination (i.e., a greater number of electrons or a higher H^+/e^- ratio) or the accumulated protons are used with greater efficiency. The chloroplasts used in our experiments are probably dark adapted or at least nearly so. Thus, the burst in ATP synthesis may result from a transient rapid transport of electrons by photosystem II into the acceptor pool which lies ahead of the rate-limiting step of electron transport [31,32]. If so, Photosystem II should be turning over faster than Photosystem I and the number of protons transported per ms of illumination would be correspondingly higher.

In contrast to the immediate development of phosphorylation capacity in the absence of ionophores, valinomycin and K^+ considerably increase the illumination period required to initiate phosphorylation. Ort and Dilley [6] first demonstrated directly the role of the electric potential difference of protons in ATP formation immediately after the beginning of illumination. They suggested that the energy for phosphorylation initially residues in an electric potential difference which is abolished by electrophoresis of ions in the field, leaving a more slowly developing proton concentration difference as the main driving force for phosphorylation during subsequent illumination. This idea that a pool somewhere must be filled is consistent with the fact that the duration of lag induced by valinomycin plus K^+ is a function of ΔG_{ATP}. The dependence of initial ATP synthesis on a membrane potential is observed both at pH 7.0 (Fig. 4) and pH 8.0 (Fig. 3). However, the extreme sensitivity of initial phosphorylation at pH 8.0 to nigericin plus K^+ implies a requirement for a pH difference across the membrane which does not exist at more acidic external pH values.

One possibility is that at pH 8.0 a sizable ΔpH is generated by the brief flash whereas at pH 7.0 the putative ΔpH formed is much smaller. The lower buffering capacity of the chloroplast lumen near pH 8.0 could allow a more rapid development of ΔpH. If so, the development of the larger pH difference at alkaline pH should occur at the expense of the mem-

brane potential, since it seems that it is the total electrochemical potential of the accumulated protons that controls the rate of electron transport [1]. If this is the situation, the prevention of the formation of a pH-difference by nigericin should lead to an increase in the electric potential to restore at least partially Δp. The kinetic traces of the electrochromic absorption change in Fig. 8, used as a relative monitor of the size of the electric potential, do not show any detectable increase in the amplitude of the absorption change upon the addition of nigericin at pH 8.0 (compare amplitudes of traces A and C at the time when the actinic light is switched off and the computer substraction labeled A–C). On the other hand, a significant increase in the amplitude of the absorption change upon the addition of nigericin was detected at pH 7.0. Although only indirect information about ΔpH can be obtained from these measurements they seem not to support the notion that there is a significant pH difference formed during a 20 ms flash at pH 8.0. Thus, it may be that even when the ΔpH is not energetically necessary a non-energetic requirement for a minimum proton activity must be fulfilled. If so, at alkaline pH light-induced proton translocation may be required whereas at lower pH the general acidity of the aqueous environment may fulfill the need.

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References

1 Mitchell, P. (1966) Biol. Rev. Cambridge Phil. Soc. 41, 445–540
2 Mitchell, P. (1968) Chemiosmotic Coupling and Energy Transduction, Glynn Research Ltd., Bodmin
3 Schuldiner, S., Rottenberg, H. and Avron, M. (1972) FEBS Lett. 28, 173–176
4 Gräber, P. and Witt, H.T. (1976) Biochim. Biophys. Acta 423, 141–163
5 Jackson,,J. and Crofts, A. (1971) Eur. J. Biochem. 18, 120–130
6 Ort, D.R. and Dilley, R.A. (1976) Biochim. Biophys. Acta 449, 95–107
7 Bakker-Grunwald, T. and Van Dam, K. (1974) Biochim. Biophys. Acta 347, 290–298
8 Van de Stadt, R.J., De Boer, B.L. and Van Dam, K. (1973) Biochim. Biophys. Acta 292, 338–349
9 Harris, D.A. and Crofts, A.R. (1978) Biochim. Biophys. Acta 502, 87–102
10 Smith, D.J., Stokes, B.O. and Boyer, P.D. (1976) J. Biol. Chem. 251, 4165–4171
11 Secrist, J.A., III, Barrio, J.R., Leonard, N.J. and Weber, G. (1972) Biochemistry 11, 3 499–3 506
12 Lundin, A. and Thore, A. (1975) Appl. Microbiol. 30, 713–721
13 Gould, J.M. and Izawa, S. (1973) Biochim. Biophys. Acta 314, 211–223
14 Witt, H.T. (1975) in Bioenergetics of Photosynthesis (Govindjee, ed.), pp. 495–555, Academic Press, New York
15 Witt, H.T. (1979) Biochim. Biophys. Acta 505, 355–427
16 Voegeli, K.K., O'Keefe, D., Whitmarsh, J. and Dilley, R.A. (1977) Arch. Biochem. Biophys. 183, 333–339
17 Shahak, Y., Chipman, D. and Shavit, N. (1973) FEBS Lett. 33, 293–296
18 Shavit, N., Skye, G.E. and Boyer, P.D. (1967) J. Biol. Chem. 242, 5125–5130
19 Shavit, N., Dilley, R.A. and San Pietro, A. (1968) Biochemistry 7, 2356–2363
20 Rottenberg, H., Grunwald, T. and Avron, M. (1972) Eur. J. Biochem. 25, 54–63
21 Vredenberg, W.J. and Tonk, W.J.M. (1975) Biochim. Biophys. Acta 387, 580–587
22 Schroder, H., Muhle, H. and Rumberg, B. (1971) in 2nd International Congress on Photosynthesis (Forti, G., Avron, M. and Melandri, A., eds.), vol. 2. pp. 919–930, Dr. W. Junk N.V., The Hague
23 Vinkler, C., Avron, M. and Boyer, P.D. (1978) FEBS Lett. 96, 129–134
24 Nishimura, M. (1970) Biochim. Biophys. Acta 197, 69–77
25 Rumberg, B. and Siggel, U. (1968) Z. Naturforsch. 23b, 239–254
26 Junge, W., Rumberg, B. and Schröder, H. (1970) Eur. J. Biochem. 14, 575–581
27 Petrack, B. and Lipman, F. (1961) in Light and Life (McElroy, W.D. and Glass, H.B., eds.), p. 621, Johns Hopkins Press, Baltimore
28 Lynn, W.S. and Straub, K.D. (1969) Proc. Natl. Acad. Sci. U.S.A. 63, 540–547
29 Melandri, B.A., Venturoli, G., De Santis, A. and Melandri, A.B. (1980) Biochim. Biophys. Acta 592, 38–52
30 Hope, A., Walker, D., Horton, P. and Ranson, D. (1980) in First European Bioenergetics Conference Short Reports, pp. 223–224, Patron Editore, Bologna, Italy
31 Kok, B. and Cheniae, G.M. (1966) Curr. Top. Bioenergetics. 1, 1–47
32 Stiehl, H.H. and Witt, H.T. (1969) Z. Naturforsch. 24b, 1588–1598