Further Studies on the Photochemical Production of Reduced Triphosphopyridine Nucleotide and Adenosine Triphosphate by Fragmented Spinach Chloroplasts [1, 2]

Charles A. Fewson [3], Clanton C. Black [4], and Martin Gibbs

Department of Biochemistry, Cornell University, Ithaca, New York

In the course of studies on the photochemical production of TPNH and ATP by fragmented spinach chloroplasts (*22*), we became interested in the stoicheiometry of the reactions. It is generally assumed that the ATP: 2 e ratio is unity (*2*) but some workers have suggested a higher value (10, 17, 18). Ratios greater than one have seldom been obtained by direct experiment but have usually been suggested on a basis of arithmetic manipulation, or from a consideration of comparative biochemistry, or have been inferred from spectrophotometric studies. Stiller and Vennesland (*20*) have recently reported a thorough investigation into the ATP: 2 e ratio during the Hill reaction with ferricyanide as electron acceptor and concluded that the over-all ratio is one. In the present paper we demonstrate that under certain conditions it is possible to obtain ATP: TPNH ratios significantly greater than unity without necessarily implying that there is more than one site of phosphorylation coupled to the electron transfer chain which leads to TPN reduction. We also show that photophosphorylation in the absence of other electron acceptors can proceed at a rate comparable to other photosynthetic processes when photosynthetic pyridine nucleotide reductase (PPNR) is added to chloroplast fragments under aerobic conditions.

Materials and Methods

Reagents. ADP and TPN were purchased from Sigma Chemical Company. P_i^{32} was purchased from the Squibb Laboratories and treated with N HCl and charcoal before use (*22*). PPNR was purified from spinach (19) and the unit of activity is that defined by San Pietro (19). Pyridine nucleotide transhydrogenase was prepared from spinach leaves through step 5 as described by Keister et al. (14). The antibody to pyridine nucleotide transhydrogenase was generously furnished by Dr. A. San Pietro.

m-Chlorocarbonyl cyanide phenylhydrazone was a gift from Dr. P. G. Heytler, Central Research Department of the E. I. du Pont de Nemours Co., Inc. A 10^{-3} M solution was prepared daily in 10^{-2} M NaOH. Crystalline 3-(*p*-chlorophenyl)-1,1-dimethylurea was obtained from Dr. E. Shantz and dissolved in 95 % ethanol. Antimycin A, obtained from the Sigma Chemical Company, was also dissolved in 95 % ethanol. Dr. J. W. Lightbown kindly provided samples of 2-heptyl-4-hydroxyquinoline-N-oxide and 2-nonyl-4-hydroxyquinoline-N-oxide. Fresh solutions of these 2 inhibitors were prepared daily in 10^{-3} M NaOH and the concentrations determined by absorption measurements at 346 mμ (*7*). Tris was purchased from Sigma Chemical Company and twice recrystallized from 95 % ethanol for use in experiments in which the effects of Tris itself were being studied.

Reaction Conditions. The preparation of spinach chloroplast fragments, chlorophyll determinations, and methods used in determining the rates of TPN reduction and the associated phosphorylation have been described in earlier papers (*5, 22*). Reaction mixtures were usually illuminated laterally in Beckman cuvettes of 1 cm light path at room temperature. The white light source was the same as that used in earlier work (*22*). The intensity of illumination was varied by altering the distance from the source. Unless otherwise noted, all samples were illuminated at a saturating intensity of 3,000 ft-c since this gave the maximum ATP: TPNH ratio (*22*). Incubation times were usually 5 or 10 minutes, since after a longer time the rate of ATP formation was found to decrease.

Results

Effects of Phosphorylating Cofactors. The stimulation of the rate of TPN reduction by Mg^{++}, P_i, and ADP (table I) was found to resemble that reported by previous workers for TPN reduction (8, 15) and ferricyanide reduction (*20*) at saturating light intensity. This stimulation of TPNH formation in the presence of Mg^{++}, P_i, and ADP oc-

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[3] Recipient of a Charles F. Kettering International Fellowship.

[4] Post-doctoral Research Fellow of the United States Public Health Service. Present address: Charles F. Kettering Research Laboratory, Yellow Springs, Ohio.

Table I

Effect of Phosphorylating Cofactors on the Rate of TPN Reduction

The complete reaction mixture contained: Tris-HCl buffer, pH 7.65, 24 μmoles; $MgCl_2$, 4 μmoles; KH_2PO_4-K_2HPO_4, pH 7.6, 1.54 μmoles (containing approx. 1 μc of P^{32}); ADP, 2 μmoles; TPN, 1.5 μmoles; PPNR, 4 units; chloroplast fragments containing 30 μg of chlorophyll; total volume 2.0 ml. The frozen chloroplast fragments were placed at −15° for 3 hours and then thawed at room temperature. Rates of TPN reduction are given as percentages of the rate in the control reaction mixture in which Mg^{++}, P_i, and ADP were omitted.

Addition	Fresh fragments 50 ft-c	Fresh fragments 3,000 ft-c	Frozen fragments 3,000 ft-c
Control*	100(10)*	100(38)*	100(25)*
Mg^{++}	173	178	223
P_i	127	206	...
ADP	86	92	...
Mg^{++}, P_i	245	204	...
Mg^{++}, ADP	171	174	...
P_i, ADP	145	214	108
Mg^{++}, P_i, ADP	277	225	190

* Control rate of TPNH formation in μmoles/mg chlorophyll hour.

curred at both low (50 ft-c) and saturating (3,000 ft-c) light intensities. The molar ratios of ATP: TPNH were 0.49 and 1.09 at 50 ft-c and 3,000 ft-c respectively. No ATP formation could be detected with the frozen chloroplasts and ADP and P_i did not stimulate the rate of TPN reduction by these preparations above the level in the presence of Mg^{++}. Frozen chloroplast fragments were also incapable of phosphorylation in the presence of methyl phenazonium methosulfate, FMN, vitamin K_3, or ferricyanide. Storage of the fragments at 4° in 0.035 M NaCl, 0.02 M Tris pH 7.5 also destroyed the capacity to phosphorylate faster than the capacity to reduce TPN. The half-life for the loss of TPN-reducing activity was 60 hours and that for ATP formation about 20 hours.

Effect of Tris Concentration. In the absence of Mg^{++}, P_i, and ADP, there was a marked effect of Tris concentration on the rate of TPNH formation (fig 1). TPN reduction was proportional to Tris concentration up to about 0.03 M in the absence of the phosphorylating cofactors, but this effect was largely overcome by the addition of Mg^{++}. At high Tris levels there was a marked uncoupling of ATP formation as reported previously by Good (11).

Effects of NaCl Concentration. The NaCl concentration of the reaction mixture was found to have a striking effect both on the absolute rates of TPNH and ATP formation and on the ATP: TPNH ratio (fig 2). This effect was due to the NaCl per se and not to the integrity of the chloroplasts since the effect depended solely on the final concentration of NaCl in the reaction mixture and not on the isolating medium used in the preparation of the chloroplasts. Thus either whole or fragmented chloroplasts (22) gave the same rates of TPNH and ATP formation when added to reaction mixtures containing any given concentration of NaCl. A low level of NaCl (0.05–0.1 M) was required for maximal rates of TPNH and ATP formation. At higher salt concentrations there was a marked inhibitory effect on TPN reduction and a smaller effect on ATP formation, resulting in a rise in the ATP: TPNH

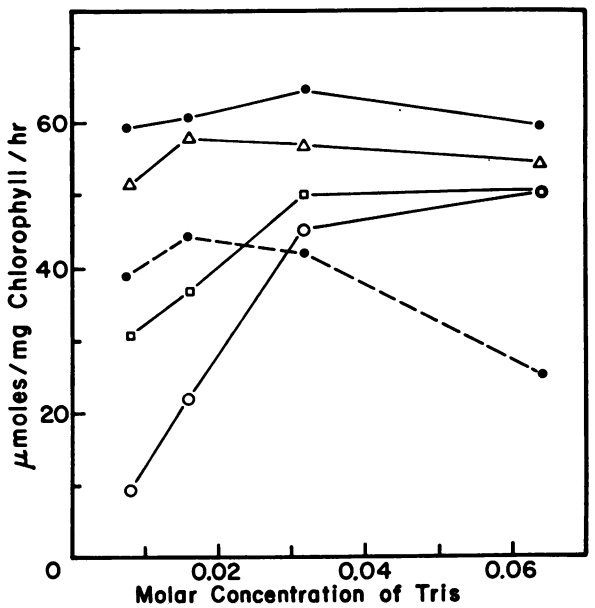


FIG. 1. The effects of Tris concentration on the rates of formation of TPNH and ATP. TPNH formation was measured in the absence of cofactors (○ — ○), or in the presence of Mg^{++} (△ — △), P_i and ADP (□ — □) or Mg^{++}, P_i and ADP (● — ●). ATP formation was measured only in the presence of all the cofactors (● - - - ●). The complete reaction mixture is described in table I.

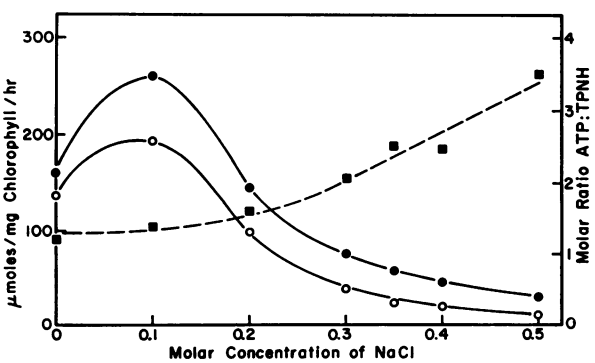
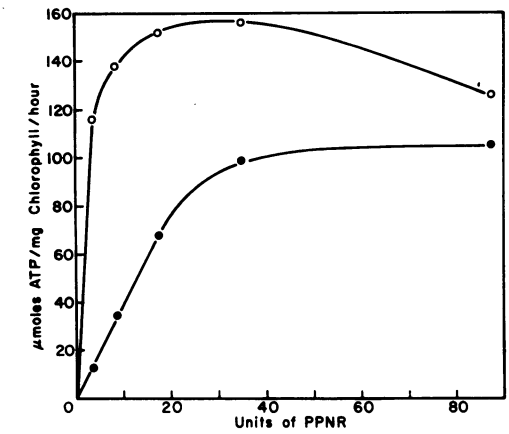


FIG. 2. The effects of NaCl concentration on the rates of formation of TPNH (○ — ○) and ATP (● — ●) and on the ATP: TPNH ratio (■ - - - ■). The reaction mixture is described in table I.



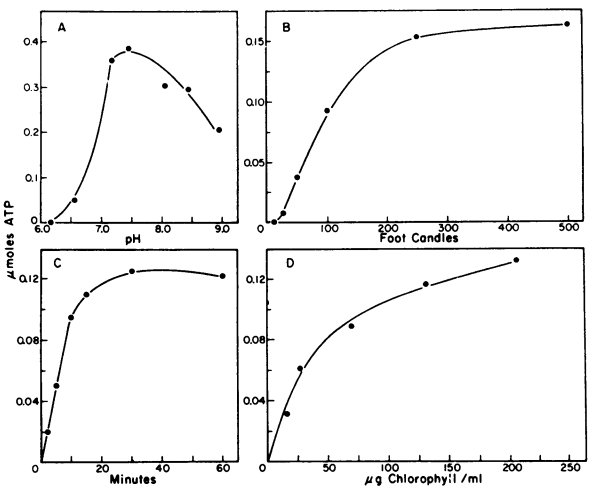


FIG. 3 (*upper*). Effects of PPNR concentration (units/2 ml) on ATP formation in the presence (○ — ○) and absence (● — ●) of exogenous TPN. The reaction mixture is described in table II except that in this experiment the total volume was 2 ml.

FIG. 4 (*lower*). Effects of various experimental conditions on the rates of ATP formation in the presence of high levels of PPNR and absence of other exogenous electron acceptors. Reaction conditions were as described in table II except for the parameters under investigation, shown along the abscissae. In figure 4A Tris-maleate buffer, 40 μmoles/ml, was used to control the pH.

ratio. A maximum molar ratio of ATP: TPNH of 3.5 was attained in a reaction mixture containing 0.5 M NaCl. An oxidation of TPNH could not be shown at any NaCl concentration.

Photophosphorylation in the Presence of High Levels of PPNR and Absence of Other Exogenous Cofactors. The effect of PPNR concentration on photophosphorylation in the presence and absence of an exogenous electron acceptor is shown in figure 3. In the presence of TPN, the phosphorylation was half-saturated at about 2 units and saturated at about 15 to 20 units of PPNR. In the absence of an exogenous electron acceptor, however, phosphorylation was half-saturated at about 12 units and saturated at about 35 to 40 units of PPNR per 2 ml reaction mixture. The maximum rate of ATP formation observed in the absence of TPN was 108 μmoles/mg chlorophyll hour.

In view of the possible physiological importance of a photophosphorylation independent of any added cofactor except PPNR, we decided to study the system in more detail. The oxygen-dependency of the reaction is illustrated in table II. The reaction was almost completely inhibited under nitrogen or helium. Pyridine nucleotide transhydrogenase usually stimulated the reaction although this was a variable effect. Transhydrogenase was shown to be involved in the reaction by the use of the enzyme antibody (14, 16) which gave inhibition of photophosphorylation (table II).

Figure 4 shows that photophosphorylation in the presence of high levels of PPNR was linear with time for at least 10 minutes and with chlorophyll concentration up to 40 μg per ml. The reaction had a pH optimum at pH 7.5. The phosphorylation showed a sigmoid response to increasing light intensity with a distinct lag up to 25 ft-c. Saturation occurred at about 250 ft-c which contrasts with the ATP formation associated with TPN reduction which is saturated only above 1,000 ft-c (22). There was an absolute requirement for a divalent metal ion (table

Table II

Effect of Various Experimental Conditions on Photophosphorylation in the Presence of High Levels of PPNR

The control reaction mixture contained: Tris-HCl buffer, pH 7.5, 8 μmoles; $MgCl_2$, 2 μmoles; KH_2PO_4-K_2HPO_4, pH 7.5, 1.54 μmoles (containing approx. 1 μc of P^{32}); ADP, 1 μmole; chloroplast fragments containing 15 μg chlorophyll; PPNR, approximately 30 units; total volume 1 ml. The experiments comparing aerobic and anaerobic conditions were made in Warburg vessels to facilitate flushing with the various gases: In these cases the vessels were illuminated from below with approximately 2,000 ft-c of white light.. Transhydrogenase (22 units) was added to the appropriate vessel. The boiled samples of PPNR, transhydrogenase, and antitranshydrogenase were prepared by heating at 100° for 10 min. The results are taken from several experiments with control rates from 52 to 83 μmoles ATP/mg chlorophyll hour.

Experimental condition	Phosphorylation as % of control
Control, aerobic	100
— PPNR	4
+ boiled PPNR	4
Nitrogen	5
Helium	7
+ transhydrogenase	100–150
+ boiled transhydrogenase	100
+ antitranshydrogenase	6
+ boiled antitranshydrogenase	93

Table III

Metal Requirement for Photophosphorylation in the Presence of High Levels of PPNR

The complete reaction mixture is described in table II.

Salt	Final molar concentration $\times 10^3$	Phosphorylation as % of maximum
None	...	6
$MgCl_2$	0.2	27
$MgCl_2$	2	80
$MgCl_2$	20	100*
$MnCl_2$	0.2	43
$MnCl_2$	2	85
$MnCl_2$	20	6
$Ca(NO_3)_2$	2	6
$ZnCl_2$	2	12
$NiCl_2$	2	37
$CdCl_2$	2	6
$Co(NO_3)_2$	2	72
$FeCl_3$	2	3
$FeSO_4$	2	2
$MgCl_2 + Ca(NO_3)_2$	2 + 2	77

* Maximum rate of ATP formation was 89 μmoles/mg chlorophyll hour.

III). Mg^{++} was the most effective ion tried but Mn^{++}, Co^{++}, and Ni^{++} could substitute to some extent.

Table IV summarizes the effects of various compounds on phosphorylation in the presence of PPNR. *m*-Chlorocarbonyl cyanide phenylhydrazone was a potent inhibitor, as it is also of phosphorylation in the presence of methyl phenazonium methosulfate and TPN (5, 12). The reaction was found to be more sensitive to Antimycin A than is either the ATP formation associated with TPN reduction or the cyclic photophosphorylation in the presence of methyl phenazonium methosulfate (5). The reaction was also sensitive to 3-(*p*-chlorophenyl)-1,1-dimethylurea, and the 2 hydroxyquinoline-N-oxide analogues. Arsenite had no effect on the reaction at the concentration tested.

Effect of Anaerobic Conditions and NaCl Concentration on the Rate of Photophosphorylation. Table V shows that 0.5 M NaCl did not inhibit phosphorylation in the absence of TPN. This is in contrast to the inhibitory effect of 0.5 M NaCl on phosphorylation accompanying TPN reduction (fig 2 and table V). These experiments also illustrate a further difference between the 2 systems. Photophosphorylation in the presence of high levels of PPNR was inhibited by anaerobic conditions (table II and V), whereas TPN reduction and the associated phosphorylation were unaffected by the absence of O_2 at low NaCl concentration. At 0.5 M NaCl, however, where the rate of TPN reduction is very low, anaerobic conditions did inhibit ATP formation by about 50 % and reduced the ATP: TPNH ratio to unity.

Table V

The Effects of Aerobiosis, Anaerobiosis and Concentration of NaCl on Photophosphorylation in the Presence and Absence of TPN

Reaction mixtures contained: Tris-HCl buffer, pH 7.6, 16 μmoles; $MgCl_2$, 4 μmoles; ADP, 2 μmoles; P_i, 2 μmoles; chloroplast fragments containing 22 μg chlorophyll; total volume, 2 ml TPN (2 μmoles), PPNR and NaCl were added to the appropriate vessels. The reactions were run in Warburg vessels to facilitate gassing. Illumination was from below with approximately 2,000 ft-c of white light and the vessels were shaken slowly. TPNH and ATP formation are given in μmoles/mg chorophyll hour.

	3 units of PPNR + TPN			30 units of PPNR − TPN
	TPNH	ATP	ATP: TPNH	ATP
Air, no NaCl added	70	53	0.76	57
N_2, no NaCl added	70	56	0.80	21
Air, 0.5 M NaCl	8	19	2.37	47
N_2, 0.5 M NaCl	10	10	1.00	28

Table IV

Influence of Various Inhibitors on Photophosphorylation in the Presence of High Levels of PPNR

The complete reaction mixture is described in table II. The reaction mixtures were incubated for 5 minutes in the dark before illumination. When the inhibitors were prepared in ethanol or NaOH, control mixtures contained equal amounts of these compounds.

Inhibitor	Final molar concentration	% inhibition of phosphorylation*
m-chlorocarbonyl cyanide phenylhydrazone	10^{-6}	100
m-chlorocarbonyl cyanide phenylhydrazone	10^{-7}	56
3-*p*-chlorophenyl-1,1-dimethylurea	10^{-5}	82
2-heptyl-4-hydroxyquinoline-N-oxide	10^{-4}	100
2-heptyl-4-hydroxyquinoline-N-oxide	10^{-5}	12
2-nonyl-4-hydroxyquinoline-N-oxide	10^{-5}	100
2-nonyl-4-hydroxyquinoline-N-oxide	10^{-6}	38
Antimycin A	10^{-5}	100
Antimycin A	10^{-6}	32
Sodium arsenite	10^{-4}	0

* Control rate was 53 μmoles ATP/mg chlorophyll hour.

Discussion

The effects of the various phosphorylating cofactors on the rate of TPN reduction are essentially in agreement with results reported by several other workers (8, 15). The pronounced interaction of the cofactors with Tris concentration illustrates the large differences that changes in the reaction mixture may have on the rates of electron transfer and photophosphorylation. Effects of this type may account for the minor discrepencies which occur in reports on the effects of individual components, such as ADP (8, 15).

The stimulation of the rate of electron transfer by the addition of Mg^{++}, P_i, and ADP at nonsaturating levels of illumination (table I) confirms that the low ATP: TPNH ratios observed at low light intensity are probably due to adenosine triphosphatase activity rather than to an absence of coupling of phosphorylation to electron transfer (C. C. Black, C. A. Fewson, M. Gibbs, and S. A. Gordon, in preparation).

Relatively little work has previously been carried out on the photophosphorylation catalyzed by chloroplasts in the absence of exogenous cofactors. Arnon et al. (1) showed that illuminated spinach chloroplasts were able, in the absence of added electron acceptors, to form about 2.5 μmoles ATP/mg chlorophyll hour. This ATP formation was shown to be O_2 dependent and approximately doubled by the addition of ascorbate. Forti and Jagendorf (9) confirmed these observations and demonstrated that the addition of PPNR stimulated the rate of phosphorylation. They also showed that the reaction could be inhibited by 3-(*p*-chlorophenyl)-1,1-dimethylurea, which is a potent inhibitor of photosynthetic O_2 evolution (13). We have now demonstrated that photosynthetic phosphorylation in the absence of other exogenous electron acceptors can proceed at a rate comparable to other photosynthetic reactions when sufficient PPNR is added to the chloroplast fragments. Thus the photophosphorylation described in this paper appears to be similar to that reported by Forti and Jagendorf (9) although the rate of ATP formation is approximately 30 times that which they obtained. The metal requirements and pH response of the phosphorylation are similar to those of other photophosphorylations (4, 23) and it is reasonable to suppose that the same coupling site is involved in both the presence and absence of an exogenous electron acceptor. The inhibition by 3-(*p*-chlorophenyl)-1,1-dimethylurea suggests that water is the ultimate source of electrons since this compound appears to inhibit the formation of O_2 from water (13). The same electron transfer sequence as normal photosynthesis appears to be operative in view of the inhibition by the analogues of hydroxyquinoline-N-oxide (3, 5). The requirement for O_2 suggests that an endogenous electron acceptor is being continuously reoxidized. This acceptor could be PPNR itself since it may be autoxidizable (21), but since the reaction is inhibited by the antibody to pyridine nucleotide transhydrogenase, this enzyme appears to be involved in the reaction (14, 16). It is, therefore, suggested that the terminal electron acceptor is a small amount of an endogenous acceptor which is undergoing a continuous reduction and oxidation. Perhaps the small amount of phosphorylation under anaerobic conditions may represent a "substrate amount" phosphorylation in which all the PPNR is reduced and then cannot be reoxidized (table II).

In none of the experiments reported in this paper is there any evidence for more than one site of phosphorylation associated with photosynthetic TPN reduction. Ratios of ATP: TPNH greater than unity have been obtained in the presence of high levels of NaCl (fig 2). Further experiments (table V) demonstrate that this excess phosphorylation is probably caused by ATP formation similar to that in the presence of high levels of PPNR and no other exogenous cofactor. For instance the additional phosphorylation was inhibited by anaerobic conditions. This is probably also the explanation of the ATP: TPNH ratio slightly greater than unity which was found by Keister et al. at high levels of PPNR (fig 6 of ref 15). With the exception of these effects, under optional conditions of time and amount of illumination, chlorophyll concentration and reaction components, the ratio of ATP: TPNH invariably approaches unity.

Summary

I. The effects of magnesium ions, inorganic phosphate, adenosine diphosphate, and tris (hydroxymethyl) aminomethane have been studied on the rates of formation of reduced triphosphopyridine nucleotide and adenosine triphosphate by fragmented spinach chloroplasts.

II. Chloroplast fragments have been shown to catalyze a phosphorylation in the presence of large amounts of photosynthetic pyridine nucleotide reductase, and no other added electron acceptor. Rates of photophosphorylation up to 108 μmoles adenosine triphosphate/mg chlorophyll hour were obtained. The phosphorylation was inhibited by anaerobic conditions and by 3-(*p*-chlorophenyl)-1,1-dimethylurea. Pyridine nucleotide transhydrogenase was shown to be involved.

III. Under most experimental conditions the molar ratio of adenosine triphosphate to reduced triphosphopyridine nucleotide was unity, although ratios as high as 3.5 were obtained in the presence of high sodium chloride concentrations. The excess phosphorylation was concluded to be similar to that found in the presence of photosynthetic pyridine nucleotide reductase and no other exogenous cofactor.

Addendum. After the submission of this paper, K. Tagawa, H. Y. Tsujimoto, and D. I. Arnon (Proc. Natl. Acad. Sci. 49, 567-72, 1963) have reported a cyclic photophosphorylation catalyzed by ferredoxin (PPNR) which shows an increased sen-

sitivity to Antimycin A. This reaction was found to proceed anaerobically in the presence of 3-(*p*-chlorophenyl)-1,1-dimethylurea under low levels of illumination. Recent work by H. E. Davenport (Proc. Roy. Soc. London, 157B, 332–45, 1963) also suggests that PPNR may be involved in cyclic photophosphorylation.

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