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STUDIES ON PHOTOSYNTHETIC PHOSPHORYLATION

III. RELATION BETWEEN PHOTOSYNTHETIC PHOSPHORYLATION AND REDUCTION OF TRIPHOSPHOPYRIDINE NUCLEOTIDE BY CHLOROPLASTS

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SUMMARY

The photochemical reduction of TPN by isolated chloroplasts was investigated. A comparison of the rate of TPN reduction with that of photosynthetic phosphorylation provided evidence that the generation of ATP in the presence of vitamin K_3 or FMN is not coupled with the reoxidation of TPNH by the oxidized product of the photolysis of water. Photosynthetic phosphorylation could proceed unimpaired under conditions in which the chloroplasts had lost their ability to reduce TPN. On the other hand, TPN reduction could be considerably stimulated by a chloroplast extract which did not affect photosynthetic phosphorylation. These results are discussed in relation to the recent finding that the reduction of TPN by chloroplasts is accompanied by ATP formation.

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INTRODUCTION

The photosynthetic reduction of carbon dioxide depends on the availability of reduced triphosphopyridine nucleotide (TPNH) and of adenosine triphosphate (ATP).

In the presence of vitamin K_3 or flavin mononucleotide (FMN), chloroplasts can convert light energy into the high-energy phosphate bonds of ATP (photosynthetic phosphorylation[1-4]). The generation of ATP by chloroplasts is linked to the recombination of the oxidized and reduced products of the photolysis of water. In previous papers[5,6] we have shown that under anaerobic conditions FMN and vitamin K_3 are involved in separate pathways for photosynthetic phosphorylation.

In recent years evidence has been presented that chloroplasts, in addition to synthesizing ATP, are able to bring about the direct photochemical reduction of pyridine nucleotides[7,8]. This result, in conjunction with the observation that TPN stimulates the formation of ATP at suboptimal concentrations of vitamin K_3 or FMN, has led some investigators to conclude that photosynthetic phosphorylation is accomplished at the expense of energy liberated by the reoxidation of TPNH by the oxidized product of photodecomposition of water[8-10].

In this paper some results of our experiments on photosynthetic phosphorylation and TPN reduction are reported; these indicate that ATP synthesis by illuminated chloroplasts in the presence of vitamin K_3 or FMN can proceed unimpaired under conditions in which the chloroplasts have lost their ability to reduce TPN. This finding suggests that TPN does not serve as an electron carrier in photosynthetic phosphorylation.

METHODS AND MATERIALS

Preparation of chloroplasts

Broken chloroplasts were prepared as previously described[6] except that tris-(hydroxymethyl)aminomethane (Tris) buffer pH 7.5 was substituted for Tris buffer pH 7.2, and the chloroplast suspension was diluted to a concentration of 0.5 mg of chlorophyll/ml. The chlorophyll content of the chloroplast suspension was determined by the method of ARNON[11].

Measurement of photosynthetic phosphorylation

The ATP formed during photosynthetic phosphorylation was used to synthesize glucose-6-phosphate by adding hexokinase, glucose and $MgCl_2$. The reaction was carried out in Warburg manometer vessels of conventional design. The reaction mixture contained: 40 μmoles of Na and K phosphate buffer, pH 7.5, 10 μmoles of $MgCl_2$, 125 μmoles of glucose, 1 μmole of ADP, 0.5 μmole of vitamin K_3 or FMN, 25 K.M. units[12] of hexokinase, 1 ml of a suspension of chloroplasts in 0.1 *M* Tris buffer, pH 7.5, (containing 0.5 mg of chlorophyll) and deionized water to give a final vol. of 3.0 ml. The vessels were attached to manometers and shaken at 15° in a refrigerated Warburg apparatus, which was illuminated from the bottom by means of two Philips neon lamps (93169 P/00–300/400 W). Anaerobic conditions were maintained by flushing the vessels with pure nitrogen and placing chromous chloride in a side-arm. The reaction was terminated after 30 min by turning off the light and adding 0.3 ml of 20% trichloroacetic acid (TCA) to each vessel. Chloroplast debris

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was removed by centrifugation, the precipitate washed with 2 % trichloroacetic acid, and the supernatant liquid and washings were neutralized with KOH and made up to 10 ml.

Glucose-6-phosphate was determined in the sample by the increase in absorption at 340 mμ with TPN and glucose-6-phosphate dehydrogenase as described previously[6].

Measurement of TPN reduction

The standard reaction mixture contained 1 ml of neutralized TPN (6 μmoles), 1 ml of a suspension of chloroplasts in 0.1 *M* Tris buffer, pH 7.5, containing 0.5 mg of chlorophyll, and 1 ml of deionized water. The reaction was carried out under anaerobic conditions in Warburg manometer vessels as described above for photosynthetic phosphorylation. The reaction was terminated after 20 min by turning off the light, adding 0.3 ml of 1 *M* Na_2CO_3, and immersing the vessels for 5 min in a water-bath at 100°. TCA could not be used in this case, because TPNH is readily destroyed at pH values below 7. The suspension was centrifuged for 15 min at 5000 × *g*, and the precipitate washed twice with 0.5 ml 0.1 *M* Na_2CO_3. The supernatant liquid and washings were neutralized with about 0.5 ml 1 *N* H_2SO_4 and made up to 10 ml with 0.1 *M* Tris buffer, pH 7.5.

TPNH was determined in the sample by the decrease in absorption at 340 mμ with glutathione and glutathione reductase[13]. To 1.9 ml of 0.1 *M* potassium phosphate buffer, pH 7.5, in a quartz cell having a light-path of 1 cm, were added 0.1 ml of oxidized glutathione (10 μmoles) and 1 ml of the unknown TPNH soln. Then 0.1 ml of glutathione reductase (400 units) was added to start the reaction. From the decrease in O.D. at 340 mμ, measured in the Unicam spectrophotometer, the amount of TPNH present was calculated. The estimation of TPNH was always performed in triplicate. With standard solutions of TPNH it was found that the extinction was directly proportional to the concentration up to 8 μmoles of TPNH/sample.

Materials

Glutathione reductase was prepared from yeast by the method of RACKER[14]. DPN-ase was isolated from *Neurospora* mats grown in a zinc-deficient medium using the method of KAPLAN[15]. *Neurospora crassa* Sear et Dodge (5297 A) was obtained from the Centraal Bureau voor Schimmelcultures, Baarn. Glucose-6-phosphate dehydrogenase and hexokinase were prepared as described previously[6].
was isolated from Neurospora mats grown in a zinc-deficient medium using the method of KAPLAN[15]. Neurospora crassa Sear et Dodge (5297 A) was obtained from the Centraal Bureau voor Schimmelcultures, Baarn. Glucose-6-phosphate dehydrogenase and hexokinase were prepared as described previously[6].

TPN, TPNH, ADP, ATP and FMN were products of the Sigma Chemical Company. Glutathione (oxidized form) was obtained from Boehringer, Mannheim.

RESULTS

Fig. 1 shows that the initial rate of TPN reduction by illuminated chloroplasts is unaffected by ascorbate. The decrease in reduction rate with time, observed when chloroplasts are illuminated for a period longer than 15–20 min, however, can be prevented to a large extent by the addition of ascorbate to the reaction mixture.

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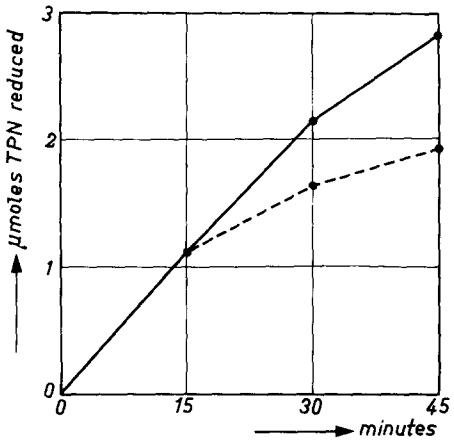

Fig. 1. Time course of aerobic and anaerobic reduction of TPN by illuminated chloroplasts in the presence (———) and in the absence (– – – –) of 20 μmoles of ascorbate.

This result could be explained by assuming that ascorbate protects some essential component of the system against photo-inactivation. Possibly this component is photosynthetic pyridine nucleotide reductase, a soluble enzyme isolated from chloroplasts by SAN PIETRO AND LANG[16], which is required for the photochemical reduction of TPN by chloroplasts. GIOVANELLI AND SAN PIETRO[17] have recently reported that ascorbate is able to protect photosynthetic pyridine nucleotide reductase against photo-inactivation.

The rate of TPN reduction and the effect of ascorbate were found to be the same whether the reaction was performed under aerobic or anaerobic conditions.

Although the rate of photosynthetic phosphorylation was found to be nearly constant over the last year (60–70 μmoles ATP/mg chlorophyll/h), the rate of TPN reduction was highly variable in our experiments. The reduction rate of the most active preparations was about 17 μmoles of TPN reduced/mg of chlorophyll/h. This rate was computed on the basis of the amount of TPN reduced during the first 20 min of the reaction.

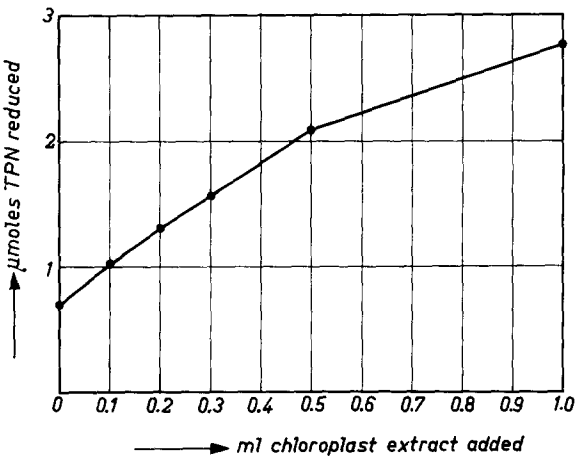

Fig. 2. Reduction of TPN by illuminated chloroplasts as a function of added chloroplast extract.

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In agreement with the findings of ARNON *et al.*[8] and SAN PIETRO *et al.*[16], the rate of TPN reduction could be increased by the addition of a chloroplast extract containing photosynthetic pyridine nucleotide reductase (Fig. 2). For this extract we used the supernatant liquid of a chloroplast suspension containing 1 mg of chlorophyll/ml and prepared as described under METHODS, after centrifugation at 20,000 × *g* for 15 min at 0°.

It is of interest to note that this chloroplast extract did not enhance the amount of ATP formed during photosynthetic phosphorylation. ARNON *et al.*[8,18] and JAGENDORF *et al.*[19,20] originally found that chloroplast extract stimulated photosynthetic phosphorylation, but JAGENDORF AND AVRON reported in an Addendum[20] that at the present time they could observe only negligible stimulation of phosphorylation by adding chloroplast extract to the reaction mixture. Probably, the extractable factor involved in the phosphorylation is not as readily lost by chloroplasts as photosynthetic pyridine nucleotide reductase, so that repeated washings of the chloroplasts are required in order to observe a distinct increase in ATP synthesis by addition of a chloroplast extract. AVRON AND JAGENDORF[21] have shown that different extractable factors are involved in photosynthetic phosphorylation and in TPN reduction. They found that the TPN-reducing factor, partially purified, had a high activity in TPN reduction but only a very low activity in the phosphorylation system. The variations in the rate of TPN reduction observed in our experiments may be due to differences in the concentration or activity of photosynthetic pyridine nucleotide reductase in the various chloroplast preparations. Actually, the greatest effect of chloroplast extract on TPN reduction was found when the TPN-reducing activity of the chloroplasts was rather low.

As shown in Fig. 3, TPN reduction has a rather sharp pH optimum at about 7.5. Accordingly, this pH was adopted for all experiments performed to compare the TPN-reducing and phosphorylating activity of illuminated chloroplasts under various conditions. Although the pH optimum of photosynthetic phosphorylation by chloroplasts was about 7.7, the rate at pH 7.5 was still high (Fig. 4).

Fig. 5 shows that TPN reduction by illuminated chloroplasts is directly proportional to the concentration of chlorophyll down to 0.5 mg/3 ml of reaction mixture. At lower concentrations of chloroplast material the rate of TPN reduction decreases rapidly, possibly because of a relatively lower content of photosynthetic pyridine nucleotide reductase.

Like MARRÈ *et al.*[22] and SAN PIETRO *et al.*[16] we could not demonstrate that ATP had any positive effect on TPN reduction. The reaction rate was also practically unaffected by addition of $MgCl_2$, ADP and inorganic phosphate or one of these components to the reaction mixture, or by addition of the glucose–hexokinase system. Cofactors of photosynthetic phosphorylation such as vitamin K_3 and FMN (0.1 μmole), however, inhibited the reaction completely.

As shown in Table I, chloroplasts oxidize TPNH rapidly both in darkness and in light when vitamin K_3 or FMN is provided (*cf.* ref. [23]). The oxidation of TPNH is also catalyzed by benzoquinone and 2,6-dichlorophenol-indophenol, but not by potassium ferricyanide and ascorbate. The reaction is only slightly inhibited by $3 \cdot 10^{-4}$ *M* *p*-chloromercuribenzoate (*p*CMB), and appears to be dependent on oxygen, for only a small amount of TPNH is oxidized under anaerobic conditions.

In the absence of an intermediate electron acceptor, chloroplasts are almost

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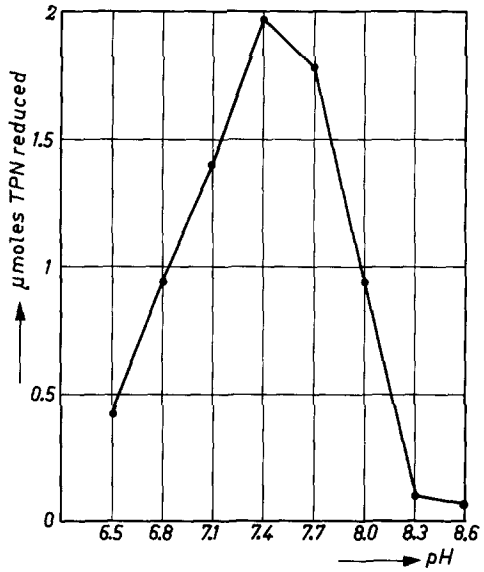

Fig. 3.

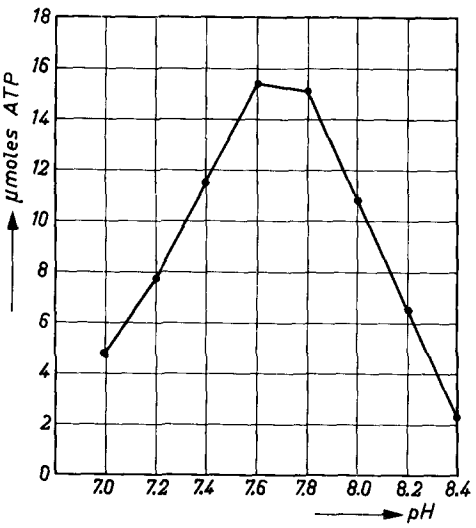

Fig. 4.

Fig. 3. Effect of pH on TPN reduction.

Fig. 4. Effect of pH on photosynthetic phosphorylation.

Fig. 5. TPN reduction as a function of chlorophyll concentration.

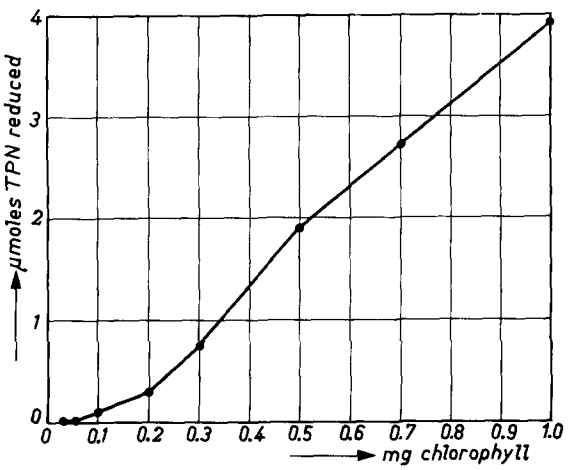

Fig. 5.

entirely unable to oxidize TPNH. When chloroplasts are illuminated, however, the rate of photoreduction of TPN may be equal to or higher than the rate of some light-induced oxidation of TPNH, so that no net oxidation of TPNH can be observed. When *p*CMB, which inhibits TPN reduction[8] but does not affect photolysis of water[24], was added TPNH was not oxidized in light; however, the oxidation of TPNH may also be blocked by *p*CMB. Alternatively, the reduced and oxidized products of photodecomposition of water may recombine rapidly when no hydrogen acceptor is added to the system. Therefore we tried to detect TPNH oxidation in light after addition to the chloroplasts of potassium ferricyanide (5 µmoles) and *p*CMB. The former did not catalyze the oxidation of TPNH by chloroplasts, but it appeared to be reduced by TPNH in a non-enzymic reaction (Table I). Hence we cannot say with certainty

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TABLE I

OXIDATION OF TPNH BY CHLOROPLASTS

The reaction mixture also included 1 ml of chloroplast suspension, containing 0.3 mg chlorophyll, in 0.1 *M* Tris buffer, pH 7.5, 1 ml of TPNH (6 μmoles), and deionized water to give a final vol. of 3 ml. The reaction was carried out under aerobic conditions at 15° in Warburg manometer vessels. TPNH was determined as described under METHODS.

Additions (μmoles)	*μmoles TPNH oxidized in 20 min*	
	dark	*light*
No addition	0.4	0.3
0.1 vitamin K_3	3.8	3.7
0.1 FMN	1.7	1.8
0.1 benzoquinone	0.9	—
0.1 2,6-dichlorophenol-indophenol	1.2	—
0.1 $K_3Fe(CN)_6$	0.3	—
10 ascorbate	0.3	0.3
0.1 vitamin K_3 + 10 ascorbate	3.7	3.7
0.1 vitamin K_3 + 1 *p*CMB	3.4	—
0.1 vitamin K_3; chloroplasts omitted	0	—
1 *p*CMB	—	0.4
5 $K_3Fe(CN)_6$	2.1	2.1
5 $K_3Fe(CN)_6$; chloroplasts omitted	1.9	—
5 $K_3Fe(CN)_6$ + 1 *p*CMB	—	2.1
0.1 vitamin K_3; anaerobic conditions	0.7	0.6

that chloroplasts are unable to oxidize TPNH under light conditions when no intermediate electron acceptor is provided.

No phosphorylation occurred when chloroplasts were exposed to light in the presence of TPNH, or in the dark upon addition of TPNH and vitamin K_3 or FMN.

Effect of inhibitors on TPN reduction and photosynthetic phosphorylation

In a previous paper[6] the influence of several inhibitors on photosynthetic phosphorylation was reported. We have now compared the effect of inhibitors on TPN reduction and on photosynthetic phosphorylation in the presence of vitamin K_3 or FMN (Table II). The reactions were carried out under anaerobic conditions at pH 7.5 and at a chlorophyll concentration of 0.5 mg/vessel, without addition of chloroplast extract or ascorbate.

o-Phenanthroline[25], 3-(4-chlorophenyl)-1,1-dimethylurea (CMU)[26] and dicumarol[27], which are known to inhibit the HILL reaction very strongly, inhibit both photosynthetic phosphorylation and TPN reduction. This result indicates that photolysis of water is a prerequisite for both photochemical processes. As shown in Table II, TPN reduction is somewhat more sensitive to *o*-phenanthroline than the phosphorylation.

The greater sensitivity of phosphorylation with FMN to KCN, NaN_3 and NH_2OH as compared to ATP synthesis in the presence of vitamin K_3 has been discussed previously[5,6]. TPN reduction is resistant to KCN and NaN_3, but is inhibited by 10^{-3} *M* NH_2OH to the same extent as photosynthetic phosphorylation with FMN. Phosphorylation and TPN reduction also responded differently to *p*CMB, 2,5-dinitrophenol and 2,4-dinitrophenol. Whereas the reduction of TPN by illuminated chloroplasts was inhibited by very low concentrations of these compounds, their effect on

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TABLE II

EFFECT OF INHIBITORS ON PHOTOSYNTHETIC PHOSPHORYLATION AND TPN REDUCTION

The values presented in this table indicate percentage of the rate observed in the absence of the inhibitor.

Inhibitor	*Photosynthetic phosphorylation*		*TPN reduction*
	with vitamin K_3	*with FMN*	
10^{-3} *M* NH_2OH	65	25	25
10^{-3} *M* NaN_3	95	30	90
10^{-4} *M* *p*CMB	35	35	0
$3.3 \cdot 10^{-5}$ *M* *p*CMB	60	60	5
$2 \cdot 10^{-5}$ *M* *p*CMB	90	90	65
10^{-5} *M* *p*CMB	100	100	95
10^{-2} *M* KCN	100	15	100
$4 \cdot 10^{-6}$ *M* CMU	45	40	45
$2 \cdot 10^{-4}$ *M* *o*-phenanthroline	15	10	10
10^{-4} *M* *o*-phenanthroline	55	50	20
$5 \cdot 10^{-5}$ *M* *o*-phenanthroline	80	85	35
$2 \cdot 10^{-2}$ *M* iodoacetamide	90	75	60
10^{-2} *M* iodoacetamide	100	90	70
10^{-4} *M* dicumarol	40	35	45
$3 \cdot 10^{-4}$ *M* dicumarol	15	15	20
$5 \cdot 10^{-4}$ *M* 2,5-dinitrophenol	45	45	5
$2 \cdot 10^{-4}$ *M* 2,5-dinitrophenol	90	95	10
10^{-4} *M* 2,5-dinitrophenol	100	100	35
$5 \cdot 10^{-5}$ *M* 2,5-dinitrophenol	100	100	55
$5 \cdot 10^{-4}$ *M* 2,4-dinitrophenol	60	30	30
$2 \cdot 10^{-4}$ *M* 2,4-dinitrophenol	90	75	40
10^{-4} *M* 2,4-dinitrophenol	100	90	50
$5 \cdot 10^{-5}$ *M* 2,4-dinitrophenol	100	100	55

photosynthetic phosphorylation was less pronounced. Phosphorylation with vitamin K_3 was somewhat more resistant to 2,4-dinitrophenol than that with FMN (*cf.* ref. [9]).

These results show that the generation of ATP by illuminated chloroplasts can still proceed at a fast rate under conditions in which TPN reduction is strongly inhibited.

Photochemical activity of chloroplast fragments obtained from digitonin extracts of chloroplasts

Evidence in support of the conclusion that photosynthetic phosphorylation can proceed independently of TPN reduction was also obtained from experiments with chloroplast fragments prepared from digitonin extracts of chloroplasts. These chloroplast fragments could still generate ATP in light, but had lost the ability to reduce TPN (Table III).

The chloroplast fragments used were prepared as follows. Whole spinach chloroplasts were prepared as described under METHODS. The chloroplasts were suspended in 40 ml of a 1% soln. of digitonin in 0.1 *M* Tris buffer, pH 7.5. The suspension was allowed to stand for 30 min at 0°, and was then centrifuged for 7 min at 1000 × *g*. The residue was discarded and the supernatant liquid centrifuged for 30 min at 20,000 × *g*. The precipitated chloroplast fragments were resuspended in the 0.1 *M* Tris buffer, and the suspension was diluted to a concentration of 1 mg of chlorophyll/ml. Photosynthetic phosphorylation and TPN reduction were measured as described

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under METHODS except that an amount of chloroplast fragments equivalent to 1 mg of chlorophyll was used in the reaction vessels.

TABLE III

PHOTOSYNTHETIC PHOSPHORYLATION AND TPN REDUCTION BY CHLOROPLAST FRAGMENTS OBTAINED FROM DIGITONIN EXTRACTS OF CHLOROPLASTS

	μmoles ATP/mg chlorophyll/h		*μmoles TPN reduced/mg chlorophyll/h*
	with vitamin K_3	*with FMN*	
Without ascorbate	8.9	6.0	0.1
40 μmoles of ascorbate added	11.2	7.7	0.1

Effect of DPN-ase on photosynthetic phosphorylation

It seemed to us that the supposition that photosynthetic phosphorylation is independent of TPN reduction could also be investigated by adding the enzyme DPN-ase, which should abolish a TPN-dependent phosphorylating mechanism.

The addition of DPN-ase in amounts up to 500 units/reaction vessel did not affect the formation of ATP by illuminated chloroplasts in the presence of vitamin K_3 or FMN. As was to be expected, TPN reduction was completely abolished under these conditions.

In the experiments with DPN-ase, the reaction mixture was incubated for 10 min at 15° before turning on the light. At 15°, 100 units of DPN-ase were found to split about 0.5 μmole of TPN in 10 min. KROGMAN[28] has recently measured the pyridine nucleotide content of isolated chloroplasts. He found a maximum value of 1 molecule of oxidized pyridine nucleotide/2500 molecules of chlorophyll. Although the amount of DPN-ase added in our experiments thus seems to be quite sufficient to split any TPN present, the possibility that some bound TPN was unaffected by the enzyme cannot be completely excluded.

DISCUSSION

A comparison of the rate of TPN reduction by illuminated chloroplasts with that of photosynthetic phosphorylation in the presence of vitamin K_3 or FMN under various conditions yielded the following results: (1) The rate of ATP synthesis was nearly constant over a period of a year, but the rate of TPN reduction was highly variable. (2) The reduction of TPN was considerably stimulated by a chloroplast extract which did not affect the phosphorylation. (3) The phosphorylation could still proceed at a high rate under conditions in which TPN reduction was strongly blocked by inhibitors. (4) Chloroplast fragments obtained from digitonin extracts of chloroplasts were still able to generate ATP in light, but had lost the ability to reduce TPN. (5) DPN-ase did not inhibit photosynthetic phosphorylation.

These results provide evidence that photosynthetic phosphorylation is independent of TPN reduction, and that ATP synthesis in the presence of vitamin K_3 or FMN is not coupled to the reoxidation of TPNH by the oxidized product of photodecomposition of water.

Since the reduction of TPN by water involves a change in standard free energy

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of about 50 kcal, which is 8 kcal more than the energy of one quantum of red light, some investigators have suggested that the additional energy could be provided by the pyrophosphate bonds of ATP. In previous papers[5,6,27] we have postulated a mechanism by which vitamin K, which has a more positive redox potential than TPN, would be directly reduced by the illuminated chloroplasts through a 1-quantum process. The reduction of pyridine nucleotides by a part of the reduced vitamin K would then follow, the energy for this reaction being supplied by ATP generated during the oxidation of residual reduced vitamin K. The generation of ATP by illuminated chloroplasts in the presence of vitamin K_3 or FMN was assumed to be linked to the reoxidation of photochemically-reduced vitamin K.

BISHOP[29] has recently found additional evidence in support of our hypothesis that vitamin K is an essential factor in the photochemical activity of isolated chloroplasts. He has shown that menadione can restore the reducing capacity towards HILL oxidants of freeze-dried chloroplasts which have been extracted with petroleum ether.

ARNON *et al.*[9] have recently shown, however, that the reduction of TPN by illuminated chloroplasts is accompanied by the formation of ATP:

$$2ADP + 2P_i + 2TPN^+ + 4H_2O \longrightarrow 2ATP + 2TPNH + 2H^+ + 2H_2O + O_2$$

Under appropriate experimental conditions, the evolution of 1 mole of O_2 was accompanied by the reduction of 2 moles of TPN and the esterification of 2 moles of inorganic phosphate (P_i). We confirmed that the reduction of TPN was accompanied by ATP formation, but the stoichiometry of the reaction was found to depend upon the amount of chloroplast extract added. As the reduction of TPN was much more stimulated by the extract than the accompanying phosphorylation, the ratio μmoles TPN reduced: μmoles ATP formed could be raised from 0.7 to 2 by adding chloroplast extract. Addition of 0.001 μmole of vitamin K_3 or FMN, on the other hand, increased ATP formation by about 100% without suppressing TPNH accumulation. In the presence of larger amounts of these cofactors TPN reduction was abolished and ATP synthesis was strongly stimulated.

The fact that the reduction of TPN by illuminated chloroplasts is accompanied by ATP formation may be explained by one of the following theories:

1. ATP is generated by the same mechanism as in the presence of vitamin K_3 or FMN. This seems rather improbable because no ATP is formed when chloroplasts are illuminated in the absence of both TPN and vitamin K_3 or FMN.

Since photosynthetic phosphorylation has been shown to be independent of TPN reduction, addition of TPN to chloroplasts seems unlikely to induce this phosphorylation process.

2. The formation of ATP is coupled to partial reoxidation of TPNH. As the rate of TPN reduction by chloroplasts was found to be much lower than the rates of the HILL reaction and of photosynthetic phosphorylation, this possibility must be considered. However, because of the difficulties encountered in detecting TPNH oxidation in light, no definite conclusion is possible at present.

3. Illuminated chloroplasts are able to attain a redox potential below the level of TPN. In a 2-quanta process sufficient energy would be available to accomplish both the reduction of 1 mole of TPN and the formation of 1 mole of ATP. BRUGGER AND FRANCK[30] have suggested a mechanism in which a chlorophyll molecule in its lowest metastable triplet state receives energy by the process of sensitized fluorescence

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from a second excited chlorophyll molecule (in its first excited singlet state), and is thereby promoted to the next higher triplet state. In this way 65–70 kcal could be stored in one chlorophyll molecule.

This explanation could account for the ability of methylviologen and benzylviologen, which have E_0^1 values at pH 7 of —0.446 and —0.359 V respectively[31], to catalyze photosynthetic phosphorylation[20].

To explain the essential function of vitamin K in the HILL reaction and the fact that redox systems with a negative E_0^1 value are scarcely reduced by illuminated chloroplasts[24,32], we may assume that the HILL oxidants are reduced via vitamin K. Reoxidation of vitamin K and of the HILL oxidant by the oxidized product of photolysis of water could prevent the attainment of a negative redox potential in this system (*cf.* ref. [33]).

In Fig. 6 a tentative scheme for the photochemical activities of chloroplasts is given, based on the assumption that in photosynthesis water is split through a 2-quanta process.

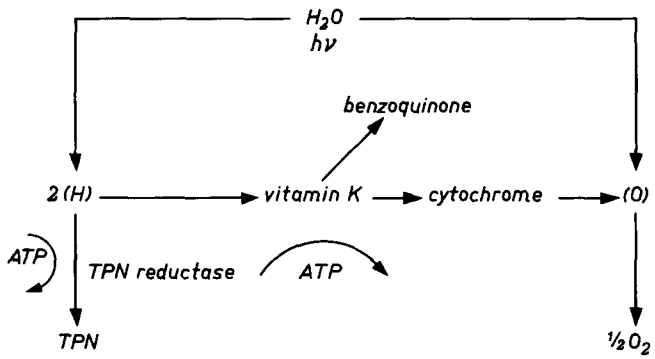

Fig. 6.

This scheme implies that the transport of electrons from the reduced [H] to the oxidized [O] product of the photolysis of water via vitamin K and the cytochromes is coupled to the formation of ATP. *In vitro*, an intermediate electron carrier like vitamin K_3 or FMN is required to restore the electron transport which may be abolished during the isolation procedure of the chloroplasts. For the sake of simplicity only one pathway for photosynthetic phosphorylation is given in Fig. 6.

In the HILL reaction, the hydrogen acceptor, *e.g.* benzoquinone, is reduced via vitamin K and oxygen is evolved. Apparently, reoxidation of hydroquinone by [O] or molecular oxygen does not compete greatly with the photochemical reduction of benzoquinone. The marked stimulating effect of phenazine methosulfate on photosynthetic phosphorylation and on the HILL reaction[20] might indicate an unusual efficiency in promoting electron transport from [H] to [O] and to the HILL oxidant, respectively, possibly owing to a fast "by-pass" around some rate-limiting site of the system.

In the absence of any cofactor of photosynthetic phosphorylation, chloroplasts are able to reduce TPN and to evolve oxygen. TPN reduction requires photosynthetic pyridine nucleotide reductase and is coupled with the formation of ATP.

It must be stressed, however, that at present it cannot be definitely stated that

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the photolytic splitting of water by chloroplasts proceeds via a 2-quanta process. With the exception of the viologens, compounds effective as cofactors for photosynthetic phosphorylation have a redox potential of around o V at pH 7, and can thus be reduced by vitamin K hydroquinone. The ability of methyl viologen (N,N'-dimethyl-γ,γ'-dipyridinium dichloride) or benzyl viologen (N,N'-dibenzyl-γ,γ'-dipyridinium dichloride) to act as cofactor in photosynthetic phosphorylation could also be explained by assuming that the reduction of the viologens, which are chemically related to the pyridine nucleotides, is accomplished by the same mechanism as the reduction of TPN.

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