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PHOTOSYNTHETIC ADENOSINE TRIPHOSPHATE FORMATION AND PHOTO-REDUCTION OF DIPHOSPHOPYRIDINE NUCLEOTIDE WITH CHROMATOPHORES OF *RHODOSPIRILLUM RUBRUM*

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SUMMARY

We have studied photosynthetic DPN reduction by succinate and ATP formation from ADP and P_i in chromatophores of *Rhodospirillum rubrum*.

Our findings are:

1. Magnesium ion accelerates DPN photoreduction most effectively at $5 \cdot 10^{-3}$ M, but is inhibitory at higher concentrations. The stimulatory effect of the ion is similar to that on the photosynthetic ATP formation.

2. Photoreduction of DPN is maximal in rate in the presence of $3 \cdot 10^{-4}$ M DPN, and is inhibited with increasing concentration.

3. In response to variations in concentration of succinate or DPN, photoreduction of DPN reaches a steady state in which approximately half of the DPN present is reduced.

4. DPNH/DPN (1 : 1) is oxidized by succinate/fumarate anaerobically in darkness, provided that the E_h value of succinate/fumarate is more positive than —0.04 V. This accords with the suggestion that there is an enzyme system which catalyzes oxidation of DPNH by fumarate anaerobically in darkness.

5. Photoreduction of DPN is inhibited in varying degree by various adenosine derivatives and pyrophosphate; *i.e.* strong inhibition with ATP, ADP and pyrophosphate; moderate with adenosine 3′-monophosphate, AMP and adenosine; none with orthophosphate.

6. Photosynthetic ATP formation with "bound" ADP is inhibited by adenosine, adenosine 3′-monophosphate and DPN, but not by ADP or TPN. The presence of ATP or pyrophosphate inhibits ATP formation either with "bound" ADP or in the presence of exogenous ADP: The lower the concentration of ADP present, the greater is the inhibition. Kinetic data show that the inhibition by ATP is competitive with orthophosphate, but non-competitive with ADP, while pyrophosphate is competitive with both ATP and orthophosphate.

Based on these findings, mechanisms for photosynthetic ATP formation and DPN reduction are suggested.

INTRODUCTION

It is generally accepted that photosynthetic bacteria produce both oxidizing and reducing systems in chromatophores upon illumination with actinic wavelengths of light, just as do the chloroplasts of green leaves in higher plants and algae.

Using the facultative photoheterotrophe, *Rhodospirillum rubrum*, Frenkel[1,2] showed that chromatophores could catalyze photoreduction of DPN in the presence of either FMNH$_2$, or succinate with an accumulation of DPNH. His findings were extended by Vernon and Ash[3,4]. Many researches have shown also that a heme protein(s) present in the cells is oxidized, upon illumination with actinic lignt[5–7].

The respiratory chain of the cells of *R. rubrum* has been studied, and characterized with respect to a sequence of components, which leads alternatively to oxygen as one terminus, and which functions in a cyclic fashion with bacteriochlorophyll as another terminus when it is photoactivated[8,9]. Electron flow through the cyclic chain is coupled with ATP formation from ADP and P_i (see ref. 10). It is not known whether pyridine nucleotide functions in the cyclic chain. There are a number of speculations about the function of DPN in bacterial photometabolism[2,10] and of TPN in green-plant photosynthesis[11]. Frenkel[2] and Vernon and Ash[3] found that photoreduction of DPN by *R. rubrum* chromatophores was significantly inhibited in the presence of ADP; the latter authors suggested that ADP-activated photophosphorylation coupled with the cyclic electron flow shunted electron flow from photoreductant to DPN. On the other hand, Nozaki *et al.*[12] reported that photoreduction of DPN appeared to accompany ATP formation; one mole of ATP was formed for each mole of DPN reduced. San Pietro[13] found that the washed chloroplasts prepared from spinach leaves, if supplemented with the photosynthetic pyridine nucleotide reductase, could restore photophosphorylation activity as well as photoreduction of TPN, which indicated that photoreduction of TPN might accompany ATP formation in the chloroplasts. More recently Krogmann and Stiller[14] have found that the ATP formation with washed chloroplasts can be restored by addition of a naturally occurring flavone-like substance. These researches are cited as a few among many which show that the co-factor requirements for photophosphorylation are still imperfectly understood.

We have used *R. rubrum* chromatophores to study photoreduction of DPN and ATP formation, in an attempt to elucidate relationships between these two processes. Kinetics of the two reactions have been compared in the presence and absence of various substances, in particular adenosine derivatives and pyrophosphate.

MATERIALS AND METHODS

R. rubrum was cultured as described previously[10]. The cells were harvested and washed once in 0.1 M Tris–HCl buffer (pH 8.0) containing 10 % (w/v) sucrose (Tris–HCl–sucrose buffer). The washed cells were suspended in approx. 5 vol. (v/w) of Tris–HCl–sucrose buffer, and ruptured by sonication in a 10-kc Kubota oscillator for approx. 2 min in the cold (0–4°). The resultant solution was centrifuged, and the fraction which sedimented between 20000 × *g* for 15 min and 64000 × *g* for 1 h was collected, washed three times with the Tris–HCl–sucrose buffer, and then suspended in a small volume of the buffer so that $A_{880\,m\mu}$ per ml of the suspension was approx. 200. This concentrated chromatophore suspension was stored in the darkness in a refrigerator (care taken not to freeze). For tests, aliquots were diluted with the Tris–HCl–sucrose buffer so that $A_{880\,m\mu}$ per ml of the test solution was approx. 50; this solution was called "chromatophores". These preparations were active in DPN photoreduction and ATP formation, and were used in most of the experiments. The chromatophores, if prepared with 0.1 M Tris–HCl buffer (pH 8.0) instead of Tris–HCl–sucrose buffer, usually lost the ability to photoreduce DPN, although the capacity for photosynthetic ATP formation was always retained. Results on photosynthetic ATP formation were essentially the same for the chromatophores prepared by the two methods, if ascorbate was used as a reducing agent instead of succinate.

ATP formation during photophosphorylation was assayed by the method of NIELSEN AND LEHNINGER[15] as modified by AVRON[16]. Standard components of the reaction mixture for photophosphorylation were as follows: 0.2 M Tris–HCl buffer (pH 8.0), 0.50 ml; 0.1 M $MgCl_2$, 0.10 ml; approx. 0.1 M [^{32}P]P_i solution (pH 8.0), 0.10 ml; 0.67 M ascorbate solution (pH 8), 0.10 ml; the chromatophores ($A_{880\,m\mu}$/ml, approx. 50), 0.10 ml; total volume adjusted to 1.50 ml by addition of water. The reaction mixture was pipetted into small test tubes (10 × 1 cm). For experiments in the dark, the tubes were completely covered with aluminum foil. The reactions were carried out at 30° with illumination from a bank of tungsten lamps; uniform intensity of illumination was obtained by means of a frosted glass located in front of the lamps. The temperature was controled adequately by the use of a large water bath provided with a rapid circulation system. The light intensity employed was 500 ft-candles. After preincubation for 3 min at 30°, the reactions were started by addition of chromatophores in most of the cases and [^{32}P]P_i in some, and stopped by adding 0.50 ml of ice-cold 30 % trichloroacetic acid, immediately followed by cooling in an ice–water bath. After standing for at least 10 min, the test tubes were centrifuged and decanted. Aliquots of the clear supernatant liquids (0.50 ml) were used for the assay of the ATP formed.

Photoreduction of pyridine nucleotide was followed by measuring the increase of $A_{340\,m\mu}$ in a Cary, model 14-R, spectrophotometer. The reactions were carried out in vacuum in Thunberg-type cuvettes (1 cm optical path) in a water bath regulated at 22°. The standard components of the reaction mixture were as follows in the main chamber: 0.1 M Tris–HCl–sucrose buffer (pH 8.0), 1.00 ml; 0.1 M $MgCl_2$, 0.20 ml; 0.005 M DPN, 0.20 ml; chromatophores ($A_{880\,m\mu}$/ml, approx. 50), 0.20 ml; total volume of cuvette adjusted to 3.00 ml with water; in the side chamber: 0.1 M succinate (pH 8), 0.20 ml. To effect anaerobic conditions, the cuvettes were evacuated until no bubbling occurred, then filled with nitrogen gas, which had been previously purified to minimize contaminating oxygen in the gas (commercial product of Nihon Sanso Co., Ltd., Osaka) by passing through dithionite solution, then pyrogallol solution. This gas-exchange procedure was repeated five times. The reactions were started by mixing the contents of both chambers, and readings were made every 5 min to measure the differential absorbancy at 340 mμ between trial and control cuvettes. The latter contained the same reaction mixture except DPN and were treated in the same manner as were the trial cuvettes. In most experiments, readings were made for 10 min in the darkness as control tests, then in the light (500–900 ft-candles) for another 30 min. The rates of the reactions in the light were essentially linear with time and could be compared under various conditions.

The oxidation of DPNH by fumarate with the chromatophores in the darkness under anaerobic conditions was followed by measuring the decrease in $A_{340\,m\mu}$. Other experimental conditions were the same as for the photoreduction of DPN. The standard components of the reaction mixture were as follows in the main chamber of a Thunberg-type cuvette: 0.1 M Tris–HCl–sucrose buffer (pH 8.0), 1.00 ml;

0.1 M $MgCl_2$, 0.20 ml; chromatophores ($A_{880\ m\mu}$/ml, approx. 50), 0.20 ml; 1.0 M succinate (pH 8), 0.20 ml; varied concentration of fumarate, 0.20 ml; total volume of a cuvette adjusted to 3.00 ml with water; in the side chamber: 0.005 M DPNH, 0.20 ml; 0.005 M DPN, 0.20 ml. The reactions were started by mixing the reaction components. After 5 min, readings were made, and continued every 5 min for 30 min. The reactions were carried out anaerobically in the darkness at 22°.

Photosynthetic pyridine nucleotide reductase was prepared from spinach leaves according to the method of SAN PIETRO AND LANG[17].

L *d*-Ascorbic acid was a commercial-sample material (Wako pure chemical Industries, Ltd., Oska). ATP, ADP (each from muscle), adenosine 3′-monophosphate, AMP and adenosine were commercial preparations (Sigma Chemical Co., St. Louis, Mo.). These reagents were dissolved in 0.1 M Tris - HCl buffer (pH 8.0) and adjusted to pH 8 with NaOH. [^{32}P]Phosphoric acid (the Radiochemical Centre, Amersham) was boiled in 1 N HCl for several hours and treated with Norite, as recommended by AVRON[16]. The resulting solution ([^{32}P]P_i solution) was supplemented with an appropriate amount of Na_2HPO_4, then adjusted to pH 8 with NaOH; the final concentration of phosphorus was approx. 0.1 M, and radioactivity (counts/min/ml) approx. 10^7 unless otherwise specified. The phosphate concentration of the [^{32}P]P_i solution was assayed according to the method of FISKE AND SUBBAROW[18].

The concentration of the adenosine derivative solutions was determined spectrophotometrically based on published values for molar extinction[19]. Molar extinctions of DPN and DPNH were taken as $18.0 \cdot 10^3$ at 260 mμ and $6.22 \cdot 10^3$ at 340 mμ, respectively[20].

RESULTS

General properties of photoreduction of DPN by chromatophores

It was found that very low intensities of light were effective in DPN photoreduction; saturation was reached at 500 ft-candles[3]. In agreement with the findings of FRENKEL[1,2] and VERNON AND ASH[3], DPN was not photoreduced by the chromatophores unless either succinate or $FMNH_2$ were present; even in their presence TPN was not photoreduced unless the reaction system was supplemented with the photosynthetic pyridine nucleotide reductase preparation[17] which had been purified up to the protamine sulfate precipitation step (see refs. 3 and 17). Magnesium ion was found to accelerate DPN photoreduction most effectively at $5 \cdot 10^{-3}$ M, but was somewhat inhibitory at higher concentrations (Fig. 1). The stimulatory effect of magnesium ion appeared to be similar for both photoreduction of DPN and ATP formation. Photoreduction of DPN also was optimal at $3 \cdot 10^{-4}$ M DPN; higher concentrations of DPN exerted a marked inhibitory effect, apparently owing to substrate inhibition (Fig. 2). This relation between concentration of pyridine nucleotide and rate of reaction was like that found previously for the DPNH–heme protein reductase of *R. rubrum* (see ref. 8), indicating that the same enzyme might be functional in both DPN reduction and DPNH oxidation.

Repeated trials showed that the chromatophores, if prepared with the use of Tris - HCl buffer instead of Tris - HCl - sucrose buffer, lost most of their DPNH-photoreducing activity with succinate, but retained it with $FMNH_2$ or ascorbate; they retained photophosphorylation activity as well if the redox-potential of the reaction system was adjusted to an optimal value with ascorbate (*cf.* ref. 10). Together with the finding that DPN-photoreducing activity so inactivated could not be restored by addition of photosynthetic pyridine nucleotide reductase with succinate as the electron donor, even if either DPN or TPN was used as the electron acceptor, it seemed plausible that electron flow from succinate to the cyclic system, possibly *via* the succinic dehydrogenase system, was hampered.

In an attempt to achieve conditions for electron flow which were as physiological as possible, a succinate/fumarate system (mixture of succinate and fumarate in an appropriate ratio in concentration) was used as an electron donor or acceptor instead of chemical oxidation-reduction reagents. In most experiments, the chromatophores prepared with Tris-HCl-sucrose buffer were used, but the ones prepared with Tris-HCl buffer gave similar results for photophosphorylation when in the presence of an appropriate concentration of ascorbate[10].

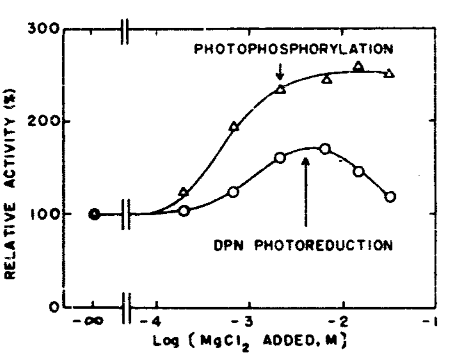


Fig. 1. Effect of magnesium ion on photosynthetic ATP formation and photoreduction of DPN. Both reactions were carried out with illumination of 500 ft-candles.

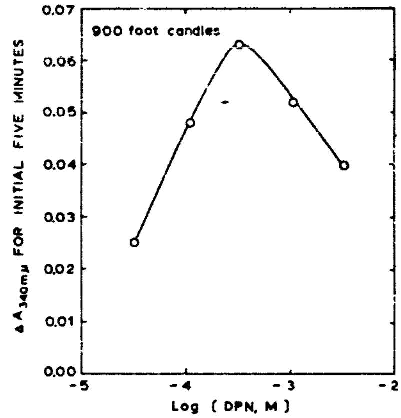


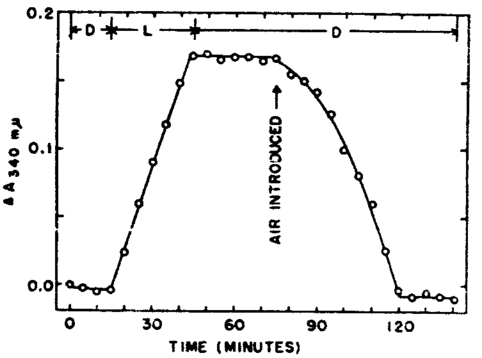
Fig. 2. Effect of concentration of DPN on photoreduction of DPN.

The DPNH once formed photosynthetically by the chromatophores in the presence of succinate was found not to be oxidized at all under anaerobic conditions in the darkness established immediately after the illumination period (Fig. 3). If air was allowed to diffuse into the reaction mixture, the DPNH was rapidly oxidized to completion. This suggested that an intermediate substance photochemically produced disappeared so that oxidation of the DPNH in darkness by the fumarate (which had been formed from the succinate during the reaction in the light) could not proceed. This possibility was ruled out by the finding that DPNH was rapidly oxidized by fumarate under anaerobic condition in darkness provided that the concentration of succinate present in the reaction mixture was lowered (see below).

Effects of adenosine derivatives and phosphates on DPN photoreduction

FRENKEL[2] and VERNON AND ASH[3] found that DPN photoreduction was markedly inhibited in the presence of ADP. As mentioned previously, the latter authors suggested that the inhibition caused by ADP owed its origin to activation of photophosphorylation, but it appears that in their experiments, the effect of externally added P_i was not tested. Although the possibility could not be excluded that a trace of P_i was still present in the chromatophores after their washing procedure and would have been available for ATP formation, it is unlike that such traces of P_i would have been sufficient.

This conclusion has been strengthened by the demonstration that the Michaelis–Menten constant for P_i in the photosynthetic ATP formation is now known to be as high as 10^{-3} M, and that there is no inhibitory effect on the ATP formation at the highest concentration tested[21] (0.1 M). It has been confirmed that P_i does not affect DPN photoreduction even in the presence of a high concentration (0.04 M). ADP is found to inhibit photoreduction of DPN both in the presence and absence of P_i; the K_m for ADP is approx. $5 \cdot 10^{-3}$ M (Fig. 4). Furthermore, ATP and PP_i inhibit the DPN photoreduction as well as does ADP. Other adenosine derivatives examined, adenosine 3'-monophosphate, AMP and adenosine, are also markedly inhibitory, but less effective than ATP, ADP and PP_i.



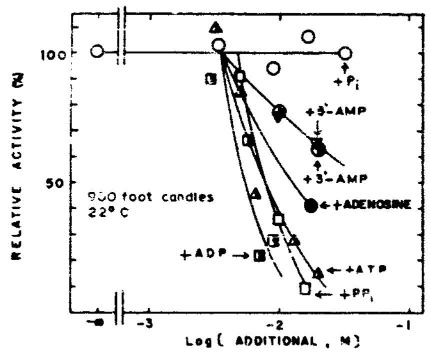


Fig. 3. Photoreduction of DPN in the presence of succinate and re-oxidation in the darkness of the DPNH thus formed. Reactions in the darkness and in the light are indicated by "D" and "L", respectively. "L" was carried out with illumination of 700 ft-candles. For the second dark period, air was introduced gently in the gas phase of the Thunberg-type cuvette containing the reaction mixture.

Fig. 4. Effect of adenosine derivatives and pyrophosphate on DPN photoreduction.

Effects of adenosine derivatives and pyrophosphate on photosynthetic ATP formation

In the presence of externally added ADP (1.33 mM), both ATP and PP_i were found to be effective inhibitors for photosynthetic ATP formation, but adenosine, adenosine 3'-monophosphate, AMP, DPN and TPN were not (Table I). On the other hand, in the absence of ADP, photosynthetic ATP formation with the bound ADP present in the washed chromatophores and externally added P_i (see ref. 21) was inhibited in the presence of adenosine, adenosine 3'-monophosphate and DPN but not AMP or TPN. The lack of inhibition with AMP could be explained by the supposition that the washed chromatophores still possessed "adenylate kinase"[22] to a small extent.

Under our experimental conditions, ATP formation was inhibited in the presence of ATP when the concentration was greater than $2 \cdot 10^{-3}$ M; the K_m for ATP was approx. $1.5 \cdot 10^{-2}$ M, or approx. 11 times as high as the concentration of ADP present (Fig. 5). A plot of reciprocals of the P_i concentrations and amounts of ATP formed according to the method of LINEWEAVER AND BURK[23] showed two straight lines crossing each other just at the point of infinite concentration of P_i added (Fig. 6). This agreed with the conventional mechanism for ATP as a competitive inhibitor, thus ATP could be pictured as displacing P_i from an active site. On the other hand, ATP appeared to be a non-competitive (uncompetitive) inhibitor[24,25] against ADP, as indicated by the Lineweaver-Burk plot which showed two straight lines parallel to each other (Fig. 7). Hence, it appeared that ATP combined with an intermediate complex of ADP and an enzyme functional in the ATP-forming process, but not with the free enzyme. Furthermore, it was found that PP_i was a competitive inhibitor with both ADP and P_i (Figs. 8 and 9).

DPN-reducing capacity produced in photoactivated chromatophores

As mentioned above, the chromatophores acquire the ability capable to reduce DPN in the presence of succinate on illumination. It is apparent that this reduction capacity can be restored as long as the chromatophores are illuminated. DPN, once reduced with the illuminated chromatophores, is not re-oxidized anaerobically in the

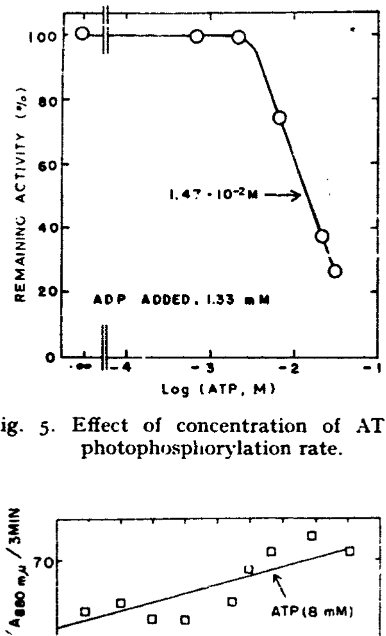


Fig. 5. Effect of concentration of ATP on photophosphorylation rate.

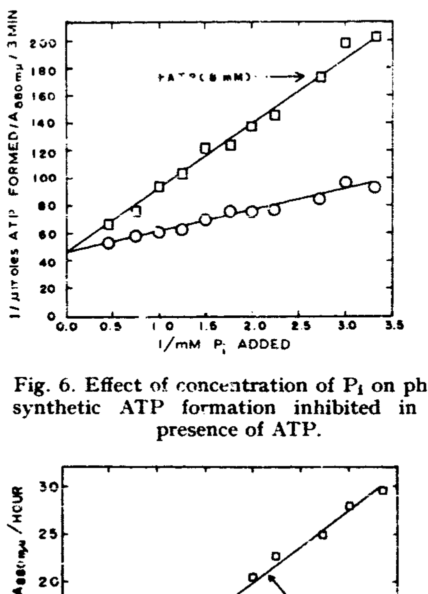


Fig. 6. Effect of concentration of P_i on photosynthetic ATP formation inhibited in the presence of ATP.

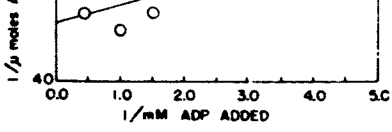


Fig. 7. Effect of concentration of ADP on photosynthetic ATP formation inhibited in the presence of ATP.

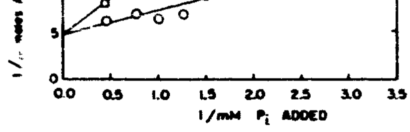


Fig. 8. Effect of concentration of P_i on photosynthetic ATP formation inhibited in the presence of pyrophosphate.

darkness immediately after the illumination period (Fig. 3). From a consideration of the normal oxidation-reduction potentials (E'_0) of the succinate/fumarate system (+ 0.024 V, see ref. 26) and the DPNH/DPN system (−0.32 V, see ref. 27), it seems most likely that the chromatophores acquire additional capacity for reduction of DPN by succinate on illumination. Under our experimental conditions, it is estimated that approximately half (40–50%) of the DPN added to the reaction mixture can be reduced in the steady state (Fig. 10). It can be concluded that the additional reducing capacity is equivalent to establishment of a potential of −0.32 V or more negative.

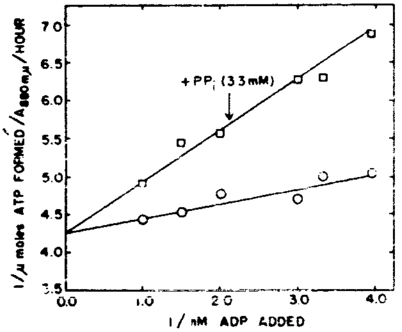


Fig. 9.

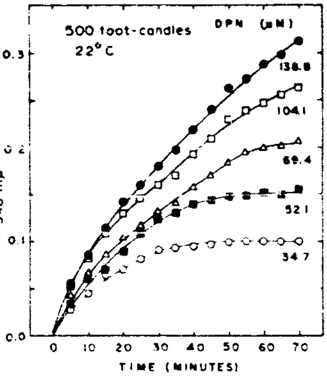


Fig. 10.

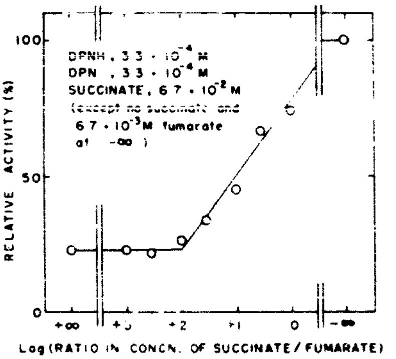


Fig. 11.

Fig. 9. Effect of concentration of ADP on photosynthetic ATP formation inhibited in the presence of pyrophosphate.

Fig. 10. Photosynthetic DPN reduction.

Fig. 11. Oxidation of DPNH/DPN (1:1) system by succinate/fumarate system anaerobically in the darkness.

This value of E_h is not detectably altered if the concentration of succinate is changed from $6.7 \cdot 10^{-3}$ M to $1.3 \cdot 10^{-1}$ M, which indicates that the E_h value photosynthetically produced is independent of the E_h value of the succinate/fumarate system which should have formed at equilibrium.

At $6.7 \cdot 10^{-3}$ M succinate and $3.47 \cdot 10^{-5}$ M DPN (see Fig. 10), $2 \cdot 10^{-14}$ M succinate only should have been dehydrogenated into fumarate if the succinate/fumarate system thus formed had attained the E_h value of −0.32 V (equivalent to the E_h value of the DPNH/DPN formed, the ratio of which was calculated to be 46/54). The value calculated from the DPNH formed could indicate that approx. $1.6 \cdot 10^{-5}$ M fumarate was actually formed ($8 \cdot 10^{8}$ times as much as the concentration calculated as above, $2 \cdot 10^{-14}$ M) and the succinate/fumarate system thus formed had the E_h value of

TABLE I

EFFECT OF ADENOSINE DERIVATIVES ON PHOTOSYNTHETIC ATP FORMATION

In one series of experiments 1.33 mM ADP was added to the reaction mixture, but not in the other. The washed chromatophores used contained approx. 5 mμmoles of bound ADP per $A_{880\,m\mu}$ (see ref. 21 for assay); hence, the average molar concentration of bound ADP in the reaction mixture was calculated to be approx. 3 μM. Reactions were started by the final addition of 0.10 ml of $[^{32}P]P_i$ solution (0.1 M, $2.3 \cdot 10^9$ counts/min/ml); reaction times, 2 min.

Additions	*mM*	*Relative activity (%)*	
		With ADP externally added	*With bound ADP*
None	—	(100)	(100)
Adenosine	22	101	—
	12	—	85
Adenosine 3'-monophosphate	22	—*	—
	6.7	96	85
AMP	22	104	—
	6.7	97	100
ATP	22	26	—**
	6.7	73	—**
	2.2	97	—**
DPN	6.7	—*	—
	2.2	100	91
TPN	6.7	—*	—
	2.2	98	99

* Assay unreliable.

** Assay apparently failed because of ADP contamination in the ATP sample.

TABLE II

A TYPICAL EXPERIMENT TO EXAMINE ATP FORMATION COUPLED WITH ANAEROBIC OXIDATION OF DPNH BY FUMARATE BY CHROMATOPHORES

Standard components of reaction mixture were as follows in the main chamber of a Thunberg-type cuvette: 1.00 ml Tris-HCl-sucrose buffer (0.1 M, pH 8.0), 0.20 ml $MgCl_2$ (0.1 M), 0.20 ml ADP (0.033 M), 0.20 ml chromatophores ($A_{880\,m\mu}$/ml, approx. 50), 0.60 ml water; in the side chamber: 0.20 ml $[^{32}P]P_i$ solution (0.1 M, $8.9 \cdot 10^8$ counts/min/ml), additions. Reactions were carried out anaerobically in the darkness at 22°, and were stopped by addition of 1.00 ml of cold 30% aq. trichloroacetic acid.

No.	*Additions in the side chamber*					*Reaction time (min)*	*$[^{32}P]P_i$ incorporated* (counts/min in excess)*
	Succinate (0.20 ml) (M)	*Fumarate (0.20 ml) (M)*	*DPNH (0.005 M, 0.20 ml)*	*DPN (0.005 M, 0.20 ml)*	*H_2O (0.20 ml)*		
1	1.0	—	−	+	+	0	55
2	1.0	—	−	+	+	30	938
3	1.0	—	+	−	+	30	938
4	0.1	—	−	+	+	30	795
5	0.1	—	+	−	+	30	869
6	—	1.0		+	+	30	1096
7	—	1.0	+	−	+	30	1168
8	—	0.1	−	+	+	30	816
9	—	0.1	+	−	+	30	938
10	1.0	0.1	−	+	−	30	1005
11	1.0	0.1	+	−	−	30	945

* The presence of externally added ADP appeared to be essential for these $[^{32}P]P_i$ incorporations.

-0.05 V. The fact that the E_h value of the DPNH/DPN system in the steady state was the same in the presence of the three different concentrations of DPN initially added (34.7, 52.1 and 69.4 μM in Fig. 10), accords with the suggestion that the E_h value, -0.32 V, approximates the E'_0 value of the photochemical reductant; similarly, the invariance to addition of varied amounts of succinate is understandable.

As remarked previously, the marked difference in E_h between DPNH/DPN and succinate/fumarate on cessation of illumination suggests that the DPNH once photosynthetically formed might have been re-oxidized by the fumarate so formed, possibly through photochemically formed intermediates, labile in the darkness. To examine this possibility, oxidation of the DPNH/DPN (1:1) system by succinate/fumarate system (in varying ratios) was studied anaerobically in the darkness. In the presence of $6.7 \cdot 10^{-2}$ M succinate and no fumarate, DPNH was rapidly oxidized when the reaction components containing the chromatophores were mixed with the DPNH/DPN ($3.3 \cdot 10^{-4}$ M in each) system, but this rapid dehydrogenation of DPNH ceased within one minute after mixing, followed by a slow dehydrogenation which continued through the experimental period. It was estimated that the amount of the DPNH rapidly dehydrogenated was approx. 2 % of the DPNH initially added. In practice, reaction rates were compared between 5 and 35 min after mixing, during which amounts of DPN formed were found to be linear in time. As the ratio in concentration of succinate (fixed at $6.7 \cdot 10^{-2}$ M)/fumarate (varied in molarity) increased, the anaerobic dehydrogenation of the DPNH/DPN system by the succinate/fumarate system decreased in rate, and when the ratio of succinate/fumarate reached about 100/1, the rapid-type dehydrogenation disappeared and the rate became the same as for the slow dehydrogenation (mentioned above) (Fig. 11). In other words, the DPNH/DPN system could not be oxidized by the succinate/fumarate system when the latter's E_h value was more negative than approx. -0.04 V. This value of E_h appeared in a good accord with that of the succinate/fumarate system formed in the DPNH photoreduction at the equilibrated state, -0.05 V (see above). This result indicated that the chromatophores possessed an enzyme system capable of catalyzing anaerobic oxidation of DPNH/DPN by succinate/fumarate in the darkness, and at the same time, explained why the DPNH photochemically formed was not re-oxidized anaerobically in the darkness by the fumarate photochemically formed.

We have not succeeded in demonstrating ATP formation from P_i and ADP coupled with the anaerobic oxidation of DPNH by fumarate, either in the presence or absence of a ATP-trapping hexokinase–glucose system (Table II).

DISCUSSION

We propose that there is a regulatory mechanism in the enzyme system which catalyzes anaerobic oxidation of DPNH/DPN by succinate/fumarate in the darkness, so that when the E_h value of the oxidant is approx. -0.04 V or more negative, electron flow from DPNH to fumarate may accumulate succinate, while if the E_h value is more positive, it may exceed the capacity of the fumarate–succinate system. It has been suggested that photosynthetic ATP formation is optimal at the E_h value of approx. zero Volt, and that a flavin is functional in the ATP-forming process[10,29]. Furthermore, it has been found that the DPNH–heme protein reductase of *R. rubrum* possesses a flavin-like substance as its co-factor[8]. These facts, as well as the analogy of the mitochondrial respiratory chain lead to the suggestion that there is a flavo-enzyme—symbolized "E"—in the electron-transferring system from DPNH/DPN to succinate/fumarate, and a photochemical reductant (E'_0 presumably around −0.32 V or lower)—symbolized X^*H—where the asterisk denotes photoactivation and "H" reduced form. The inhibitory effect on the DPN photoreduction by ATP, ADP, AMP, adenosine and PP_i as well as by DPN itself, suggests that at the site of this regulatory mechanism there is an affinity for adenosine and PP_i, but not P_i. X^*H can be postulated to reduce an intermediate catalyst functional in the system from the electron-transferring system from DPNH/DPN to succinate/fumarate, possibly "E".

The kinetic study on the inhibitory effect of adenosine derivatives and PP_i on photosynthetic ATP formation suggests possible functional or inhibitory intermediate complexes formed prior to the ATP formation as follows: E_1–P_i, E_1–ATP (see Fig. 6), E_2–PP_i, E_2–ADP (see Fig. 9), E_3–P_i, E_3–PP_i (see Fig. 8), E_4–ADP and E_4–ADP–ATP (see Fig. 7), where E_1, E_2, E_3 and E_4 represent active sites functional in the ATP-forming processes. Because chromatophores tightly bind a trace but definite amount of ADP, called "bound" ADP (see ref. 21), despite repeated washing, and photosynthetic ATP formation with the bound ADP is inhibited in the presence of PP_i, adenosine, adenosine 3′-monophosphate, but not with the ADP externally added, it is possible to reduce the several forms of the complexes as follows: as an equivalent form, $E_1 = E_2$–ADP $= E_3 = E_4$–ADP. If either E_2 or E_4 is regarded as E, then E–ADP–P_i, E–ADP–ATP, E–PP_i, E–ADP and E–ADP–PP_i remain as the functional or inhibitory intermediate complexes in addition to E–DPN (= E–ADP–ribose-pyridine), E–AMP and E-adenosine (see Table I).

In summary, the following reactions are proposed for the photosynthetic ATP formation and DPN reduction (*cf.* refs. 29 and 30 for oxidative phosphorylation).

In photosynthetic ATP formation:

$$2H^+ + 2e^- + nX \longrightarrow nX\cdot H_2 \ (n,\ 1 \text{ or } 2) \tag{1}$$
$$nX\cdot H_2 + h\nu \longrightarrow nX^*\cdot H_2 \tag{2}$$
$$nX^*\cdot H_2 + E\text{–ADP} \longrightarrow nX + EH_2\text{–ADP} \tag{3}$$
$$EH_2\text{–ADP} + P_i + Y \longrightarrow E\text{–ADP} \sim P_i + YH_2 \tag{4}$$
$$E\text{–ADP} \sim P_i + \text{ADP} \longrightarrow E\text{–ADP} + \text{ATP} \tag{5}$$
$$YH_2 \longrightarrow 2H^+ + 2e^- + Y \tag{6}$$

$$\text{Sum: ADP} + P_i + h\nu \longrightarrow \text{ATP}$$

where X and Y represent substances such as bacteriochlorophyll in its ground state and RHP or cytochrome c_2 (see refs. 32 and 33), respectively (see ref. 10).

In photosynthetic DPN reduction:

$$\text{succinate} + E' \longrightarrow \text{fumarate} + E'H_2 \tag{7}$$
$$E'H_2 + Y \longrightarrow E' + YH_2 \tag{8}$$
$$YH_2 \longrightarrow 2H^+ + 2e^- + Y \tag{9}$$
$$2H^+ + 2e^- + nX \longrightarrow nX\cdot H_2 \tag{10}$$
$$nX\cdot H_2 + h\nu \longrightarrow nX^*\cdot H_2 \tag{11}$$
$$E\text{–ADP} + \text{DPN}^+ \longrightarrow E\text{–DPN}^+ + \text{ADP} \tag{12}$$
$$E\text{–DPN}^+ + nX^*\cdot H_2 \longrightarrow EH_2\text{–DPN}^+ + nX \tag{13}$$
$$EH_2\text{–DPN}^+ + \text{ADP} \longrightarrow E\text{–ADP} + \text{DPNH} + H^+ \tag{14}$$

$$\textbf{Sum: succinate} + \textbf{DPN}^+ + h\nu \longrightarrow \textbf{fumarate} + \textbf{DPNH} + \textbf{H}^+$$

where E' represents a flavoprotein such as succinic dehydrogenase[34]. The Michaelis–Menten constant for ADP in the photosynthetic ATP formation is now known to be approx. $2 \cdot 10^{-5}$ M (see ref. 21). The ATP formation is stimulated in rate with increasing concentration of ADP externally added to the reaction mixture up to the highest concentration examined, $7 \cdot 10^{-3}$ M (see ref. 21). On the other hand, the DPN photoreduction is markedly inhibited in the presence of ADP; *e.g.*, 80 % inhibition with $7 \cdot 10^{-3}$ M ADP. This fact strongly supports the suggestion that DPN is situated outside the cycle of electron transport coupled with photosynthetic ATP formation.

Using chloroplasts from spinach, Avron and Jagendorf[35] studied effects of arsenate on the ferricyanide reduction in the Hill reaction which could be accelerated by addition of ADP and P_i, and found that arsenate plus ADP but not arsenate alone could replace ADP plus P_i, leading to the suggestion that an "energy-rich" intermediate compound prior to ATP formation was formed with ADP but not with P_i. Later in time, Schulz and Boyer[36] found that an oxygen was lost from P_i in the photosynthetic formation of ATP from ADP and P_i as catalyzed by spinach chloroplasts, arguing against the participation of an adenosine diphosphoryl intermediate in photosynthetic phosphorylation. Recently, Kahn and Jagendorf[37] have isolated and purified an enzyme catalyzing the exchange of P_i between ATP and ADP from spinach chloroplasts. The discrepancy among these findings might be rationalized in the hypothetical scheme for the photosynthetic ATP formation mentioned above.

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