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Donor function of phosphate ions in photosystem 2

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Abstract

Evidence was obtained for the interaction between the photosystem 2 (PS2) reaction centre (RC) chlorophyll (Chl) P680 and inorganic phosphate, P_i. The light-induced endogenous basal electron transport to ferricyanide in PS2 depended on endogenous P_i. The electron transport in phosphate deficient chloroplasts was absent, and could be resumed upon the addition of exogenous P_i or of the exogenous electron donor, diphenylcarbazide. Some chloroplast Chl molecules were apparently bound with P_i to a complex *via* the magnesium atom that was detected by the increase in absorbance in the Chl *a* absorption maximum at 435 nm observed after the consumption of endogenous P_i in the photophosphorylation reactions. The electron paramagnetic resonance (EPR) Signal I, found in the spectra at 77 K after irradiation of frozen samples in chloroplasts poor in endogenous P_i, was the sum of $P700^+$ and $P680^+$ signals. The $P680^+$ signal disappeared after addition of P_i, diphenylcarbazide or diuron to the chloroplasts before freezing. In addition, the EPR doublet signal of the phosphate anion radicals was recorded at 77 K after irradiation in the ethanol solutions of Chl *a* containing potassium phosphate. The same doublet signal was discovered in the difference EPR spectrum "chloroplasts minus chloroplasts with diuron" at 77 K after irradation. The results are a possible evidence of the participation of phosphate ions in the primary light reactions of PS2.

Additional key words: chloroplasts; diuron inhibition; EPR spectrum of chloroplasts; ferricyanide reduction; methylamine; NH_2Cl; phosphate anion radical; photosynthetic electron transport.

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Abbreviations: Arg - arginine; Chl - chlorophyll; diuron - 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DPC - diphenylcarbazide; EPR - electron paramagnetic resonance; FeCy - ferricyanide, $K_3(Fe(CN)_6)$; MV - methyl viologen; P680 - photosystem 2 reaction centre chlorophyll *a* molecule; $P680^+$ - cation radical of P680; P700 - photosystem 1 reaction centre chlorophyll *a* molecule; $P700^+$ - cation radical of P700; P_i - inorganic phosphate; PS - photosystem; RC - reaction centre.

Introduction

In model systems, Chl *a* and Chl (*a*+*b*) in ethanol solutions and Chl adsorbates on aluminium or silicon oxides in aqueous media can photosensitize the electron transfer from phosphate ions (Goncharova and Goldfeld 1990). The phosphate anion radical formed is broken down to hydroxyl radical and short-lived metaphosphate ion which phosphorylates ADP to ATP or phosphate to pyrophosphate. The ability of phosphate ion to be converted to anion radical has been demonstrated by Sukharukov *et al.* (1966), Huber and Hayon (1968), Black and Hayon (1970), Subramanian *et al.* (1970), and Ginns and Symons (1975). Sukharukov *et al.* (1966) suggest that phosphate anion radical may be a precursor in ATP formation in biochemical reactions. The idea that Chl can take part in the ATP formation has been first formulated by Calvin (1962). However, the proposed mechanism has not taken into account the ability of phosphate ions to react with Chl as the electron donor. In the present work we asked whether Chl of the PS2 RC in chloroplasts, $P680^+$, could accept electron from phosphate ions as it was observed in the model systems.

The nature of the endogenous electron donor for $P680^+$ is not clear. The endogenous electron donor exists in a complex with P680 and can reduce the cation radical $P680^+$ formed on the light at liquid nitrogen temperature. Therefore, at this temperature the radical of the oxidized endogenous electron donor might be detected. However, all the known experiments have been made at temperatures above or at 0 ° C. For example, at room temperature the changes of chloroplast EPR spectrum in the region of EPR Signal II (Weaver 1968), designated Signal II_f and Signal II_{vf}, were discovered (Blankenship *et al.* 1975, O'Malley and Babcock 1984), and also a tyrosine radical was observed (Gerken *et al.* 1988). Periodic changes of the oxidation state of manganese, included in oxygen-evolving complex, were determined at room temperature on irradiation with short flashes (Wydrzynski and Sauer 1980) and at cryogenic temperatures after irradiation at room temperature (Dismukes and Siderer 1981). In several experiments the samples were irradiated at 190 K, then warmed to 0 °C for 30 min, and again frozen at 77 K for the measurements (De Paula *et al.* 1985). Radicals observed under these conditions belong to the oxygen-evolving complex.

At present it is believed in conformity with the chemiosmotic hypothesis of Mitchell (1967) that ATP formation proceeds under the influence of membrane H^+-ATPase, and the electron transport chain creates only a membrane electrochemical gradient. However, the ATP formation may occur also directly in the electron transport chain. Such possibility is realized if the phosphate ions participate in the transport of electrons as electron donors. The electron transport from phosphate ions may be connected with ATP formation at the photophosphorylation site at the donor side of PS2. The ATP formation in PS2 can precede oxygen evolution (Goncharova *et al.* 1993b). The present paper describes the results of the study of electron donor function of phosphate ions in PS2 of chloroplasts.

Materials and methods

Plants of *Phaseolus vulgaris* L. cv. Russian black were grown in a greenhouse at 20/15 °C (day/night). For the ferricyanide (FeCy) photoreduction experiments, chloroplasts poor in endogenous P_i were used. In order to prepare such chloroplasts, the plants before the chloroplast isolation were irradiated at 10 °C in a climatic chamber for some hours to some days depending on irradiance (1000-100 W m^{-2}). On intense irradiation the content of endogenous P_i dropped more rapidly to an undetectable concentration [less than 10 mmol kg^{-1}(Chl)], but simultaneously the irreversible inactivation of the photochemical apparatus occurred. At lower irradiances the concentration of endogenous P_i decreased more slowly, but the irreversible photoinactivation was less, and the back reactivation of electron transport by phosphate was higher. We irradiated the plants at 10 °C because this method gave active chloroplasts containing below 10 mmol(endogenous P_i) kg^{-1}(Chl) in sufficient quantities. Irradiance in a climatic chamber modelled solar radiation. Temperature 10-15 °C and solar radiation are natural conditions for plants, these irradiances are often observed in morning hours at the beginning of summer. In our initial experiments we took leaves of bean plants grown in sun in the garden near the greenhouse in the morning. Under these conditions dark reactions in the plant slowed down and light reactions proceeded at the same rate as at 20 °C. As a result, P_i utilization in light reactions was not compensated by P_i intake from the conductive system, and P_i deficiency developed in chloroplasts.

Chloroplasts of "type B" in the classification of Hall were isolated as described by Reeves and Hall (1973, 1980) and Goncharova and Gol'dfel'd (1983). 10 g leaves in 100 cm^3 of medium cooled to 0 °C, containing 0.4 M sorbitol, 0.2 mM $MgCl_2$, and 35 mM Tris-HCl, pH 6.5, were ground in a homogenizer for 15 s (three times for 5 s each). The suspension was squeezed through a layer of cotton wool between two layers of gauze, and the filtrate was centrifuged at 2000×*g* for 30 s at 0 °C. The chloroplast pellet was washed by centrifugation with a medium containing 0.4 M sorbitol, adjusted to pH 7.5 with Tris. The final chloroplast pellet was suspended in 3 cm^3 of medium containing 0.4 M sorbitol, 10 mM NaCl, 5 mM $MgCl_2$, and 35 mM Tris-HCl, pH 7.6, and kept at 0 °C. Chloroplast fragments ("type E" chloroplasts) were isolated in a hypotonic medium: 2.5 g leaves were ground in a plexiglass mortar in 100 cm^3 0.1 M Tris-HCl, pH 7.6, in four portions. The suspension was filtered and centrifuged as described above, and the chloroplast pellet was suspended in 2 cm^3 of the same Tris buffer.

For the measurement of endogenous P_i, 7.5 cm^3 H_2O and 1.5 cm^3 20 % trichloroacetic acid were added to 1.0 cm^3 chloroplasts. The sediment was removed by centrifugation, and the supernatant was analysed by the method of Fiske and Subbarow, as modified by Allen (1940).

Photoreduction of ferricyanide (FeCy) was determined spectrophotometrically, $\varepsilon_{420} = 1.04 \times 10^3$, in a medium containing in a total volume of 2.0 cm^3 chloroplasts equivalent to 30-40 μg Chl, 0.1 M sorbitol, 4 mM $MgCl_2$, 20 mM NaCl, 2 mM FeCy, 0.05 M Tris-HCl, pH 8.0, and, where indicated, K_2HPO_4, DPC, NH_4Cl, or

methylamine. A 750 W lamp with condenser, a water filter, and a red radiation filter, $\lambda \geq 610$ nm, were used for irradiation. The FeCy photoreduction and the Chl spectra were measured on a *Specord* spectrophotometer (Germany) in cuvettes of 1 cm optical pathlength. The absorption spectra of the chloroplasts were recorded on a *Hitachi-356* spectrophotometer (Japan). The evolution of oxygen was estimated at 20 °C by an amperometric method using an *OH-105* polarograph (Hungary) in a 1.9 cm^3 cell.

Photophosphorylation was measured by an isotopic method with $^{32}P_i$ using the technique of Avron (1960). The reaction medium contained, in a total volume of 3.0 cm^3, chloroplasts equivalent to 300 μg Chl, 0.02 M Tris-HCl, pH 7.6, 3.3 mM K_2HPO_4, 0.1 mM MV chloride, 2 mM ADP or, where indicated, 10 mM arginine, creatine, or leucine.

Samples used for EPR spectroscopic studies were frozen with liquid nitrogen under weak background irradiation (50 W m^{-2}) to get the maximum EPR Signal II which did not change in frozen samples. To register the maximum EPR Signal I, frozen samples were irradiated for 10 s before the measurement at 77 K in liquid nitrogen with "white light" of a 500 W lamp (400 W m^{-2}). The EPR spectra were measured on a spectrometer *R-1306* (Russia) and a *Bruker ER 220 D* spectrometer (Germany).

Chl *a* was isolated by the method of Iriyama *et al* (1974). For the EPR measurements, solid Chl *a* was dissolved in 0.1 cm^3 ethanol, then 0.025 cm^3 0.4 M K_2HPO_4 was added; the solution was mixed, and 0.9 cm^3 ethanol with a small amount of HCl was added for the creation of necessary pH. This mixture in quartz tubes was frozen in liquid nitrogen. The Chl concentration, determined by the method of Wintermans and De Mots (1965), was about 3 kg m^{-3}.

The chloroplasts with normal amount of P_i for EPR measurements of the phosphate anion radical were isolated by the usual method, and suspended in 1 cm^3 0.1 M potassium phosphate, pH 7.5 or 8.0. One part of these chloroplasts (0.5 cm^3) was a control; diuron to 10^{-5} M concentration was added to other part. Both chloroplast suspensions were immediately frozen in liquid nitrogen. The operations with chloroplasts were carried out at 4 °C. The Chl concentration in samples was about 4.5 kg m^{-3}.

Results

PS2 electron transport depends on phosphate: Fig. 1*A* shows the photoreduction of FeCy by P_i-deficient chloroplasts [the contents of endogenous P_i were less than 10 mmol kg^{-1}(Chl)]. The reaction medium did not contain adenine nucleotides and other acceptors of the phosphoryl group. The chloroplasts did not reduce FeCy without addition of P_i or DPC, and reduced it when P_i or DPC was added. The DPC was more active. When DPC and P_i were added to the chloroplasts simultaneously, the FeCy reduction rate was equal to that observed after addition of only P_i. The DPC had no effect on the reduction of FeCy in chloroplasts with the normal contents of endogenous P_i (Fig. 1*B*).

Vernon and Shaw (1969) have discovered that DPC can be an electron donor for PS2 in chloroplasts washed with 0.2 M Tris buffer, pH 8.0, and in PS2-enriched subchloroplast fragments prepared by treatment of chloroplasts with *Triton X-100*, where the electron transfer from endogenous electron donors is absent. Fig. 1 demonstrates that DPC has a similar effect in P_i-deficient chloroplasts.

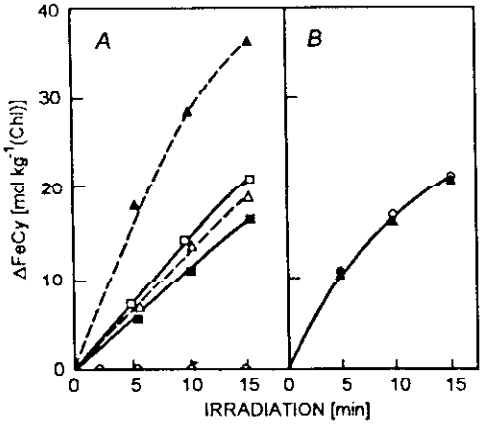


Fig. 1. Photoreduction of ferricyanide (FeCy) by chloroplasts. *A*: Chloroplasts deficient in endogenous P_i [less than 10 mmol kg^{-1}(Chl)]. *B*. Normal chloroplasts [135 mmol (endogenous P_i) kg^{-1}(Chl)]. Curves: ○: medium [100 mM sorbitol, 50 mM Tris-HCl, pH 8.0, 0.1 mM $MgCl_2$, 0.1 mM NaCl, 2 mM ferricyanide, and chloroplasts containing 10 g(Chl) m^{-3}(reaction medium); □: medium + 1.7 mM P_i; ■ medium + 0.83 mM P_i; ▲: medium + 0.17 mM DPC; Δ: medium + 0.17 mM DPC + 0.83 mM P_i.

Activation of the FeCy photoreduction with DPC, the specific electron donor to PS2, in chloroplasts poor in endogenous P_i indicated that under these conditions the chloroplasts became deficient in endogenous electron donors. On the other hand, since P_i, similar to DPC, activates the electron transport to FeCy, we cannot exclude a possibility of its participation in electron transport as an electron donor.

West and Wiskich (1968) and Reeves and Hall (1973) have shown that photosynthetic oxygen evolution depends on the presence of P_i. We also observed that the P_i-deficient chloroplasts, which did not reduce FeCy, did also not evolve oxygen. If phosphate was added in the reaction medium of such chloroplasts, the oxygen evolution was resumed simultaneously with the FeCy photoreduction. However, we did not study oxygen evolution in detail because it has been discovered that P_i resumes electron transport to FeCy also in chloroplast fragments (chloroplasts of "type E" in the classification of Hall) prepared in a hypotonic medium, although these chloroplast fragments do not evolve oxygen due to the loss of some components of the oxygen evolution system.

The chloroplast reactions are followed usually in a hypotonic medium in ordre to rupture the chloroplast envelope and enable penetration into chloroplasts of adenine nucleotides or other phosphoryl group acceptors and electron acceptors. Chloroplast E fragments are prepared at once in a hypotonic medium. They lose the oxygen evolution activity and are inactivated during storage at 0 °C more rapidly than chloroplasts of "type A" and "type B" but retain completely the phosphorylation activity. These facts indicate that electron transport from phosphate ions and the phosphorylation precede oxygen evolution. Table 1 shows the photophosphorylation by chloroplast fragments with the depletion of endogenous P_i in the presence of the

electron acceptor MV, exogenous phosphate, and various acceptors of the phosphoryl group.

Table 1. Photophosphorylation [mmol(P_i) kg^{-1}(Chl) s^{-1}] catalyzed by P_i-deficient chloroplast fragments in the presence of methyl viologen (time of irradiation 5 min). Details as in Materials and methods.

Addition	Reaction product	Photophosphorylation
P_i, ADP	ATP	25.6
P_i, arginine	Phosphoarginine	10.4
P_i, creatine	Phosphocreatine	5.2
P_i, leucine	Pyrophosphate	1.1
P_i	Pyrophosphate	1.7

Table 2. Photoreduction of ferricyanide (FeCy) [mmol kg^{-1}(Chl) s^{-1}] by chloroplasts in the presence of endogenous P_i [mmol kg^{-1}(Chl)] and various additions. Reaction time 5 min. The average error of the determination of FeCy reduction is ±1.4 mmol kg^{-1}(Chl) s^{-1}. Details as in Materials and methods, and, where indicated, 1.7 mM K_2HPO_4, pH 8.0, 0.17 mM DPC, 0.83 mM NH_4Cl, and 3 mM methylamine were added.

Experiment	Endogen. P_i	FeCy reduction				
		No additions	P_i	DPC	NH_4Cl	Methylamine
1	10^3	33.6	33.6	33.6	65.6	82.8
2	<10	25.6	30.3	31.9	49.7	52.8
3	<10	17.5	27.2	25.6	40.0	48.9

Our investigations of the effect of uncouplers (NH^+ and methylamine) in P_i-deficient chloroplasts showed that uncouplers increased the electron transport rate only in the presence of P_i. Table 2 demonstrates the rate of the photoreduction of FeCy in control chloroplasts with the normal content of endogenous P_i and in *chloroplasts containing less than 10 mmol(P_i) kg^{-1}(Chl), in which, however, the* initial electron transport is present. Experiment *1* is control. Chloroplasts contained 10^3 mmol(endogenous P_i) kg^{-1}(Chl), and the addition of exogenous P_i or DPC did not affect the rate of FeCy reduction. Uncouplers, NH_4Cl and methylamine, increased the rate to 2 and 2.5-fold, respectively. The effect of uncouplers on the FeCy reduction was similar both in chloroplasts which evolved oxygen and in chloroplast fragments of "type E" which did not evolve oxygen. Chloroplasts with endogenous P_i deficiency were used in experiment *2*. Addition of P_i or DPC restored the rate of electron transport to the level of the control experiment. Therefore the RCs were not destroyed in spite of the low concentration of endogenous P_i. However, the effect of NH_4Cl and methylamine was considerably lower than in the control. In experiment *3* the addition of P_i or DPC restored the rate of the electron transport to the level of experiment *2*, but the rate with uncouplers was lower than there.

FeCy can accept electrons at the level of both PS1 and PS2. The addition of 10^{-5} M diuron (an inhibitor of the electron transport between the PS2 primary electron

acceptor Q and plastoquinone) to chloroplasts abolished the reduction of FeCy in all these experiments.The sensitivity to diuron indicated the participation of PS2 in these reactions. Thus a point of the phosphate effect was located in the PS2 donor side and endogenous basal electron transport depended on endogenous phosphate. Such results have also been discussed by Goncharova and Gol'dfel'd (1983) and Goncharova *et al.* (1990).

The utilization of endogenous P_i results in a damage of the RCs: Irradiation of plants under conditions promoting the consumption of endogenous P_i can damage the chloroplast photochemical apparatus. The irradiation of plants at 10 °C (400 W m^{-2}) resulted in the decrease in phosphate contents in chloroplasts from 1 mol to less than 10 mmol per kg(Chl) (Fig. 2). This process was accompanied by the decrease in the total Chl content and in the amount of the RCs determined directly in leaves. The amount of the RCs was estimated at 77 K by the integral intensity of EPR Signals I and II (Weaver 1968) per unit leaf surface; the method was described by Chetverikov (1983). The EPR Signal I is mainly due to $P700^+$, while the EPR Signal II is a complex signal that appears during the action of PS2, and correlates with the number of the PS2 RCs.

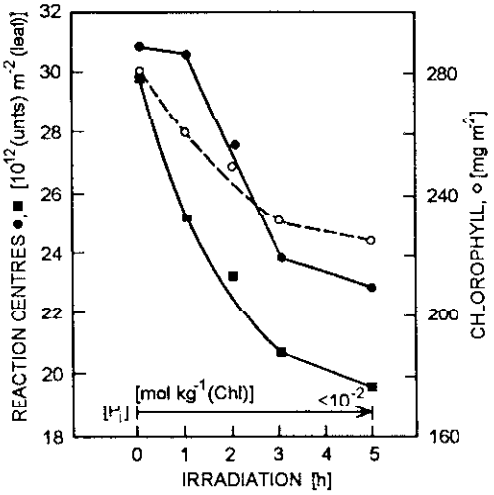


Fig. 2. The amounts of reaction centres of PS1 (●) and PS2 (■) and the total chlorophyll contents (○) per unit leaf surface as a function of the time of irradiation of bean plants in a climatic chamber (400 W m^{-2}, 10 °C). Concentration of endogenous P_i [mol kg^{-1}(Chl)] in isolated chloroplasts is shown in lower part of the figure.

The amount of the PS2 RCs and the total Chl amount decreased at once, while the amount of the PS1 RCs decreased with a certain delay, *i.e.*, the depletion of endogenous P_i resulted in the first place in the damage of the PS2 RCs. The decrease in the Chl contents was apparently connected with bleaching of the light-harvesting Chl as a result of the absence of the radiant energy transfer to RCs owing to their damage (see also Goncharova *et al.* 1990).

The damage of the RCs often proceeds simultaneously with the endogenous P_i consumption, and it is difficult to prepare active chloroplasts with initial zero electron transport. Therefore we usually used chloroplasts in which a small electron

transport to FeCy was present, and the addition of P_i or DPC activated the available electron transport.

The absorption spectrum of chloroplasts depends on endogenous P_i: If P_i reacts with the PS2 RC Chl molecule on light as an endogenous electron donor, it should be in a complex with Chl. The isolated Chl forms complexes *via* the magnesium atom with oxygen atoms of various compounds (Evstigneev *et al.* 1950): in organic solvents, such a complex is displayed by the decrease of absorbance at 430 nm, in the blue component in the doublet structure of the blue-violet absorption band, in the presence of alcohols, water, and O_2.

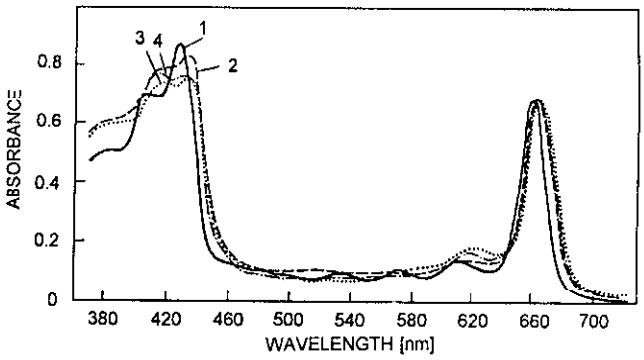


Fig. 3. Influence of the solvent and inorganic phosphate on the absorption spectrum of chlorophyll *a*. *1* - diethyl ether; *2* - 96 % ethanol and 96 % ethanol + 2.5 % water (by volume); *3* - 96 % ethanol + 2.5 % water + K_2HPO_4, pH 7.6; *4* - the same as *3*, but pH 5.5.

Fig. 3 presents the absorption spectrum of Chl *a* in diethyl ether, in ethanol, and in ethanol with a small amount of water, and in the same aqueous ethanol solution containing K_2HPO_4. In ethanol the maximum of the blue component in region at 430 nm was lower than in diethyl ether. In the aqueous ethanol solution containing phosphate this maximum was lower than in ethanol or in the aqueous ethanol solution without phosphate. The formation of a complex between the Mg atom of Chl and the oxygen atoms of phosphate has been detected by infra-red spectroscopy in aqueous acetone solutions by Leicknam *et al.* (1974).

Fig. 3 demonstrates the region where changes may be expected when endogenous P_i is consumed in chloroplasts. The protein-lipid environment and other pigment molecules can also form a complex with the Mg atom of Chl. In order to reveal the effect only of endogenous P_i, we varied the reaction medium to create the situation when endogenous P_i was consumed or not consumed.

Irradiation of chloroplasts in the presence of the phosphoryl group acceptor caused an increase in the maximum at 435 nm (Fig. 4; see also Goncharova and Gol'dfel'd 1988). As an acceptor of the phosphoryl group we used Arg, that is not a potential source of P_i. On the contrary, ADP was converted to AMP and P_i under the influence of adenylate kinase and ATPase. As shown in Table 1, Arg could be phosphorylated in chloroplasts.

The irradiation of chloroplasts in the medium containing MV and Arg produced during the first 12 min an increase in absorbance at 435 nm (Fig. 4*B*). Further irradiation caused the decrease in absorbance owing to the pigment bleaching. In

experiments with diuron (Fig. 4*C*), when electron transport through PS2 was inhibited and the consumption of P_i in photophosphorylation was absent, the absorbance at 435 nm did not increase, but decreased due to the pigment bleaching.

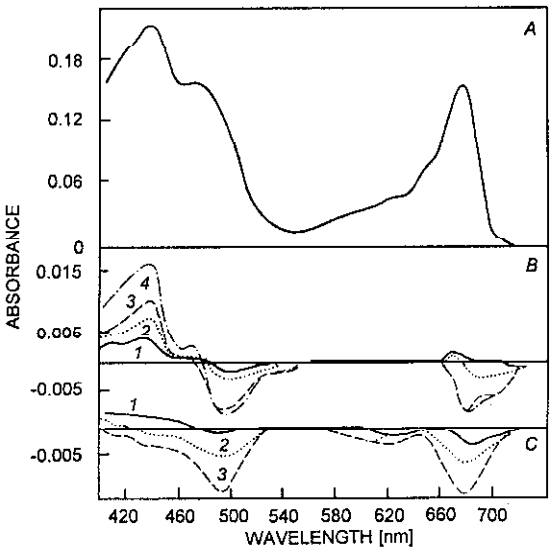


Fig. 4. Changes in the absorption spectra of chloroplasts caused by irradiation-induced consumption of endogenous P_i. *A*: initial absorption spectrum of chloroplasts [1 mol (endogenous P_i) kg^{-1}(Chl)]; medium: 100 mM sorbitol, 50 mM Tris-HCl, pH 8.0, 1 mM NaCl, 0.5 mM $MgCl_2$, and chloroplasts 4.4 g(Chl) m^{-3}(medium)]. *B,C*: differential spectra "light minus darkness"; *B* - medium + 0.7 mM arginine (Arg) + 0.125 mM methyl viologen (MV); *C* - medium + Arg + MV + 10^{-5} M diuron. Time of irradiation: *1* - 2.5 min; *2* - 5 min; *3* - 10 min; *4* - 12.5 min.

In another series of experiments (Table 3), the increase in absorbance at 435 nm proceeded in experiment *1*, when MV and Arg were present, *i.e.*, when endogenous P_i was consumed, and in experiments *5* and *6*, when the uncoupler was also present. The increase in absorbance at 435 nm did not proceed when the endogenous P_i consumption was absent, *i.e.*, when exogenous P_i was present (experiment *2*), the inhibitor of PS2, diuron, was present (experiment *3*), or MV and Arg were absent (experiment *4*).

Table 3. Effect of the composition of reaction medium upon the increase in absorbance at 435 nm in chloroplasts. Details as in Fig. 4 and, where indicated, 1.2 mM K_2HPO_4, pH 8.0, 10^{-5} M diuron, 0.83 mM NH_4Cl, and 3 mM methylamine were added.

Experiment	Additions	Maximum A_{435} increase	Irradiation [min]
1	Arg, MV	0.010	5
2	Arg, MV, P_i	-	-
3	Arg, MV, diuron	0.001	2.5
4	-	0.003	2.5
5	Arg, MV, NH_4Cl	0.014	2.5
6	Arg, MV, methylamine	0.021	2.5

The action of uncouplers, NH_4Cl and methylamine, is of special interest. The increase in absorbance at 435 nm proceeded very rapidly, and during 2.5 min reached a higher value than in photophosphorylation (experiment *1*). Then absorbance at 435

nm decreased owing to the irreversible bleaching of the pigment apparatus. Uncouplers inhibited the ATP formation, increased the electron transport rate, and, as Table 2 shows, their effect upon the electron transport depended on endogenous P_i. Therefore, the rapid growth in absorbance at 435 nm indicated that rapid electron transport in the uncoupler presence was accompanied by the rapid P_i consumption, although phosphorylated products were not formed. Most likely, NH_4Cl and methylamine formed some rapidly splitting products with metaphosphate ions in the phosphorylation process.

In addition, the chloroplasts with endogenous P_i deficiency always had a higher ratio of maxima at 435 and 675 nm than the chloroplasts with the normal content of endogenous P_i. The addition of exogenous P_i to P_i-deficient chloroplasts was readily followed by a decrease in absorbance at 435 nm.

The increase in the absorbance at 435 nm observed upon utilization of endogenous phosphate was not associated with the increase in the radiation scattering, which was a consequence of the turbid media and depended on the particle size, firstly, because there was no increase in the absorbance at 550 nm, where photosynthetic pigments absorbed weakly; secondly, all the experiments with chloroplasts were conducted in the hypotonic medium where they had broken envelopes. To completely exclude the possibility of such an explanation, we did additional measurements. In experiments with the largest increase in absorbance, the emission spectrum showing the level of the radiation scattering in the reaction medium with chloroplasts was recorded from the side surface of cuvette. This emission did not exceed 0.004 absorbance units and did not change during irradiation. Consequently, our results indicated that some Chl molecules formed a complex with P_i: this could also explain why endogenous P_i was not washed out when the chloroplasts were destroyed.

Characteristics of EPR Signal I in chloroplasts depend on P_i: As mentioned above, the EPR Signal I in chloroplasts belongs to $P700^+$. $P680^+$ is not observed in EPR spectra because it is immediately reduced by the electron donor that complexes with it. However, if the endogenous electron donor is absent, one can expect the appearance of the $P680^+$ signal in the EPR spectra of chloroplasts. The $P680^+$ EPR spectrum (g = 2.0025, width = 0.9 mT - Goldfield *et al.* 1978, Hoganson and Babcock 1989) differs from the $P700^+$ EPR spectrum (g = 2.0023, width = 0.75 mT; Weaver 1968) in the width, and the appearance of $P680^+$ signal in the EPR spectra of chloroplasts should result in broadening of the EPR Signal I.

Really (Fig. 5), if a deficiency in P_i contents arose in chloroplasts and the FeCy photoreduction was activated by exogenous P_i or DPC, the EPR Signal I characteristics at 77 K (both a signal amplitude and a width increase) were altered which indicated the appearance of $P680^+$ signal. These characteristics may return to the normal values that the EPR Signal I, containing only the $P700^+$ signal, has in P_i-normal chloroplasts, if P_i or DPC is added to P_i-deficient chloroplasts before freezing in liquid nitrogen. A similar effect was observed with diuron which excluded the PS2 primary electron acceptor from the reaction, and thus prevented the $P680^+$ formation. A similar result of the action of P_i, the PS2 electron donor DPC and the PS2 acceptor

side inhibitor diuron indicated probably the presence of $P680^+$ signal in the EPR spectrum of the P_i-deficient chloroplasts.

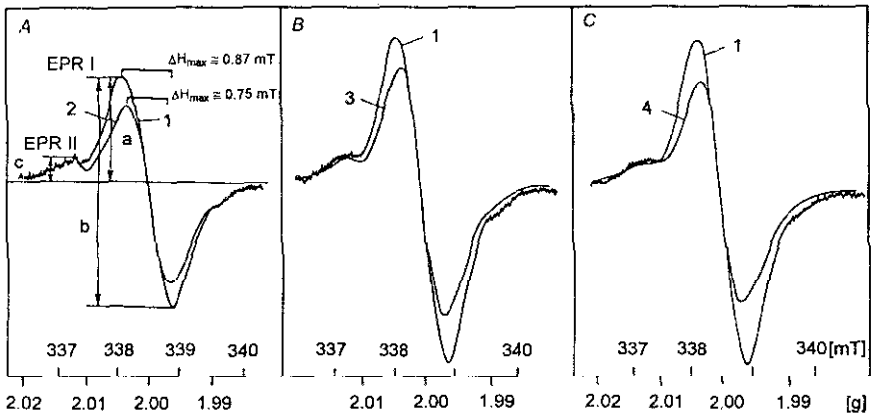


Fig. 5. EPR spectra of P_i-deficient chloroplasts at 77 K. *1* - P_i-deficient chloroplasts [less than 10 mmol(P_i) kg^{-1}(Chl)]; *2* the same chloroplasts after the addition of 0.01 M P_i, pH 7.8 (*A*); *3* 10^{-3} M DPC was added (*B*); *4* - 10^{-5} M diuron was added (*C*); EPR conditions: 0.5 mW microwave power; 0.1 mT modulation amplitude. Parameters *a,b,c* of the EPR Signal I and Signal II are used in Fig. 6. In Figs. 5-7 the samples contained 0.5 kg(Chl) m^{-3} of the medium (100 mM sorbitol, 50 mM Tris-HCl, pH 7.8, 0.1 mM $MgCl_2$; 0.1 mM NaCl).

Besides, the chloroplasts with normal P_i contents and the P_i-deficient chloroplasts differed in the microwave power saturation of the EPR Signal I at 77 K. The curve of the microwave power saturation of P_i-deficient chloroplasts (Fig. 6) was distin-

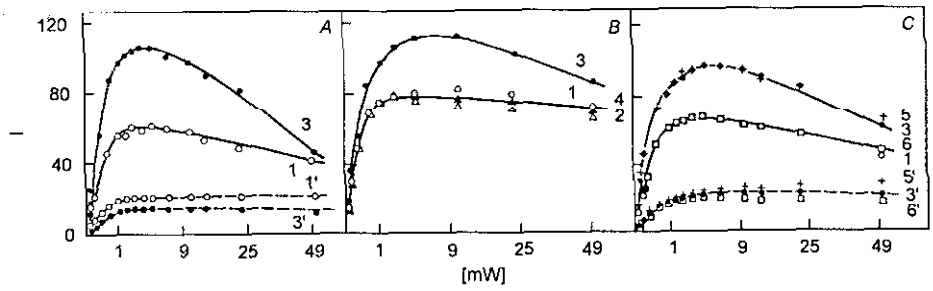


Fig. 6. Curves of the microwave power saturation of EPR Signal I and Signal II of normal and P_i-deficient chloroplasts. Modulation amplitude of 0.1 mT was used in recording the spectra. *A*: curves of the microwave power saturation of EPR Signal I (parameter *a*) - *1* and *3*, and Signal II (parameter *c*) - *1'* and *3'*; *1* and *1'* - normal chloroplasts, *3* and *3'* - P_i-deficient chloroplasts. *B*: curves of the microwave power saturation of EPR Signal I (parameter *b*) of the same chloroplasts as in *A*; *1* - normal chloroplasts ; *2* - the same chloroplasts as *1* but in the presence of 10^{-5} M diuron ; *3* - P_i-deficient chloroplasts; *4* - the same chloroplasts as *3* but in the presence of 10^{-5} M diuron. *C*: curves of microwave power saturation of EPR Signal I (parameter *a*) - *1*, *3*, *5* and *6*, and Signal II (parameter *c*) - *3'*, *5'* and *6'*. *1* - normal chloroplasts; *3* and *3'* - P_i-deficient chloroplasts; *5* and *5'* - P_i-deficient chloroplasts in the presence of 0.2 M NaCl; *6* and *6'* - P_i-deficient chloroplasts in the presence of 0.01 M P_i. In P_i-deficient chloroplasts used in experiments *A* and *B* the ferricyanide photoreduction was activated by exogenous P_i 3 times, and in those used in *C* 2 times.

guished from the curve characteristic for normal chloroplasts having only $P700^+$. The curve of the microwave power saturation of P_i-deficient chloroplasts became similar to the curve of normal chloroplasts having only $P700^+$ after addition of P_i or diuron before freezing. Comparison of the microwave power saturation curves of the P_i-deficient chloroplasts from different preparations (Fig. 6*A,C*) showed that the larger activation of the FeCy photoreduction by P_i (in Fig. 6*A* the activation was 3 times, in Fig. 6*C* 2 times) was followed by a larger distinction of the curve of P_i-deficient chloroplasts from the curve of the normal chloroplasts, and, consequently, a larger fraction of $P680^+$ without an endogenous electron donor might be present. The EPR Signal II microwave power saturation at 77 K was the same in P_i-deficient and normal chloroplasts.

The differential EPR spectra of P_i-deficient chloroplasts and of these chloroplasts after the addition of P_i, DPC, and diuron represented a singlet signal close to that of $P680^+$ (Fig.7).

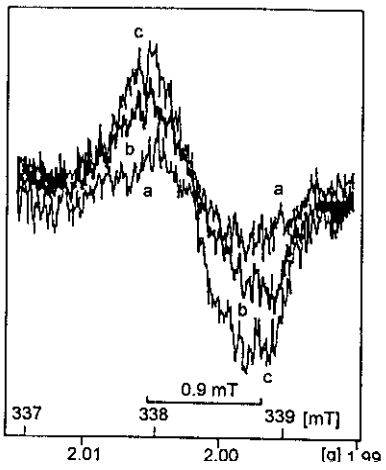


Fig. 7. EPR differential spectra of chloroplasts: *a, b*, and *c* between EPR spectra represented in Fig. 5*A, B, C*.

The above results indicate that under P_i deficiency the EPR Signal I is the sum of the $P700^+$ and $P680^+$ signals. These results have also been discussed by Goncharova *et al.* (1991).

The EPR spectrum of the phosphate anion radical: If phosphate is an endogenous electron donor, the primary product of the interaction of phosphate ions and Chl is the phosphate anion radical. Earlier, the EPR spectra of phosphate radical were measured at 77 K in crystals (Subramanian *et al.* 1970) and in aqueous glasses (Ginns and Symons 1975) irradiated with ^{60}Co γ-rays. However, to compare the EPR spectrum of the oxidized electron donor to $P680^+$ with the EPR spectrum of the phosphate anion radical, it is necessary to measure the latter under conditions similar to those supposed in chloroplasts, *i.e.*, at the state of a complex with the Mg atom of Chl. Therefore, we recorded first the phosphate anion radical by the EPR spectroscopy method in ethanol solution of Chl, containing phosphate added in a small amount of water, at 77 K after irradiation, and then tried to detect the EPR

signal of the phosphate anion radical in the EPR spectra of chloroplasts also at 77 K after irradiation.

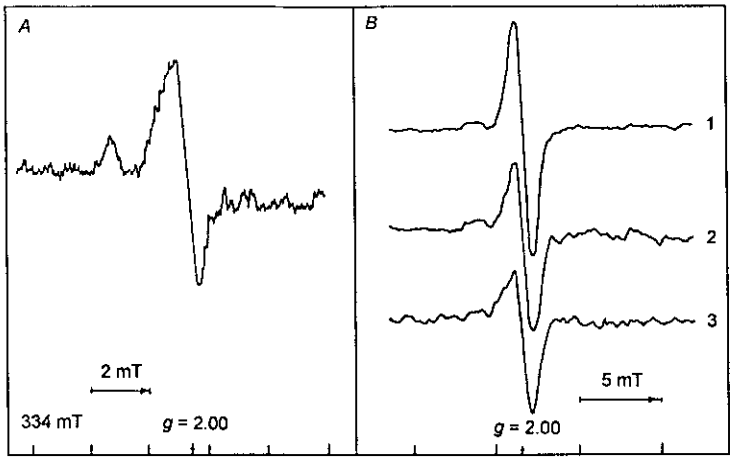


Fig. 8. EPR spectra of the aqueous ethanol solutions of chlorophyll *a* containing 0.01 M potassium phosphate at 77 K after irradiation. *A*: pH 7.6; EPR conditions: 5 mW microwave power; 0.5 mT modulation amplitude. *B*. pH 8.0; microwave power 10 mW (*1*), 50 mW (2), and 100 mW (*3*); modulation amplitude 0.5 mT.

The EPR signals of the phosphate anion radical in the aqueous ethanol solutions of Chl at 77 K after irradiation of frozen samples are demonstrated in Fig. 8. At room temperature, pyrophosphate is formed in this system (Goncharova and Goldfeld 1990). Air oxygen acts as the final electron acceptor. At 77 K, only the electron transfer, photosensitized by Chl, from phosphate to oxygen occurred, and the phosphate radical was generated. Best of all, the phosphate anion radical was formed at pH 7.6 when the pyrophosphate formation had a pH optimum at room temperature. The EPR signals disappeared upon thawing of the samples. In the control experiments without phosphate no EPR signals were found in the ethanol solutions of Chl *a* at 77 K in the presence of air oxygen at the same pH after irradiation.

The EPR spectra of ethanol Chl solutions at 77 K have been studied in detail (Rikhireva *et al.* 1964, Evstigneev *et al.* 1971). In the presence of electron acceptor, *e.g.*, air oxygen, Chl by irradiation sensitizes electron transfer from various donors (including ethanol molecules) to air oxygen. The EPR signal of the Chl radical is not observed in such conditions. One can see the Chl radical, if air oxygen is absent in ethanol solution, for example, after it is pumped out. The forming ethanol radicals (Kholmogorov *et al.* 1963) and paramagnetic oxygen molecules and their radicals (Beringer and Castle 1951, Wertz and Bolton 1972) give multiline EPR signals of low intensity which are poorly seen in the EPR spectra. Therefore, the EPR spectra represented in Fig. 8 may belong to the phosphate anion radical (see also Goncharova *et al.* 1993a).

In γ-irradiated crystal samples, the EPR spectrum of the phosphate radicals is the doublet arising from an interaction between an unpaired electron of oxygen and a magnetic nucleus of ^{31}P (Subramanian *et al.* 1970). The doublet signal has the small anisotropy of *g*-tensor and hyperfine coupling constant. However, in solutions (Ginns and Symons 1975) owing to the spontaneous position of molecules, the spectrum lines broaden, and the *g*-tensor and the hyperfine coupling constant have average

values: the *g*-tensor in the region of 2.00, and the coupling constant in the range of 2-4 mT. The phosphate radicals are stable at 77 K but rapidly disappear at room temperature. The EPR doublet signal is a characteristic of many phosphate radicals. However, several calcium and magnesium phosphate radicals give EPR signals consisting of a single broad line, *e.g.*, $MgHPO_4 \times 3\ H_2O$ gives the EPR spectrum characterized by a very broad single line with the width of approximately 1.2 mT (Subramanian *et al.* 1970).

The EPR spectra represented in Fig. 8*A,B* show that the doublet, characteristic for the phosphate radicals, is better seen at pH 7.6. The EPR signal has the *g*-tensor 2.00 and the hyperfine coupling constant at about 2 mT. At pH 8.0 this signal is almost completely transformed in a single line with the width of approximately 1.1 mT which is easily saturated by the increase in the microwave power. Probably, in a more alkaline medium the interrelation of phosphate and the magnesium atom of Chl increases, and this leads to alterations in the signal form.

If $P680^+$ reacts with phosphate ions as electron donors, the EPR signal of the phosphate anion radical must always be present in the chloroplast EPR spectra. On the other hand, this signal must be absent if the electron transfer through P680 ceases owing to the inhibition of the primary electron acceptor of PS2, Q, by diuron. Actually, in the difference spectrum "chloroplasts minus chloroplasts with diuron" the doublet signal was apparent which had characteristics similar to the EPR signal of the phosphate anion radical: the *g*-tensor 2.00, and the hyperfine coupling constant of about 2 mT (Fig. 9*A,B*). The EPR spectra of chloroplasts were measured at 77 K after irradiation of frozen samples in liquid nitrogen. To record the difference EPR spectrum, the EPR spectra of chloroplasts were made equal in EPR Signal II which did not change on irradiation at 77 K. The doublet signal in chloroplasts (Fig. 9*A,B*), as in ethanol solutions of Chl *a* (Fig. 8*A,B*), was better seen at pH 7.5; at 8.0 the quota of the single line with the width of approximately 1.1 mT increased. The single EPR signal was also easier saturated by the increase in the microwave power.

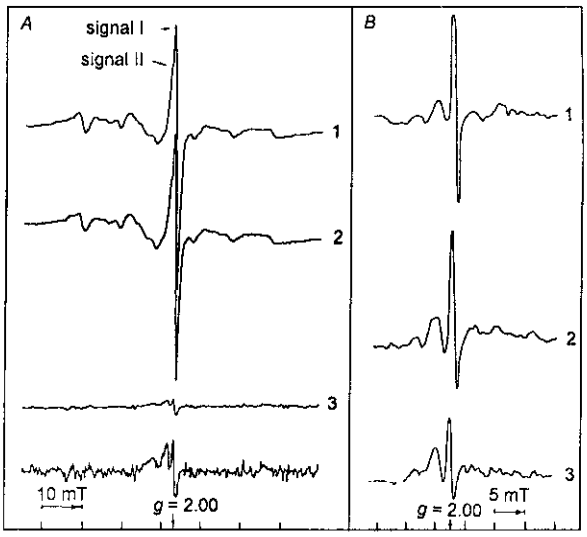
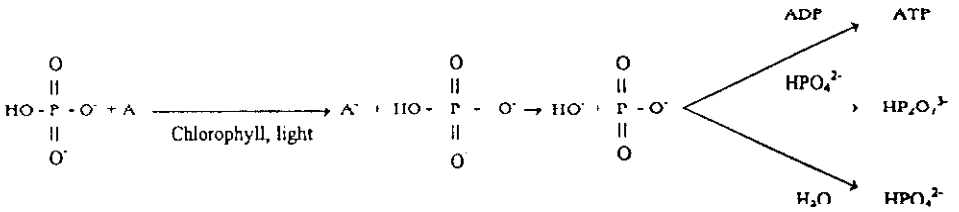


Fig. 9. EPR spectra of chloroplasts at 77 K after irradiation. *A*: pH 7.5; *1*- control chloroplasts, *2* - chloroplasts with diuron, *3* - the difference spectrum "chloroplasts minus chloroplasts with diuron"; *4* - the same as *3* but increased to 4 times. EPR conditions: 5 mW microwave power, 0.2 mT modulation amplitude. *B*: EPR difference spectra "chloroplasts minus chloroplasts with diuron", pH 8.0. Microwave power 10 mW (*1*), 50 mW (*2*), and 100 mW (*3*); modulation amplitude 0.5 mT.

The difference EPR spectrum "chloroplasts minus chloroplasts with diuron" allows to remove the EPR signals of the radicals arising after irradiation at the PS2 acceptor side, and also in PS1, and all dark EPR signals. In the experiments with diuron, the reduction of the primary electron acceptors before the site of diuron inhibition occurs during the freezing of chloroplasts by the action of weak background radiation. When the small pool of primary electron acceptors is filled, the electron transfer through P680 stops. At the same time, diuron does not affect the primary electron transport in PS1 and the dark EPR signals.

Discussion

Our study is based on the experiments with Chl in model systems. They showed that Chl in simple chemical systems, containing phosphate and an electron acceptor, photosensitized the pyrophosphate bonds formation (Goncharova and Goldfeld 1990):



The idea of the Chl and phosphate interaction permitted to discover new facts in experiments with chloroplasts: (*1*) In the absence of endogenous P_i, the light-induced basal electron transport estimated by the FeCy reduction depends on exogenous phosphate; (*2*) phosphate can be replaced with the electron donor to PS2 diphenylcarbazide, and both reactions are inhibited by diuron; (*3*) decrease in the contents of endogenous phosphate results in the damage of RCs, first of PS2, and then of PS1, and light-harvesting Chl; (*4*) endogenous P_i consumption causes the increase in absorbance at 435 nm in absorption spectrum of chloroplasts, which indicates the existence of a complex between P_i and Chl; (*5*) under deficiency of endogenous P_i in chloroplasts, the EPR Signal I at 77 K is the sum of the $P700^+$ and $P680^+$ signals as can be seen from the increase of the amplitude and the width of Signal I, and from the changes in the Signal I microwave power saturation; all changes disappear after addition of P_i, DPC, or diuron before freezing the samples; (*6*) after irradiation at 77 K, in native chloroplasts the EPR signal similar to that of the phosphate anion radical is observed. These results indicate that phosphate has an electron donor function in PS2.

As demonstrated in the scheme, in the model systems the transfer of just a single electron from the phosphate ion is necessary for the pyrophosphate bond formation. We suppose that such reaction mechanism acts in PS2 of chloroplasts. Hydroxyl radicals detached from phosphate anion radicals perhaps may serve as a substrate for the oxygen-evolving system (Goncharova *et al.* 1993b).

The value of the redox potential of phosphate ions cannot be directly measured, because the product of the phosphate ion oxidation, phosphate anion radical, has a half-life of about 300 μs at room temperature (Huber and Hayon 1968). However, Sukhorukov *et al.* (1966) have shown that many organic electron acceptors, *e.g.*, *p*-benzoquinone (E_0' = + 0.28 V), methyl blue (E_0' = - 0.06 V), riboflavin (E_0' = - 0.21 V), and also tetra-cyanoethylene and chloranil, can accept an electron from phosphate ions to yield one-electron reduced forms, registered by the EPR method. Therefore, the redox potential of phosphate ions may be in the region below zero. In this connection the free energy, released by the phosphate ions oxidation by many compounds including Chl $P680^+$ and other electron carriers, is enough to form ATP.

According to the above scheme, the transfer of one electron in PS2 must be followed by the formation of one ATP molecule. Reeves and Hall (1973) and other investigators have obtained in chloroplasts the ratio of P/2e equal 2. This indicates that one ATP molecule may be formed per one electron if even two phosphorylation sites (at the donor side of PS2, and between PS2 and PS1) take part in the reaction, because some amounts of metaphosphate ions are inevitably lost by the interaction with water molecules, and, as a result, phosphate is formed again. For example, Shavit *et al.* (1967) have demonstrated that ^{18}O from water molecules is incorporated into phosphate on light. Besides, the ATP formation proceeds on each flash (Gol'dfel'd *et al.* 1978, Goncharova *et al.* 1993b), *i.e.*, the transfer of a single electron is sufficient for the formation of ATP.

As the scheme indicates, the hydroxyl radical is formed simultaneously with metaphosphate ion. Hydroxyl radicals are the rapid oxidizing agents, and they must be eliminated, *e.g.*, by the reduction to H_2O under the influence of reduced electron carriers (in bacterial photophosphorylation, in a phosphorylation site between PS2 and PS1, and also in mitochondrial oxidative phosphorylation) or by the conversion into O_2 in the system of oxygen evolution.

Phosphate does not compete with water in PS2. On the contrary, the water molecule is oxidized with the participation of phosphate, because the water molecule is attached to metaphosphate, and gives phosphate ion which is oxidized. The phosphate participation in primary reaction of PS2 can account for the isotopic composition of molecular oxygen evolved in photosynthesis: when CO_2 fixation is absent, *e.g.*, in experiments of Radmer and Ollinger (1980), the evolved O_2 includes oxygen only from water; but when the CO_2 fixation is present, *e.g.*, in experiments of Metzner *et al.* (1979), the evolved O_2 includes also oxygen from CO_2, because it is incorporated into phosphate through the pentose phosphate cycle of CO_2 fixation. In addition, oxygen from phosphate has also been found in oxygen evolved in photosynthesis (Roux *et al.* 1961).

The results presented in our article give a new additional information about the electron transport and the ATP formation in chloroplasts which permits to find one more step in complete overall picture of the mechanism of the conversion of solar energy to chemical bound energy in photosynthesis.

May we consider our results in light of the current understanding of the photochemical reactions of PS2 (for reviews, see Debus 1992, Miller and Brudvig 1991)? Actual investigations are in progress not on native chloroplasts, as in our

experiments, but on membrane preparations obtained by the use of detergents. Probably, detergents are attached to the Mg atom of Chl, as it is known for detergent solutions of Chl in model systems, and remove endogenous phosphate which is also attached to Chl. Membrane preparations obtained by the action of high concentration of detergent do not contain endogenous phosphate and cannot evolve oxygen. When detergents are used in low concentrations, a portion of endogenous phosphate remains attached to Chl molecules, and may participate in photochemical reactions initiating the oxygen evolution during several seconds to several minutes as observed in experiments with preparations of PS2 RCs (Ghanotakis and Yocum 1986). Detergents cannot be removed completely by dialysis, and enter the reaction medium.

Detergents have an uncoupling effect, and inhibit the formation of ATP (Avron and Neumann 1968). They increase the rate of electron transport and oxygen evolution but phosphorylation rate, depending on the detergent concentration, either decreases or is absent. According to the chemiosmotic hypothesis, the uncouplers increase the membrane permeability for hydrogen ions, and break down the transmembrane proton gradient; therefore the formation of ATP stops. However, it is impossible to ignore another explanation: uncouplers form with metaphosphate ion, arising as a result of the phosphate activation, a rapidly splitting compound, and so release the RC for next act. For example, the results of Tables 2 and 3 indicate such a possibility for NH_4Cl and methylamine.

The various reducing agents, for example NH_2OH, dithionite, and ascorbate, are often added to the membrane preparations. In the presence of such strong reducing agents, phosphate is not oxidized. Thus, in experiments with the membrane preparations containing PS2 RCs, the conditions are created in which the endogenous electron donor of PS2, in our opinion endogenous phosphate, can not be discovered.

The preparations of PS2 RCs can oxidize on irradiation another available electron donors, for example, reduced cytochrome b_{559} and Chl molecules surrounding a RC as shown by Koulougliotis *et al.* (1994). When all possible electron donors cease, P680 is oxidized irreversibly, and is destroyed.

In native chloroplasts, the oxidation of endogenous phosphate ions at room temperature must proceed in the nanosecond time range. In this range the appearance of the tyrosine radical is also observed. However, it is not implied that tyrosine is an electron donor of $P680^+$. Tyrosine (Y_Z and Y_D) incorporates into the proteins D1 and D2 of the PS2 RC, and is situated near the manganese atoms of the oxygen-evolving complex (for review see Debus 1992). The tyrosine redox conversion is most likely associated with the redox processes on the manganese atoms.

The participation of the endogenous phosphate ions in the electron transport as an electron donor of $P680^+$ in native chloroplasts accounts for the nature of the basal electron transport and its connection with photophosphorylation that has remained vague from the first works in this area.

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