BBA 45900

THE SITE OF PHOSPHORYLATION ASSOCIATED WITH PHOTOSYSTEM I

GÜNTER A. HAUSKA, RICHARD E. MCCARTY AND EFRAIM RACKER
Section of Biochemistry and Molecular Biology, Cornell University, Ithaca, N.Y. 14850 (U.S.A.)
(Received September 19th, 1969)

SUMMARY

1. Small particles prepared from spinach chloroplasts after treatment with digitonin, exhibited Photosystem I reactions, including phosphorylation, at rates as high as those in chloroplasts, whereas electron flow from water to $NADP^+$ or ferricyanide through Photosystem II was completely lost. Mediators of cyclic electron flow, such as pyocyanine, or *N*-methylphenazonium methosulfate in red light, had to be reduced to support photophosphorylation.

Diaminodurene at high concentrations catalyzed cyclic phosphorylation under anaerobic conditions without addition of a reductant. In fact, addition of ascorbate gave rise to a marked inhibition which was released by addition of a suitable electron acceptor such as methylviologen.

2. Under aerobic conditions a low O_2 uptake, observed in the presence of diaminodurene, was stimulated several-fold upon addition of methylviologen and was stimulated again several-fold on further addition of ascorbate. The rate of phosphorylation, however, remained the same. The low P/2e ratio obtained under these conditions was not decreased at lower light intensities.

3. These findings suggest a phosphorylation site associated with cyclic electron flow through Photosystem I without participation of the electron carriers of Photosystem II. A non-cyclic electron flow to O_2 can be induced in this system by addition of methylviologen which effectively competes with the electron acceptors of cyclic flow. This non-cyclic electron flow still involves the same phosphorylation site. A scheme for electron transport and for the location of phosphorylation sites in chloroplasts is proposed.

INTRODUCTION

Electron flow from water to $NADP^+$ which requires Photosystems I and II supports phosphorylation[1] which is referred to as non-cyclic photophosphorylation. This reaction is characterized by its high sensitivity to dichlorophenyl-1,1-dimethylurea (DCMU). A cyclic electron flow which takes place in the presence of suitable electron carriers[2], such as *N*-methylphenazonium methosulfate (PMS), and which is resistant to DCMU, is also associated with phosphorylation. This process is referred to as cyclic photophosphorylation. The possibility that the site of cyclic phosphoryla-

Abbreviations: PMS, *N*-methylphenazonium methosulfate; DCIP, dichlorophenolindophenol; DCMU, dichlorophenyl-1,1-dimethylurea; Tricine, tris(hydroxymethyl)methylglycine.

tions differs from that involved in non-cyclic phosphorylation was raised by the findings that several uncouplers[3,4] and heptane treatment of chloroplasts[5] impair one type of phosphorylation more than the other. WITT *et al.*[6], ARNON *et al.*[7] and others[8,9] have suggested that cyclic electron flow with the associated phosphorylation takes place in a separate Photosystem I unit.

A non-cyclic electron flow to $NADP^+$ which is insensitive to DCMU can be induced in Photosystem I by addition of ascorbate and a suitable electron carrier such as dichlorophenolindophenol (DCIP)[10] or diaminodurene[11]. It has been questioned, however[12], whether phosphorylation observed under these conditions is indeed linked to the non-cyclic electron flow or reflects a superimposed cyclic electron flow which takes place in the presence of these carriers.

It is the purpose of this paper to describe experiments with subchloroplast particles which have lost Photosystem II. A non-cyclic electron flow was induced in Photosystem I by addition of methylviologen which was accompanied by phosphorylation. Under these conditions, there was little or no cyclic electron flow. The relationship of this phosphorylation site to that of cyclic electron flow and to that of DCMU-sensitive non-cyclic phosphorylation will be discussed.

MATERIALS AND METHODS

Preparation of chloroplasts and subchloroplast particles

Chloroplasts were prepared from spinach leaves as previously described[13]. Digitonin subchloroplast particles were prepared according to ANDERSON AND BOARDMAN[14]. The chloroplasts were suspended in 0.4 M sucrose, 0.05 M phosphate buffer (pH 7.2) and 0.01 M NaCl at 0° and 2 % digitonin in the same medium was added to a final concentration of 0.5 %. The final chlorophyll concentration was 0.7–0.8 mg/ml. After 30 min of incubation at 0° with stirring, a fraction sedimenting between 1000 and 10000 $\times g$ (calculated for the bottom of the tube), called the D-10 particle, and a fraction sedimenting between 50000 and 144000 $\times g$, called the D-144 particle, were separated using the Beckman Spinco 50-R rotor. Both pellets were suspended in 1 M sucrose and 2 mM tris(hydroxymethyl)methylglycine (Tricine) (pH 8.0) and stored in 0.1-ml aliquots in liquid N_2 for months without loss of photochemical activity.

Preparations of proteins

Ferredoxin, plastocyanin, ferredoxin–NADP reductase, chloroplast coupling factor 1 (CF_1)[15] and coupling factor 2 (CF_2)[16] were prepared as described previously[17]. Bovine serum albumin (Fraction V) was defatted by the procedure of CHEN[18].

Analytical methods

Protein was determined by the spectrophotometric method of WARBURG AND CHRISTIAN[19].

Total chlorophyll in chloroplasts was determined by the method of ARNON[20]. Chlorophylls *a* and *b* and total chlorophyll in 80 % acetone extracts of digitonin subchloroplast particles were calculated according to VERNON[21]. The Hill reaction with ferricyanide was determined spectrophotometrically by the method of JAGENDORF AND SMITH[22].

NADPH formation was measured with the glutathione, NADPH–glutathione reductase system as published elsewhere[17].

$^{32}P_i$ esterification was measured as described previously[13]. If carried out

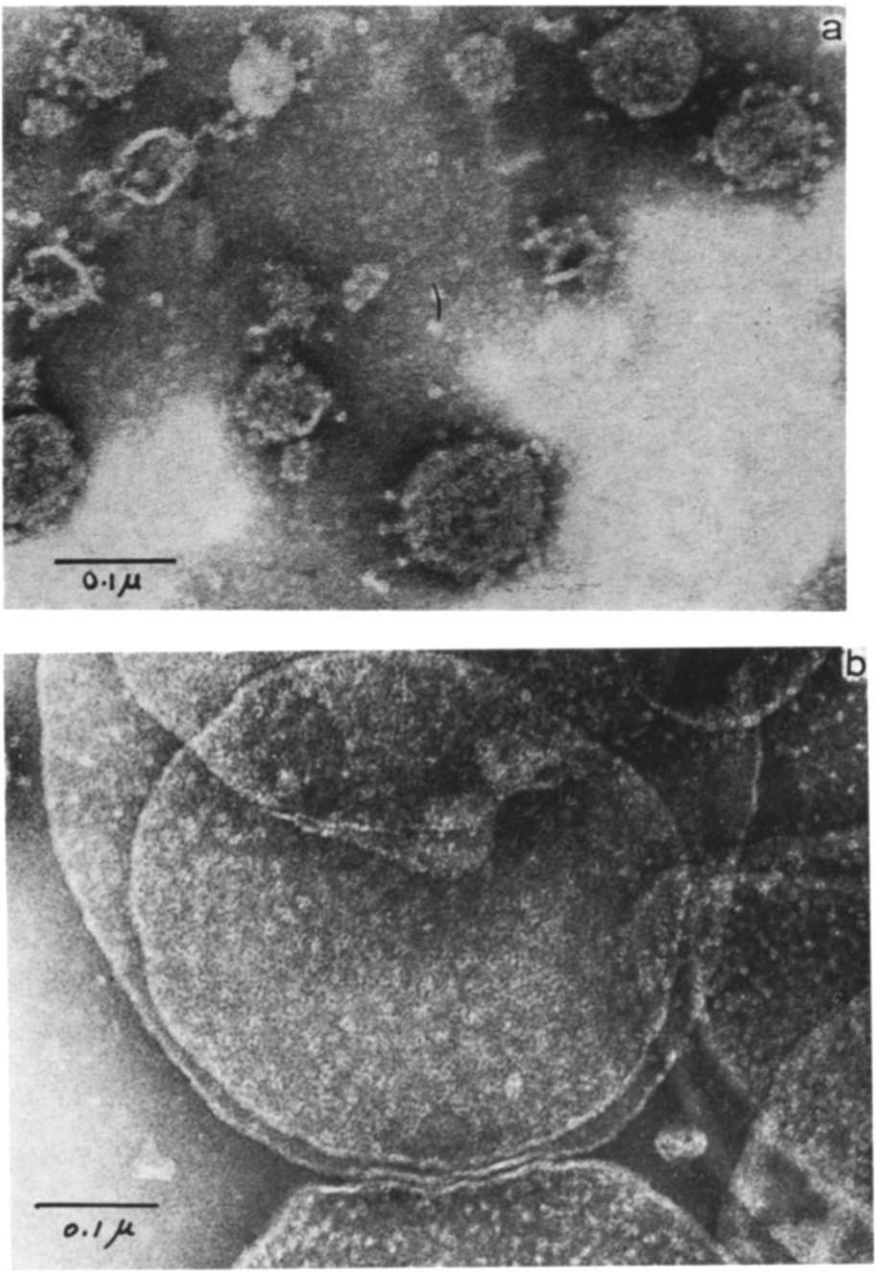

Fig. 1. Electron micrograph of subchloroplast particles prepared by incubation with digitonin. Negative stain with 2 % phosphotungstate (pH 6.8). Final magnification × 160000. a. D-144 particles. b. D-10 particles.

anaerobically the incubation was performed in Thunberg tubes which were flushed 3 times with argon before illumination with a light intensity of $2 \cdot 10^6$ ergs/cm^2 per sec at room temperature for 2 min. Photophosphorylation under aerobic conditions was assayed in a 1.8-ml cell at 25°. The illumination in this case was for 1 min with an intensity of $5 \cdot 10^5$ ergs/cm^2 per sec. O_2 uptake was measured with a Clark-type electrode covered with a Teflon membrane and attached to a Gilson Medical Electronics oxygraph. Light intensity was measured with a YSI-Kettering radiometer. ATPase activity, activated by trypsin, was assayed by the method of VAMBUTAS AND RACKER[15].

MATERIALS

Digitonin was purchased from Sigma Chemical Co. and was recrystallized from ethanol. PMS, glutathione and glutathione reductase were obtained from Sigma. Tricine was purchased from General Biochemicals. Methylviologen was obtained from Mann Research Laboratories. DCMU was obtained from K and K Chemicals and was recrystallized twice. Pyocyanine was prepared from PMS by the method of JAGENDORF AND MARGULIES[23]. Diaminodurene was a generous gift from Dr. N. E. Good.

RESULTS

Properties of the digitonin subchloroplast particles

The properties of the D-10 and D-144 particles were essentially as described by ANDERSON AND BOARDMAN[14]. The ratio of chlorophyll *a* to chlorophyll *b* was 2.3 for the D-10 and 5.5 for the D-144 particle, starting with chloroplasts with a ratio of 2.5. Fig. 1 shows electron micrographs of the particles negatively stained with 2 % phosphotungstate. Characteristic 90-Å spheres[15,24] were seen in the D-144 particles which had an average diameter of 700 Å. The average diameter of the D-10 particles was about 5000 Å. The trypsin-activated, Ca^{2+}-dependent ATPase[15] was as high in the D-144 particles as in chloroplasts (per mg chlorophyll). Cyclic phosphorylation was also as rapid as in chloroplasts and no stimulation by addition of CF_1 and CF_2 was observed. As found by ANDERSON AND BOARDMAN[14], and confirmed by ARNON *et al.*[25], the D-144 particles were enriched in Photosystem I as measured by composition, cyclic phosphorylation and photoreduction of $NADP^+$ with ascorbate, but were inactive in catalyzing non-cyclic electron flow from water with ferricyanide or $NADP^+$ as electron acceptor. The D-10 particles showed the same activities, including the ATPase, as the original chloroplasts, but at a rate of about one third to one sixth.

Cyclic phosphorylation in D-144 particles under anaerobic conditions

Data on cyclic phosphorylation in D-144 particles and chloroplasts under anaerobic conditions with various mediators, are recorded in Table I. The rate of phosphorylation catalyzed by PMS or diaminodurene was almost as high in D-144 particles as in chloroplasts. However, pyocyanine, which gave a high rate of cyclic phosphorylation in chloroplasts, was virtually inactive in D-144 particles. In confirmation of previous findings[23], it was observed that in chloroplasts DCMU strongly inhibited phosphorylation in the presence of pyocyanine, whereas the rate with PMS

TABLE I

CYCLIC PHOTOPHOSPHORYLATION IN D-144 PARTICLES AND CHLOROPLASTS

The reaction mixture contained in a final volume of 1 ml: 50 mM Tricine–NaOH buffer (pH 8.0), 10 mM NaCl, 5 mM $MgCl_2$, 2 mM phosphate (pH 8.0) containing $2 \cdot 10^5$–$10 \cdot 10^5$ counts $^{32}P_i$/min per μmole, 3 mM ADP, 1 mg defatted bovine serum albumin and 10 μg of chlorophyll. It further contained, where indicated, 0.05 mM PMS, 0.05 mM pyocyanine, 1 mM diaminodurene, 0.5 mg borohydride, 2 mM dithiothreitol, 5 mM ascorbate and 0.02 mM DCMU. Illumination was performed in Thunberg tubes as described under MATERIALS AND METHODS. Reduction of the cyclic mediators was performed in the side arm of the Thunberg tube, then the solutions in the side arm and in the bottom of the tube were mixed. For illumination with red light (> 620 nm) a red glass filter (Corning glass No. 2403) was used. The red light intensity was $4 \cdot 10^5$ ergs/cm^2 per sec.

Additions	*Rate of phosphorylation (μmoles P_i esterified per mg chlorophyll per h*			
	D-144		*Chloroplasts*	
	Control	*+ DCMU*	*Control*	*+ DCMU*
White light				
PMS	532	560	694	413
Pyocyanine	3	3	615	107
Diaminodurene	389	403	586	762
Pyocyanine + BH_4^-	275	—	643	630
Pyocyanine + dithiothreitol	150	—	670	615
PMS + BH_4^-	520	—	710	590
PMS + dithiothreitol	520	—	450	450
Red light				
PMS	70	—	507	98
PMS + ascorbate	575	—	579	560

was much less affected. DCMU had no effect on phosphorylation in D-144 particles. These observations are readily explained by the finding that reduction of pyocyanine by either borohydride or dithiothreitol restored phosphorylation in D-144 particles and eliminated the inhibition by DCMU in chloroplasts. The contribution of Photosystem II to cyclic phosphorylation can therefore be attributed to a reduction of pyocyanine. With PMS a reducing agent was not required since it was shown by JAGENDORF AND MARGULIES[23] that white light caused a fast non-enzymatic reduction of PMS, whereas red light did not. Accordingly, cyclic phosphorylation with PMS in red light was found to be very low in the D-144 particles. If, however, ascorbate was added the rate of phosphorylation approached that in white light. In chloroplasts, PMS-dependent phosphorylation in red light was as sensitive to DCMU as pyocyanine-dependent phosphorylation in white light. Reduction of PMS with ascorbate reversed this inhibition. It should be pointed out that ascorbate reduces PMS but does not reduce pyocyanine which requires stronger reducing agents such as dithiothreitol or borohydride. Diaminodurene, which was added in its reduced form, required no reducing agents. FMN and menadione gave low rates of cyclic phosphorylation which were also stimulated several-fold by reducing agents (not shown in Table I).

It is apparent from these findings that a mediator of cyclic phosphorylation must be reduced so that it can donate electrons for cyclic electron flow. Alternatively, electrons for cyclic flow in Photosystem I can be supplied by Photosystem II.

Inhibition of diaminodurene-mediated cyclic phosphorylation by ascorbate under anaerobic conditions

As shown in Table II, ascorbate strongly inhibited diaminodurene-mediated cyclic phosphorylation in chloroplasts and D-144 particles. In chloroplasts the inhibition was less pronounced in the presence of DCMU. The inhibition by ascorbate was released by addition of an electron acceptor for Photosystem I such as methylviologen. Since the additional amount of ATP formed exceeded the amount of methylviologen added, it is probable that methylviologen participates in cyclic electron flow. However, methylviologen did not catalyze cyclic phosphorylation without diaminodurene, presumably because it remained in its oxidized form even in the presence of ascorbate.

TABLE II

EFFECT OF ASCORBATE ON THE DIAMINODURENE-DEPENDENT CYCLIC PHOSPHORYLATION IN D-144 PARTICLES AND CHLOROPLASTS

The conditions and the concentrations during the incubation were the same as described for Table I. Additionally, where indicated, the mixture contained 0.1 mM methylviologen, 20 μg (1 ×) or 200 μg (10 ×) ferredoxin, 0.7 μg ferredoxin–NADP$^+$ reductase and 0.25 μmole NADP$^+$. For the photoreduction of NADP$^+$ the mixture further contained 2.5 mM glutathione and 0.5 μg glutathione reductase.

Additions	*Rate of phosphorylation (μmoles P_i esterified per mg chlorophyll per h)*		
	D-144	*Chloroplasts*	
		Control	*+ DCMU*
Diaminodurene	547	587	762
Diaminodurene + ascorbate	89	50	240
Diaminodurene + ascorbate + pyocyanine	650	875	745
Diaminodurene + ascorbate + methylviologen	530	850	795
Diaminodurene + ascorbate + ferredoxin (1 ×)	190	50	208
Diaminodurene + ascorbate + ferredoxin (10 ×)	500	—	—
Diaminodurene + ascorbate + ferredoxin + ferredoxin–NADP$^+$ reductase + NADP$^+$	193	122	204

It is concluded from these observations that when diaminodurene is very rapidly reduced by an external electron donor, cyclic electron flow of Photosystem I and the associated phosphorylation is markedly inhibited. In line with this conclusion, it was found that suitable electron acceptors released the inhibition by ascorbate. Pyocyanine, which, as pointed out above, did not catalyze cyclic phosphorylation in D-144 particles with ascorbate alone, reversed the inhibition by ascorbate to give rise to phosphorylation at an even higher level than that in the presence of diaminodurene and in the absence of ascorbate. Ferredoxin also reversed the inhibition of phosphorylation by ascorbate. The presence of ferredoxin–NADP$^+$ reductase and NADP$^+$ did not improve the efficiency of this reversal at low concentrations of ferredoxin. On the other hand in chloroplasts, ferredoxin reversed the inhibition by ascorbate only if ferredoxin–NADP$^+$ reductase and NADP$^+$ were present and DCMU was absent. It is apparent, therefore, that the often confusing variability of chloroplasts and subchloroplast particles in their response to different agents that affect

their electron transport system is dependent on their intrinsic capabilities of oxido-reductions, and particularly on the functioning of Photosystem II.

Diaminodurene-mediated Photosystem I activities under aerobic conditions

In view of the above observations, it was of interest to measure Photosystem I activities under aerobic conditions. Diaminodurene was shown to be an electron donor for $NADP^+$ reduction by Photosystem I provided both ferredoxin and ferredoxin–$NADP^+$ reductase are present[11]. However, ferredoxin and ferredoxin–$NADP^+$ reductase are not required for the reduction of methylviologen. The reaction with methylviologen represents therefore a simpler system. Moreover, reduced methylviologen reacts rapidly with O_2 to form H_2O_2, thus the reaction can be followed by measuring O_2 polarographically[26].

In D-144 particles a considerable O_2 uptake can be observed on addition of diaminodurene without methylviologen. This O_2 uptake, which must be due to auto-oxidation of a natural electron acceptor (probably X^-, the reductant of Photosystem I), decreased quite rapidly with time, presumably because oxidized diaminodurene accumulated and competed with O_2 for electrons from X^-. In line with this interpretation is the finding (Table III) that when diaminodurene was kept reduced by ascorbate a constant rate of O_2 uptake was maintained. Methylviologen increased

TABLE III

REACTIONS OF PHOTOSYSTEM I IN THE PRESENCE OF DIAMINODURENE UNDER AEROBIC CONDITIONS IN D-144 PARTICLES AND CHLOROPLASTS

The incubation under aerobic conditions was performed as described under MATERIALS AND METHODS. The basic reaction mixture was the same as described for Table I. In addition the mixture contained, where indicated, 3.3 mM ascorbate, 0.7 mM diaminodurene, 0.07 mM methylviologen and 2 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). 20 mμmoles of DCMU were added when chloroplasts were used. The O_2 uptake in the light is corrected for any reaction in the dark. If the rate levelled off during the illumination time, the average rate is given in the table. I_1, I_2 and I_3 represent light intensities of $5 \cdot 10^5$, $1 \cdot 10^5$ and $1.3 \cdot 10^4$ ergs/cm^2 per sec, respectively.

Additions	*Electron transport (μmoles O_2 reduced per mg chlorophyll per h)*			*Rate of phosphorylation (μmoles P_i esterified per mg chlorophyll per h)*			*Ratio P/2e*		
	I_1	I_2	I_3	I_1	I_2	I_3	I_1	I_2	I_3
D-144									
Diaminodurene	305	279	108	437	262	48	1.43	0.94	0.44
Diaminodurene + methylviologen	1430	1130	341	394	249	67	0.27	0.22	0.19
Diaminodurene + ascorbate	1280	1165	376	156	150	44	0.12	0.13	0.12
Diaminodurene + ascorbate + methylviologen	4040	2740	485	447	276	70	0.11	0.10	0.13
Diaminodurene + ascorbate + methylviologen + CCCP	3950	—	—	95	—	—	0.024	—	—
Chloroplasts									
Diaminodurene	216	120	60	729	242	26	3.37	2.00	0.43
Diaminodurene + methylviologen	850	432	150	652	248	21	0.77	0.57	0.14
Diaminodurene + ascorbate	715	270	132	352	135	24	0.49	0.50	0.32
Diaminodurene + ascorbate + methylviologen	3460	1820	354	780	322	24	0.23	0.18	0.07

TABLE IV

EFFECT OF METHYLVIOLOGEN ON O_2 UPTAKE AND PHOSPHORYLATION IN D-144 PARTICLES IN THE PRESENCE OF DIAMINODURENE UNDER AEROBIC CONDITIONS

The incubation under aerobic conditions was performed as described under MATERIALS AND METHODS. The basic reaction mixture is described in the legend for Table I. It further contained, where indicated, 0.7 mM diaminodurene and varying concentrations of methylviologen. The average rate of O_2 uptake during the illumination time of 1 min is given. The rate of electron transport represents the reduction of O_2 to H_2O_2 involving two electrons.

Additions	*Electron transport (μmoles O_2 reduced per mg chlorophyll per h)*	*Rate of phosphorylation (μmoles P_i esterified per mg chlorophyll per h)*	*Ratio P/2e*
Diaminodurene	404	349	0.87
Diaminodurene + 0.007 mM methylviologen	568	364	0.64
Diaminodurene + 0.07 mM methylviologen	895	330	0.36
Diaminodurene + 0.7 mM methylviologen	1385	356	0.26

the rate of O_2 uptake several-fold, but again a decrease in the rate with time was observed which was eliminated by ascorbate.

The competition between oxidized diaminodurene (cyclic electron flow) and methylviologen (non-cyclic electron flow) was also apparent from measurements of phosphorylation. Whereas in the presence of diaminodurene alone the P/2e ratio was relatively high because of contribution by both cyclic and non-cyclic electron flow, on addition of increasing amounts of methylviologen (Table IV) the phosphorylation remained constant, but since O_2 uptake was enhanced, the P/2e ratio decreased.

The phosphorylation rate was decreased in the presence of ascorbate (Table III) but not as markedly as under anaerobic conditions (Table II), and was completely restored on addition of methylviologen. As pointed out above, O_2 uptake under these conditions remained linear with time both in D-144 particles and in chloroplasts. Since the P/2e ratio in D-144 particles with diaminodurene and ascorbate was almost the same with and without methylviologen, it seems likely that a P/2e ratio of about 0.1 represents the degree of coupling for this type of non-cyclic electron flow in D-144 particles. Even at low light intensities similar P/2e ratios were observed. 10-fold higher concentrations of methylviologen had no further effect either on O_2 uptake or on phosphorylation. The addition of carbonyl cyanide *m*-chlorophenylhydrazone had no effect on the rate of O_2 uptake but inhibited phosphorylation. It appears from these findings that cyclic electron flow in Photosystem I can be readily converted into a non-cyclic electron flow without affecting the net rate of phosphorylation.

Effect of variations of light intensity on diaminodurene-linked reactions of Photosystem I under aerobic conditions

To show more conclusively that phosphorylation was linked to non-cyclic electron flow conditions of effective competition of non-cyclic with cyclic electron flow in Photosystem I were induced by lowering the light intensity. Values for two lower light intensities are given in Table III. In D-144 particles with diaminodurene *plus* ascorbate or with diaminodurene *plus* ascorbate *plus* methylviologen, when

presumably non-cyclic electron flow in Photosystem I predominated, the P/2e ratio stayed fairly constant over the range of light intensities tested. With diaminodurene alone, when cyclic phosphorylation predominated, the P/2e ratio dropped with decreasing light intensity. The well-known dependence of cyclic phosphorylation on high light intensities probably reflects the light-dependent and rate-limiting oxidation of the mediator diaminodurene which serves in its oxidized form as the acceptor of electrons from X^- (see Fig. 2) in cyclic electron flow. In non-cyclic electron flow the concentration of the electron acceptor, such as methylviologen or O_2, is independent of light. Therefore, if both types of electron flow occur simultaneously, the non-cyclic type must be favored at lower light intensities and the P/2e ratio must drop. In line with this explanation is the observation that at low light intensities the inhibition of diaminodurene-linked phosphorylation by ascorbate disappeared. The addition of methylviologen stimulated phosphorylation at low light intensities. Presumably the back-reaction of X^- with the oxidant of Photosystem I becomes effective at low light intensities and is supressed by methylviologen.

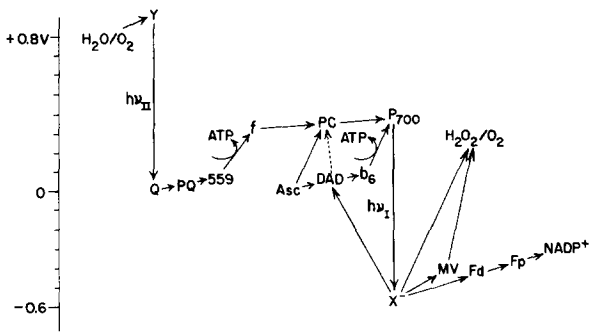

Fig. 2. Scheme for electron flow and phosphorylation in chloroplasts, roughly correlated to the scale of redox potentials. The symbols used are: Y, oxidant of Photosystem II; Q, reductant of Photosystem II (quencher of chlorophyll fluorescence); PQ ,plastoquinone; 559, cytochrome 559; *f*, cytochrome *f*; PC, externally added plastocyanin; Asc, ascorbate; b_6, cytochrome b_6; P_{700}, oxidant of Photosystem I; X^-, reductant of Photosystem I; MV, methylviologen; Fd, ferredoxin; Fp, ferredoxin–$NADP^+$ reductase; DAD, diaminodurene.

The effect of plastocyanin on electron transport and phosphorylation in D-144 particles under aerobic conditions

Plastocyanin has been shown to serve as an electron carrier between Photosystem I and II[27]. Since D-144 particles are lacking Photosystem II and have been claimed to be deficient in plastocyanin[25], it was of interest to examine the effect of plastocyanin on the reactions of Photosystem I in D-144 particles. It can be seen from Table V that plastocyanin stimulated electron flow markedly only at low concentrations of diaminodurene or with ascorbate alone. In no case a stimulation of the rate of phosphorylation by plastocyanin was observed. Since the O_2 uptake in the presence of ascorbate and plastocyanin was not linked to phosphorylation, it appears that electrons from external plastocyanin enter Photosystem I *via* a pathway that by-passes the site of phosphorylation.

TABLE V

THE EFFECT OF PLASTOCYANIN ON THE REACTIONS OF PHOTOSYSTEM I IN D-144 PARTICLES IN THE PRESENCE OF DIAMINODURENE UNDER AEROBIC CONDITIONS

The incubation was performed as described for Table III. 2 nmoles plastocyanin were added where indicated. All the other concentrations were the same as given in the legend for Table III.

Additions	*Electron transport (μmoles O_2 reduced per mg chlorophyll per h)*	*Rate of phosphorylation (μmoles P_i esterified per mg chlorophyll per h)*
0.7 mM diaminodurene		
None	290	336
+ plastocyanin	345	345
+ ascorbate + methylviologen	4080	395
+ ascorbate + methylviologen + plastocyanin	4810	340
0.035 mM diaminodurene		
None	109	40
+ plastocyanin	145	35
+ ascorbate + methylviologen	340	38
+ ascorbate + methylviologen + plastocyanin	1180	33
Controls		
Ascorbate	20	0
Ascorbate + plastocyanin	310	0
Ascorbate + methylviologen	20	0
Ascorbate + methylviologen + plastocyanin	845	0

DISCUSSION

The redox state of cyclic mediators

The importance of the redox state of mediators for cyclic phosphorylation was pointed out by TREBST AND ECK[28] and confirmed by others[29,30]. It was shown[28] that reduction of menadione which was used as mediator markedly stimulated cyclic phosphorylation.

A particularly clear demonstration of the importance to maintain a proper balance of the oxidized and the reduced form of the mediator in cyclic electron flow has emerged from experiments with D-144 particles reported in this paper. Oxidized mediators, such as pyocyanine or PMS in red light, were virtually inactive and were reactivated by external reductants. In chloroplasts, Photosystem II could supply electrons for Photosystem I. This explains why the difference in the behavior of PMS and pyocyanine was not fully appreciated until subchloroplast particles were used[17] which were lacking electron flow from Photosystem II. Although such particles have been previously studied by other investigators, they have used PMS as mediator. Since PMS is non-enzymatically reduced in white light[23], an external reducing agent was not required. The requirement of reduction also explains older observations[23] on the inhibition of cyclic phosphorylation in chloroplasts by chlorophenyl-1,1-dimethylurea. This was confirmed in this paper with DCMU and it was shown that strong inhibition only occurs with pyocyanine or in red light with PMS. The inhibition was reversed by reducing agents such as ascorbate for PMS or borohydride for pyo-

cyanine. It is thus apparent that for the operation of cyclic phosphorylation, some electrons must be introduced either externally or from Photosystem II.

In the presence of a reduced mediator such as diaminodurene, addition of ascorbate resulted in a marked inhibition of phosphorylation, which was released by a suitable electron acceptor such as methylviologen. It was shown previously[31] and confirmed in this paper that DCMU stimulates cyclic phosphorylation with diaminodurene in chloroplasts. Thus, it is apparent that a continuous flow of reducing equivalents either from an external donor or from Photosystem II inhibits phosphorylation, presumably by impairing the cyclic flow due to a diminished concentration of the oxidized form of the mediator. This phenomenon of "overreduction" has been observed also in other photophosphorylating systems[32,33].

The question remains which of the steps in cyclic electron flow through Photosystem I becomes rate limiting when overreduction takes place. In the presence of ascorbate and diaminodurene the most logical step is between X^- and diaminodurene (Fig. 2) because insufficient amounts of oxidized diaminodurene are available. Introduction of a suitable electron acceptor for X^-, *e.g.* methylviologen, permits continuation of electron flow. Pyocyanine could also be used to release the inhibition by ascorbate. This experiment is meaningful since ascorbate does not reduce pyocyanine, and because oxidized pyocyanine does not catalyze photophosphorylation in D-144 particles. Considerations of the stoichiometry of ATP formation mentioned previously suggest that pyocyanine as well as methylviologen not only accept electrons from X^- but also act as mediators of cyclic electron flow. The fact that ascorbate inhibited cyclic phosphorylation with diaminodurene but not with PMS is likely due to a much higher affinity of the latter for X^-. The need for a comparatively higher concentration of diaminodurene for cyclic phosphorylation is in line with the postulated lower affinity of this mediator for X^-.

Electrons from Photosystem II also inhibit cyclic phosphorylation with diaminodurene. This is most likely due to an overreduction of P_{700}. Thus in this case the rate-limiting step would be between diaminodurene and P_{700} rather than between X^- and diaminodurene (see Fig. 2). Although overreduction by ascorbate and by Photosystem II results in a similar inhibition, the possible difference in the rate-limiting step may be useful for the design of future experiments. This difference in the rate-limiting step may also help to explain why in chloroplasts the inhibition of cyclic phosphorylation by ascorbate and by Photosystem II is additive (see Table II).

The location of the phosphorylation site of Photosystem I

There is considerable controversy with regard to the phosphorylation sites of Photosystem I and II. Evidence is accumulating that there are at least two different sites in operation. Recently[5], a distinction between the two sites was achieved by a differential inactivation of non-cyclic phosphorylation associated with $NADP^+$ reduction by treatment with heptane. Differences in susceptibility to uncouplers[3,4] also favor multiple phosphorylation sites.

An exact location of these sites has not been achieved. From cross-over point experiments[34] it was concluded that in non-cyclic phosphorylation involving Photosystem II the phosphorylation site is prior to the reduction of cytochrome *f*. It is widely assumed that this site is between plastoquinone and cytochrome *f*, a position quite analogous to the second phosphorylation site in oxidative phosphorylation.

However, recent experiments[35] with reductants of Photosystem II suggest the possibility of a phosphorylation site prior to the reduction of plastoquinone.

Equally controversial is the location of the phosphorylation site in Photosystem I. Based on experiments on phosphorylation accompanying the reduction of $NADP^+$ by Photosystem I with ascorbate *plus* DCIP as mediator, it has been proposed[12] that this electron transport is not directly linked to phosphorylation and that all the accompanying phosphorylation comes from the superposition of cyclic electron flow with a phosphorylation site between X^- and the mediator. Other investigators, however, have obtained evidence for phosphorylation during electron transport from ascorbate to $NADP^+$, but assumed that the phosphorylation site is the same which operates in electron flow from water[36].

It is apparent from the experiments reported in this paper that the phosphorylation site associated with Photosystem I must be in a carrier system that by-passes external plastocyanin. The simplest formulation places the phosphorylation site between the mediator and P_{700}. This localization explains why methylviologen, which under aerobic conditions causes a transition of cyclic to non-cyclic electron flow in Photosystem I, does not inhibit diaminodurene-linked phosphorylation (see Fig. 2). These experiments appear to rule out a phosphorylation site between X^- and the mediator since in this case methylviologen would be expected to inhibit phosphorylation by diverting electrons from the phosphorylation site. An alternative explanation for these findings rests on the assumption that cyclic phosphorylation operates at the phosphorylation site between plastoquinone and cytochrome *f* and that a second, non-phosphorylating system of electron flow operates with the mediation of plastocyanin (*cf.* ref. 35). This formulation could indeed explain most of the findings reported in this paper but is not favored because of the widely accepted notion that cytochrome *f* and plastocyanin operate in series rather than in parallel[37,38], and because it was shown that cytochrome *f*-deficient mutant of *Chlamydomonas reinhardii* catalyzed cyclic phosphorylation with PMS at rates as high as those observed in the wild type.

It is quite clear that in D-144 particles, externally added plastocyanin has no effect on photophosphorylation as was observed previously[25]. On the other hand, it was reported[37] that in a plastocyanin-deficient mutant of *C. reinhardii* cyclic phosphorylation with PMS was impaired. Recent studies with *Anabaena variabilis*[39], however, are in line with our conclusion that externally added plastocyanin is not involved in cyclic phosphorylation.

In Fig. 2 we have tentatively included cytochrome b_6 as a component of cyclic electron flow in Photosystem I. Several other investigators[40,41] have also placed it in a cyclic pathway, and indeed this cytochrome appears to be closely associated with Photosystem I (ref. 42). The potential difference of 0.4 V between cytochrome b_3 and P_{700} is sufficient to allow for ATP generation.

ACKNOWLEDGMENTS

This work was supported by Public Health Service Research Grant CA-03463-08 from the National Cancer Institute, and by Grant GM-14409-03 from the National Institutes of General Medical Sciences. R. E. McCarty is a Career Development

Awardee of the National Institutes of Health. G. A. Hauska is a recipient of a travel grant under the Fulbright-Hays Act.

NOTE ADDED IN PROOF (Received February 10th, 1970)

Since this paper was submitted we have succeeded in preparing an antibody in rabbits against plastocyanin. With the aid of this antibody, it was possible to demonstrate (a) that digitonin particles[14] contain considerable amounts of plastocyanin (contrary to the claim of ARNON *et al.*[25]) and (b) that this internal plastocyanin (in contrast to externally added plastocyanin) participates in cyclic phosphorylation. It therefore appears that in the scheme presented above, cyclic phosphorylation should include as a component of Photosystem I plastocyanin located at a site of the membrane inaccessible from the outside.

REFERENCES

1 D. I. ARNON, F. R. WHATLEY AND M. B. ALLEN, *Biochim. Biophys. Acta*, 32 (1959) 47.
2 D. I. ARNON, M. B. ALLEN AND F. R. WHATLEY, *Nature*, 174 (1954) 394.
3 M. AVRON AND N. SHAVIT, *Biochim. Biophys. Acta*, 109 (1965) 317.
4 Z. GROMET-ELHANAN AND M. AVRON, *Plant Physiol.*, 40 (1965) 1060.
5 C. C. BLACK, *Biochem. Biophys. Res. Commun.*, 28 (1967) 985.
6 H. T. WITT, A. MUELLER AND B. RUMBERG, *Nature*, 197 (1963) 987.
7 D. I. ARNON, H. Y. TSUJIMOTO AND B. D. SWAIN, *Nature*, 214 (1967) 562.
8 W. TANNER, E. LOOS AND O. KANDLER, in J. B. THOMAS AND J. C. GOEDHEER, *Currents in Photosynthesis*, Donker, Rotterdam, 1966, p. 243.
9 W. TANNER, M. LOFFLER AND O. KANDLER, *Plant Physiol.*, 44 (1969) 422.
10 L. P. VERNON AND W. S. ZAUGG, *J. Biol. Chem.*, 235 (1960) 2728.
11 A. TREBST, E. PISTORIUS AND E. ELSTNER, in J. B. THOMAS AND J. C. GOEDHEER, *Currents in Photosynthesis*, Donker, Rotterdam, 1966, p. 409.
12 Z. GROMET-ELHANAN, *Biochim. Biophys. Acta*, 131 (1967) 526.
13 R. E. MCCARTY AND E. RACKER, *J. Biol. Chem.*, 242 (1967) 3435.
14 J. M. ANDERSON AND N. K. BOARDMAN, *Biochim. Biophys. Acta*, 112 (1966) 403.
15 V. K. VAMBUTAS AND E. RACKER, *J. Biol. Chem.*, 240 (1965) 2660.
16 A. LIVNE AND E. RACKER, *J. Biol. Chem.*, 244 (1969) 1339.
17 M. M. ANDERSON AND R. E. MCCARTY, *Biochim. Biophys. Acta*, 189 (1969) 193.
18 R. F. CHEN, *J. Biol. Chem.*, 243 (1967) 173.
19 O. WARBURG AND W. CHRISTIAN, *Biochem. Z.*, 310 (1941) 384.
20 D. I. ARNON, *Plant Physiol.*, 24 (1949) 1.
21 L. P. VERNON, *Anal. Chem.*, 32 (1960) 1144.
22 A. T. JAGENDORF AND M. SMITH, *Plant Physiol.*, 37 (1962) 135.
23 A. T. JAGENDORF AND M. MARGULIES, *Arch. Biochem. Biophys.*, 90 (1960) 184.
24 J. S. C. WESSELS, in J. B. THOMAS AND J. C. GOEDHEER, *Currents in Photosynthesis*, Donker, Rotterdam, 1966, p. 129.
25 D. I. ARNON, H. Y. TSUJIMOTO, B. D. SWAIN AND R. K. CHAIN, *Comp. Biochem. Biophys. Photosynthesis*, University of Tokyo Press, Tokyo, 1967, p. 113.
26 S. IZAWA, T. N. CONOLLY, G. D. WINGET AND N. E. GOOD, *Brookhaven Symp. Biol.*, 19 (1966) 169.
27 D. C. FORK AND W. URBACH, *Proc. Natl. Acad. Sci. U.S.*, 53 (1965) 1307.
28 A. TREBST AND H. ECK, *Z. Naturforsch.*, 16b (1961) 455.
29 J. S. C. WESSELS, *Biochim. Biophys. Acta*, 65 (1962) 561.
30 Z. GROMET-ELHANAN AND M. AVRON, *Biochemistry*, 3 (1964) 365.
31 A. TREBST AND E. PISTORIUS, *Z. Naturforsch.*, 20b (1965) 143.
32 S. K. BOSE AND H. GEST, *Proc. Natl. Acad. Sci. U.S.*, 49 (1963) 337.
33 D. L. KEISTER, *J. Biol. Chem.*, 240 (1965) 2673.
34 M. AVRON AND B. CHANCE, *Brookhaven Symp. Biol.*, 19 (1966) 149.
35 H. BOEHME AND A. TREBST, *Biochim. Biophys. Acta*, 180 (1969) 137.
36 A. TREBST AND E. PISTORIUS, *Biochim. Biophys. Acta*, 131 (1967) 580.
37 D. S. GORMAN AND R. P. LEVINE, *Proc. Natl. Acad. Sci. U.S.*, 54 (1965) 1665.
38 G. HIND, *Biochim. Biophys. Acta*, 153 (1968) 235.
39 S. S. LEE, A. M. YOUNG AND D. W. KROGMANN, *Biochim. Biophys. Acta*, 180 (1969) 130.
40 G. HIND AND J. M. OLSON, *Brookhaven Symp. Biol.*, 19 (1966) 188.
41 T. HIYAMA, M. NISHIMURA AND B. CHANCE, *Plant Physiol.*, 44 (1969) 527.
42 N. K. BOARDMAN AND J. M. ANDERSON, *Biochim. Biophys. Acta*, 143 (1967) 187.