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Studies on the Energy-coupling Sites of Photophosphorylation

II. TREATMENT OF CHLOROPLASTS WITH NH_2OH PLUS ETHYLENEDIAMINETETRAACETATE TO INHIBIT WATER OXIDATION WHILE MAINTAINING ENERGY-COUPLING EFFICIENCIES[1]

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DONALD R. ORT AND SEIKICHI IZAWA
Department of Botany and Plant Pathology, Michigan State University, East Lansing, Michigan 48824

ABSTRACT

Artificial electron donors to photosystem II provide an important means for characterizing the newly discovered site of energy coupling near photosystem II. However, water oxidation must be completely abolished, without harming the phosphorylation mechanism, for these donor reactions and the associated phosphorylation to withstand rigorous quantitative analysis. In this paper we have demonstrated that treatment of chloroplasts with hydroxylamine plus EDTA at pH 7.5 in the presence of Mg^{2+} followed by washing to remove the amine is a highly reliable technique for this purpose. The decline of the Hill reaction and the coupled phosphorylation during the treatment were carefully followed. No change in the efficiency of phosphorylation (P/e_2 1.0–1.1) was observed until the reactions became immeasurable. Photosystem I-dependent reactions, such as the transfer of electrons from diaminodurene or reduced 2,6-dichlorophenolindophenol to methylviologen, and the associated phosphorylation were totally unaffected. It is clear that the hydroxylamine treatment is highly specific, with no adverse effect on the mechanism of phosphorylation itself. Benzidine photooxidation via both photosystems II and I in hydroxylamine-treated chloroplasts (electron acceptor, methylviologen; assayed as O_2 uptake) supports phosphorylation with the same efficiency as that observed for the normal Hill reaction (P/e_2 = 1.1). An apparent P/e_2 ratio of 0.6 was computed for the photooxidation of ascorbate.

The recent papers from this and other laboratories (15, 21, 25–28), which dealt with partial reactions of chloroplast electron transport, strongly indicated the existence of a site of energy coupling in the vicinity of photosystem II (most probably before plastoquinone), in addition to the well recognized site of phosphorylation between plastoquinone and cytochrome *f* (4, 5). Consequently, the question has arisen as to the exact location of this second site of phosphorylation. We must seriously consider here the possibility that an energy-conservation reaction is coupled to the process of water oxidation or to photoact II itself, since available thermodynamic data (10) seem somewhat unfavorable to the existence of an energy-conservation reaction between photosystem II and plastoquinone.

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As an approach to the problem of mapping the location of this site, investigations of the quantitative relationships between electron transport and phosphorylation supported by artificial donors to photosystem II become quite important. This approach calls for a specific and complete inhibition of water oxidation. Yamashita and Butler (31, 32), using their "tris-washed" chloroplasts, and Böhme and Trebst (6), using mildly heat-treated chloroplasts, have already shown that the donor reactions mediated by photosystem II can support phosphorylation with various efficiencies (P/e_2[2] ratios) depending upon the electron donor used. It is clear, however, that more extensive studies are required, if one is to draw decisive conclusions as to the site of phosphorylation, paying careful attention to possible adverse effects of these treatments or of the electron donors used or both on the machinery of phosphorylation itself. For instance, we have noticed that our chloroplasts are somewhat resistant to tris treatment, and our attempts to totally abolish water oxidation without appreciably impairing the phosphorylation mechanism have not been successful. Even greater difficulties in terms of the inhibition specificity were encountered with the heat treatment.

Hydroxylamine is a potent inhibitor of water oxidation. Cheniae and Martin (8) have shown that its effect on isolated chloroplasts is specific and irreversible, involving a release of Mn from the chloroplast membranes. No inhibition of photosystem I-mediated electron transport was found. The use of hydroxylamine as an electron transport inhibitor for photophosphorylation studies have been shunned because of the ambiguous results one would expect from its possible uncoupling effect as an amine or its ability to serve as an electron donor to photosystem II (22, 29). Wessels (30) did employ NH_2OH in his very early studies on photophosphorylation. The effect of hydroxylamine-O-sulfonate on photophosphorylation has recently been studied by Elstner *et al.* (11). We have examined the effect of hydroxylamine on chloroplast reactions under various conditions and found a simple method of treating chloroplasts with this amine which allows total inhibition of water oxidation without any detectable damage to the mechanism of phosphorylation. This paper describes details of the method and some preliminary results of photophosphorylation experiments with the hydroxylamine-treated chloroplasts.

MATERIALS AND METHODS

Chloroplast Isolation. Chloroplasts were isolated from commercial spinach (*Spinacia oleracea* L.). Leaves were washed

[2] Abbreviations: P/e_2: the ratio of the number of ATP molecules formed to the number of pairs of electrons transported; DAD: diaminodurene (2,3,5,6-tetramethyl-*p*-phenylenediamine); DCIP: 2,6-dichlorophenolindophenol; MV: methylviologen.

with cold-distilled water and ground in a Waring Blendor for 5 sec in a medium consisting of 0.3 M NaCl, 30 mM Tricine-NaOH buffer (pH 7.8), 3 mM $MgCl_2$, and 0.5 mM EDTA. The homogenate was filtered through eight layers of cheesecloth, and the chloroplasts were sedimented at 2500*g* for 2 min. The chloroplast pellet was then resuspended in a medium containing 0.2 M sucrose, 5 mM HEPES-NaOH buffer (pH 7.5), 2 mM $MgCl_2$, and 0.05% bovine serum albumin. After a 45-sec centrifugation at 2000*g* to remove cell debris, the chloroplasts were spun down again (2000*g* 4 min) and finally suspended in a few milliliters of the above suspending medium.

Chemicals. A stock solution of 0.1 M NH_2OH was made by dissolving the hydrochloride salt in 0.05 N HCl and stored at 0 C. Fresh solutions were prepared every 3 to 4 days. When necessary, the pH of the NH_2OH solution was adjusted to desired pH values immediately before use. D-Ascorbate solution (0.1 M; pH adjusted to 6.5 with NaOH) was stored at −20 C in small, tightly sealed vials. Diaminodurene dihydrochloride and benzidine dihydrochloride were recrystalized from charcoal-treated aqueous alcoholic and aqueous solutions, respectively, by adding excess HCl at 0 C. The completely colorless crystals thus obtained were stored at −20 C. Fresh aqueous solutions of these compounds were made up daily and kept at 0 C during the experiment.

Hydroxylamine Treatment. NH_2OH and EDTA (when used) were added to the suspending medium described above, pH adjusted with NaOH to 7.5, and used immediately. The treatment was done in the dark at either room temperature (21 C) or at 0 C as indicated. The Chl concentration during the NH_2OH treatment was approximately 100 μg/ml. Upon the completion of the prescribed treatment period, the chloroplasts were spun down (2000*g*, 4 min) and washed twice with suspending medium to remove the NH_2OH. The Chl concentrations of final stock suspensions were determined by the method of Arnon (2).

Electron Transport and Phosphorylation Assays. The ferricyanide Hill reaction was assayed as O_2 evolution, and the MV Hill reaction as O_2 uptake resulting from aerobic reoxidation of reduced MV. Electron transport from artificial donors to MV was assayed as O_2 uptake (20). A membrane-covered Clark-type oxygen electrode was used for these O_2 assays. When artificial donors were used, the observed rate of electron transport was corrected for the slow rate of dark autooxidation of the donors which ranged from 5 to 20% of the rate in the light. In no case was it necessary to add a H_2O_2 trap to the reaction mixture, since the chloroplast preparations used were free from catalase activity. The intensity of actinic light (600–700 nm) was approximately 600 kergs·sec^{-1} cm^{-2}. The reaction temperature was 19 C. Phosphorylation was measured as the residual radioactivity after the extraction of the ^{32}P-labeled orthophosphate as phosphomolybdic acid in butanol-toluene (3). Radioactivity was determined by Čerenkov radiation as described by Gould *et al.* (14).

RESULTS

Effect of NH_2OH Added in the Reaction Mixture of Phosphorylation. To test the potency of NH_2OH as an uncoupler, we have examined the effect of NH_2OH added in the reaction mixture on postillumination phosphorylation (X_E) (17) and on the steady state phosphorylation supported by the transfer of electrons from DAD to MV (Table I). In the X_E experiments, hydroxylamine was present only during the dark phosphorylation stage. The inhibition of X_E thus observed has been shown to be a sensitive indicator of uncoupling (16). The concentrations of NH_2OH used in these experiments are those commonly used for inhibition of O_2 evolution. Clearly, the uncoupler action of NH_2OH is weak, as one would predict from the low basicity of this amine (pK_a = 6) (13, 18). Weak as it is, this side effect of NH_2OH is definitely undesirable when a rather precise assessment of phosphorylation efficiency is required. As described below, the uncoupling effect of NH_2OH can be completely eliminated by washing the NH_2OH-treated chloroplasts, without relieving the desired inhibition of water oxidation.

Table I. *Effect of NH_2OH on Postillumination ATP Formation (X_E) and Steady State Photophosphorylation (Test for Uncoupler Action of NH_2OH)*

The X_E experiments were carried out as described before (19). NH_2OH was present only in the dark phosphorylation stage. The reaction mixture for the steady state photophosphorylation (2 ml) contained 0.1 M sucrose, 50 mM Tricine-NaOH buffer (pH 8.0), 2 mM $MgCl_2$, 0.75 mM ADP, 5 mM $Na_2H^{32}PO_4$, 0.5 mM DAD, 1 mM ascorbate, 50 μM MV, 1 μM DCMU, chloroplasts equivalent to 20 μg of Chl, and indicated concentrations for NH_2OH.

NH_2OH	Postillumination ATP Formation (X_E)	Steady State Phosphorylation (DAD → MV)		
		Electron transport	ATP	P/e_2
mM	*nmoles/100 µg Chl*	*µeq/hr·mg Chl*	*µmoles/hr·mg Chl*	*ratio*
0	8.3	2840	586	0.41
0.1	8.1			
2.0	6.0			
3.0		2660	442	0.35
5.0	4.0	2600	395	0.30

Pretreatment of Chloroplasts with NH_2OH and EDTA. In all of the following experiments, chloroplasts were treated with NH_2OH under a variety of conditions and then washed twice with a large volume of amine-free suspending medium (see "Materials and Methods") at 0 to 4 C. The data are for these washed chloroplasts.

Figure 1A shows that the NH_2OH inhibition of water oxidation proceeds much more slowly at 0 C than at 21 C. Cheniae and Martin (8) reported a Q_{10} of 2.43 for the development of NH_2OH inhibition. The effect of increasing concentrations of NH_2OH is shown in Figure 1B for both 0 C and 21 C treatments. Notably, in both figures, there are significant rates of electron transport and phosphorylation remaining (typically 40–60 μeq or 20–30 μmoles ATP hr^{-1}·mg Chl^{-1}) when NH_2OH alone is relied upon to abolish water oxidation. This residual electron flow and accompanying phosphorylation are obliterated when the treatment includes 1 mM EDTA. The above residual rates of electron transport (ferricyanide reduction) and phosphorylation are still in a ratio of approximately 2 (*i.e.* P/e_2 = 1). The rate of photosystem I-dependent electron transport from DAD to MV and the efficiency of the associated phosphorylation are totally unaffected (inset, Fig. 1B). These results clearly indicate that the weak uncoupling effect of NH_2OH is completely eliminated when NH_2OH-treated chloroplasts are washed as described.

Since the inclusion of EDTA seemed to be highly effective in achieving the complete inhibition of water oxidation, and the difference between 0 and 21 C treatments seems to be only in the rate of the development of inhibition, we have examined more closely the effect of pretreatment of chloroplasts with NH_2OH plus EDTA at 21 C (Fig. 2). The P/e_2 ratio of noncylic photophosphorylation (H_2O → MV) remains the same until the rates of electron transport and phosphorylation become immeasurable. The photosystem I-dependent electron

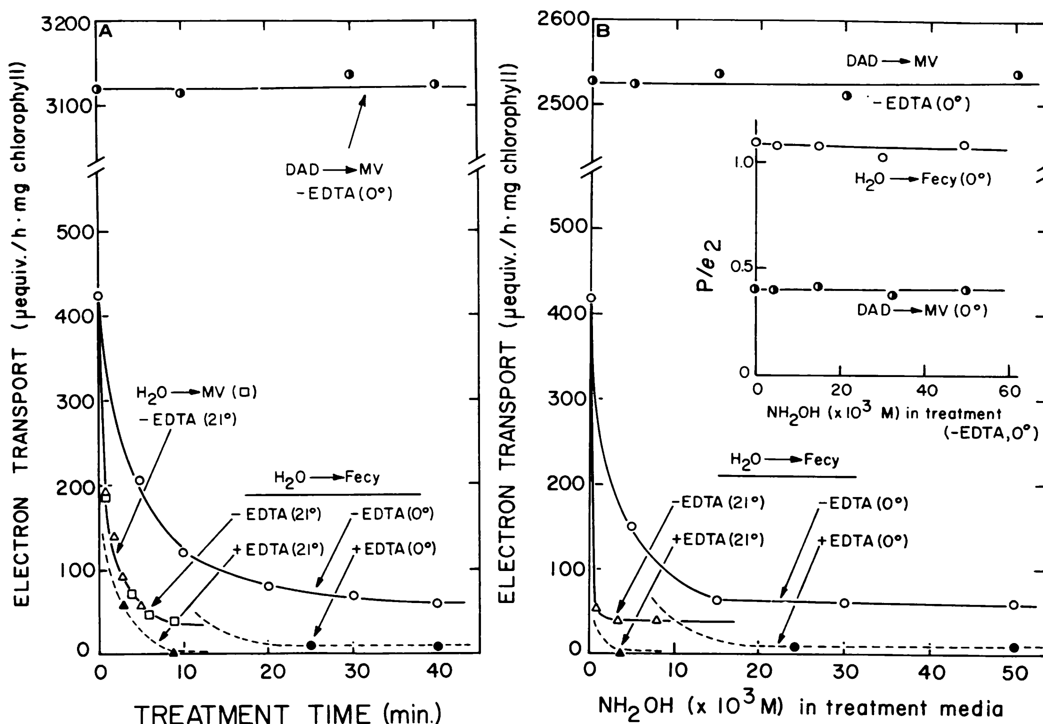

FIG. 1. A: Effect of time of hydroxylamine pretreatment of chloroplasts on the Hill reaction and photosystem I-dependent donor reactions. Chloroplasts were pretreated for the indicated periods of time with NH_2OH dissolved in the suspending medium (see "Materials and Methods"). The Chl concentration at this stage was approximately 100 μg/ml. The concentrations of NH_2OH were 5 mM for 0 C and 3 mM for 21 C treatment. EDTA was 1 mM when included. After the treatment, the chloroplasts were spun down, washed twice with amine-free suspending medium, and finally suspended in a few ml of the same medium. These procedures were carried out at 0 to 4 C. The basic ingredients of the reaction mixture (2 ml) were: 0.1 M sucrose, 50 mM Tricine-NaOH buffer (pH 8.0), 2 mM $MgCl_2$, 0.75 mM ADP, 5 mM $Na_2H^{32}PO_4$, and chloroplasts containing 40 μg of Chl (or 10 μg of Chl for the DAD → MV system). The concentrations of MV and Fecy (ferricyanide) were 50 μM and 0.4 mM, respectively. The DAD → MV reaction was run in the presence of 1 μM DCMU and 2.5 mM ascorbate. The concentration of DAD was 0.5 mM. For assay conditions, see "Materials and Methods." The temperatures indicated in the figure are for pretreatment periods. B: Effect of varied concentrations of hydroxylamine (in pretreatment medium) on the Hill reaction and photosystem I-mediated donor reactions. The treatment time was 9 min in both 0 and 21 C treatment. For other conditions, see A.

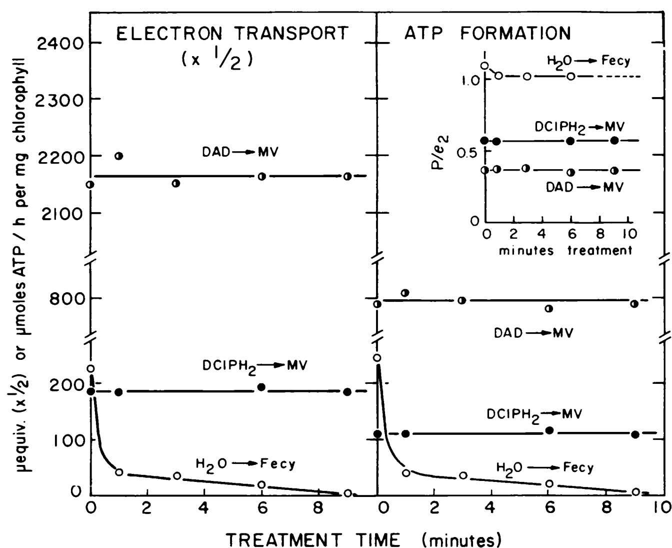

FIG. 2. Effect of time of pretreatment of chloroplasts (at 21 C) with hydroxylamine plus EDTA on the Hill reaction, photosystem I-mediated donor reactions, and associated phosphorylation. The reaction mixture for the $DCIPH_2$ → MV reaction contained 0.4 mM DCIP, 2.5 mM ascorbate, 50 μM MV, 1 μM DCMU. For other conditions see Fig. 1A.

transport (DAD → MV or $DCIPH_2$ → MV) and the coupled phosphorylation are totally unaffected. Therefore it seems quite safe to conclude that brief 21 C (room temperature) treatment of chloroplasts with NH_2OH plus EDTA at pH 7.5 in the presence of Mg^{2+} and subsequent washing at 0 to 4 C with NH_2OH-free media, provide a highly reliable method of abolishing the water-oxidizing ability of chloroplasts without impairing any other functions of chloroplasts including phosphorylation.

Photosystem II-mediated Donor Reactions in NH_2OH-treated Chloroplasts. Figure 3 shows the photooxidation of D-ascorbate and the associated phosphorylation in chloroplasts which have been pretreated with NH_2OH plus EDTA as described above. The electron acceptor used was methylviologen. The rate of electron transport was calculated according to the formula: ascorbate + O_2 → dehydroascorbate + H_2O_2 (for each pair of electrons transported). The stoichiometric disappearance of ascorbate was confirmed by titration with DCIP at acidic pH (Table II). The stoichiometric formation of dehydroascorbate has also been reported by Böhme and Trebst (6), who found a P/e_2 ratio of 0.5 for this donor reaction in heat-treated chloroplasts. Our data show a P/e_2 ratio of 0.6 at an optimal pH of 8.0 to 8.5. The remote possibility that these low P/e_2 ratios may be due to weak uncoupling by ascorbate has been ruled out by X_E experiments (Table III) which failed to detect any uncoupling action of ascorbate up to 30 mM.

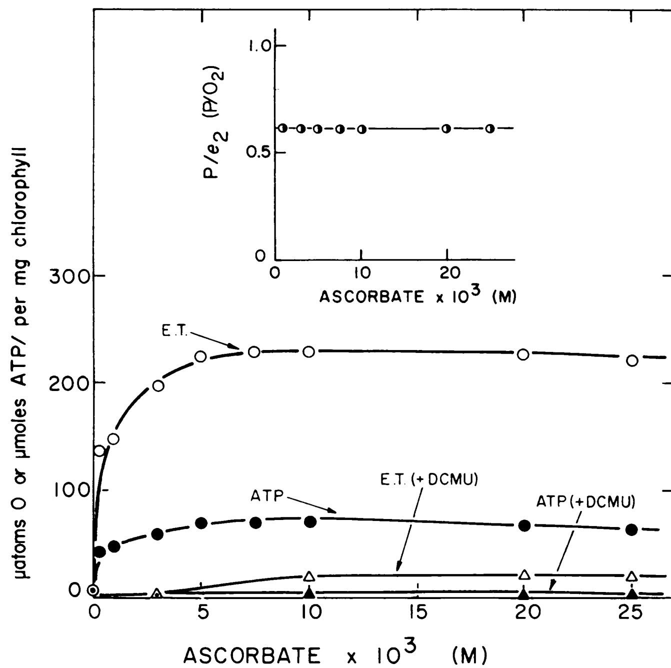

FIG. 3. Photooxidation of ascorbate with MV as the electron acceptor in chloroplasts pretreated with hydroxylamine plus EDTA. Chloroplasts were pretereated for 9 min at 21 C with 3 mM NH_2OH plus 1 mM EDTA as described in Fig. 1A. The basic ingredients of the reaction mixture are also given in Fig. 1A. The concentration of DCMU (when used) was 1 μM. 0.4 mM KCN was included so as to minimize the autoxidation of ascorbate. Note that the reactions contain practically no DCMU-insensitive components.

Table II. *Photooxidation of Ascorbate in NH_2OH-treated Chloroplasts as Assayed by O_2 Uptake and by Titration with DCIP*

Basic reaction conditions were as in Figure 3. The concentration of ascorbate used was 1.0 mM. After 3 to 4 min illumination, during which O_2 uptake was continuously monitored, the reaction mixture was acidified to pH 6 with MES buffer (containing DCMU to prevent further oxidation in room light), centrifuged, and the resultant supernatant was titrated quickly using a microburette with a standardized 1 mM DCIP solution. The DCIP solution, freshly prepared, was standardized spectrophotometrically at pH 8.0 using the molecular extinction coefficient (E_{600} = 21,800 $M^{-1}\cdot cm^{-1}$) of Armstrong (1), and also by titration with a fresh solution of ascorbate according to the stoichiometry-DCIP + ascorbate → $DCIPH_2$ + dehydroascorbate. Both methods gave the same value for the DCIP concentration within an error of 5%. A good agreement between the values obtained by O_2 assay and by titration indicates that under the conditions employed ascorbate was photooxidized only to the level of dehydroascorbate.

Experiment	Ascorbate Consumption	
	O_2 uptake	DCIP titration
No.	*neq/2 ml reaction mixture*	
1	235	251
2	235	229
3	223	285

Thus it seems that the apparent phosphorylation efficiency of ascorbate photooxidation via Photosystems II and I is close to half of the efficiency when water is photooxidized (see "Discussion"). Also worthy of note here is the fact that with these NH_2OH-treated chloroplasts the ascorbate oxidation contains practically no DCMU-resistant component (Fig. 3). A significant rate of DCMU-insensitive ascorbate photooxidation was observed for tris-washed chloroplasts (32), suggesting that ascorbate could be an electron donor for photosystem I, depending on the integrity of the chloroplast membranes (24).

Figure 4 shows that benzidine, unlike ascorbate, supports phosphorylation with a P/e_2 ratio of 1.1 which is almost the same as the value for normal noncyclic photophosphorylation involving water oxidation (P/e_2 = 1.0 to 1.2). In this experiment a low level of ascorbate (0.2 mM) was present in the reaction mixture to eliminate the possibility of a cyclic reaction with oxidized benzidine. Nearly identical data (not shown) were obtained without ascorbate, indicating that no significant cyclic electron flow occurred during the short illumination period employed (1–2 min).

Table III. *Effect of Ascorbate on Postillumination Phosphorylation (X_E)*

The procedure and conditions for the experiments were as described before (19). Ascorbate was present only in the dark phosphorylation stage. Note that ascorbate had no effect on the dark phosphorylation while the known uncoupler methylamine (also present at the dark stage only) greatly diminished the yield of ATP.

Addition at Dark Stage	ATP formed
mM	*nmoles/100 µg Chl*
None	6.8
Ascorbate (3)	8.0
Ascorbate (30)	7.5
Methylamine (5)	1.6

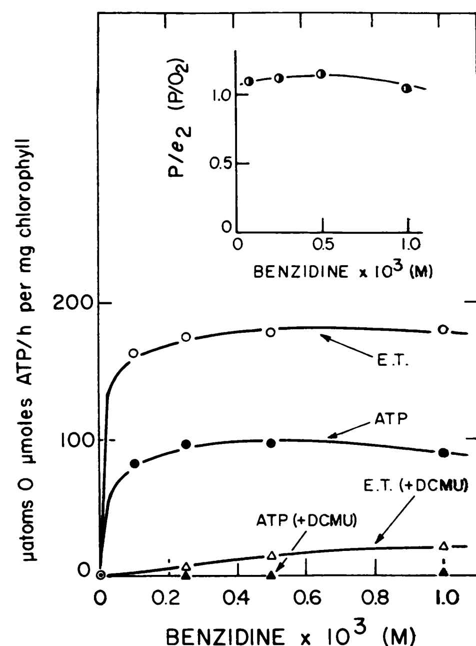

FIG. 4. Photooxidation of benzidine with MV as the electron acceptor in chloroplasts pretreated with hydroxylamine plus EDTA. Chloroplasts were pretreated for 9 min at 21 C with 3 mM NH_2OH plus 1 mM EDTA as described in Fig. 1A. The basic ingredients of the reaction mixture are also given in Fig. 1A. Ascorbate (0.2 mM) was included to keep benzidine in its reduced state, and 0.4 mM KCN to help prevent the autoxidation of ascorbate. Note that the phosphorylation efficiency of this reaction system is the same as that of normal noncyclic photophosphorylation (P/e_2 = 1.1).

DISCUSSION

Currently, the most widely employed procedures for inhibiting O_2 production are the tris treatment of Yamashita and Butler (31, 32) and the mild heating of chloroplasts (to 50 C for several minutes) (6, 24). However, both of these methods are not quite satisfactory in dealing with critical experiments on photophosphorylation, as briefly mentioned earlier in this paper. Well coupled chloroplasts are rather resistant to Cl^--removal treatment (7) which is also known to inhibit water oxidation (22). Repeated washings with Cl^- free media combined with room-temperature treatment did severely suppress the Hill reaction, but their secondary effects were quite appreciable.

In this paper, we have presented a highly effective procedure for abolishing the water-splitting reaction in isolated chloroplasts which can be extremely useful for photophosphorylation studies utilizing artificial electron donors for photosystem II. This procedure involves the treatment of chloroplast with NH_2OH plus EDTA in the presence of Mg^{2+}. No signs of uncoupling or inhibition of energy coupling were observed after treated chloroplasts were washed with NH_2OH-free medium. Actually, we found NH_2OH to be a rather poor uncoupler of photophosphorylation, and therefore it is possible that some uncritical phosphorylation experiments may be carried out in the presence of a few millimolar NH_2OH which is sufficient to suppress water oxidation.

The effectiveness of EDTA in facilitating the complete inhibition of water oxidation by NH_2OH suggests an interesting possibility concerning the mechanism of extraction of Mn from the lamellar membranes by NH_2OH. Since EDTA *per se* has no effect on water oxidation nor is able to release Mn from the membrane (9) (in the absence of Mg^{2+}, EDTA-uncoupling [23] gives very high rates of the Hill reaction), the observed effect of EDTA on water oxidation is probably indirect. It seems possible that a portion of the Mn extraction by NH_2OH is reversible, and the binding of released Mn^{2+} by EDTA does not allow the reversal to occur.

The apparent P/e_2 ratio of 0.6 found for the ascorbate photooxidation in NH_2OH-EDTA-treated chloroplasts is similar to the value of 0.5 Böhme and Trebst (6) found for heat-treated chloroplasts. They interpreted the data to suggest that the donation of electrons by ascorbate may have occurred after one of two sites of phosphorylation, the site which they suggest to be associated with the water-oxidation step. Although this is certainly the simplest and the most attractive interpretation, the validity of the widely used method for computing the electron flux in this donor reaction based on O_2 uptake data may be in error (12). A more comprehensive assessment of this complication is presented in a subsequent publication. In this respect the data for benzidine photooxidation may be of more importance. The constant P/e_2 of 1.0 to 1.1 observed over a wide range of benzidine concentrations confirms and greatly strengthens the brief data of Yamashita and Butler (31). These authors, using tris-washed chloroplasts, found a P/e_2 ratio of 0.97 with benzidine (33 μM) as electron donor and $NADP^+$ as acceptor. These P/e_2 ratios are indeed very close to that of the normal Hill reaction. It seems unlikely, therefore, that there is a phosphorylation site specifically associated with the mechanism of water oxidation. However, the possibility still remains that an energy conservation reaction is linked to some step of oxidoreduction reactions on the water-oxidizing side of photosystem II, a step which is involved both in water oxidation and the oxidation of artificial reductants. Research is now in progress testing various donor reactions in NH_2OH-treated chloroplasts in an attempt to locate more precisely the photosystem II-associated site of phosphorylation.

Note. After submission of this manuscript, a paper by J. F. Allen and D. O. Hall (Biochem. Biophys. Res. Commun. 52: 856–862) has appeared in which the authors demonstrated that the aerobic photooxidation of ascorbate by (untreated) chloroplasts involves a nonbiological oxidation of ascorbate by superoxide radicals. Therefore, it is almost certain that the assumption $2e^- = O_2$ used for computing electron flux and P/e_2 in Figure 3 is incorrect.

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LITERATURE CITED

1. Armstrong, J. 1964. The molecular extinction coefficient of 2,6-dichlorophenolindophenol. Biochim. Biophys. Acta 86: 194–197.
2. Arnon, D. I. 1949. Copper enzymes in isolated chloroplasts: polyphenoloxidase in *Beta vulgaris*. Plant Physiol. 24: 1–15.
3. Avron, M. 1960. Photophosphorylation by Swiss chard chloroplasts. Biochim. Biophys. Acta 40: 257–272.
4. Avron, M. and B. Chance. 1966. Relation of phosphorylation to electron transport in isolated chloroplasts. Brookhaven Symp. Biol. 19: 149–160.
5. Böhme, H. and W. A. Cramer. 1972. Localization of a site of energy coupling between plastoquinone and cytochrome *f* in the electron transport chain of spinach chloroplasts. Biochemistry 11: 1155–1160.
6. Böhme, H. and A. Trebst. 1969. On the properties of ascorbate photooxidation in isolated chloroplasts. Evidence for two ATP sites in noncyclic photophosphorylation. Biochim. Biophys. Acta 180: 137–148.
7. Bové, J. M., C. Bové, F. R. Whatley, and D. I. Arnon. 1963. Chloride requirement for oxygen evolution in photosynthesis. Z. Naturforsch. 18b: 683–688.
8. Cheniae, G. M. and I. F. Martin. 1971. Effects of hydroxylamine on photosystem II. I. Factors affecting the decay of O_2 evolution. Plant Physiol. 47: 568–575.
9. Cheniae, G. M. and I. F. Martin. 1966. Studies on the function of manganese in photosynthesis. Brookhaven Symp. Biol. 19: 406–417.
10. Cramer, W. A. and W. L. Butler. 1969. Potentiometric titration of the fluorescence yield of spinach chloroplasts. Biochim. Biophys. Acta 172: 503–510.
11. Elstner, E. F., A. Heupel, and S. Vaklinova. 1970. Über die Hemmung des photosynthetischen Electrononentransports in isolierten Chloroplasten durch Hydroxylamin. Z. Pflanzenphysiol. 62: 173–183.
12. Elstner, E. F., A. Heupel, and S. Vaklinova. 1970. Über die Oxidation von Hydroxylamin durch isolierte Chloroplasten und die mögliche Funktion einer Peroxidase aus Spinatblättern bei der Oxidation von Ascorbinsäure und Glykolsäure. Z. Pflanzenphysiol. 62: 184–200.
13. Good, N. E. 1960. Activation of the Hill reaction by amines. Biochim. Biophys. Acta 40: 502–517.
14. Gould, J. M., R. Cather, and G. D. Winget. 1972. Advantages of the use of Čerenkov counting for determination of P^{32} in photophosphorylation research. Anal. Biochem. 50: 540–548.
15. Gould, J. M. and S. Izawa. 1973. Photosystem II electron transport and phosphorylation with dibromothymoquinone as the electron acceptor. Eur. J. Biochem. 37: 185–192.
16. Hind, G. and A. T. Jagendorf. 1965. Effect of uncouplers on the conformational and high energy states in chloroplasts. J. Biol. Chem. 240: 3202–3209.
17. Hind, G. and A. T. Jagendorf. 1965. Separation of light and dark stages in photophosphorylation. Proc. Nat. Acad. Sci. U.S.A. 49: 715–722.
18. Hind, G. and C. P. Whittingham. 1963. Reduction of ferricyanide by chloroplasts in presence of nitrogenous bases. Biochim. Biophys. Acta 75: 194–201.
19. Izawa, S. 1970. The relation of post-illumination ATP formation capacity (X_E) to H^+ accumulation in chloroplasts. Biochim. Biophys. Acta 223: 165–173.
20. Izawa, S., T. N. Connolly, G. D. Winget, and N. E. Good. 1966. Inhibition and uncoupling of photophosphorylation in chloroplasts. Brookhaven Symp. Biol. 19: 169–187.
21. Izawa, S., J. M. Gould, D. R. Ort, P. Felker, and N. E. Good. 1973. Electron transport and photophosphorylation in chloroplasts as a function of the electron acceptor III. A dibromothymoquinone-insensitive phosphorylation reaction associated with Photosystem II. Biochim. Biophys. Acta 305: 119–128.
22. Izawa, S., R. L. Heath, and G. Hind. 1969. The role of chloride ion in photosynthesis III. The effect of artificial electron donors upon electron transport. Biochim. Biophys. Acta 180: 388–398.
23. Jagendorf, A. T. and M. Smith. 1962. Uncoupling phosphorylation in spinach chloroplasts by absence of cations. Plant Physiol. 37: 135–141.
24. Katoh, S. and A. San Pietro. 1967. Ascorbate supported NADP photo-

reduction by heated Euglena chloroplasts. Arch. Biochem. Biophys. 122: 144–152.

25. Kraayenhof, R., S. Izawa, and B. Chance. 1972. Use of uncoupling acridine dyes as stoichiometric energy probes in chloroplasts. Plant Physiol. 50: 713–718.
26. Ouitrakul, R. and S. Izawa. 1973. Electron transport and photophosphorylation in chloroplasts as a function of the electron acceptor II. Acceptor-specific inhibition by KCN. Biochim. Biophys. Acta 305: 105–118.
27. Saha, S., R. Ouitrakul, S. Izawa, and N. E. Good. 1971. Electron transport and photophosphorylation in chloroplasts as a function of the electron acceptor. J. Biol. Chem. 246: 3204–3209.
28. Trebst, A. and S. Reimer. 1973. Properties of photoreduction by photosystem II in isolated chloroplasts. An energy-conserving step in the photoreduction of benzoquinones by photosystem II in the presence of dibromothymoquinone. Biochim. Biophys. Acta. 305: 129–139.
29. Vaklinova, S., N. Tomova, E. Nikolova, and M. Sečenska. 1965. The effect of certain factors in the photooxidation of hydroxylamine in isolated chloroplasts. C. R. Acad. Bulg. Sci. 18: 659–662.
30. Wessels, J. S. C. 1958. Studies on photosynthetic phosphorylation II. Photosynthetic phosphorylation under aerobic conditions. Biochim. Biophys. Acta 29: 113–123.
31. Yamashita, T. and W. L. Butler. 1968. Inhibition of the Hill reaction by tris and restoration by electron donation to photosystem II. Plant Physiol. 44: 435–438.
32. Yamashita, T. and W. L. Butler. 1968. Photoreduction and photophosphorylation with tris-washed chloroplasts. Plant Physiol. 43: 1978–1986.