PHOTOSYNTHESIS BY ISOLATED CHLOROPLASTS

IV. GENERAL CONCEPT AND COMPARISON OF THREE PHOTOCHEMICAL REACTIONS

by

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In previous communications from this laboratory[1,2,3] the view has been put forward that the chloroplast is "a photosynthetic unit" *i.e.* a specialized cytoplasmic structure in which is localized the complete process of photosynthesis of green cells. This concept, although dominant in plant physiology at one time, was largely abandoned in the last twenty years (see review[2]). The substitute view which gained ascendancy on the strength of new experimental evidence regarded chloroplasts as structures "much simpler than required for photosynthesis"[4] and ascribed to them *in vivo* only the limited functions of photolysis of water and the transfer of electrons and protons from water to such acceptors as pyridine nucleotides[5]. Other reactions in photosynthesis were, in this view, linked to the reoxidation of the photochemically reduced pyridine nucleotides by enzyme systems situated outside the chloroplasts, as for instance in the generation, by oxidative phosphorylation, of adenosine triphosphate (ATP)** by mitochondria[6] and in CO_2 fixation by reductive carboxylations of the type exemplified by the malic enzyme reaction[5,7].

Chloroplasts were known to evolve oxygen when illuminated in the presence of an artificial electron acceptor (Hill reaction) in accordance with Equation 1,

$$\text{Light} + A + H_2O \longrightarrow H_2A + \tfrac{1}{2} O_2 \qquad (1)$$

in which A represents an electron or hydrogen acceptor other than CO_2. Reaction 1 has until recently represented the only known photochemical activity of isolated chloroplasts and has, for this reason, sometimes been called "the chloroplast reaction" (HILL[8]; WHITTINGHAM[9]). However, recent experiments in our laboratory have provided a new basis for the old concept of the localization of all photosynthetic reactions in the chloroplasts, namely, the discovery of two additional photochemical reactions of isolated chloroplasts: photosynthetic phosphorylation[1,10,11,12] (Equation

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** The following abbreviations will be used: AMP, adenosine 5-phosphate; ADP, ATP, adenosine di- and triphosphate; P_i, orthophosphate; FMN, flavin mononucleotide; Tris, tris (hydroxymethyl) aminomethane.

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2) and reduction of CO_2 to the level of carbohydrate with a simultaneous evolution of oxygen[1,13] (Equation 3):

$$\text{Light} + \text{ADP} + P_i \longrightarrow \text{ATP} \quad (2)$$

$$\text{Light} + CO_2 + H_2O \longrightarrow (CH_2O) + O_2 \quad (3)$$

This paper presents a general concept of photosynthesis by isolated chloroplasts based on a study of the interrelations of their three photochemical reactions. The discussion will be largely confined to evidence obtained from experiments with whole chloroplasts. The companion paper[14] reports on photosynthesis by broken chloroplasts. A historical survey of extracellular photosynthesis has been presented elsewhere[2] and an extensive discussion of the literature on phosphorus metabolism in photosynthesis is being reserved for a separate treatment[15].

METHODS

The general plan of the investigation was to use chloroplasts, prepared by a standard procedure in three parallel series of experiments in which photolysis (Hill reaction), photosynthetic phosphorylation, and CO_2 fixation were measured separately on portions of the same chloroplast preparation. Certain experiments were also carried out with chloroplasts prepared by a different technique, and with chloroplast fragments and other cytoplasmic particles.

Preparation of chloroplasts and other cytoplasmic particles

Standard procedure. Whole chloroplasts were isolated from spinach leaves (*Spinacea oleracea*, Var. Viroflay). Spinach plants were grown in a synthetic nutrient solution[16,17] and fully matured leaves were harvested a short time before each experiment. The leaves were washed with distilled water, shaken to remove excess liquid and placed in a plastic bag in the refrigerator to maintain their turgidity. The midribs were removed from the fully turgid leaves and the leaf blades were weighed just prior to grinding. The leaf blades were then quickly sliced into pieces about 0.5 cm^2 to facilitate grinding. In a number of experiments whole spinach plants were purchased from commercial sources; the mature undamaged leaves were cut off from the roots and handled as described above.

100–200 g of sliced leaf blades were ground in a large ice-cold mortar with 100–250 ml ice-cold 0.35 *M* NaCl and cold sand to assist in the grinding. The slurry was squeezed through a double layer of cheesecloth and the green juice centrifuged for 1 min at 0° C and 200 *g* to remove sand, leaf debris, and whole cells. The green supernatant liquid (leaf homogenate) was carefully decanted and centrifuged in the cold for 7 min at 1000 *g*. This sedimented the whole chloroplasts, leaving broken chloroplast fragments and smaller cellular particles in the supernatant liquid (S_1), which was decanted. The sedimented whole chloroplasts were washed by suspending them in *ca.* 100 ml ice-cold 0.35 *M* NaCl. A stirring rod with a piece of absorbent cotton at the end was used to resuspend gently the sedimented chloroplasts so as to give an even suspension. The suspension was centrifuged again for 7 min at 1000 *g* in the cold, and the pale supernatant liquid was discarded. The sedimented whole chloroplasts were finally suspended in *ca.* 25 ml 0.35 *M* NaCl. Aliquots of this suspension (P_1) were used in the experiments as a source of whole chloroplasts. It was found very important to maintain the solutions and apparatus ice-cold during all the manipulations. Warming of the suspensions to room temperature for even brief periods results in considerable loss of activity. 0.5 *M* glucose was used instead of 0.35 *M* NaCl in the preparations of the chloroplast suspensions used in some early experiments[10].

Chloroplast fragments together with other small cellular particles were isolated, when needed, by further centrifugation at 0° C of the supernatant liquid, S_1, which was usually discarded during the preparation of whole chloroplasts. S_1 was first centrifuged for 1 min at 18,000 *g* and the sediment, consisting chiefly of whole chloroplasts not previously removed, was discarded. The green supernatant fluid was centrifuged in a Spinco Model L centrifuge at 140,000 *g* for 10 min. The clear supernatant fluid was discarded and the residue (P_2), consisting of chloroplast fragments and other small particles including mitochondria, was resuspended in 0.35 *M* NaCl. The microscopic appearance of whole chloroplasts (P_1) and chloroplast fragments (P_2) is shown in Fig. 1.

Preparation of chloroplasts and other cytoplasmic particles in citrate-sorbitol-borate buffer

The isolated chloroplasts prepared by the previous procedure were found to have a capacity for

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photosynthetic phosphorylation but not for oxidative phosphorylation[2,3]. OHMURA[18] has recently described, however, chloroplasts prepared in a citrate sorbitol borate buffer which carry out oxidative phosphorylation. His solution was therefore used, as described below, in the preparation of chloroplasts and other cytoplasmic particles for a further comparison of oxidative and photosynthetic phosphorylation (Table IV).

70 g freshly harvested spinach leaves were cut into small pieces and macerated for 1 minute in a Waring blendor at 0° C with 100 ml of a solution containing 162 g sucrose, 33 ml 0.3 *M* ethylenediaminetetraacetate, pH 7.2, 8.8 g sodium citrate and 14.3 g sorbitol borate buffer (Atlas Powder Company) pH 7.0, made up to 1 liter with glass-distilled water. The green slurry was filtered through two layers of cheesecloth and centrifuged for 1 min at 200 *g* to remove whole cells and cell debris. The residue was discarded. The green supernatant fluid (F_1) was centrifuged for 7 min at 1000 *g*. The sedimented chloroplasts (whole chloroplasts) were divided into 2 portions. One was suspended in 0.35 *M* NaCl for measurements of photosynthetic phosphorylation and the other in the citrate-sorbitol-borate mixture for measurements of oxidative phosphorylation. The supernatant fluid (F_2) was used for the separation of a particulate fraction consisting of chloroplast fragments and other small particles. This fraction is designated in Table IV as "remaining particles". To obtain it, F_2 was first centrifuged for 1 min at 18,000 *g* to sediment the whole chloroplasts which were not removed by the 7 min centrifugation. These were discarded and the supernatant fluid (F_3) was centrifuged for 15 min at 18,000 *g* to sediment the remaining small particles. The "remaining particles" were divided into 2 batches. One was suspended in 0.35 *M* NaCl and the other in the citrate-sorbitol-borate mixture.

For comparison, all the particles (whole chloroplasts plus "remaining particles") in the F_1 homogenate were collected by centrifugation at 18,000 *g* for 15 min (Table IV). The sedimented "all particles" were again divided into two batches and suspended either in 0.35 *M* NaCl or in the citrate-sorbitol-borate solution.

The chlorophyll content of each particulate preparation was determined[17] and, unless otherwise noted, aliquots containing 0.5 mg chlorophyll (approx. 4 mg total protein) were used in each reaction vessel.

Measurement of photochemical reactions

Photolysis (the Hill reaction) was measured manometrically as oxygen evolution. Recrystallized *p*-benzoquinone was used as the hydrogen acceptor (Equation 1). Other conditions were as previously described[16] except that 0.2 ml of 0.2 *M* Tris**, pH 7.2 was substituted for the phosphate buffer and ice-cold 0.35 *M* NaCl was used to bring the volume of the reaction mixture to 3 ml.

Measurements of "organic phosphate" were used to follow the esterification of inorganic phosphate into ATP during photosynthetic phosphorylation[10]. (ATP was previously shown to be the "organic phosphate" product of the reaction[10].) The reaction was carried out in conical manometer vessels of about 18 ml capacity. To the main compartment of the ice-chilled vessels were added, in micromoles: Tris, pH 7.4, 40; a mixture of K and Na phosphate, (1:1), pH 7.2, 20; Na ascorbate, 10; neutralized AMP**, 20; $MgCl_2$, 10; FMN**, 0.1; and vitamin K_3 (2-methyl-1,4-naphthoquinone)[12] dissolved in 0.05 ml methanol, 0.03. In the sidearm was placed 5 μmoles of the Na-K phosphate buffer containing $5-10 \cdot 10^5$ c.p.m. of ^{32}P. An aliquot of P_1 or P_2 was then pipetted into the main compartment and the reaction mixture brought to a final volume of 3 ml by the addition of 0.35 *M* KCl. The chilled vessels were then attached to manometers, filled with nitrogen gas, and shaken at 15° C in a refrigerated bath, provided with a source of light[13]. The reaction was started by pouring the radioactive phosphate solution from the sidearm into the main compartment of the vessel and turning on the light. The reaction was terminated (usually after 1 hour) by turning off the light and adding 0.3 ml 20% trichloracetic acid to each vessel. The acidified reaction mixture was centrifuged and a 1.0 ml aliquot of the supernatant fluid was taken for analysis of "organic phosphate". "Organic phosphate" was determined as described previously[10]. Because of the high rates of photosynthetic phosphorylation the use of ^{32}P was not essential in recent experiments. The esterification of inorganic phosphate could be easily followed by the conventional molybdenum blue method of FISKE AND SUBARROW[19] with non-radioactive phosphorus.

CO_2 fixation was carried out at 15° C in conical manometer flasks flushed with nitrogen gas prior to turning on the light. In addition to chloroplasts the reaction mixture contained, in micromoles: Tris buffer, pH 7.4, 40; Na ascorbate, 10; $MnCl_2$, 2; $Na_2{}^{14}CO_3$ (containing *ca.* $1.5 \cdot 10^6$ c.p.m. of ^{14}C) 1.5; and 0.35 *M* NaCl to give a final volume of 3.0 ml. The reaction was stopped by pouring 0.3 ml of 0.5 *M* HCl or 20% trichloracetic acid from the sidearm into the main compartment of the vessel. Further details are given elsewhere[13].

Measurement of oxidative phosphorylation

The reaction mixture contained, in micromoles: Tris buffer, pH 7.4, 40; potassium sodium phosphate buffer, pH 7.2, 20; neutralized AMP, 2; $MgSO_4$, 10; plus 30 mg glucose and 75 units of hexokinase[20], in 1 ml total volume. To this was added the particulate suspension (Table IV)

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containing 0.5 mg chlorophyll in 2 ml of the citrate-sorbitol-borate mixture. The reaction was carried out in the dark at 15° in Warburg manometer vessels, with 10% KOH in the center well to absorb carbon dioxide. After equilibration with air, carrier free ^{32}P (650,000 c.p.m.) was added from the sidearm. Oxygen uptake was measured manometrically and esterification of inorganic phosphate was measured after stopping the reaction by addition of trichloracetic acid as described above for photosynthetic phosphorylation.

RESULTS

Whole chloroplasts as the site of three photochemical reactions

The preparations previously used in this laboratory[7,16] for the study of the photochemical reactions of isolated chloroplasts were not whole chloroplasts but chloroplast fragments. The chloroplast fragments were capable of carrying out the Hill reaction at a vigorous rate[16] and of being linked in a coupled reaction to other enzyme systems[5,7]. It became of interest therefore to determine the capacity of chloroplast fragments for the two new photochemical reactions found with whole chloroplasts: photosynthetic phosphorylation[1,10] and CO_2 fixation[1,13].

Fig. 1. Photomicrograph of whole spinach chloroplasts (P_1), below, and chloroplast fragments (P_2), above. Particles fixed with osmic acid and stained with safranin. The diameter of the whole chloroplasts is 3–5 microns.

The photomicrograph in Fig. 1 illustrates the different appearance of the whole chloroplasts and chloroplast fragments used in the present experiments. A comparison of their photochemical activities is shown in Fig. 2. Whole chloroplasts (P_1) were

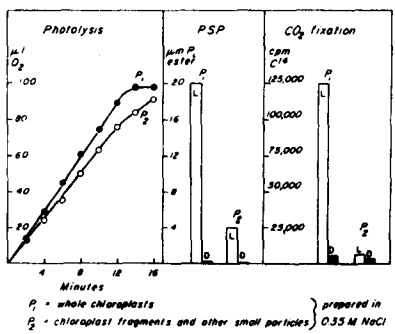

Fig. 2. A comparison of the capacity of whole chloroplasts (P_1) and chloroplast fragments (P_2) for photolysis (Hill reaction), photosynthetic phosphorylation (PSP), and CO_2 fixation. Experimental conditions are given in the text.

found capable of carrying out all three photochemical reactions but chloroplast fragments (P_2), which had virtually the same capacity for photolysis, showed only feeble phosphorylation and practically no CO_2 fixation. These results, which are representative of a number of similar experiments, suggested that while the capacity for photolysis is a prerequisite, it is not the sole condition for photosynthetic phosphorylation and CO_2 fixation by chloroplast preparations. Support for this conclusion was found in the inhibition experiments described below.

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The results shown in Fig. 2 corroborate those reported earlier[5,7] that chloroplast fragments were by themselves unable to fix CO_2. In these experiments the CO_2-fixing enzyme system originally present in whole chloroplasts[13] was lost and CO_2 fixation by chloroplast fragments occurred only on addition of the "malic enzyme" and TPN. However, the products of CO_2 fixation by whole chloroplasts[13] and the recent experiments with broken chloroplasts[14] leave no doubt that the malic enzyme cannot be equated with the complete enzyme system responsible for CO_2 fixation by whole chloroplasts. CO_2 fixation by chloroplast fragments fortified with the malic enzyme must therefore be regarded as an artificial model system.

Inhibition of the three photochemical reactions of chloroplasts by o-phenanthroline

In these, as in all subsequent experiments, whole chloroplasts (P_1) were used in the study of the photochemical reactions. The dependence of photosynthetic phosphorylation and CO_2 fixation on photolysis was demonstrated by the use of *o*-phenanthroline, a powerful inhibitor of photolysis[21,16]. As shown in Fig. 3, *o*-phenanthroline was also an inhibitor of photosynthetic phosphorylation and CO_2 fixation. These results are consistent with the view that photolysis of water is the basic reaction involved in the conversion of light into chemical energy. Light energy is consumed in the decomposition of water into a reduced and an oxidized product. In accordance with our general concept of photosynthesis by chloroplasts[2] (see also discussion below) the subsequent fate of the products of the photodecomposition of water determines whether the captured light energy will be stored in the pyrophosphate bonds of ATP or in the molecular configuration of starch, sugars and other products of CO_2 fixation.

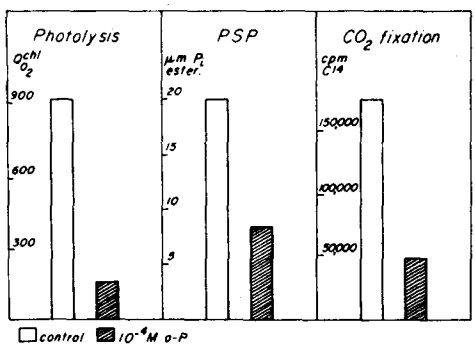

Fig. 3. Effect of *o*-phenanthroline on photolysis (Hill reaction), photosynthetic phosphorylation (PSP), and CO_2 fixation by whole chloroplasts. The inhibitor was added to the chloroplasts in the reaction mixture 15 minutes before turning on the light. Experimental conditions are given in the text.

Fig. 3 suggests that *o*-phenanthroline is a more powerful inhibitor of photolysis than of photosynthetic phosphorylation or CO_2 fixation. These results probably reflect the fact that in these experiments the capacity for photolysis was greater than that for the subsequent reactions of photosynthetic phosphorylation and CO_2 reduction. If with higher rates of photosynthetic phosphorylation and CO_2 reduction photolysis became the rate-limiting reaction, a similar degree of inhibition by *o*-phenanthroline of all three reactions would be expected.

Effect of sulfhydryl group inhibitors on the photochemical activity of chloroplasts

Iodoacetamide. This inhibitor was found to be without effect on the photolysis reaction. The effect of iodoacetamide on photosynthetic phosphorylation and CO_2 fixation is shown in Table I. CO_2 fixation was sharply inhibited by a concentration of $5 \cdot 10^{-3} M$ whereas photosynthetic phosphorylation was scarcely affected by a concentration of $10^{-2} M$.

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TABLE I

EFFECT OF IODOACETAMIDE (IAA) ON PHOTOSYNTHETIC PHOSPHORYLATION AND CO_2 FIXATION BY WHOLE CHLOROPLASTS

The inhibitor was added to the chloroplasts in the reaction mixture 30 min before turning on the light. Experimental conditions are given in the text.

IAA conc.	*P_i esterified µmoles*	*$^{14}CO_2$ fixed c.p.m.*
0	18.0	216,000
$1 \cdot 10^{-3} M$	18.1	179,000
$5 \cdot 10^{-3}$	—	45,000
$1 \cdot 10^{-2}$	16.2	—
$2 \cdot 10^{-2}$	—	5,800

TABLE II

EFFECT OF ARSENITE ON PHOTOLYSIS, PHOTOSYNTHETIC PHOSPHORYLATION AND CO_2 FIXATION BY WHOLE CHLOROPLASTS

Experimental conditions are given in the text.

Arsenite conc.	*Q_{O_2} µl/th/mg chl.*	*µmoles P_i esterified*	*$^{14}CO_2$ fixed c.p.m.*
0	1000	8.0	123,700
$2 \cdot 10^{-3} M$	1000	7.4	1,250
$1 \cdot 10^{-2} M$	560	2.9	1,200

Sodium arsenite. Arsenite, at a concentration of $2 \cdot 10^{-3} M$, had no effect on photolysis, inhibited photosynthetic phosphorylation slightly, but almost completely abolished CO_2 fixation (Table II). Inhibition of photolysis and photosynthetic phosphorylation was obtained only at a very high concentration of the inhibitor ($10^{-2} M$).

p-*Chloromercuribenzoate* (*CMB*). As shown in Fig. 4, $1.5 \cdot 10^{-4} M$ CMB completely inhibited CO_2 fixation and photosynthetic phosphorylation without affecting photolysis. These results are similar to those previously reported[1] in showing no effect of CMB at this concentration on photolysis while CO_2 fixation was abolished. The relative inhibition of photosynthetic phosphorylation was, however, greater in the current experiments, in which much higher rates of phosphorylation (14 μM P_i esterified per hour) were obtained anaerobically[11,12], than in the earlier experiments[1] on photosynthetic phosphorylation carried out under aerobic conditions (1.5 μM P_i esterified per hour). The residual phosphorylation (*ca.* 0.6 μM) in the inhibited system was appreciable in relation to the low total phosphorylation under aerobic conditions but was negligible in relation to the high phosphorylation under anaerobic conditions.

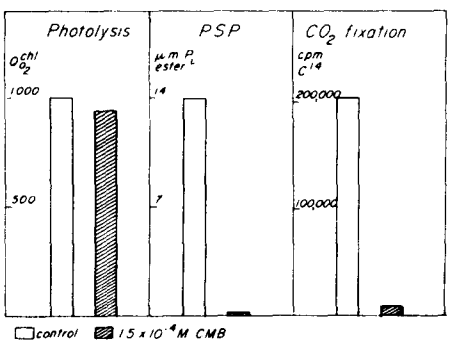

Fig. 4. Effect of *p*-chloromercuribenzoate (CMB) on photolysis, photosynthetic phosphorylation (PSP) and carbon dioxide fixation by whole chloroplasts. The inhibitor was added to the chloroplasts in the reaction mixture 30 minutes before turning on the light. Experimental conditions are given in the text.

Reversal of arsenite and CMB inhibition by glutathione

All three sulfhydryl group inhibitors sharply inhibited CO_2 fixation, whereas only CMB was inhibitory to photosynthetic phosphorylation. These results suggested a probable participation of one or more sulfhydryl compounds in the chain of enzymic reactions leading to CO_2 fixation and a possible participation in the chain of reactions concerned with photosynthetic phosphorylation. Further support for the idea that sulfhydryl compounds participate in the photochemical reactions of chloroplasts (exclusive of photolysis) is found in the substantial reversal of CMB and arsenite inhibition by glutathione (Table III).

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TABLE III

EFFECT OF GLUTATHIONE (GSH) IN REVERSING THE INHIBITION OF PHOTOSYNTHETIC PHOSPHORYLATION (PSP) AND CO_2 FIXATION BY SULFHYDRYL GROUP INHIBITORS

The inhibitors were incubated with the chloroplasts in reaction mixture 15–20 min before adding GSH. Experimental conditions are given in the text.

	PSP *μmoles P_i esterified*		*CO_2 fixation* *c.p.m. ^{14}C*	
	no GSH	*10^{-3} M GSH*	*no GSH*	*10^{-3} M GSH*
Control	12.6	13.0	352,000	344,000
$3 \cdot 10^{-4}$ M *p*-chloromercuribenzoate	2.0	12.8	—	—
$2 \cdot 10^{-4}$ M sodium arsenite	—	—	60,000	180,000

With the improved experimental conditions glutathione was, by itself, not inhibitory in the concentrations used in the current experiments. The inhibitory effect of cysteine, however, was found as in the earlier experiments[1].

Effect of other inhibitors

Dinitrophenol. The effects of 2–4 dinitrophenol ($8 \cdot 10^{-4}$ M) on the three photochemical reactions of chloroplasts is shown in Fig. 5. Dinitrophenol was a strong inhibitor of photosynthetic phosphorylation and CO_2 fixation but its effect on photolysis was less pronounced.

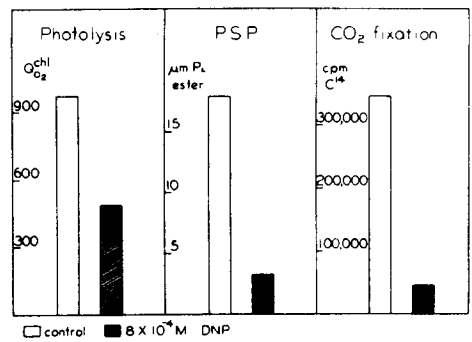

Fig. 5. Effect of 2,4-dinitrophenol on photolysis, photosynthetic phosphorylation (PSP), and carbon dioxide fixation by whole chloroplasts. The inhibitor was added to the chloroplasts in the reaction mixture 15 min before turning on the light. Experimental conditions are given in the text.

The inhibition of photosynthetic phosphorylation by dinitrophenol is of special interest in view of its well-known[22,13] uncoupling action in oxidative phosphorylation: it suppresses phosphorylation without inhibiting the hydrogen or electron transfer from substrate to molecular oxygen during respiration. It is possible that the inhibitory action of dinitrophenol in photosynthetic phosphorylation is also confined to the phosphorylating steps proper leading to ATP synthesis. This conclusion is supported by the rather mild effect of dinitrophenol (Fig. 5) on the Hill reaction, which is a measure of a hydrogen or electron transfer divorced from phosphorylation (no esterification of inorganic phosphate was obtained in the course of the Hill reaction). In photosynthetic phosphorylation, in contrast to oxidative phosphorylation, it is not experimentally possible to follow the effect of dinitrophenol by measuring oxygen uptake. During photosynthetic phosphorylation molecular oxygen is neither consumed nor produced[10].

In intact cells HOLZER[24] has shown that concentrations of dinitrophenol which do not inhibit oxygen consumption by *Chlorella pyrenoidosa* in the dark are sharply inhibitory to photosynthesis. A further discussion of these results will be given elsewhere[15].

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Methylene blue. A clear-cut distinction between photolysis and the other two photochemical reactions was provided by methylene blue, which is known to act as an uncoupling agent in oxidative phosphorylation[25]. $10^{-5}M$ methylene blue almost completely inhibited photosynthetic phosphorylation and CO_2 fixation but had no effect on photolysis (Fig. 6).

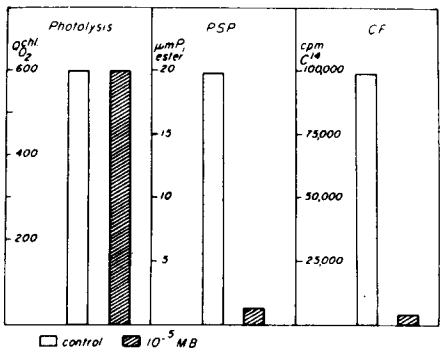

Fig. 6. Effect of $10^{-5}M$ methylene blue (M.B.) on photolysis, photosynthetic phosphorylation (PSP), and CO_2 fixation by whole chloroplasts. The inhibitor was added to the chloroplasts in reaction mixture 15 min before turning on the light. Experimental conditions are given in the text.

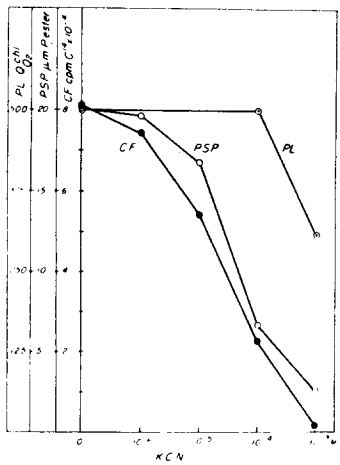

Fig. 7. Effect of KCN on photolysis (PL), photosynthetic phosphorylation (PSP), and carbon dioxide fixation (CF). PL is expressed as $Q_{O_2}{}^{Chl}$ (mm^3 O_2 evolved per hour per mg chlorophyll). Experimental conditions are given in the text.

Cyanide. The three photochemical reactions of chloroplasts showed a differential sensitivity to cyanide (Fig. 7). CO_2 fixation was sensitive to even very low concentrations of cyanide (10^{-6} to $10^{-5}M$), photosynthetic phosphorylation somewhat less so and an effect of cyanide on photolysis was obtained only at concentrations higher than $10^{-4}M$. Although there is doubt as to the suitability of cyanide as an inhibitor of photolysis when benzoquinone is the hydrogen acceptor[21], the sensitivity of the other two photochemical reactions, and particularly of CO_2 fixation, to this inhibitor appears to be unequivocal and of special interest in the light of the recent report of WARBURG and coworkers[26] of the extreme sensitivity to cyanide of that phase of photosynthesis in *Chlorella* cells which is concerned with CO_2 fixation.

Antimycin A and gramicidin. The effect of these two substances, known to inhibit oxidative phosphorylation[25,27-30], was tested on photosynthetic phosphorylation. Antimycin A (10 γ/3 ml), which was kindly supplied by Dr. P. A. ARK, was without effect on photosynthetic phosphorylation whereas 1 γ/3 ml was found to inhibit oxidative phosphorylation[25,30]. Gramicidin (40 γ/3 ml) was also without effect on photosynthetic phosphorylation (*cf.* LEHNINGER *et al.*[25]).

Oxidative phosphorylation by cytoplasmic particles from leaves

Photosynthetic phosphorylation provides a mechanism for generating ATP in light independently of respiration[2,3]. Since in strong light the rate of photosynthesis is many times greater than that of respiration it seems reasonable to expect that during periods of strong illumination the energy requirements of green cells could be fully

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met without oxidative phosphorylation. It is not suggested that photosynthetic phosphorylation would, by itself, render the oxidative reactions of respiration unnecessary during photosynthesis *in vivo*. The oxidative reactions of respiration not only liberate energy but are also necessary to supply essential intermediates in cellular metabolism (KREBS[31]). However, for the generation of ATP, photosynthetic phosphorylation must, in strong light, greatly overshadow the oxidative reactions of respiration.

Effective as photosynthetic phosphorylation may be as a mechanism for generating ATP in light, it obviously cannot function in the dark when an oxidative phosphorylation mechanism must take over. The question arises whether in green cells photosynthetic phosphorylation, which generates ATP anaerobically[11,12] in light, and oxidative phosphorylation, which generates ATP aerobically independently of illumination, occur at the same or at different sites. Several observations suggested that chloroplasts are adapted solely to photosynthetic phosphorylation. Photosynthetic phosphorylation by chloroplasts was found to be strictly light-dependent[1-3,10]. In the dark whole chloroplasts exhibited practically no endogenous oxygen uptake[32] nor was any oxygen absorption observed when they were incubated with 10 micromoles of each of the following substrates: acetate, pyruvate, malate, succinate, citrate, ascorbate, and malate plus pyruvate.. The addition of ATP and of pyridine nucleotides was also without effect.

This whole question was reexamined following a recent report by OHMURA[18] that chloroplast fragments prepared in a citrate-sorbitol-borate buffer had a capacity for oxidative phosphorylation. Chloroplasts were prepared in this buffer solution and their capacity for oxidative and photosynthetic phosphorylation compared. The results are summarized in Table IV.

TABLE IV

PHOTOSYNTHETIC AND OXIDATIVE PHOSPHORYLATION BY PARTICULATE FRACTIONS FROM LEAVES

	Photosynthetic phosphorylation (gas phase nitrogen)	*Oxidative phosphorylation (gas phase air)*	
	P_i esterified μmoles	*P_i esterified μmoles*	*O_2 absorbed μ atoms*
Whole chloroplasts	17.6	0.2	0.5
Remaining particles	3.2	6.2	3.0
All particles	11.6	1.2	1.0

Whole chloroplasts prepared in citrate buffer showed vigorous photosynthetic phosphorylation under anaerobic conditions but only negligible oxidative phosphorylation. An interesting reversal of the relative activities for the two types of phosphorylation was found in the particulate fraction identified as "remaining particles" (Table IV). This fraction represented the smaller particles left in suspension after the removal of whole chloroplasts (see METHODS) and consisted of chloroplast fragments and other cytoplasmic particles, presumably mitochondria. There are several independent lines of evidence pointing to the association of small non-chloroplast particles, corresponding to mitochondria, with the "chloroplast fragments" fraction when centrifugal separations similar to the ones described here are used[32,33].

The "remaining particles" had an appreciable capacity for oxidative phosphorylation but a feeble one for photosynthetic phosphorylation. When whole chloroplasts

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were combined with the "remaining particles" the activity of this mixed preparation ("all particles", Table IV) for the two types of phosphorylation was intermediate between whole chloroplasts and "remaining particles".

It seems reasonable to conclude that in the intact cell the capacity for photosynthetic phosphorylation resides in chloroplasts alone whereas the capacity for oxidative phosphorylation is limited to the smaller particles, presumably mitochondria, which in our preparative technique were associated with chloroplast fragments. The capacity of any particulate fraction from green leaves for the two types of phosphorylation would thus depend on the proportion of the active particles of each type present.

DISCUSSION

Our results suggest that chloroplasts are specialized cytoplasmic organs which contain multienzyme systems divided into three main groups, each controlling a distinct phase of photosynthesis: photolysis of water, photosynthetic phosphorylation, and CO_2 fixation. The evidence suggests an increasing order of complexity for the three photochemical reactions. Photolysis could be carried out by preparations incapable of photosynthetic phosphorylation and CO_2 fixation. In turn, photosynthetic phosphorylation was found to proceed unimpaired in preparations which could not fix carbon dioxide. CO_2 fixation, however, has been observed only in chloroplast preparations capable of active photolysis and phosphorylation.

This postulated increasing order of complexity for the three photochemical reactions is supported by their experimental separation through differential inhibition or variations in the technique for the preparation of chloroplasts. Chloroplast fragments had a capacity for photolysis almost equal to that of whole chloroplasts but lost the ability to accomplish photosynthetic phosphorylation and CO_2 fixation. In whole chloroplasts it was possible to inhibit a more complex phase of photosynthesis without affecting the simpler one which preceded it and, conversely, inhibition of a simpler phase of photosynthesis was invariably paralleled by an inhibition of the more complex phase which followed it. Thus iodoacetamide and arsenite inhibited CO_2 fixation but not photosynthetic phosphorylation or photolysis. Methylene blue inhibited both CO_2 fixation and photosynthetic phosphorylation but not the photolysis reaction. On the other hand, *o*-phenanthroline, which inhibited photolysis, also inhibited photosynthetic phosphorylation and CO_2 fixation.

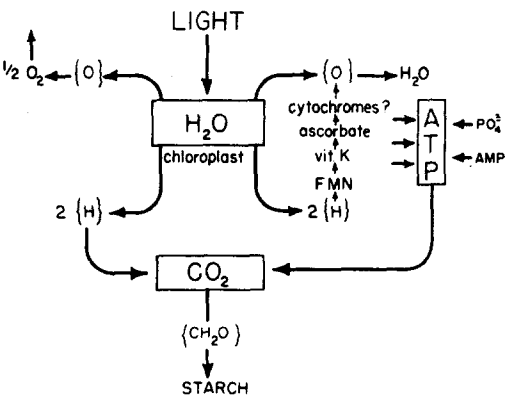

Fig. 8. Scheme for photosynthesis by isolated chloroplasts. Photolysis of water (center) leading either to ATP synthesis and the reconstitution of water (right) or to CO_2 reduction (below) linked with oxygen evolution (upper left).

The suggested interrelation of the three photochemical reactions of chloroplasts is shown in Fig. 8. Photolysis of water is identified with the primary conversion of

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light into chemical energy and is thus a prerequisite for both photosynthetic phosphorylation and CO_2 fixation. *In vivo*, photolysis of water is considered to be always linked either with phosphorylation, resulting in the production of ATP and the reconstitution of water, or with CO_2 fixation, resulting in the evolution of oxygen and the reduction of CO_2 to the level of carbohydrate. Only in CO_2 fixation is the oxygen of water released as a free gas, while the hydrogen becomes a part of the sugar molecule. In photosynthetic phosphorylation the photolytic cleavage of water is followed by its reconstitution before any evolution of oxygen takes place.

According to this scheme, CO_2 fixation or complete photosynthesis, requires the participation of all three groups of enzymes, phosphorylation requires two, whereas photolysis of water can proceed without the others *provided* an artificial hydrogen acceptor is supplied. The last process, the well-known Hill reaction (Equation 3), provides a convenient method for measuring the activity of the enzymes concerned in the photolysis of water, under non-physiological conditions when neither photosynthetic phosphorylation nor CO_2 fixation takes place.

It was suggested earlier[1] that a competitive relation exists between photosynthetic phosphorylation and CO_2 fixation. This may result from a competition between the two pathways for the reduced product of water photolysis, [H] in the proposed scheme (Fig. 8). Further support for this idea is found in the consistent inhibition of CO_2 fixation, not only by inorganic phosphate as reported earlier[1], but also by one of the cofactors of photosynthetic phosphorylation, vitamin K_3. It is conceivable that since normal cellular regulators may be absent from isolated chloroplasts, the addition, under our particular experimental conditions, of inorganic phosphate and vitamin K_3 diverts the flow of [H] away from the path of CO_2 fixation in favor of photosynthetic phosphorylation.

As shown in Fig. 8, it is envisaged that in photosynthetic phosphorylation the recombination of the products of photolysis of water proceeds in several successive steps, which together constitute an "electron ladder" analogous to that discussed for respiration by LIPMANN[34]. Of the catalysts of photosynthetic phosphorylation[11,12], Mg^{++} probably has a function in the transfer of phosphate, whereas FMN, vitamin K, and ascorbate could serve as electron carriers in the "electron ladder" shown in Fig. 8 (see discussion in reference [2]). The identity of the electron carriers above ascorbate is unknown, but they may very likely prove to be components of a cytochrome system[35–37]. The observed cyanide inhibition (Fig. 7) supports this possibility, but the lack of inhibition with antimycin A suggests again that some of the cofactors of photosynthetic phosphorylation, including components of the cytochrome system, may be different from those in oxidative phosphorylation[25,27–30].

The data presented in Table IV support the conclusion that the generation of ATP in green cells occurs at two distinct sites: in the mitochondria, adapted to phosphorylation of the oxidative type[38] needed to maintain cellular activity during periods of darkness, and in the chloroplasts, adapted to the direct conversion of light energy into pyrophosphate bond energy during photosynthesis.

Other lines of evidence also point to the special properties of photosynthetic phosphorylation which distinguish it from oxidative phosphorylation. Photosynthetic phosphorylation is an anaerobic process[11,12]; molecular oxygen is neither produced nor consumed. This was established by manometric measurements. The lack of any oxygen evolution was confirmed by the very sensitive luminous bacteria method for

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the detection of molecular oxygen[13]. Equation 2 for photosynthetic phosphorylation is visualized as the sum of Equations 4 and 5:

$$\text{Light} + H_2O \longrightarrow 2\,[H] + [O] \quad (4)$$

$$2\,[H] + [O] + ADP + P_i \longrightarrow H_2O + ATP \quad (5)$$

The oxygen and hydrogen in brackets represent an oxidized and a reduced product of photolysis of water (*not* molecular oxygen or hydrogen). In reaction 5 these photodecomposition products recombine with the aid of several catalysts as depicted in Fig. 8 to reconstitute water, and the energy liberated thereby is stored in the pyrophosphate bonds of ATP.

A similar conclusion about the special nature of photosynthetic phosphorylation as distinguished from oxidative phosphorylation has been reached by KANDLER[39] on the basis of experiments with whole *Chlorella* cells. A fuller discussion of this and other work[40] will be presented elsewhere[15].

The nature of the enzyme systems participating in the three phases of photosynthesis by isolated chloroplasts is currently under investigation. Some recent findings are discussed in the companion paper[14]. The results of the experiments with the sulfhydryl group inhibitors presented here suggest that sulfhydryl compounds, either enzymes or cofactors, participate in photosynthetic phosphorylation and especially in CO_2 fixation.

It has recently been proposed[41-44] that a sulfhydryl compound, 6-thioctic acid, is involved in the primary conversion of light into chemical energy. The lack of inhibition of the photolysis reaction in our experiments by iodoacetate, *p*-chloromercuribenzoate, and especially arsenite (which inhibited other reactions of chloroplasts) argues against this proposal. In photosynthesis by chloroplasts sulfhydryl compounds appear to be concerned not with the early but with the later phases of the photosynthetic process.

The attainment of complete extracellular photosynthesis by chloroplasts is considered significant because it offers special opportunities for the study of the photosynthetic process in a cell-free system without the attendant complications of extraneous metabolic reactions. Extracellular photosynthesis by chloroplasts proceeds in the absence of respiration, oxidative phosphorylation or other cellular processes requiring the consumption of molecular oxygen. It would be premature, however, to equate at this time photosynthesis by isolated chloroplasts with photosynthesis in intact cells. It seems best now to explore the characteristics of extracellular photosynthesis as a separate process and to test later their validity for photosynthesis in whole cells.

SUMMARY

1. Procedures are described for the preparation of chloroplasts capable of carrying out three photochemical reactions, each representing an increasingly complex phase of photosynthesis: photolysis of water (Hill reaction), esterification of inorganic phosphate into adenosine triphosphate (photosynthetic phosphorylation) and the reduction of carbon dioxide to the level of carbohydrates with a simultaneous evolution of oxygen.

2. The three photochemical reactions were separable by variations in the technique for preparation of chloroplasts and by differential inhibition by several reagents. Inhibition of a more complex phase of photosynthesis does not affect the simpler one which precedes it and, conversely, the inhibition of a simpler phase of photosynthesis is paralleled by an inhibition of the more complex phase which follows.

3. Reversible inhibition of CO_2 fixation and photosynthetic phosphorylation, but not of photolysis, by sulfhydryl group inhibitors suggests that sulfhydryl compounds (enzymes, cofactors,

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or both) are involved in phosphorylation and CO_2 fixation, but not in the primary conversion of light into chemical energy as measured by the Hill reaction.

4. Evidence is presented in support of the conclusion that the synthesis of ATP by green cells occurs at two distinct sites: anaerobically in chloroplasts, by photosynthetic phosphorylation, and aerobically in smaller cytoplasmic particles, presumably mitochondria, by oxidative phosphorylation independent of light.

5. A general scheme of photosynthesis by chloroplasts, consistent with these findings, is presented.

REFERENCES

1 D. I. ARNON, M. B. ALLEN AND F. R. WHATLEY, *Nature*, 174 (1954) 394.
2 D. I. ARNON, *Science*, 122 (1955) 9.
3 D. I. ARNON, M. B. ALLEN, F. R. WHATLEY, J. B. CAPINDALE AND L. L. ROSENBERG, *3rd Intern. Congr. Biochem. Brussels, 1955, Confér. et Rapports*, 227–232.
4 R. LUMRY, J. D. SPIKES AND H. EYRING, *Ann. Rev. Plant Physiol.*, 5 (1954) 271.
5 W. VISHNIAC AND S. OCHOA, *J. Biol. Chem.*, 195 (1952) 75.
6 W. VISHNIAC AND S. OCHOA, *J. Biol. Chem.*, 198 (1952) 501.
7 D. I. ARNON, *Nature*, 167 (1951) 1008;
D. I. ARNON AND G. HEIMBÜRGER, *Plant Physiol.*, 27 (1952) 828.
8 R. HILL, *Symposia Soc. Exptl. Biol.*, 5 (1951) 223.
9 C. P. WHITTINGHAM, *Biol. Revs.*, 30 (1955) 40.
10 D. I. ARNON, F. R. WHATLEY AND M. B. ALLEN, *J. Am. Chem. Soc.*, 76 (1954) 6324.
11 F. R. WHATLEY, M. B. ALLEN AND D. I. ARNON, *Biochim. Biophys. Acta*, 16 (1955) 605.
12 D. I. ARNON, F. R. WHATLEY AND M. B. ALLEN, *ibid.*, 16 (1955) 607.
13 M. B. ALLEN, D. I. ARNON, J. B. CAPINDALE, F. R. WHATLEY AND L. J. DURHAM, *J. Am. Chem. Soc.*, 77 (1955) 4149.
14 F. R. WHATLEY, M. B. ALLEN, L. L. ROSENBERG, J. B. CAPINDALE AND D. I. ARNON, *Biochim. Biophys. Acta*, 20 (1956) 463.
15 D. I. ARNON, *Ann. Rev. Plant Physiol.*, 7 (1956).
16 D. I. ARNON AND F. R. WHATLEY, *Arch. Biochem.*, 23 (1949) 141.
17 D. I. ARNON, *Plant Physiol.*, 24 (1949) 1.
18 T. OHMURA, *Arch. Biochem. Biophys.*, 57 (1955) 187.
19 C. H. FISKE AND Y. SUBARROW, *J. Biol. Chem.*, 66 (1925) 375.
20 M. KUNITZ AND M. R. MCDONALD, *J. Gen. Physiol.*, 29 (1946) 393.
21 O. WARBURG, *Heavy Metal Prosthetic Groups and Enzyme Action*, Clarendon Press, Oxford, 1949.
22 W. F. LOOMIS AND F. LIPMANN, *J. Biol. Chem.*, 173 (1948) 807.
23 R. CROSS, J. TAGGERT, G. CORO AND D. E. GREEN, *J. Biol. Chem.*, 177 (1949) 655.
24 H. HOLZER, *Z. Naturforsch.*, 6b (1951) 424; *Angew. Chem.*, 66 (1954) 65.
25 A. L. LEHNINGER, M. UL HASSAN AND H. C. SUDDUTH, *J. Biol. Chem.*, 210 (1954) 911.
26 O. WARBURG, G. KRIPPAHL AND W. SCHRODER, *Z. Naturforsch.*, 9b (1954) 667.
27 K. AHMAD, H. G. SCHNEIDER AND F. M. STRONG, *Arch. Biochem.*, 28 (1950) 281.
28 V. R. POTTER AND A. E. RIEF, *J. Biol. Chem.*, 194 (1952) 287.
29 B. CHANCE, *Nature*, 169 (1952) 215.
30 G. F. MALEY AND H. A. LARDY, *J. Biol. Chem.*, 210 (1954) 903.
31 H. A. KREBS, in *Chemical Pathways of Metabolism*, Vol. I., D. M. Greenberg (editor), Academic Press, New York, 1954.
32 D. I. ARNON AND F. R. WHATLEY, *Physiol. Plantarum*, 7 (1954) 602.
33 J. H. MCCLENDON, *Am. J. Botany*, 39 (1952) 275.
34 F. LIPMANN, in *Currents in Biochemical Research*, D. E. Green (editor), Interscience Publishers, New York, 1946, p. 145.
35 H. E. DAVENPORT AND R. HILL, *Proc. Roy. Soc. London*, B, 139 (1952) 137.
36 H. LUNDEGARDH, *Physiol. Plantarum*, 7 (1954) 375.
37 L. N. M. DUYSENS, *Nature*, 173 (1954) 692.
38 A. MILLERD, J. BONNER, B. AXELROD AND R. BANDURSKI, *Proc. Natl. Acad. Sci. U.S.*, 37 (1951) 855.
39 O. KANDLER, *Z. Naturforsch.*, 10b (1954) 38.
40 A. FRENKEL, *J. Am. Chem. Soc.*, 76 (1954) 5568.
41 M. CALVIN AND J. A. BARLTROP, *J. Am. Chem. Soc.*, 74 (1952) 6153.
42 M. CALVIN, *Chem. Eng. News*, 31 (1953) 1735.
43 D. F. BRADLEY AND M. CALVIN, *Arch. Biochem. Biophys.*, 53 (1954) 99.
44 M. CALVIN, *Federation Proc.*, 13 (1954) 697.

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