Oxidative Photosynthetic Phosphorylation by Spinach Chloroplasts*

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In an earlier note, evidence was presented for the occurrence of a new type of chloroplast-catalyzed photosynthetic phosphorylation called “oxidative photophosphorylation” (1). When spinach chloroplasts are illuminated in the presence of orthophosphate, adenosine diphosphate, and a dye such as trichlorophenol indophenol, adenosine triphosphate is generated provided oxygen is available, but not in the absence of oxygen. This report describes the characteristics of the system in detail.

EXPERIMENTAL

Chloroplasts were prepared from market spinach by the method of Jagendorf and Avron (2), washed once, and used immediately after preparation. Chloroplasts prepared in 0.35 M sodium chloride according to the procedure of Arnon (3) were practically identical with regard to the activities measured but appeared to be considerably less stable. Ferricyanide reduction was measured by the method of Krogmann and Jagendorf (4).

All phosphorylation reactions were conducted at 15°C in an illuminated bath with shaking. The light intensity at the surface of the reaction vessel was 2000 foot-candles. Light was provided from a bank of 300-watt incandescent photoflood bulbs located below the bath.

The 2,3′,6-trichlorophenol indophenol was obtained from Eastman Organic Chemicals. ADP was purchased from Pabst Laboratories and DPNH from the Sigma Scientific Company. The DCMU was a gift from Dr. N. Bishop. O-Phenanthroline and oleic acid were products of the Fisher Scientific Company.

Standard Assay System—For convenience in reporting the results, most of the data given in the present paper were obtained with a standard reaction system which included chloroplasts equivalent to 0.1 mg. of chlorophyll, 2 μmoles of orthophosphate, 2.5 μmoles of ADP, 10 μmoles of MgCl₂, 0.75 μmoles of 2,3′,6-trichlorophenol indophenol, and 50 μmoles of Tris buffer of pH 7.8 in a final volume of 1.5 ml. The reaction mixtures were incubated in 50-ml. Erlenmeyer flasks. When an atmosphere other than air was used, the flasks, during shaking, were flushed with gas for 10 minutes before illumination. The reactions were initiated and terminated by turning the lights on and off. After an illumination period of 10 minutes, trichloroacetic acid was added, the protein was removed by centrifugation, and the supernatant was analyzed for inorganic phosphate by the method of Fiske and SubbaRow (5). Chlorophyll was determined by the method of Arnon (6).

RESULTS

Requirements for Oxidative Phosphorylation by Illuminated Chloroplasts—As indicated in the previous report (1) oxygen, light, trichlorophenol indophenol, and ADP as well as inorganic phosphate are necessary components for oxidative phosphorylation by spinach chloroplasts. Table I illustrates the requirement for each of these components. It can be seen that an atmosphere of nitrogen or helium suppresses phosphate esterification, whereas a pure oxygen atmosphere gives the same result obtained when the reaction is run in air. The data also show that added Mg greatly stimulates the reaction.

A study was made of the effect of varying the concentration of each reactant independently, with the other reactants at optimal concentration. Fig. 1 illustrates the optimal chloroplast concentration, in terms of the chlorophyll content, for the reaction conditions employed. Fig. 2 shows the effect of added MgCl₂ on the rate of phosphate esterification. The effect of varying the concentration of Tris buffer at pH 7.8 is shown in Fig. 3.

Fig. 4 illustrates the effect of varying the ADP concentration. It is apparent that ADP becomes inhibitory at the higher concentrations tested. When 10 μmoles of ATP were added to the standard assay system, oxidative phosphorylation was inhibited by 30 per cent. The same amount of AMP had no effect. The inhibitory effects of ATP and ADP are reminiscent of the ATP inhibition of ferricyanide reduction by illuminated chloroplasts (7).

The effect of trichlorophenol indophenol concentration is seen in Fig. 5. The apparent inhibition at high dye concentration is in all likelihood due to excessive light absorption by the oxidized dye. Fig. 6 illustrates the effect of phosphate concentration on the rate of the phosphate esterification. In these experiments the amount of phosphate esterified was determined by using charcoal to absorb the ATP produced, according to the method of Crane and Lipmann (8). It is of interest to note that the reaction is saturated with phosphate at 3.3 × 10⁻² M. This is to be compared with the saturation at 10⁻² M phosphate of both the cyclic photosynthetic phosphorylation (2) and the stoichiometric photophosphorylation accompanying ferricyanide reduction (7).

The pH optimum for oxidative photophosphorylation, illustrated in Fig. 7, is very similar to those determined for cyclic photophosphorylation (9) and stoichiometric phosphorylation (7) by illuminated chloroplasts.
Role of Trichlorophenol Indophenol in Oxidative Photosynthetic Phosphorylation—Inasmuch as the work of Avron et al. (7) had indicated the possibility that ATP synthesis might occur during the reduction of trichlorophenol indophenol, experiments were devised to check this possibility. First, illuminated chloroplasts were allowed to reduce large quantities of the dye under a nitrogen atmosphere. The dye was added to the illuminated reaction mixture in 0.5-μmole aliquots, each succeeding aliquot being added after the preceding one had been reduced, so as to avoid excessive light absorption by the oxidized dye. In this way, up to 5 μmoles of dye were photoreduced in the standard assay system, with no detectable uptake of inorganic phosphate. These results together with the oxygen requirement of this system,

TABLE I
Requirements for oxidative phosphorylation by illuminated chloroplasts

<table>
<thead>
<tr>
<th>Reaction mixture</th>
<th>Atmosphere</th>
<th>Phosphate uptake (μmoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>Air</td>
<td>1.6</td>
</tr>
<tr>
<td>Complete</td>
<td>Nitrogen</td>
<td>0.0</td>
</tr>
<tr>
<td>Complete</td>
<td>Helium</td>
<td>0.0</td>
</tr>
<tr>
<td>Complete</td>
<td>Oxygen</td>
<td>1.6</td>
</tr>
<tr>
<td>Minus light</td>
<td>Air</td>
<td>0.0</td>
</tr>
<tr>
<td>Minus ADP</td>
<td>Air</td>
<td>0.0</td>
</tr>
<tr>
<td>Minus Mg</td>
<td>Air</td>
<td>0.2</td>
</tr>
<tr>
<td>Minus trichlorophenol indophenol</td>
<td>Air</td>
<td>0.0</td>
</tr>
<tr>
<td>Complete, boiled chloroplasts</td>
<td>Air</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Fig. 1. Effect of chloroplast concentration on oxidative photophosphorylation. The assays were performed as described under Experimental.

Fig. 2. Effect of MgCl₂ on oxidative photophosphorylation. The assays were performed as described under Experimental.

Fig. 3. Effect of buffer concentration on oxidative photophosphorylation. The assays were performed as described under Experimental.

Fig. 4. Effect of ADP concentration on oxidative photophosphorylation. The assays were performed as described under Experimental.

Fig. 5. Effect of trichlorophenol indophenol concentration on oxidative photophosphorylation. The assays were performed as described under Experimental.
indicated that the phosphorylation accompanied the oxidation of the dye rather than its reduction. This conclusion was supported by an independent line of evidence obtained through the use of the inhibitors DCMU and o-phenanthroline.

Avron and Jagendorf (10) had found that cyclic photosynthetic phosphorylation with N-methyl phenazonium methosulfate as catalytic cofactor could proceed unimpaired at concentrations of 3-chlorophenyl-1,1-dimethylurea or o-phenanthroline sufficient to suppress practically all trichlorophenol indophenol reduction by illuminated chloroplasts. This suggested the possibility that amounts of inhibitor sufficient to suppress photoreduction of the dye might still permit photophosphorylation provided the dye was reduced by an external reducing agent. This line of reasoning proved to be correct. Inhibition of the phosphorylation reaction by DCMU or by o-phenanthroline could be reversed by reducing the dye chemically with cysteine, glutathione, or ascorbic acid (1). It was not possible, however, to determine the stoichiometric relationship between the amount of reducing agent oxidized and the amount of phosphate esterified, because the chloroplast preparations caused a rapid photooxidation of these reducing agents, regardless of the presence or absence of the components of the phosphorylation system.

A search was made for a reagent which would reduce the dye but which was not rapidly photooxidized in the presence of illuminated chloroplasts. Jagendorf had reported the presence in chloroplast preparations of enzymes which transfer electrons from reduced pyridine nucleotides to indophenol (11). This observation was confirmed and exploited. Either DPNH or TPNH could be employed to reverse the inhibitory effects of DCMU or o-phenanthroline on the photophosphorylation reaction. An experiment illustrating the maximal rates of phosphorylation achieved when DCMU inhibition of dye reduction was counteracted by the addition of DPNH is shown in Fig. 8. The curves show the dual nature of the inhibition by DCMU. At lower concentrations of inhibitor the inhibition is completely reversible, but as the concentration of the inhibitor is increased, the effectiveness of the reducing agent diminishes until a concentration is reached at which the addition of dye-reducing substances will not restore phosphorylation. This is reminiscent of the results of Avron and Jagendorf (10) who found that higher concentrations of 3-chlorophenyl-1,1-dimethylurea were required to inhibit cyclic photophosphorylation than to suppress photoreduction. It is also apparent from Fig. 8 that 1 µmole of phosphate is esterified for every 0.5 µmole of DPNH added. Similar experiments with essentially identical results were performed in which o-phenanthroline at a concentration range between 10⁻⁵ m and 10⁻⁴ m was substituted for DCMU.

**Oxygen Requirement**—Manometric techniques were used for measurement of the oxygen consumption of the system. Inasmuch as the conventional conical Warburg flasks present a small surface area for illumination, it was necessary to prolong the time of incubation and increase the amount of reactants.

Table II gives the data from an experiment in which both oxygen uptake and phosphate esterification were determined for each reaction mixture. The effectiveness of DCMU as an inhibitor diminished if the DCMU was preincubated with the reaction mixture before illumination. To avoid this difficulty, the inhibitor was added from the side arm.

With the complete reaction mixture, 4 µmoles of ATP were synthesized and only slight gas uptake was observed. This probably reflects a Mehler reaction (12). The lack of significant net oxygen consumption was presumably due to the fact that an atom of oxygen was evolved per mole of dye reduced and then reabsorbed when the dye was reoxidized. When dye reduction was inhibited by o-phenanthroline or DCMU, no phosphate was

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**Fig. 6.** Effect of orthophosphate concentration on oxidative photophosphorylation. The assays were performed as described under Experimental.

**Fig. 7.** The effect of pH on oxidative photophosphorylation. The assays were performed as described under Experimental.

**Fig. 8.** Reversal of DCMU inhibition by DPNH. The assays were performed as described under Experimental. It was necessary to add the inhibitor and DPNH immediately before illumination.
esterified nor was any oxygen consumed. When DPNH was added with the inhibitor, however, 1 μm of oxygen was consumed and 2 μmoles of phosphate were esterified per μmole of DPNH added. If dye, phosphate, ADP, Mg, or light were omitted, oxygen consumption was unimpaired, although there was no phosphorylation.

The experiment shown in Table II was one of four similar experiments, all of which gave equivalent results. The P:O ratio calculated from these experiments was close to 2. It is believed that this represents the best result that could be achieved with the techniques employed. Attention is called, nevertheless, to the fact that the total amounts of oxygen consumed were too small for accurate measurement. It is also not excluded that conditions might be discovered in which the oxygen consumption was dependent on the components of the phosphorylation system. This possibility requires further exploration.

Effect of "Uncoupling" Treatment on Oxidative Photosynthetic Phosphorylation—in a previous report, a treatment was described which uncouples the esterification of inorganic phosphate from ferricyanide reduction by illuminated chloroplasts (13). This treatment consists in diluting the chloroplast suspension in 0.35 M NaCl of pH 6.2 to a final chlorophyll concentration of 0.01 mg. per ml., after which the chloroplasts are recovered by centrifugation and resuspended in the isolation medium. Chloroplasts thus treated are able to photoreduce ferricyanide in the absence of a phosphate acceptor system at a greatly increased rate, and their ability to synthesize ATP in the presence of an acceptor system is largely lost. The effect of this treatment on ferricyanide reduction and oxidative phosphorylation is indicated in Table III.

Inhibitors—Several reagents known to inhibit cyclic photophosphorylations and the stoichiometric phosphorylation accompanying ferricyanide reduction were examined in the oxidative photosynthetic phosphorylation test system. Table IV lists the inhibitory compounds tested and the concentrations required for 50 per cent inhibition. Ammonium chloride and arsenate inhibit oxidative photophosphorylation at approximately the same concentrations reported for the other types of photosynthetic phosphorylation (14). Similarly, dinitrophenol inhibition of oxidative photophosphorylation occurs at concentrations previously reported for cyclic and stoichiometric phosphorylation as well as for the Hill reaction (14). Oleic acid, also reported as a potent inhibitor of the Hill reaction (15) likewise inhibited oxidative photophosphorylation. Potassium cyanide at concentrations up to 10⁻⁴ M had no effect on this reaction.

Other Factors—To test for the participation of soluble components in the oxidative photophosphorylation reaction, unwashed chloroplasts were compared with chloroplasts that had been washed four times in the sucrose-Tris-NaCl medium routinely used in these experiments. No significant difference in activity was observed between the two preparations.

As an alternate electron donor, dichlorophenol indophenol was found to be 60 per cent as effective as trichlorophenol indophenol on a mole for mole basis. Methylene blue was completely inactive as a cofactor for this reaction.

The components of the reduced cytochrome c photooxidase described by Nieman and Vennesland had no effect on this reaction nor had they any oxidative photophosphorylation activity in themselves (16). Also, catalase or catalase plus ethanol had no effect on the oxidative photophosphorylation reaction.

**DISCUSSION**

Two types of photophosphorylation have been described previously (17). These may be differentiated for the purpose of this discussion as (a) "cyclic" photophosphorylation, and (b) photophosphorylation coupled to a Hill reaction. In both cases, the chemical events of the process have been pictured in terms of the initial generation of a reductant, XH₂, and an oxidant, YOH, formed as a result of the photolysis of water. The conversion of ADP and orthophosphate to ATP is then regarded as a consequence of an electron flow presumably occurring in the dark and associated with either (a) the reoxidation of XH by YOH, as mediated by a catalytic amount of a cofactor, or (b)
the reoxidation of $XH$ by a Hill oxidant, accompanied by the regeneration of $Y$ in a process whereby molecular oxygen is eliminated. Both processes can be visualized in terms of the diagram shown in Fig. 9.

There is a simple way in which oxygen might stimulate the photophosphorylation system diagrammed in Fig. 9. Suppose that a catalytic amount of riboflavin phosphate is used as a cofactor and that the slow, rate-limiting step in the cyclic photophosphorylation is the reoxidation of reduced riboflavin phosphate by $YOH$. It is conceivable that reduced riboflavin phosphate should first be formed by a Hill reaction coupled to a phosphorylation, and that the autoxidation of it by $O_2$ would occur while $YOH$ is converted to $O_2$. This would constitute an $O_2$-dependent photophosphorylation reaction which may be regarded as a hybrid of the two types of photophosphorylation just described. It is not necessary to modify the scheme shown in Fig. 9 to explain such an oxygen effect. The stimulatory effects of $O_2$ which Wessels (18) has described could possibly be attributed to this type of “mixed” reaction. Avron and Jagendorf (10) have presented this line of reasoning in a recent publication and give supporting evidence for it.

Since the photophosphorylation with trichlorophenol indophenol described in the present paper is dependent on oxygen, the question arises whether this dye system may not also be regarded as a “mixed” reaction in which there occurs a photoreduction of dye coupled to a phosphorylation, followed by oxidation of the dye by oxygen. The facts above, however, that this is definitely not the case. The photophosphorylation with the dye occurs when the reduced dye is oxidized and not during the photoreduction of the dye. The dye is readily photoreduced, but without any coupled phosphorylation; and an increase in the concentration of reduced dye does not make the system independent of oxygen.

Thus it is not possible to explain the oxidative photophosphorylation with trichlorophenol indophenol in terms of the simple scheme shown in Fig. 9. An addition or an essential modification must be made in this picture. It is therefore appropriate to classify “oxidative” photophosphorylation as a distinct reaction type, different from the two types of photophosphorylation previously described.

The problem now arises of how “oxidative” photophosphorylation may be explained. This problem has not been solved, in the sense that the facts do not permit a definitive choice among a number of possible explanations.

One group of possible explanations for “oxidative photophosphorylation” would invoke a different site of phosphorylation from that (or those) which operates with other cofactors or Hill reagents. Although this is a possibility, the facts which are pertinent suggest rather that the same site is operative in all the various types of photophosphorylation. Thus all the photophosphorylation reactions of spinach chloroplasts are sensitive to uncoupling by dilution of the chloroplasts at pH 6.2, all have about the same pH optimum, and all are similarly sensitive to arsenate, NH$_3$, dinitrophenol, o-phenanthroline and DCMU. One difference, which is not striking, is the lower optimal orthophosphate concentration required by the oxidative photophosphorylation system with indophenol. This may well mean, however, that a different step is rate-limiting, not that the phosphorylation reaction is fundamentally different from cyclic photophosphorylation.

The unique features of oxidative photophosphorylation should probably be sought not in a different kind of phosphorylation reaction, but in the different manner in which the indophenol interacts with the oxidation-reduction compounds present in the chloroplast. It was previously postulated that trichlorophenol indophenol might be reduced at a site on the chloroplast electron transport chain different from the site of ferriyanide and TPN reduction (7). This seems to be the case inasmuch as phosphorylation accompanies the reduction of ferriyanide and TPN but accompanies the oxidation of indophenol. In this connection it is also of interest, as pointed out by Witt et al. (19), that dichlorophenol indophenol and some related dyes cause a large increase in the rate of a spectrophotometrically measured dark reaction which follows illumination of grana. This behavior is not exhibited by Hill reagents such as quinone and ferriyanide. This special behavior of dichlorophenol indophenol may have a relationship to the special characteristics which it exhibits in the photophosphorylating system.

Perhaps the central question in connection with the mechanism of the “oxidative photophosphorylation” pertains to the identity of the energy source for the ATP formation. Under the conditions in which a P:O ratio of 2 was measured, there was ample energy released by the dark oxidation of the DPNH to form the high energy phosphate bonds. Nevertheless, the reaction gave no phosphorylation unless the system was illuminated, even though the light caused no increase in the rate of oxygen consumption. The relation is not known between the reactions induced by photon absorption and the reactions leading to oxygen consumption. Under these circumstances, it is well to reserve conclusions regarding the significance of the P:O ratio.

The role of light in photophosphorylation reactions has hitherto been mainly visualized as being limited to the provision of reducing and oxidizing power from the products of the photolysis of water. Wessels (18), however, has suggested the involvement of a light-requiring step distinct from photolysis. In the experiments described here, light does not necessarily supply the reducing power for phosphorylation, and if $O_2$ supplies the oxidizing power, there does not appear to be any need for the products of water photolysis. Perhaps the reduced dye serves as an electron donor for a phosphorylation reaction sequence activated by light with $O_2$ as a terminal electron acceptor. Such a scheme demands the action of light for more than the photolysis of water. Perhaps, on the other hand, the reduced dye serves as a trap for the photolytically formed oxidant and allows the photolytic redundant to reduce oxygen in a phosphorylating reaction sequence. This latter interpretation preserves the economy of one photochemical step.
It is not difficult to construct diagrams of the possible chemical reactions involved in such a way as to accommodate the experimental data and thereby "explain" oxidative photophosphorylation; but this cannot be done without making a number of *ad hoc* assumptions as well as changes in or additions to the diagram shown in Fig. 9. We prefer to leave the question of mechanism open at this time.

**SUMMARY**

A reaction has been described in which illuminated spinach chloroplasts catalyze the synthesis of adenosine triphosphate from adenosine diphosphate and inorganic phosphate in the presence of 2,3',6-trichlorophenol indophenol and oxygen. By the use of inhibitors to suppress selectively the reduction of the dye by the chloroplast, the stoichiometry of the phosphorylation reaction was ascertained. Per mole of reduced dye oxidized or per atom of oxygen consumed, 2 moles of adenosine triphosphate are synthesized. The reaction characteristics and inhibitor susceptibility indicate that the phosphorylation step is very similar to or identical with that step in the other chloroplast phosphorylation reactions.

**REFERENCES**
