Further Studies on Oxidative Photosynthetic Phosphorylation*

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(Received for publication, June 30, 1960)

In a previous report from this laboratory, the process of oxidative photosynthetic phosphorylation by spinach chloroplasts was described in detail (2). This phosphorylation, elicited by indophenol dyes, is unique in its absolute dependence on atmospheric oxygen. Here, further experiments are described which may better characterize this reaction.

EXPERIMENTAL PROCEDURE

The technique and reagents employed in these experiments, in general, were the same as previously reported (2). The chloroplast digitonin fragments were prepared by the method of Koukol et al. (3).

The carvacrol indophenol, 1-naphthol-2-sodium sulfonate indophenol, o-cresolindo-2, 6-dichlorophenol, and m-carboxyphenolindo-2,6-dibromophenol, all products of the British Drug Houses, Ltd., were a generous gift of Dr. R. Hill. The 2,6-dichlorophenol indophenol and 2,6-dibromophenol indophenol were purchased from Eastman Organic Chemicals and the phenol indophenol from the Allied Chemical and Dye Corporation. Crystal-line catalase and alcohol dehydrogenase were obtained from the Sigma Chemical Company. Crude D-amino acid oxidase was purchased from the Worthington Biochemical Corporation and used without further purification.

Catalase activity was measured by the procedure of Chan-trene as described by Lavorel (4) as well as by manometric methods. Catalase activity is expressed in the units described by Sumner and Somers (5). D-Amino acid oxidase activity was measured manometrically and by the spectrophotometric assay described by De Luca et al. (6). Acetaldehyde was measured enzymatically according to the procedure of Racker (7). Control experiments showed that good recovery of acetaldehyde was achieved only if the samples were kept below 20° at all times. The photosynthetic pyridine nucleotide reductase was a crude preparation obtained from an acetone powder of leaf extract prepared according to the procedure of San Pietro and Lang (8). This preparation also served as a source of spinach TPNH diaphorase, an enzyme described by Neish (11). Neish (11) had reported the association of catalase with the chloroplast fraction of a number of species. Jagendorf (12) found very little catalase in the chloroplast fraction of spinach when the fractionation was carried out in media of varying density. Since the preparation of chloroplasts in these experiments

The values for indophenol concentration obtained in this fashion were checked by ascorbic acid titration in those cases in which the dye was readily reduced by ascorbic acid.

RESULTS

Various Indophenol Dyes As Catalysts For Oxidative Photosynthetic Phosphorylation—In all the experiments reported to date, as in the bulk of those reported here, the dye 2,3',6-trichlorophenol indophenol has been used as the cofactor for oxidative photophosphorylation. When the dye was purified according to the procedure of Savage (10), essentially the same results were obtained as with the crude preparation. A variety of other indophenol dyes have been examined for activity in the oxygen-dependent photophosphorylation and the results of these experiments are reported in Table I. The concentration of the dye which gives a maximal rate of photophosphorylation is listed with the rate of phosphorylation achieved. Phosphate esterification with these dyes was, in every case, completely suppressed when the reaction was carried out in a nitrogen atmosphere. However, when the dye, phenol blue, which is structurally quite similar to the indophenols, was used, the concentration optimum and the rate of phosphorylation were identical to those values given for phenol indophenol, but this phosphate esterification was unaffected by incubation under a nitrogen atmosphere.

Light Saturation of Oxidative Photosynthetic Phosphorylation—The light intensity required for maximal photophosphorylation with trichlorophenol indophenol was determined and the resulting data are reported in Fig. 1. Variations in light intensity were achieved by the insertion of neutral density filters between the reaction vessels and the light source. The phosphate esterification reaction is nearly saturated with light at an intensity of approximately 1000 foot-candles.

Oxygen Saturation of Oxidative Photosynthetic Phosphorylation—Fig. 2 presents data from an experiment in which the oxygen saturation for this oxygen-requiring photophosphorylation was determined. The reaction is half-saturated at 1% oxygen in the gas phase.

Catalase and Hydrogen Peroxide in Oxidative Photosynthetic Phosphorylation—Inasmuch as several of the suggested mechanisms for oxygen participation in photophosphorylation require the participation of catalase, it seemed pertinent to measure the level of this enzyme's activity in the chloroplast preparations used. Neish (11) had reported the association of catalase with the chloroplast fraction of a number of species. Jagendorf (12) found very little catalase in the chloroplast fraction of spinach when the fractionation was carried out in media of varying density. Since the preparation of chloroplasts in these experiments

* A preliminary report of this work has been presented before the meeting of The Federation of American Societies for Experimental Biology in April 1960 (1). This investigation was aided by a grant from the National Science Foundation to B. Vennesland.

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The complete reaction mixture contained in μmoles: Tris buffer of pH 7.8, 50; orthophosphate, 1.5; ADP, 1.5; MgCl₂, 10; 2,3',6-trichlorophenol indo-2,6-dibromophenol, 0.5; Tris buffer at pH 7.8, 50; and chloroplasts equivalent to 0.1 mg of chlorophyll in a final volume of 1.5 ml. The oxygen concentrations were obtained by mixing liquid pumped nitrogen with mixtures of either 8 or 2% oxygen in nitrogen prepared by the National Cylinder Gas Company. The desired mixture was achieved by regulating the gas flow from each cylinder with Vickers cylinder gas flow regulator units. The reaction vessels were preflooded with the gas mixtures for 3 minutes in the dark with shacking and flushed continuously during the illumination. The phosphorylation at each concentration of oxygen was compared with an identical reaction mixture incubated in air. The values reported are the average of triplicate determinations which varied no more than 10% from one another.

Table II

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Catalase activity (units/mg chlorophyll)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole leaf homogenate</td>
<td>24.4, 26.7</td>
</tr>
<tr>
<td>Unwashed chloroplasts</td>
<td>6.8, 7.2, 6.4</td>
</tr>
<tr>
<td>Chloroplasts washed once</td>
<td>6.8, 6.1, 6.7</td>
</tr>
<tr>
<td>Chloroplasts washed 5 times</td>
<td>2.8, 3.1, 2.5</td>
</tr>
<tr>
<td>Chloroplast digitonin fragments</td>
<td>1.0, 1.2</td>
</tr>
</tbody>
</table>

Fig. 1. Effect of light intensity on oxidative photophosphorylation. The reaction mixture contained in μmoles: orthophosphate, 1.5; ADP, 1.5; MgCl₂, 10; 2,3',6-trichlorophenol indo-2,6-dibromophenol, 0.5; Tris buffer at pH 7.8, 50; and chloroplasts equivalent to 0.1 mg of chlorophyll in a final volume of 1.5 ml. The light intensity was varied by inserting neutral density filters beneath the reaction vessels. The light intensity at the flask level was measured with a General Electric light meter.

Fig. 2. Effect of oxygen concentration on oxidative photophosphorylation. The reaction mixture contained in μmoles: orthophosphate, 1.5; ADP, 1.5; MgCl₂, 10; 2,3',6-trichlorophenol indo-2,6-dibromophenol, 0.5; Tris buffer at pH 7.8, 50; and chloroplasts equivalent to 0.1 mg of chlorophyll in a final volume of 1.5 ml. The oxygen concentrations were obtained by mixing liquid pumped nitrogen with mixtures of either 8 or 2% oxygen in nitrogen prepared by the National Cylinder Gas Company. The desired mixture was achieved by regulating the gas flow from each cylinder with Victor meter gas flow regulator units. The reaction vessels were preflooded with the gas mixtures for 3 minutes in the dark with shaking and flushed continuously during the illumination. The phosphorylation at each concentration of oxygen was compared with an identical reaction mixture incubated in air. The values reported are the average of triplicate determinations which varied no more than 10% from one another.

Table I

Various indophenol dyes as cofactors for oxidative photosynthetic phosphorylation

<table>
<thead>
<tr>
<th>Dye</th>
<th>Optimal concentration 10⁻⁴ M</th>
<th>Rate of phosphorylation μmole/hr/mg chlorophyll</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,3',6-Trichlorophenol indophenol</td>
<td>2</td>
<td>60</td>
</tr>
<tr>
<td>2,6-Dichlorophenol indophenol</td>
<td>5</td>
<td>86</td>
</tr>
<tr>
<td>2,6-Dibromophenol indophenol</td>
<td>6.6</td>
<td>137</td>
</tr>
<tr>
<td>m-Carboxyxylenol indophenol</td>
<td>2.6</td>
<td>110</td>
</tr>
<tr>
<td>e-Cresol indophenol</td>
<td>6.3</td>
<td>110</td>
</tr>
<tr>
<td>1-Naphthol-2-sodium sulfonate indophenol</td>
<td>2.3</td>
<td>120</td>
</tr>
<tr>
<td>Guaiacol indophenol</td>
<td>3.2</td>
<td>115</td>
</tr>
<tr>
<td>Carvacrol indophenol</td>
<td>3</td>
<td>6</td>
</tr>
</tbody>
</table>

Should oxygen be reduced to hydrogen peroxide either by the illuminated chloroplasts or by photoreduced indophenol and should this product be dismutated by the endogenous catalase to oxygen and water, the significance of oxygen consumption and production described in an earlier report (14) would be achieved. To test the possibility that oxygen participation in indophenol-catalyzed photophosphorylation proceeds via the Mehler reaction, the catalase-ethanol system for trapping hydrogen peroxide was used. In this system, catalase acts peroxidatically with use of hydrogen peroxide to oxidize ethanol to acetaldehyde. This reaction was first described by Keilin and Hartree (15) and was used by Mehler in his elucidation of oxygen reduction by illuminated chloroplasts (13). The acetaldehyde produced was measured in our experiments with DPNH and alcohol dehydrogenase.

The presence of catalase in the chloroplast preparations forces a consideration of the possible participation of this enzyme in the over-all reactions of oxidative photosynthetic phosphorylation. Mehler described an ability of chloroplasts to reduce atmospheric oxygen to hydrogen peroxide (15), and the reaction sequence, as usually experienced with unsupplemented chloroplasts, is described by the following equations:

\[ 2 \text{H}_2\text{O} \xrightarrow{\text{light}} 2\text{H} + 2\text{OH} \]

\[ 2\text{OH} \rightarrow \text{H}_2\text{O} + \frac{1}{2}\text{O}_2 \]

\[ 2\text{H} + \text{O}_2 \rightarrow \text{H}_2\text{O}_2 \]

\[ \text{H}_2\text{O}_2 \xrightarrow{\text{catalase}} \text{H}_2\text{O} + \frac{1}{2}\text{O}_2 \]

Should oxygen be reduced to hydrogen peroxide either by the illuminated chloroplasts or by photoreduced indophenol and should this product be dismutated by the endogenous catalase to oxygen and water, the significance of oxygen consumption and production described in an earlier report (14) would be achieved. To test the possibility that oxygen participation in indophenol-catalyzed photophosphorylation proceeds via the Mehler reaction, the catalase-ethanol system for trapping hydrogen peroxide was used. In this system, catalase acts peroxidatically with use of hydrogen peroxide to oxidize ethanol to acetaldehyde. This reaction was first described by Keilin and Hartree (15) and was used by Mehler in his elucidation of oxygen reduction by illuminated chloroplasts (13). The acetaldehyde produced was measured in our experiments with DPNH and alcohol dehydrogenase.
First, it seemed imperative to test the efficiency of the catalase-ethanol trap for hydrogen peroxide under the circumstances of oxidative photophosphorylation. To do this, D-amino acid oxidase, instead of illuminated chloroplasts, was used as a hydrogen peroxide generator. The over-all reaction sequence is outlined by the following equations:

\[ \text{D-alanine} + \text{O}_2 \xrightarrow{\text{D-amino acid oxidase}} \text{pyruvate} + \text{H}_2\text{O}_2 + \text{NH}_3 \]

\[ \text{H}_2\text{O}_2 + \text{ethanol} \xrightarrow{\text{catalase}} \text{acetaldehyde} + \text{H}_2\text{O} \]

\[ \text{acetaldehyde} + \text{DPNH} \xrightarrow{\text{alcohol dehydrogenase}} \text{ethanol} + \text{DPN} \]

\[ \text{pyruvate} + \text{DPNH} \xrightarrow{\text{lactic dehydrogenase}} \text{lactate} + \text{DPN} \]

This enzymatic generation of hydrogen peroxide affords the advantage of a readily measured product other than hydrogen peroxide against which the efficiency of the catalase-ethanol trapping system may be checked. The products of this enzymatic oxidation of D-alanine are hydrogen peroxide and pyruvate, and the pyruvate is readily measured by the DPNH-lactic dehydrogenase reaction. The pyruvate produced by the D-amino acid oxidase reaction should be equivalent to the hydrogen peroxide formed, regardless of the fate of that hydrogen peroxide. Data from such a control experiment are presented in Table III. The values for hydrogen peroxide trapped by the catalase-ethanol system, as measured either by oxygen consumption or acetaldehyde produced, agree rather well with the value for pyruvate recovered. In the absence of ethanol, the net oxygen consumption is decreased by half, as expected from the catalatic destruction of hydrogen peroxide. The lower pyruvate recovery seen in the final line of Table III might result from the nonenzymatic

### Table III

**Recovery of hydrogen peroxide**

These assays were performed with conventional Warburg apparatus. The reaction mixture contained 100 μmoles of Tris buffer at pH 7.8; 5 μmoles of inorganic phosphate; 5 μmoles of ADP; 10 μmoles of MgCl₂; 0.5 μmole of 2,3',6-trichlorophenol indophenol; 0.04 mg of catalase; 1.1 mg of ethanol; chloroplasts equivalent to 0.2 mg of chlorophyll, and water to a final volume of 1.5 ml. The flasks were equilibrated for 5 minutes in the dark, then the reaction was initiated by turning on the lights. The reaction mixtures were illuminated with shaking for 30 minutes at 15° with a light intensity of 2000 foot-candles.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Reaction mixture</th>
<th>O₂ consumed</th>
<th>H₂O₂ recovered as acetaldehyde</th>
<th>Pyruvate recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μmoles</td>
<td>μmoles</td>
<td>μmoles</td>
<td>μmoles</td>
</tr>
<tr>
<td>1. Complete</td>
<td>0.7</td>
<td>0.69</td>
<td>3.8</td>
<td></td>
</tr>
<tr>
<td>Dye omitted</td>
<td>0.6</td>
<td>0.66</td>
<td>3.8</td>
<td></td>
</tr>
<tr>
<td>Ethanol omitted</td>
<td>0</td>
<td>0</td>
<td>3.8</td>
<td></td>
</tr>
<tr>
<td>Light omitted</td>
<td>0.4</td>
<td>0</td>
<td>3.8</td>
<td></td>
</tr>
</tbody>
</table>

### Table IV

**Generation of hydrogen peroxide by phosphorylating chloroplasts**

These assays were performed with the conventional Warburg apparatus. The reaction mixture contained 50 μmoles of Tris buffer at pH 7.8; 5 μmoles of inorganic phosphate; 5 μmoles of ADP; 10 μmoles of MgCl₂; 0.5 μmole of 2,3',6-trichlorophenol indophenol; 0.04 mg of catalase; 1.1 mg of ethanol; chloroplasts equivalent to 0.2 mg of chlorophyll, and water to a final volume of 1.5 ml. The flasks were equilibrated for 5 minutes in the dark, then the reaction was initiated by turning on the lights. The reaction mixtures were illuminated with shaking for 30 minutes at 15° with a light intensity of 2000 foot-candles.

These experiments indicated that the catalase-ethanol system can be made to trap 75 to 80% of the hydrogen peroxide generated under the circumstances of oxidative photophosphorylation.

Table IV contains data from experiments in which the abilities of chloroplast preparations to reduce oxygen to hydrogen peroxide and to carry out oxidative photophosphorylation are compared. It is apparent that hydrogen peroxide production occurs at only a fraction of the rate of ATP synthesis, and unlike the phosphorylation, is independent of the presence of indophenol dye. It would appear that the oxygen consumption accompanying oxidative photophosphorylation does not necessarily proceed through hydrogen peroxide as an intermediate.

**Experiments with TPN**—The possibility of substituting some other oxidant for oxygen was suggested by an experiment of Jagendorf (16). Jagendorf had found that p-chlorophenyl-dimethyurea inhibition of TPN reduction by illuminated chloroplasts could be reversed by the addition of ascorbic acid and indophenol dye. We were able to repeat this observation by using either 3-(3,4-dichlorophenyl)-1,1-dimethylurea as an inhibitor or by exposing the chloroplasts to a high concentration of Tris buffer, a treatment which eliminates the ability of chloroplasts to carry out a net Hill reaction (17). That photophosphorylation might occur under such circumstances of inhibition and reversal is confirmed in Table V. Chloroplasts washed with 0.4 M Tris buffer, of pH 7.8, were used, so that reduction of indophenol or TPN and phosphorylation accompanying the reduction of the latter compound were inhibited. In the absence of TPN, photophosphorylation with ascorbate-reduced indophenol was found to be oxygen-dependent, as in previous experiments. However, in the presence of TPN, photophosphorylation occurred under either air or a nitrogen atmosphere. Phosphate esterification and TPN reduction are seen to require both ascor-
bic acid and indophenol dye in the final experiment reported in this table. In separate experiments, the necessity for added photosynthetic pyridine nucleotide reductase was established. In nonilluminated controls there was neither phosphate esterification nor TPN reduction. Cysteine or reduced glutathione substitute equally well for ascorbate in these experiments, indicating that the function of these compounds is merely to supply reduced indophenol dye. That the concentration of reducing agent is critical is indicated by Fig. 3. It has been our general experience that an excess of the indophenol-reducing agent will inhibit photophosphorylation. As can be seen from this graph, at low concentrations of ascorbic acid, the ratio of phosphate esterified to TPN reduced exceeds 1.

**DISCUSSION**

The ability to support oxidative photosynthetic phosphorylation seems to be a general characteristic of the variously substituted indophenol dyes. Why photophosphorylation does not accompany the reduction of indophenols as it does with other Hill reagents is an intriguing question to which a satisfactory answer has yet to be found. The indophenol dyes used in this survey show a span in the standard oxidation reduction potential at pH 7 of from 0.123 volts for 1-naphthol-2-sodium sulfonate indophenol to 0.250 volts for m-carboxyphenol indo-2,6 dibromo phenol. As these two compounds, at the extremes of the range of potentials examined, both gave rather high rates of oxidative photophosphorylation, it would seem that there is no obvious relation between the potential of these dyes and their ability to support phosphorylation.

The intensity of light needed to half saturate oxidative photophosphorylation, approximately 500 foot-candles, provides a point for comparison between this reaction and other photochemical activities of isolated chloroplasts. Jagendorf and Avron (18) have reported that the reduction of trichlorophenol indophenol dye by the Hill reaction is half-saturated at a light intensity of 700 foot-candles. The above mentioned authors found that photophosphorylation with flavin mononucleotide as a cofactor is half-saturated with light at approximately 200 to 300 foot-candles, and with phenazine methosulfate as cofactor, photophosphorylation increases with increasing light intensity up to 5000 foot-candles and perhaps beyond. Bishop et al. (19) have reported half saturation of the spinach chloroplast cytochrome c photooxidase reaction at about 400 foot-candles. The peculiar absence of linearity between light intensity and phosphate esterification with indophenol in the region below 500 foot-candles is not generally experienced with these other chloroplast reactions, and its meaning in this case is obscure.

The oxygen response curve for oxidative photophosphorylation shows the reaction to be half-saturated at 1% oxygen. This oxygen saturation curve is similar to the one obtained with mammalian cytochrome oxidase preparations (20). In general, flavoproteins show a lower affinity for oxygen and so respond with an increasing rate to much higher percentages of oxygen (21).

A consideration which might militate against the accuracy of the results in the experiments involving peroxide measurement is the observation of Lavorel (4) that indophenol enhances a photoinhibition of the catalase activity of chloroplasts. We have been able to observe a photoinhibition of chloroplast catalase in the presence of indophenol, but only at very low levels of catalase such as encountered in Lavorel's experiments. With the higher level of endogenous catalase in our reaction mixture and the supplementary addition of crystalline enzyme, no photoinhibition could be detected under the experimental conditions for oxidative photophosphorylation.

The elimination of hydrogen peroxide as a participant in an aerobic chloroplast reaction is at best a difficult task. The major interest in these studies was to compare the rate of hydrogen peroxide formation in these reaction mixtures with the rate of oxygen exchange measured with isotopic oxygen as described in an earlier publication (14). When chloroplasts were illuminated under conditions for oxidative photophosphorylation, an indophenol-dependent oxygen exchange reaction proceeding at a rate of 50 atoms of oxygen consumed and evolved per mg of chlorophyll per hour was observed. In the experiments reported here, hydrogen peroxide production never exceeded a rate of 7 µmoles per mg of chlorophyll per hour, a figure so low as to make hydrogen peroxide only a minor contributor to the oxygen exchange. Since this hydrogen peroxide production is independent of the presence of indophenol dye, the indophenol dye-catalyzed oxygen exchange does not appear to be the mechanism by which the catalase activity is inhibited.
exchange and the generation of hydrogen peroxide would appear to be independent processes. As noted previously, the magnitude of the oxygen exchange reaction is such that it could serve stoichiometrically in oxidative photophosphorylation. The rate of hydrogen peroxide formation seems to be too low to act in a stoichiometric fashion with oxidative photophosphorylation. Inasmuch as oxygen does not appear to be reduced to hydrogen peroxide, it is suggested that the presence of reduced indophenol dye permits the reduction of oxygen to water. Hill has described an autoxidizable cytochrome in chloroplasts, designated $b_1$ (22). This component might well serve as the terminal electron donor to oxygen. However, this reaction with an attendant photophosphorylation appears possible only in the presence of reduced indophenol dye.

In 1954, Vernon and Kamen (23) described simultaneous oxidation and reduction reactions of illuminated chloroplasts. The interpretation developed by these authors of simultaneous and interdependent trapping of both the reducing and oxidizing products of chloroplast photolysis is essentially similar to the interpretation we have derived for oxidative photophosphorylation. In their original work, Vernon and Kamen found an equivalence of indophenol-mediated ascorbate oxidation and acetaldehyde production in the presence of catalase and ethanol. However, Habermann (24) has described a stimulation of oxygen exchange in the presence of ascorbic acid alone at concentrations to those employed by Vernon and Kamen. Perhaps the resolution of this discrepancy in acetaldehyde production can be attributed to ascorbic acid.

The experiments reported here in which TPN was presumed to act as a substitute for oxygen are by no means unambiguous. It is clear from the work of Jagendorf, and the work of Vernon (25) that photoreduction can be made dependent on a photooxidation of reduced indophenol dye. It is now evident that photophosphorylation can be made to accompany these interdependent electron transfer processes. These experiments do not localize the site of the phosphorylation as accompanying either the reduction of TPN or the oxidation of reduced indophenol. We had previously suggested that oxygen served in this system to trap the photochemically generated reductant, permitting reduced indophenol dye to trap the photochemically generated oxidant and so support phosphorylation. In the experiments cited here, phosphorylation is possible only when the chloroplasts can reduce either oxygen or TPN on the one hand and simultaneously oxidize reduced indophenol dye on the other.

**SUMMARY**

The oxidative photosynthetic phosphorylation reaction of spinach chloroplasts has been further characterized with regard to responses to variously substituted indophenol dyes, to variations in light intensity, and variations in oxygen concentration. Experiments have been described which would seem to eliminate hydrogen peroxide as a participant in this reaction. Finally, experiments have been cited which suggest that triphosphopyridine nucleotide may act as a substitute for oxygen in maintaining oxidative photophosphorylation.

**Acknowledgment**—The author is indebted to Dr. Birgit Ven-nesland for her encouragement and for her many helpful discussions and suggestions during the course of this investigation.

**REFERENCES**
