Interactions of Catalytic Cofactors for Photosynthetic Phosphorylation with Hill Reaction Oxidants*†

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Oxidation-reduction reactions in chloroplasts were first observed when Hill (1) discovered photolysis by isolated chloroplasts. In the Hill reaction electron transport proceeds from water to the artificial oxidant which can be any one of a number of compounds (2-4).

A cyclic series of oxidation-reduction reactions is postulated as the basis for photosynthetic phosphorylation by spinach chloroplasts (5). In phosphorylation water is presumably cleaved and then regenerated. In order for the regeneration of water to occur an oxidation-reduction cofactor must be added in catalytic amounts, and again a large variety of compounds will function (6, 7).

Our primary question is whether the Hill reaction and cyclic phosphorylation share all or most of the same electron transport pathways in chloroplasts, or whether there is a different set of phosphorylation pathways in chloroplasts. In the Hill reaction electron transport proceeds from water to the artificial oxidant which can be any one of a number of compounds.

In experiments where TPN or ferricyanide was reduced at the same time that phosphate uptake occurred, it was possible to obtain a P/2e ratio. This will be defined as the moles of inorganic phosphate esterified per 2 x microequivalents of oxidant reduced.

If the time course of a reaction were to be studied, the reaction mixture was flushed with nitrogen gas continuously while the light was on. At the intervals shown 0.5 ml. aliquots were removed from the reaction mixture by means of a syringe and a long, thin needle inserted through the gas outlet tube.

Photosynthetic pyridine nucleotide reductase (14) was a 35-fold purified preparation very kindly supplied by Dr. A. San Pietro. The amount used was sufficient to catalyze the reduction of a large excess of TPN, under our reaction conditions.

EXPERIMENTAL

Spinach was purchased from local groceries; chloroplasts were prepared as described previously (7) and either washed once or not at all. Phosphorylation reactions were performed with a 1.5 ml. reaction mixture in Erlenmeyer flasks under nitrogen, at 15°, and at approximately 4000 foot candles. No “extractable factor” (11) was used in the experiments reported here. The stimulation due to this cofactor has never amounted to more than 40 per cent in the present system while often it has been absent. In the interest of simplicity, therefore, the “extractable factor” was omitted. The reaction mixture contained 25 to 40 μg. of chlorophyll, and 2.5 μmoles each of ADP and phosphate. Other reagents were in the proportions previously used (7).

Disappearance of inorganic phosphate was measured by the method of Tausky and Shorr (12) unless ferricyanide was present in the reaction mixture. To avoid the interference due to ferricyanide, a slightly modified Borel and Chain procedure (13) was used. The modification consisted of decreasing the amounts of all components so that the final volume of blue phomolybdate complex was 5.0 ml. Optical density was measured at 740 mμ in a spectrophotometer. The reduction of ferricyanide was measured by the loss of absorption at 420 mμ in a separate aliquot of the denatured reaction mixture. TPN reduction was estimated from the increase of absorption at 340 mμ. When phosphate uptake was measured associated with TPN reduction, the phosphate concentration was lowered to either 0.75 or 1.0 μmole per flask.

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RESULTS AND DISCUSSION

Phosphorylation Accompanying Net Reduction of TPN or Ferricyanide—The uptake of phosphate accompanying reduction of TPN is shown in Table I. This confirms the findings of the Berkeley group (8). In the two experiments shown the P/2e ratios are 0.71 and 0.87. In our experiment this ratio has varied between 0.3 and 0.9, evidently approaching a limit of 1.0.

Fig. 1 shows a time course experiment, in which 15 μmoles of phosphate, ADP, and ferricyanide were present in the reaction mixture. It can be seen that the reduction of ferricyanide was complete by 15 minutes. Phosphate uptake also stopped at the end of 15 minutes, even though only 50 per cent of the phosphate present had been esterified. This shows that ferricyanide is quite incapable of acting as a catalytic cofactor; that although ferricyanide can be reduced, ferrocyanide cannot be reoxidized by the chloroplasts in light. The P/2e ratio is very close to 1.0 throughout the reaction.

The reduction of TPN and its accompanying phosphorylation require a distinct enzyme, called photosynthetic pyridine nucleotide reductase (14, 15). Table II shows, in confirmation of the findings of Arnon et al. (10), that ferricyanide reduction is independent of the enzyme, even after the chloroplasts have been washed a number of times. Although there is some loss in fer-
TABLE I

**TPN reduction accompanied by phosphate esterification**

<table>
<thead>
<tr>
<th>Phosphate uptake</th>
<th>TPN reduced</th>
<th>P/2e</th>
</tr>
</thead>
<tbody>
<tr>
<td>μmoles/mg. chloro-phyll/hr.</td>
<td>μmoles/mg. chloro-phyll/hr.</td>
<td></td>
</tr>
<tr>
<td>Experiment 1</td>
<td>13.5</td>
<td>15.6</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>16.5</td>
<td>23.0</td>
</tr>
</tbody>
</table>

TABLE II

**Relation of TPN or ferricyanide reduction and phosphorylation to photosynthetic pyridine nucleotide reductase**

<table>
<thead>
<tr>
<th>Chloroplasts</th>
<th>TPN Reduction</th>
<th>Ferricyanide Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unwashed</td>
<td>17.5</td>
<td>5.1</td>
</tr>
<tr>
<td>Unwashed plus PPNR</td>
<td>17.9</td>
<td>6.4</td>
</tr>
<tr>
<td>5 times washed</td>
<td>2.5</td>
<td>1.5</td>
</tr>
<tr>
<td>5 times washed PPNR</td>
<td>12.1</td>
<td>6.1</td>
</tr>
</tbody>
</table>

* All values expressed as μmoles per mg. of chlorophyll per hour. Reaction time, 10 minutes. For TPN reduction, 1 μmole of TPN, 0.75 μmole of phosphate, 1.0 μmole of ADP per 1.5 ml. total volume. 178 μg. of chlorophyll of unwashed chloroplasts or 198 μg. of washed chloroplasts per flask. For ferricyanide reduction, 5 μmoles of ferricyanide, 2.5 μmoles of phosphate, 4.0 μmoles of ADP per 1.5 ml. reaction volume. 135 μg. of chlorophyll of unwashed chloroplasts, or 132 μg. of chlorophyll of washed chloroplasts, per flask.

† PPNR refers to photosynthetic pyridine nucleotide reductase, 35-fold purified preparation. To the washed chloroplasts with ferricyanide, TPN and TPNH diaphorase were added with the PPNR.

**TABLE III**

**Independence of cyclic phosphorylation with riboflavin phosphate from photosynthetic pyridine nucleotide reductase**

All values shown are in μmoles/mg. of chlorophyll/hour. The reaction time was 5 minutes, and each flask contained 0.100 mg. of chlorophyll in the chloroplasts added. The reaction mixture contained 10 μmoles of ascorbate, which was found recently by San Pietro and Giovanelli (personal communication) to act as a protective agent for the reductase. Total volume was 3.0 ml. The higher specific activities seen in this table for TPN reduction can be ascribed in part to the lower concentration of chloroplasts, and in part to the presence of ascorbate.

Reaction mixtures contained 10 μmoles each of phosphate and ADP, and 5 X 10⁻⁶ c.p.m. of P³⁻. ATP formation was measured by adsorption of ATP on charcoal, then subsequent hydrolysis of the radioactive phosphate with 1.0 N HCl (16).

**Fig. 1.** Time course of phosphate esterification associated with ferricyanide reduction. Reaction mixture contained 300 μg. of chlorophyll, 120 μmoles of Tris, 210 μmoles of NaCl, 15 μmoles of phosphate, 24 μmoles of ADP, 15 μmoles of potassium ferricyanide, and 30 μmoles of magnesium. Total volume was 9.0 ml. Notice that phosphate uptake stops at 50 per cent of the possible total amount, and the P/2e ratio is approximately 1.0.

Cyclic phosphorylation with riboflavin phosphate as the cofactor is similarly independent of added reductase, TPN, and TPNH diaphorase, either before or after washing (Table III).

**Relationship of Hill Reaction Oxidants to Cyclic Phosphorylation**—Phosphate uptake accompanies the reduction of ferricyanide but not the reduction of 2,3,6-trichlorophenolindophenol dye, in our experience. The interactions between these two Hill oxidants and cyclic phosphorylation mediated by phenazine methosulfate were studied in mixture experiments.

Fig. 2 shows the time course of phosphate uptake in a reaction mixture containing both phenazine methosulfate and the indophenol dye. The reaction is characterized by a lag period whose length depends directly on the amount of indophenol added (compare the various curves of Fig. 2).

During the course of these reactions any indophenol present is being reduced. By measuring residual optical density at 620 mg in each aliquot of the time course before measuring phosphate, it could be seen that the lag ended only when the dye was almost
Fig. 2. The effect of increasing amounts of indophenol dye on cyclic phosphorylation with phenazine methosulfate as cofactor. Final concentrations of dye shown on the graph. Reaction mixture contained 200 μg of chlorophyll; concentrations of phosphate, ADP, magnesium, Tris buffer, sodium chloride as in Table I; and phenazine methosulfate 0.2 μmole in a total volume of 6.0 ml.

completely reduced. No matter what the original concentration of dye added, the lag ended when the concentration of oxidized dye reached approximately $5 \times 10^{-4}$ M. We can conclude that the oxidized form of the dye inhibits cyclic phosphorylation completely at concentrations only slightly higher than $5 \times 10^{-4}$ M.

The reduced dye is an inhibitor, although it is not nearly so effective as the oxidized dye. In other experiments (not shown) in which indophenol dye was added together with an excess of ascorbate to keep it completely reduced, 50 per cent inhibition occurred at $1.5 \times 10^{-4}$ M and 100 per cent inhibition at $0 \times 10^{-4}$ M. No lag period was seen with the reduced dye.

One possible interpretation of the striking inhibition by oxidized indophenol dye is that it drains electrons from the chain at a rapid rate, thus preventing their passage to phenazine methosulfate. Although superficially attractive, this interpretation can be ruled out by experiments with CMU. CMU is an inhibitor of the Hill reaction (18) but not of phenazine methosulfate-mediated phosphorylation (19). One might therefore expect the addition of CMU to a mixture as in Fig. 2 to inhibit dye reduction by the Hill reaction, have no effect on electron flow to phenazine methosulfate, and thereby relieve the inhibition of phosphate uptake caused by indophenol dye. This is not the case, however, as seen in Fig. 3.

CMU at $1 \times 10^{-4}$ M had a small inhibitory effect on phosphate uptake in Fig. 3 (although the 1 minute determination appeared to indicate a lag in uptake, in many other experiments with CMU no lag phenomenon was observed). However when this much CMU was added together with $0.55 \times 10^{-4}$ M indophenol dye, a 16 second lag period caused by the dye alone was lengthened to almost 2 minutes. Optical density determinations at 620 mp showed that it took 2 minutes for the dye to be reduced, in the presence of CMU. Similarly $1.1 \times 10^{-4}$ M indophenol dye by itself caused a lag in phosphorylation of approximately 40 seconds, but the addition of CMU prolonged the lag to over 4 minutes. CMU had again lengthened the time needed to effect reduction of the indophenol dye, and phosphorylation resumed only when the dye was reduced. It is therefore evident that inhibition is due to the actual presence of oxidized indophenol dye and not to the rapidity with which it is reduced.

Fig. 4 shows that ferricyanide also is an inhibitor for cyclic phosphorylation mediated by phenazine methosulfate. Curve A shows phosphate uptake due to ferricyanide reduction. The rate here is about 25 per cent of that in Curve B, where cyclic phosphorylation is mediated by phenazine methosulfate. The amount of ferricyanide was chosen so that it would be fully reduced in about half the time allowed for the reaction, and so that only one-third of the phosphate would be esterified at that time. In the mixture of ferricyanide and phenazine methosulfate (Curve C) the initial rate of phosphate uptake is approximately equal to that in the flask with ferricyanide alone. As soon as ferricyanide is totally reduced, however, the rate of phosphorylation proceeds as rapidly as it did initially in the flask with phenazine methosulfate.

Again in this experiment it is the actual presence of ferricyanide and not its rapid rate of reduction that inhibits cyclic phosphorylation. Curve D shows that $5 \times 10^{-4}$ M CMU has no effect on the cyclic phosphorylation with phenazine methosulfate; Curve E shows a severe inhibition of ferricyanide phosphoryla-

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1 The abbreviations used are: CMU, p-chlorophenyldimethylurea; Tris, tris(hydroxymethyl)aminomethane.
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Fig. 4. Time courses of phosphorylation with a mixture of ferricyanide and phenazine methosulfate, and the effect of CMU on the reaction. Flask A, ferricyanide only; Flask B, phenazine methosulfate and ferrocyanide; Flask C, phenazine methosulfate plus ferrocyanide; Flask D, phenazine methosulfate, ferrocyanide, and 5 x 10^-4 M CMU; Flask E, ferrocyanide plus CMU; Flask F, ferrocyanide, phenazine methosulfate, and CMU. Ferrocyanide or ferrocyanide, 12 μmoles; phosphate, 20 μmoles; ADP, 25 μmoles; magnesium, 30 μmoles; phenazine methosulfate, 0.3 μmoles; total volume 9.0 ml. 220 μg. of chlorophyll per flask. Tris and NaCl in the usual proportions.

Fig. 5. Interaction of riboflavinphosphate (FMN) with phenazine methosulfate at various concentrations. Phenazine methosulfate and riboflavin phosphate as shown, other reagents in the usual concentrations. Time in light 5 minutes.

Discussion

We were able to confirm the observations of Arnon et al. (10) that phosphorylation may occur as a consequence of electron flow to the Hill oxidants, TPN, or ferricyanide. This discovery, together with the similarity in action spectra (9, 10), makes it appear quite reasonable that the cyclic phosphorylation is essentially a cyclic variant of the Hill reaction. The essence of this concept is that cyclic phosphorylation will occur if a Hill oxidant can be reoxidized by a component on the oxidized side of the cleaving of water. Since catalytic cofactors for cyclic phosphorylation do not have to be added for the Hill reaction to occur, the cofactors must function after the point at which Hill oxidants are reduced.

Additional evidence for the concept comes from the competition experiments reported here, which show that 2,3,6-trichloroindophenol and ferricyanide inhibit cyclic phosphorylation. As long as the inhibition can be ascribed to interfering with electron transport rather than to "uncoupling" or inhibiting phosphate transfer, then the competition makes it quite likely that oxidants and cofactors receive electrons from the same chain.

It seems obvious that ferricyanide, at least, inhibits by stopping electron flow rather than by interference with the actual mechanism of ATP formation. The situation might be more questionable with indophenol dye where reduction in the course of the Hill reaction does not lead to appreciable ATP formation. However, the facts that the oxidized dye is a much more effective inhibitor than the reduced dye, and that the inhibition is reversed very rapidly if the dye is reduced during the course of the reaction, suggest strongly that the dye also inhibits electron transport reactions rather than phosphate transfers.

Inhibition by either ferricyanide or the indophenol dye is in-
dependent of the rate of reduction. It seems likely therefore that they inhibit by virtue of a firm binding to the enzymatic site(s) at which they are normally reduced. Presumably they would be displaced as soon as they are reduced, and thus would no longer be inhibitors.

This effect of the Hill oxidants is the opposite of that observed by Arnon et al. (10) in which net TPN reduction was inhibited by the addition of riboflavin phosphate as a cyclic cofactor. We also find that TPN reduction is inhibited by adding phenazine methosulfate. The inhibition of net TPN reduction by cyclic cofactors could easily occur by reoxidation of TPNH; either non-enzymatically in the case of phenazine methosulfate, or through TPNH diaphorase in the case of riboflavin phosphate.

Arnon et al. (10) have pictured TPN as an obligate electron-carrying intermediate before riboflavin phosphate. Their evidence for this is a TPN and photosynthetic pyridine nucleotide reductase requirement at suboptimal concentrations of riboflavin phosphate (15). However with optimal amounts of riboflavin phosphate, we see quite clearly that addition of TPN, photosynthetic pyridine nucleotide reductase, and TPNH diaphorase has no effect on the cyclic phosphorylation (Table III). If TPN is an obligate intermediate before riboflavin phosphate, one might reasonably expect a requirement for larger amounts of TPN at optimal levels of riboflavin phosphate than at low levels. Another possible interpretation of the data of Arnon et al. (15) and one consistent with our observations is that the pathway through the reductase and TPN to riboflavin phosphate is an alternate one which does not occur when high levels of riboflavin phosphate are used in the absence of exogenous TPN.

Our experiments show that neither riboflavin phosphate-mediated cyclic phosphorylation, nor the reduction of ferricyanide requires the addition of TPN, its photosynthetic reductase, or TPNH diaphorase. The experiments do not rule out, of course, the possible participation of internally bound, catalytic amounts of these components. The fact that exogenous TPN may be reduced when the reductase is added, however, is not proof for the function of any such internal components.

Our conclusions concerning electron transport pathways on the basis of experiments reported to date are summarized in the scheme shown below (Fig. 6).

The diagram is intended to show several possibilities, each starting with the same early intermediates from the cleaving of water. Either a Hill reaction occurs and oxygen is evolved, or else a cyclic phosphorylation occurs in which water is regenerated. If the Hill oxidant is TPN, the reductase (photosynthetic pyridine nucleotide reductase) is required for its reduction. If the Hill oxidant is ferricyanide photosynthetic pyridine nucleotide reductase is not required, and in addition ferricyanide blocks electron flow to the cyclic cofactor. Indophenol dye as a Hill oxidant is not shown. Since no phosphorylation is detectable during its reduction we think it likely to be reduced at some point before the phosphorylating step. The exact nature of the catalytic cofactor for the cycle is not specified, because a fairly large number of oxidation-reduction compounds is able to satisfy this function in the present experimental system (6, 7, 21).

This scheme is basically similar to ones already presented (8, 20–23). It differs in that it merges the Hill reaction and cyclic phosphorylation into a common framework. In addition, we have tried to organize a minimal scheme which is either definitely required or strongly supported by the facts available to us at the present time, rather than to present a more complete but somewhat hypothetical scheme based on partial evidence.

**SUMMARY**

1. Phosphate esterification can be demonstrated to accompany stoichiometrically the reduction of triphosphopyridine nucleotide or ferricyanide by spinach chloroplasts in the light.
2. It can be shown that neither riboflavin phosphate cyclic phosphorylation nor ferricyanide reduction requires the addition of photosynthetic pyridine nucleotide reductase, whereas triphosphopyridine nucleotide reduction does.
3. Evidence is presented to show that ferricyanide and trichloroindophenol dye prevent electron flow to phenazine methosulfate, used as the catalytic cofactor in cyclic phosphorylation. The inhibition is specific for the oxidized form of both Hill oxidants, and is not affected by the rate at which they drain reducing power from the chloroplasts.
4. A minimal scheme is presented to indicate the sequence in which Hill reaction oxidants and catalytic cofactors interact with the electron transport chain in chloroplasts.

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**REFERENCES**
