The process of formation of oxygen by illuminated chloroplast preparations in presence of added H-acceptors resembles the converse of the process of the oxidation of H-donors by oxygen with the Keilin-Hartree muscle preparation derived from mitochondria (9). In the former process the H-transport is against the chemical potential owing to transformation of the light energy. The discoveries of the photosynthetic phosphorylation with chloroplast preparations by Arnon, Whatley and Allen (2) and with the chromatophores from a photosynthetic bacterium by Frenkel (4) were of supreme significance in the development of the biochemical study of photosynthesis. Furthermore they indicated a similarity of the chloroplast to the mitochondria which was already partly apparent from the comparative study of cytochrome components in the two subcellular structures (10, 11). In oxidative phosphorylation with mitochondria the H-transport is dependent upon the presence of both inorganic phosphate and an acceptor for the phosphate group. A similar condition has been shown to operate in the chloroplast by Arnon, Whatley and Allen (6). The reduction of an H-acceptor and concurrent O₂ production by the illuminated chloroplast system is to a very significant extent coupled with phosphorylation. Moreover the phosphate acceptor—adenosine diphosphate (ADP)—is the same for the chloroplasts and the mitochondria. With mitochondria the coupling of H-transport and phosphorylation can be removed either by the presence of certain reagents or by the treatment used for the Keilin-Hartree preparation. In such a case active H-transport is shown without any concurrent phosphorylation. An analogous process of uncoupled H-transport is shown by the chloroplast preparations (4, 16, 17) either by addition of reagents or by a physical treatment. The important difference between chloroplasts and mitochondria apparently is in the direction of hydrogen transport and it might be legitimate to term the phosphorylation with chloroplasts as "reductive" in contrast to the oxidative phosphorylation with mitochondria. It has been shown that phosphorylation can occur with chloroplasts in light without any O₂ production and without the corresponding stoichiometric reduction of any H-acceptor. Thus a situation, named by Arnon "cyclic phosphorylation," would seem to involve a reduced product being constantly oxidized not by molecular oxygen but by a possible photochemically produced precursor of this gas. In the earlier experiments of Arnon and Whatley O₂ appeared to be necessary. In this case, presumably, a reduced product was being reoxidized by molecular oxygen. Thus in order to obtain the phosphorylation reaction with chloroplast preparations one essential condition must be to establish the photochemically driven H-transport. This condition can be met by adding certain "cofactors" to the washed chloroplast preparations.

The present investigation was undertaken in order to see how specific the two reagents used by Arnon (K₃ and FMN) were in relation to the phosphorylation reaction; and to see if the activity could be related to reactions of chloroplast with H-acceptors. A preliminary report of this work was given at a meeting of the Biochemical Society. (15).

Materials and Methods

Plant Material: During the period January 14 to April 14, 1957, sugar beet leaves from greenhouse material were made available to us through the kindness of Dr. G. D. H. Bell and the staff of the Plant Breeding Station. Also we are grateful to Dr. J. Swabey, Research Station of Plant Protection Ltd., for a batch of spinach plants. During the period July 1 to 21 of the same year leaves of spinach beet (Swiss chard) were available from plants sown in the open near Cambridge in May. The plants were used when possible at a stage between 8 to 12 leaves. The leaves used were those which seemed to have reached a point slightly less than their full size and when they could be distinguished by their minimum resistance to crushing. In the Swiss chard leaves used no starch could be detected but when the leaf extracts were centrifuged at low speeds a white deposit consisting of calcium oxalate crystals was always obtained.

Reagents: Phenazine methosulphate was made from phenazine by dissolving in hot xylene (dried over K₂CO₃) 15 parts, adding slightly more than 1 equivalent of freshly redistilled dimethysulphate and heating to 115 to 120°C until the maximum yellow precipitate was obtained (about 30 mins). The mixture was cooled, the solid filtered off and washed with dry ether, followed by a very little absolute alcohol. As far as possible, all contact with moist air was avoided. The crude product was recrystallized from alcohol with the addition of ether. Pyocyanine was prepared according to McLlwain (13) and purified by repeated passage from chloroform to acid, and isolated as the crystalline free pig-

Received March 25, 1959.

1 Report of work forming part of program supported by the Agricultural Research Council of the U.K.

2 Lately Imperial Chemical Industries Research Fellow; present address: Department of Botany, Queen Mary College, University of London.
ment. The preparation was kept at \(-15^\circ\text{C}\). Resorcin blue or Lacmoid was purified by dissolving at pH 7.5 and salting out with the addition of NaCl. The following abbreviations are used: Adenosine diphosphate, ADP; Adenosine triphosphate, ATP; Flavine nucleotide, FMN; 2-methyl-1,4-naphthoquinone, Ks.

**Chloroplasts:** Chloroplasts were prepared by a method similar to that of Arnon (4) in a buffered solution containing 0.35 M NaCl, 0.001 M MgCl\(_2\), and M/15 2-amino-2-hydroxymethylpropane-1,3-diol (TRIS) at pH 7.4. For some experiments the MgCl\(_2\) was omitted and added to the reaction mixtures as required. Washed leaves (25 to 30 g) from which the midribs had been removed were illuminated in tap water for about 1 hour. They were then shaken dry, shredded into a mortar and rapidly pulped in 50 to 60 ml of buffer. The pulp was squeezed in a double thickness of muslin and the expressed juice centrifuged at low speed (average 500 G) for 1 minute. The supernatant was retained and centrifuged again (1000 G) for 10 minutes. The 2nd supernatant was discarded and the chloroplasts uniformly resuspended in about 10 ml of buffer. This suspension was brought to about 60 ml by the addition of more buffer and then centrifuged for an additional 10 minutes (1000 G). The supernatant was again discarded and the chloroplast pellets resuspended as before but using either buffer or distilled water to give a final volume of 10 ml. All the apparatus and the solutions used were kept at about 0\(^{\circ}\)C throughout.

Microscopic examination showed that the final suspensions consisted largely of intact chloroplasts. In addition there were some smaller green fragments and a few very small particles which may have been mitochondria. When distilled water, rather than the buffer solution, had been used as the suspending medium the chloroplasts appeared to be less definite in outline and soon disintegrated.

Reactions were carried out in 1.5 \times 10 \text{cm} glass centrifuge tubes with rounded ends. Each tube contained 0.1 ml of chloroplast suspension (about 0.05 mg chlorophyll), 0.1 ml of TRIS-NaCl buffer at pH 7.4, 1 \text{mM} ascorbate together with ADP, orthophosphate and other reactants as specified, in a final volume of 0.3 ml. A few seconds of centrifugation at low speed ensured that any small drops of added reactants remaining on the sides of the tubes were drawn to the ends. In the winter and early spring 1.5 \text{mM} of orthophosphate was used in each tube. In the summer this was increased to 2.5 \text{mM} of ADP and 2.0 \text{mM} of phosphate to facilitate measurements with more active chloroplast preparations.

In experiments designed to compare the activity of “broken” chloroplasts (resuspended in distilled water) with “whole” chloroplasts (resuspended in TRIS-NaCl buffer) additional buffer was added to reaction mixtures containing the “broken” chloroplasts in order that the final concentrations of TRIS-NaCl were the same in each case. In all cases tubes were chilled before the addition of the chloroplasts.

**Illumination.** Eighteen tubes were attached with “terry” clips to the rim of a cork disk fastened at its center to a motor driven stirring rod. The stirrer was adjusted so that the disk was held at an angle of about 20° to the surface of the water in a glass bath. The lower ends of the tubes were immersed in the water in the bath which was illuminated by one 200-watt bulb (above) and four 150-watt bulbs (below). When the stirring rod was rotated the motion of the inclined disk caused the contents of the tubes to swirl round in a thin film facilitating light saturation, mixing and temperature maintenance (see fig 1). Dark controls were carried out in tubes painted black.

For some anaerobic experiments the tubes were evacuated prior to illumination. This was done with a mercury pump attached to a hypodermic needle which was inserted for 90 seconds through a rubber bung in the mouth of each tube. The contents of the tubes were vigorously agitated during evacuation. In other experiments the reaction mixtures were increased 10-fold and illuminated in closed 50 ml conical flasks through which oxygen-free nitrogen was passed for 10 minutes before illumination. In these experiments inorganic phosphate was determined in an 0.3 ml sample of the reaction mixture.

**Inorganic Phosphate Estimation** (after Allen (1)) After illumination reactions were stopped either by the addition of a small volume of 60 % perchloric acid or preferably by the addition of 9.5 ml of water containing perchloric acid, sodium metabisulphite and amidol. In either case the additions were such that the tubes contained the reaction mixture, 0.15 ml of 60 % perchloric acid and 0.15 ml of a solution containing sodium bisulphite (20 % w/v) and amidol (2,4-diaminophenol hydrochloride, 1 % w/v) in a final volume of 9.8 ml. Ammonium molybdate (0.15 ml of 8.3 % w/v (NH\(_4\))\textsubscript{2}MoO\(_4\)\textsubscript{4}H\(_2\)O) was then added and the tubes centrifuged to remove the protein precipitated by the acid. After 5 minutes the intensity of the blue color which developed in the supernatant was determined by readings at 660 m\u. The quantity of phosphate was then obtained by reference to a standard curve.

The disappearance of inorganic phosphate has been taken to represent ATP formation from ADP. The inorganic phosphate content of reaction mixtures hydrolyzed for 7 minutes in \(N\) HCl before and after illumination was the same. The observed decrease in inorganic phosphate before hydrolysis therefore corresponds to an increase in “7 min” organic phosphate.

In the plants used chloroplast preparations showed no ATPase activity. In contrast, chloroplast preparations from leaves of barley seedlings have a high ATPase activity. The supernatant from a chloroplast preparation from spinach also gave smaller particles, when spun at 20,000 G, which showed a marked ATPase activity.
Fig. 1 (top, left). Water bath and illumination.

Fig. 2 (top right). Varying concentrations of added cofactors. The rate is expressed as a percentage of the maximum in each set (150 to 200 μM/phosphate/mg chlorophyll/hr). For reaction mixture see Materials and Methods.

Fig. 3 (bottom left). Progress curve of disappearance of inorganic phosphate. Reaction mixtures contained in a total volume of 0.3 ml, inorganic phosphate, 1.65 μM; ADP, 1.5 μM; FMN, 0.003 μM, ascorbate, 1 μM; chloroplast suspension, equivalent to 0.05 mg chlorophyll; TRIS-NaCl buffer at pH 7.2. A shows increase of phosphate in dark; B shows initial level of phosphate; and C difference between A and final level of inorganic phosphate.

Fig. 4 (bottom right). Activity of phosphorylation in relation to pH. For reaction mixtures see Materials and Methods. Curve 1: whole chloroplasts with pyocyanine, 10 min illumination; 2: whole chloroplasts with FMN + K₃ 10 min illumination; 3: broken chloroplasts stored for 4 hours at 0° C with pyocyanine 6 min illumination; 4: broken chloroplasts with pyocyanine 4 min illumination; and 5: broken chloroplasts with FMN + K₃ 4 min illumination.
Results

The activity of a cofactor, such as K₃ or FMN in relation to phosphorylation shows an optimum concentration in the region 10⁻⁴ M, and after some preliminary trials the experiments were carried out with a series of substances at this range. The results of experiments in air are shown in Table I. The very wide range of oxidation-reduction potential in the

<table>
<thead>
<tr>
<th>Activating agent</th>
<th>Potential</th>
<th>Rate</th>
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<tbody>
<tr>
<td>1,2-Naphthoquinone-4-sulphonate</td>
<td>+0.245</td>
<td>37</td>
</tr>
<tr>
<td>1,4-Naphthoquinone-3-sulphonate</td>
<td>+0.167</td>
<td>49</td>
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<tr>
<td>Ascorbate</td>
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<td>Juglone</td>
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<td>2-Methyl-1,4-naphthoquinone</td>
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<td>60</td>
</tr>
<tr>
<td>Methylene blue</td>
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<tr>
<td>2,7-Dichlororesorufin</td>
<td>-0.006</td>
<td>61</td>
</tr>
<tr>
<td>Pyocyanine</td>
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<td>100</td>
</tr>
<tr>
<td>Resorcin blue</td>
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<td>28</td>
</tr>
<tr>
<td>Napthazarine</td>
<td>-0.105</td>
<td>53</td>
</tr>
<tr>
<td>FMN</td>
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<tr>
<td>Dimethyl safranin sulphonate</td>
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<td>53</td>
</tr>
<tr>
<td>Anthraquinone-2-sulphonate</td>
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<td>52</td>
</tr>
<tr>
<td>Methyl viologen</td>
<td>-0.455</td>
<td>54</td>
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</table>

The potential is the characteristic potential in volts at pH 7. The rate is expressed as a percentage of the maximum (80 µM phosphate/mg chlorophyll/hr).

characteristic potentials of the reagents at pH 7 is shown by data in the last column. Of all the substances tried phenazine methosulphate appeared to be the most active. (This was also found independently by Avron and Jagendorf (12)). We noticed however that the dilute stock solution (10⁻⁴ M) rather rapidly became purple or brownish when standing even in quite a dull light. This in no way affected the activity. A sample was allowed to stand in the light, the pyocyanine extracted in chloroform, purified and added in a concentration corresponding to the original, phenazine methosulphate. The activity of the pyocyanine was the same as that given by the phenazine methosulphate, either before or after exposure to light. The formation of pyocyanine in light from phenazine methosulphate requires O₂, so that in order to see if the phenazine methosulphate was active it was necessary to test it in absence of O₂. The removal of oxygen to a degree of completeness required when a minute amount of the phenazine methosulphate at a concentration of 10⁻⁴ M is involved proved impracticable in a time during which the activity of the chloroplast preparation could be maintained. We were thus led to run the phosphorylation reaction in the presence of catalase and ethanol relying on the Mehler reaction (14) to remove the last traces of O₂ by forming acetaldehyde. The presence of catalase and ethanol in vacuo did not affect the rate of phosphorylation with pyocyanine or with preilluminated phenazine methosulphate as compared with the rate in air.

| Reactants | ADP and inorganic phosphate, 1.5 µM; MgCl₂, 10⁻¹ M; TRIS, 0.05 M, pH 8.1; ethanol, finally 3 %; catalase, 0.03 × 10⁻³ M; ascorbate 10 µM; phenazine methosulphate or pyocyanine, 10⁻⁴ M; chloroplasts, broken, 0.0075 mg chlorophyll; in a final volume of 0.3 ml.

* A value of 1000 has been obtained at 15° using similar reaction mixtures.

If the experiment was set up in the dark and the O₂ removed as completely as possible then no increase in photosynthetic phosphorylation was obtained with phenazine methosulphate unless it had been previously exposed to light in the presence of air.

A further experiment has recently been carried out by Dr. F. L. Bendall with chloroplasts of Chenopodium bonus-henricus L. the result of which is shown in Table II.

The data given in Table III show a comparison between FMN and phenazine methosulphate. There is an indication of a short lag period in the case of the latter of about 1 minute which could represent the conversion to pyocyanine.

The effect of varying concentrations of added cofactors for phosphorylation is shown in Figure 2. In the case of the magnesium ion the chloroplast preparation was not free from magnesium so that the effect of addition of Mg²⁺ is only shown at the higher concentrations. The broken curve is extrapolated.

In Figure 3 a progress curve is shown and the slight increase of inorganic phosphate which may have been released from ADP in the corresponding dark experi-

<table>
<thead>
<tr>
<th>Time (MIN)</th>
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<th>B</th>
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<tr>
<td>2</td>
<td>2.0</td>
<td>2.8</td>
</tr>
<tr>
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<td>11.2</td>
</tr>
<tr>
<td>30</td>
<td>24.4</td>
<td>12.6</td>
</tr>
</tbody>
</table>

Results as µM phosphate esterified/mg chlorophyll. Broken Swiss chard chloroplasts. A: 10⁻₅ phenazine methosulphate. B: 10⁻₅ FMN and 10⁻₅ K₃. Other reactants as indicated in Materials and Methods.
ment is shown by the sloping line lying above that indicating the theoretical total uptake for the added ADP. The disappearance of inorganic phosphate, which corresponds with the formation of ATP from ADP is nearly linear until all the added ADP is converted to ATP.

The effect of changes in pH on the amount of inorganic phosphate esterified in a short interval of time is shown in figure 4. With broken and unbroken chloroplasts there is 1 maximum with FMN and K3 while with pyocyanine the curves suggest a superposition of 2 maxima, one corresponding with that in the former curves at pH 7.7 the other, showing a higher activity at pH 7. When the broken chloroplasts were kept at 0°C (in absence of ascorbate) for 4 hours the activity measured with FMN and K3 was practically zero. The same sample measured in the presence of pyocyanine showed activity with a single maximum at pH 7.7. The curves suggest that there may be more than 1 site for the phosphorylation reaction as suggested by Wessels (16,17) from his study of inhibitors with FMN and K3. This, however, would not be the only possible interpretation of the present data.

DISCUSSION

The results obtained by addition of various substances as co-factors for phosphorylation agree with the work (actually carried out simultaneously) of Jagendorf and Avron (12). Further we found that the addition of ascorbate was not always necessary though it did diminish the rate of loss of activity on storage of the chloroplast preparations at 0°C. Here again there is agreement in the results obtained in both Arnon's (5) and Jagendorf's (12) laboratories and with those of Wessels (16,17).

The question as to whether phenazine methosulphate itself is active or whether the activity is due to its photochemical transformation to pyocyanine (which requires O2) is still controversial. Our evidence, which was obtained using concentrations in the region of 10⁻⁴ M phenazine methosulphate, is in favor of the active agent being pyocyanine, rather than phenazine methosulphate. The concentration of pyocyanine for half activation is greater than that for FMN or K3, but the maximum rate of phosphorylation for pyocyanine with some chloroplast samples is significantly higher. We were not able to observe any direct reduction of pyocyanine with chloroplast preparations, based on a bleaching of the color. There is, however, indirect evidence (Davenport, private communication) that pyocyanine can be rapidly reduced by illuminated chloroplast preparations; we are led to the conclusion that it can also be rapidly reoxidized by a photochemically induced precursor of free O2 in the chloroplast system and hence, as will be made clear later, it would be supposed to have ideal properties for establishing the photophosphorylation—termed by Arnon as cyclic—which is dependent neither on the presence of O2 nor on the production of O2.

It now therefore becomes clear, as indeed Arnon has already emphasized (6) that there is no necessity for the direct utilization of molecular oxygen in the so called “cyclic phosphorylation.” Originally (9) it was proposed that the reduced product formed in the light might be reoxidized by molecular oxygen to give a system similar to that operating in chemosynthetic organisms. Now, the important discovery by Arnon’s group that O2 production and reduction of H-acceptors is coupled with phosphorylation dispose of the need for a separate reoxidation system supplying the active phosphate groups. Also this discovery tends to show that, apart from Mg2⁺, the co-factors used in the artificially constituted phosphorylating system need not be necessarily specific, just as in the case of the original H-acceptors for oxygen production. In other words, they are not in the strict sense “co-factors” at all. The phosphorylation reaction itself now appears as a part of the normal photochemically induced H-transfer. Of course it is essential to find out what are the substances in the cell responsible for establishing the photochemically driven transport, but until the nature of the coupling between reduction and phosphorylation has been analyzed the present argument remains unaffected. It does not follow from the argument that all substances capable of being reduced by illuminated chloroplast preparations would be capable of initiating phosphorylation. The coupling between reduction and phosphorylation can be abolished and an active agent can become inhibitory at higher concentrations so that the affinities relating to the chloroplast system have to be in a suitable range.

In the original experiments of Arnon, Whatley and Allen (2) with phosphorylation in the presence of ascorbate and magnesium ion only, O2 was found to be necessary. Our experiments confirmed this, and also showed that the initial activity was maintained only for a short period. We did not determine whether the loss of activity was due to a disturbance of an endogenous H-acceptor or inactivation of the whole system. In the more active systems when FMN and K3 were added, the phosphorylation reaction was found by Arnon’s group (3,18) to progress better or slightly better in the absence of O2. More recently, systems have been used (that with pyocyanine may serve as example) where no difference is observed between the rates observed in the presence and absence of O2. On the basis of a requirement for establishing H-transport in the illuminated chloroplast system by catalytic amounts of added material, these differences could readily be explained as follows. The added material can be represented by X which is reduced to XH2 in light and has to be continuously oxidized to X again so as to maintain the H-transport in the system. If XH2 can only be oxidized in the presence of free O2 then the system will require O2 for activity. If XH2 can be oxidized rapidly by the photochemical system in a stage prior to the liberation of O2 then the system would be equally active in air or anaerobic conditions. If XH2 is also oxidized by O2 and gives H2O2 this will result in a “Mehler reaction.” The H2O2 is likely to cause loss of activity if -SH groups become oxidized. Thus in this case even in the pres-
ence of ascorbate, an improvement could result when O₂ is removed.

It may perhaps seem to some workers too sweeping a generalization to regard the phosphorylation reaction as forming a part of the photochemically induced H-transport system in the chloroplast. This would imply that differences in behavior of the so-called co-factors for phosphorylation are being governed by their reaction with the terminal H-donor in the chloroplast preparation. The problem is obviously simplified when phosphorylation can be studied in relation to a stoichiometric reduction of an H-acceptor. When a catalytic amount of an H-acceptor is used it introduces the further problem of reoxidation which can be supposed to occur by alternative mechanisms. The conclusion presented by Geller and Gregory (8) in relation to hydrogen transport in their work on phosphorylation with Rhodospirillum extracts may in part be applicable to the chloroplast system in the green plant.

**Summary**

Several substances have been found to increase phosphorylation at low concentration (10⁻³ M) in experiments carried out in air.

Pyocyanine and phenazine methosulphate were found to be the most active of the substances tested. Pyocyanine required a concentration of 6.3 × 10⁻⁴ M to give one half the maximum activity which was given with 1 × 10⁻⁴ M. FMN and K3 were found to give full activity at 10⁻⁴ M and one half activity at 5 × 10⁻⁴ M. It was concluded that the activity observed with phenazine methosulphate was due to its rapid conversion to pyocyanine in light. The effect of changes in pH and of storage times of chloroplast preparations in the system containing pyocyanine showed marked differences from the system containing FMN and K3.

The authors would like to thank Professor D. Kelin for a catalase preparation and for a sample of phenazine. They would also like to thank Dr. D. E. Green for a small quantity of phenazine methosulphate and Dr. S. Aronoff for the naphthoquinone sulphonates.

**Literature Cited**