Studies on the Hydrolysis of Adenosine Triphosphate by Spinach Chloroplasts

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(Received for publication, June 11, 1964)

During studies on photophosphorylation in cell free preparations of the blue-green alga, Anabaena variabilis, a photohydrolysis of ATP was found to be induced by high concentrations of cysteine or GSH, while photophosphorylation became inhibited (1, 2). A similar photohydrolysis was also reported with chloroplasts from spinach leaves, but still higher sulfhydryl concentrations were required to inhibit ATP synthesis and to induce ATP hydrolysis. Both light-dependent reactions (2) required an electron carrier and Mg++ and both were inhibited by ammonium ions and n-butyl 3,5-diiodo-4-hydroxybenzoate.

This report describes some additional characteristics of the light-dependent ATPase in spinach chloroplasts. The studies support the conclusion that ATP synthesis and hydrolysis in chloroplasts are closely interrelated and suggest that at least a part of the light-induced electron transport pathway is common to both processes. During this study it was found that illumination of the system in the absence of ATP, followed by addition of the substrate in the dark, still resulted in ATP hydrolysis. The data suggest that an intermediate is generated in the light phase which supports ATP hydrolysis in the dark.

EXPERIMENTAL PROCEDURE

Materials

Reduced lipoic acid was prepared by a procedure based on that reported by Gunsalus and Razzell (3). Borohydride was added until the solution appeared colorless, for which a 10-fold excess was usually required. The benzene extract of the free acid was washed several times with cold water to remove salts which had formed during the reduction; it was then dried with anhydrous Na2SO4. The benzene was removed under vacuum.

PREPARATION OF CHLOROPLASTS—Spinach chloroplasts were prepared essentially as described by Jagendorf and Avron (5). Trimmed spinach leaves (110 g) were ground for 30 seconds, in a Waring Blender, with 350 ml of a mixture containing 0.40 M sucrose, 0.05 M Tris, pH 7.8, and 0.01 M NaCl. The chloroplasts were separated by centrifugation (5), and finally suspended, at a concentration of 3 to 4 mg of chlorophyll per ml, in a sucrose-Tris-NaCl solution which contained 40 volume % ethylene glycol. The activity was retained for 1 week when stored at −20°. The ethylene glycol prevented freezing and did not affect the activity. Chloroplasts prepared from spinach grown in different regions varied in activity. Best results were obtained with Adirondack or Long Island spinach.

Standard Assay for Photophosphorylation and ATP Hydrolysis—Photophosphorylation and photolysis were both followed by measuring the change in P1 which occurred during incubations under standardized conditions. Reactions were carried out in flat-bottomed test tubes; the tubes were incubated at 25° under nitrogen, in a modified Warburg apparatus equipped with four photo-flood bulbs which delivered 30,000 foot-candles at the bottom of the tubes. Incubations were usually carried out either for 15 minutes in continuous light or for 13 minutes in the dark, following a 2-minute initial incubation in light. The reaction was terminated with 0.3 ml of 50% trichloroacetic acid, added through the air outlet hole of the rubber stopper. The precipitate was filtered off in the cold, and P1 was measured in the filtrate by the method of Lohmann and Jendrassik (6). Appropriate zero time and dark-incubated controls were included with each experiment. The reaction mixture for the standard photophosphorylation assay contained the following in 2.7 ml: 7.4 mM potassium phosphate, pH 7.8; 7.4 mM ADP; 7.4 mM MgCl2; 74 mM Tris buffer, pH 7.8; 0.056 mM PMS; and approximately 0.06 mg of chlorophyll. The reaction mixture purchased from Mann Research Laboratories, Inc. No difference between the two preparations was found in our experiments. DIB was purchased from Aldrich Chemical Company, Inc. PMS, ATP, and ADP were obtained from Sigma Chemical Company. We are grateful to Dr. P. G. Heytler for a generous gift of carbonyl cyanide p-trifluoromethylphenylhydrazone and carbonyl cyanide m-chlorophenylhydrazone.

Methods

The abbreviations used are: DIB, n-butyl 3,5-diiodo-4-hydroxybenzoate; PMS, N-methylphenazonium methosulfate; CCP, carbonyl cyanide m-chlorophenylhydrazone.
employed to follow ATP hydrolysis contained the following in 2.7 ml: 5.6 mM ATP; 7.4 mM MgCl₂; 7.4 mM reduced lipoic acid; 0.74 mM NH₄Cl; 0.056 mM PMS; 74 mM Tris buffer, pH 8.0; and approximately 0.20 mg of chlorophyll. NH₄Cl was usually omitted from assays in which ATP hydrolysis was carried out in the dark following a 2-minute initial incubation in light. Variations from these amounts or addition of other components are indicated for each reported experiment. Each determination for ATP hydrolysis was corrected by the Pi formed in dark controls. Chlorophyll was determined according to Arnon (7) on aliquots of the diluted chloroplast suspension used for each experiment. Adenine nucleotides were determined spectrophotometrically following separations on Dowex 1-chloride columns as described by Cohn and Carter (8). Reduced lipoic acid was estimated according to Ellman (9).

**RESULTS**

The rates of photophosphorylation and photohydrolysis were compared in chloroplasts that were prepared as described above and chloroplasts which were purified by the glycerol gradient technique, which eliminates mitochondria (10). This purification usually did not alter photohydrolysis activity. In those cases where some activity was lost, the ratio of photophosphorylation to photohydrolysis activity remained constant for both preparations. These observations indicate that light-dependent ATP hydrolysis is a chloroplast activity.

**Effect of Sulfhydryl Compounds**—The dependence of photohydrolysis on high concentrations of a sulfhydryl derivative was much more pronounced with spinach chloroplasts than with the algal system. It has now been found that reduced lipoic acid is much more active than either GSH or cysteine, which had been used in earlier studies. Fig. 1 compares the relative effectiveness of reduced lipoic acid and cysteine as activators for ATP hydrolysis. Maximum rates were obtained with 3 to 4 mM reduced lipoic acid, whereas 20 times this level of cysteine was required to approach the same rate. 1,3-Dimercaptopropanol was as active as reduced lipoic acid but 2,3-dimercaptopropanol was inactive under the same conditions.

Fig. 2 shows the inverse effect of reduced lipoic acid on photophosphorylation and photohydrolysis. Concentrations of reduced lipoic acid which induced maximum ATP hydrolysis strongly inhibited synthesis. Maximum rates were obtained with 3 to 4 mM reduced lipoic acid, whereas 20 times this level of cysteine was required to approach the same rate. 1,3-Dimercaptopropanol was as active as reduced lipoic acid but 2,3-dimercaptopropanol was inactive under the same conditions.

**Effect of Ammonium Ions**—It was previously reported that ammonium ions, at concentrations near 3 mM, strongly inhibited both photophosphorylation and photohydrolysis. It has now been found, however, that ammonium ions at approximately 0.74 mM are usually required for maximum rates of hydrolysis. Thus, 0.74 mM NH₄Cl stimulated ATP hydrolysis, but inhibited ATP synthesis, whereas 3 mM NH₄Cl inhibited both reactions. The inhibitory effects were consistently observed, but the stimulatory effects varied from 20 to 800% increase in activity; usually a 3-fold activation was observed. Similar effects were obtained with equivalent concentrations of (NH₄)₂SO₄.

The possibility was considered that the stimulation of photohydrolysis by NH₄Cl was related to its inhibitory effect on photophosphorylation. Under some conditions both light-induced reactions may proceed simultaneously. The measured change in Pₐ, at any time, would then be the resultant of the two opposing reactions. Since the rate of ATP synthesis is considerably faster than that of ATP hydrolysis, it may be
anticipated that maximum rates of hydrolysis would require complete inhibition of ATP synthesis.

Table I shows that photophosphorylation is inhibited 73% by 0.74 mM NH₄Cl, 78% by 5.6 mM reduced lipoic acid, and 100% by the combination of the two. In the same experiment, the addition of 0.74 mM NH₄Cl in the presence of 5.6 mM reduced lipoic acid produced a 3-fold stimulation of the hydrolysis observed with reduced lipoic acid alone. When the concentration of reduced lipoic acid was increased to 14.8 mM, photophosphorylation was found to be completely inhibited even without added NH₄Cl and 0.74 mM NH₄Cl did not stimulate photophosphorylation. In the absence of reduced lipoic acid no ATP was hydrolyzed even in the presence of NH₄Cl at concentrations which completely inhibited photophosphorylation. Thus, complete inhibition of photophosphorylation was itself insufficient to elicit hydrolysis indicating a more specific function for the sulfhydryl derivative.

Experiments carried out with different preparations of reduced lipoic acid and chloroplasts were associated with considerable variability in the above observations, but after many experiments the interrelationship between the ammonium ion effect and the sulfhydryl concentration became apparent and is illustrated in Fig. 3.

The rate of hydrolysis as a function of reduced lipoic acid concentration, both in the presence and absence of NH₄Cl, is shown in Fig. 3A. It is apparent that without NH₄Cl considerably more reduced lipoic acid was required for maximum activity. The stimulation by NH₄Cl was most pronounced at approximately 3 mM reduced lipoic acid. Lower concentrations were insufficient to support hydrolysis, even in the presence of NH₄Cl although the combination inhibited photophosphorylation 95%. The stimulation by NH₄Cl decreased as the sulfhydryl concentration was increased. This is apparent in Fig. 3B, in which the effect of NH₄Cl on the rate of hydrolysis with 3 mM and 14.8 mM reduced lipoic acid is described. At the lower sulfhydryl concentration, a 9-fold activation was found at the optimum NH₄Cl concentration of 0.74 mM. No significant stimulation was apparent at the higher sulfhydryl concentration. Higher concentrations of NH₄Cl were inhibitory at both sulfhydryl concentrations. ATP hydrolysis was completely inhibited by NH₄Cl at concentrations which completely inhibited photophosphorylation.

Stoichiometry of Hydrolysis—Reduced lipoic acid and Pi were determined in zero time and dark controls, and in reaction mixtures which had been incubated in light. No reduced lipoic acid

Table I

<table>
<thead>
<tr>
<th>Reduced lipoic acid (mM)</th>
<th>ATP synthesis</th>
<th>ATP hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;NH₄Cl</td>
<td>+NH₄Cl</td>
<td>&lt;NH₄Cl</td>
</tr>
<tr>
<td>0</td>
<td>580</td>
<td>160</td>
</tr>
<tr>
<td>5.6</td>
<td>126</td>
<td>0</td>
</tr>
<tr>
<td>14.8</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Fig. 3. Interrelationship between the effect of NH₄Cl and reduced lipoic acid on photophosphorylation of ATP in spinach chloroplasts. A, the effect of increasing the concentration of reduced lipoic acid on the rate of hydrolysis in the absence and presence of 0.74 mM NH₄Cl. The assays were performed as described under ‘Methods’ except that the concentrations of NH₄Cl and reduced lipoic acid were varied as indicated. B, the effect of increasing the concentration of NH₄Cl on the rate of hydrolysis in the presence of 3.0 or 14.8 mM reduced lipoic acid. The assays were performed as described under ‘Methods’ except that the concentrations of NH₄Cl and reduced lipoic acid were varied as indicated.
Table II
Stoichiometry of photohydrolysis reaction

<table>
<thead>
<tr>
<th>Incubation conditions</th>
<th>ATP</th>
<th>ADP</th>
<th>AMP</th>
<th>Pi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Found</td>
<td>4.618</td>
<td>0.107</td>
<td>0</td>
<td>0.02</td>
</tr>
<tr>
<td>Dark</td>
<td>4.454</td>
<td>0.228</td>
<td>0.061</td>
<td>0.14</td>
</tr>
<tr>
<td>Difference</td>
<td>-0.164</td>
<td>+0.118</td>
<td>+0.061</td>
<td>+0.12</td>
</tr>
<tr>
<td>Difference corrected for myokinase</td>
<td>-0.225</td>
<td>+0.240</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Light</td>
<td>2.390</td>
<td>2.176</td>
<td>0.215</td>
<td>2.44</td>
</tr>
<tr>
<td>Difference</td>
<td>-2.228</td>
<td>+2.009</td>
<td>+0.215</td>
<td>+2.42</td>
</tr>
<tr>
<td>Difference corrected for myokinase</td>
<td>-2.443</td>
<td>+2.499</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Difference corrected for dark</td>
<td>-2.218</td>
<td>+2.289</td>
<td></td>
<td>2.30</td>
</tr>
</tbody>
</table>

was found to be utilized during the hydrolysis of 11.2 μmoles of ATP. Evidently, the sulfhydryl derivative acts catalytically and is not stoichiometric with ATP. The possibility is not eliminated that the dithiol reduces a trace chloroplast component.

In other experiments, the formation of Pi was correlated with changes in adenine nucleotides. Typical data, shown in Table II, indicate that ADP and not AMP is the reaction product. The small amount of AMP which did form was probably derived from the ADP via myokinase activity. If this assumption is made, a correction for myokinase activity may be calculated, based on the AMP change, together with the established stoichiometry for the myokinase reaction. With this correction, excellent agreement was obtained between the ATP utilized in light and the ADP and Pi which formed.

Separation of Photohydrolysis of ATP into Light and Dark Stages—The effect of light intensity was compared for ATP synthesis and hydrolysis. Reduction from 30,000 to 3,000 foot-candles decreased photophosphorylation to one-fourth but photohydrolysis to three-fourths of the rate found at high light intensity. Further studies concerning the role of illumination revealed that light was not required throughout the incubation period, but was necessary only to initiate a reaction which then continued in the dark. Fig. 4 shows that after 2 minutes of illumination at 30,000 foot-candles, ATP was hydrolyzed for at least 30 minutes at the same constant rate in the dark or in continued illumination.2

When the hydrolysis is carried out in the dark following a 2-minute initial incubation in light, it is termed light-activated hydrolysis, in order to distinguish it from hydrolysis in continuous light. This two-stage technique was employed to determine the requirements for the light phase of hydrolysis. Single components, omitted during the initial incubation in light, were added as the lights were extinguished. ATP hydrolysis was measured for the subsequent dark incubation of the completed system.

As shown in Table III, the electron carrier, reduced lipoic acid, and also Mg++ are essential for the light phase of the reaction. However, ATP added as the lights are extinguished is hydrolyzed as rapidly as if it had been present during the light phase. In the experiment described in Table III, hydrolysis was indeed higher with delayed addition of ATP; this was frequently but not always observed.

A low activity was sometimes seen without added PMS when incubations were carried out in air; such an activity was highest in freshly prepared, unwashed chloroplasts, but it was lost almost completely if washed chloroplasts were stored at -20° for 3 days. The activity in the absence of PMS is most likely produced by small amounts of endogenous electron carrier and the requirement for oxygen may be analogous to that found for endogenous photophosphorylation (11). Under anaerobic conditions the reaction was usually fully dependent on PMS.

The observations suggest that an intermediate, formed in the light phase, supports ATP hydrolysis in the dark. The sta-

![Fig. 4. Rate of ATP hydrolysis in the dark following a 2-minute initial incubation in light and in continuous light. The reaction mixture was the same as that described for the standard assay except that NH₄Cl was omitted only from the tubes which were incubated under light-dark conditions. The tubes were incubated in continuous light (X-X), or in the dark following 2 minutes of illumination (O-O), or in the dark (●-●). The assays were completed as described under "Methods." The values in the upper curve were corrected by subtracting the dark controls.](http://www.jbc.org/)

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2 We have reported the separation of ATP hydrolysis into light and dark stages at the Gordon Conference, Tilton, New Hampshire, July 1963.
TABLE III
Requirements for light phase of ATP hydrolysis

The complete reaction mixture was the same as that used for the standard assay without NH₄Cl. The tubes were incubated for 2 minutes in light in the absence of the indicated component, which was added as the lights were extinguished. The completed system was incubated for 13 minutes in the dark, and the analyses were performed as described under "Methods."

<table>
<thead>
<tr>
<th>Component omitted during light phase</th>
<th>ATP hydrolyzed (pmoles/hr/mg chlorophyll)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>90</td>
</tr>
<tr>
<td>PMS</td>
<td>0</td>
</tr>
<tr>
<td>Reduced lipoic acid</td>
<td>0</td>
</tr>
<tr>
<td>Chloroplasts</td>
<td>0</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>3</td>
</tr>
<tr>
<td>ATP</td>
<td>138</td>
</tr>
</tbody>
</table>

Fig. 5. Decay of the light-generated intermediate associated with ATP hydrolysis. A series of tubes were prepared containing the full system as described for the standard assay, minus ATP; they were incubated for 2 minutes in the light; the light was extinguished and ATP was added to the tubes in sequence, at 5-second intervals; the incubation was continued for 13 minutes in the dark. The assays were completed as described under "Methods." The time in seconds is the interval between extinguishing the lights and the addition of ATP.

Fig. 6. Rate of formation (at 30,000 foot-candles) of the intermediate in ATP hydrolysis. The reaction mixture was the same as that described for the standard assay without NH₄Cl. The tubes were incubated in the light for the indicated period of time, followed by a 15-minute incubation in the dark. Each value was corrected both for the Pi formed during the light period alone and the equivalent incubation in dark only. Thus, the activities plotted refer to the ATP hydrolyzed during the 15-minute dark incubation following the indicated period of illumination.
varied with different chloroplast preparations. Consequently, it appeared that valid comparisons could be made only among samples which had been prepared with the same materials and incubated within 1 hour of each other. Generally, two incubations were carried out in rapid succession. The first incubation, in continuous light, included all samples for both ATP synthesis and hydrolysis. The second incubation was carried out in the dark, following a 2-minute initial incubation in light and included samples in which the test compound was added before or after the light phase. The effects found with several inhibitors are illustrated in Table IV.

As described above, 3 mM NH₄Cl inhibited both photophosphorylation and photolysis, whereas 0.74 mM NH₄Cl inhibited photophosphorylation approximately 80%, but strongly stimulated hydrolysis in continuous light, particularly at suboptimal levels of reduced lipoic acid. The stimulation of hydrolysis by NH₄Cl appeared to be indirect. NH₄Cl, at 0.74 mM, partially inhibited photophosphorylation and thus eliminated the contribution of ATP synthesis to the over-all change in Pᵢ. Further support for this explanation of the ammonium ion effect is shown in Experiment 1 of Table IV. When hydrolysis was carried out in the dark following a 2-minute incubation in light, the contribution by photophosphorylation was negligible and maximum rates of hydrolysis were observed without added NH₄Cl. In fact, the same concentration of NH₄Cl which stimulated hydrolysis in continuous light, inhibited the light-activated ATPase in the light phase only. Rather varied effects were obtained when NH₄Cl was added in the dark phase. Usually a small stimulation was obtained as reported by Hoch and Martin (12), but often no effect was found and occasionally hydrolysis was inhibited.

Experiment 2 shows that oxidized lipoic acid mimics almost all of the effects observed with NH₄Cl. It inhibited both photophosphorylation and the light phase of ATP hydrolysis. The dark phase was not affected by the same concentration of oxidized lipoic acid but higher concentrations usually did inhibit. Hydrolysis in continuous light was completely inhibited by oxidized lipoic acid at concentrations which completely inhibited photophosphorylation. However, at concentrations which only partially inhibited photophosphorylation, oxidized lipoic acid markedly stimulated hydrolysis in continuous light as did NH₄Cl. The stimulatory effects were dependent on the sulphydril concentration as described above for NH₄Cl. Further, the effects of NH₄Cl and oxidized lipoic acid appeared to be additive. The combination of oxidized lipoic acid and NH₄Cl, each at its optimum concentration when tested alone, together inhibited hydrolysis, as if the combination acted like an increased concentration of either alone. Oxidized lipoic acid could not substitute for reduced lipoic acid under any conditions.

The marked effects of oxidized lipoic acid on photolysis indicate the necessity for using completely reduced lipoic acid in these studies. The optimum concentration for both NH₄Cl and reduced lipoic acid varied within narrow limits with different chloroplast and reduced lipoic acid preparations and explains, at least in part, the variability mentioned above.

Heytler reported that derivatives of carbonyl cyanide phenylhydrazone were potent inhibitors of photophosphorylation, as well as uncouplers of oxidative phosphorylation (14, 15). The effects found with CCP in the present study are described in Experiment 3 of Table IV. Both ATP synthesis and hydrolysis were completely inhibited by 3.3 × 10⁻⁵ m CCP. At a concentration of 2.2 × 10⁻⁵ m CCP, photophosphorylation was still approximately 80% inhibited but hydrolysis in continuous light was markedly stimulated. Again, the stimulation was obtained only at specific levels of reduced lipoic acid and was abolished in the presence of NH₄Cl. Thus, CCP behaved like NH₄Cl when tested in continuous light. However, the light-activated ATPase was much more sensitive to inhibition by the CCP derivatives than was either hydrolysis in continuous light or photophosphorylation. Further the inhibitor was just as effective when it was added after the lights were extinguished. Similar observations were found with the p-trifluoromethoxy derivative but, compared with CCP, several-fold lower concentrations were required to produce the same effects.

Another inhibitor of oxidative and photophosphorylation, n-butyl 3,5-diido-4-hydroxybenzoate, was previously reported also to inhibit photophosphorylation (1, 2). The effect of this compound on photolysis was reinvestigated in view of the findings described above. In contrast to the other inhibitors of photophosphorylation, this compound did not significantly stimulate hydrolysis in continuous light at any concentration tested, together with several different levels of reduced lipoic acid. As indicated in Experiment 4, the compound inhibited both photophosphorylation and photolysis. It was, however, a little less inhibitory in the absence of NH₄Cl.

### Table IV

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>ATP synthesis (μmoles/mg chlorophyll)</th>
<th>ATP hydrolysis (μmoles/mg chlorophyll)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Continuous light</td>
<td>Light-activated</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NH₄Cl</td>
<td>+NH₄Cl</td>
</tr>
<tr>
<td>1. NH₄Cl</td>
<td>0.00</td>
<td>600</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>0.74</td>
<td>130</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>3.00</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2. Oxidized lipoic acid</td>
<td>0.00</td>
<td>600</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>0.14</td>
<td>120</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>0.28</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3. CCP</td>
<td>0.000</td>
<td>980</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>0.022</td>
<td>214</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>0.033</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4. DIB</td>
<td>0.000</td>
<td>570</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>0.025</td>
<td>189</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>0.050</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5. Arsenite</td>
<td>0.00</td>
<td>540</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>1.85</td>
<td>540</td>
<td>0</td>
</tr>
</tbody>
</table>
Arsenite had no effect on photophosphorylation, whereas ATP synthesis was completely inhibited by 1.85 mM arsenite, even in the presence of 14.8 mM reduced lipoic acid. This indicated that the inhibition of photophosphorylation by reduced lipoic acid occurred because a large part of the arsenite was bound by the added reduced lipoic acid. The effective arsenite concentration may be many-fold lower.

The same concentration of arsenite was without effect when added in the dark phase of hydrolysis. However, arsenite inhibited the dark phase at concentrations which approached that of reduced lipoic acid, suggesting that reduced lipoic acid participates both in the light and dark phase of hydrolysis.

Table IV describes four different types of inhibitor effects. For clarity these effects are summarized in Table V. The stimulation of hydrolysis in continuous light, indicated in Table V for NH₄Cl, oxidized lipoic acid, and CCP was found only at suboptimal levels of reduced lipoic acid and only at concentrations which inhibited photophosphorylation approximately 50%. All three compounds inhibited hydrolysis in continuous light at concentrations which completely inhibited photophosphorylation.

Table V

<table>
<thead>
<tr>
<th>Compound</th>
<th>ATP synthesis continuous light</th>
<th>Light-activated ATP hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Light stage</td>
</tr>
<tr>
<td>NH₄Cl or oxidized lipoic acid</td>
<td>Inhibits</td>
<td>Stimulates</td>
</tr>
<tr>
<td>CCP</td>
<td>Inhibits</td>
<td>Stimulates</td>
</tr>
<tr>
<td>DIB</td>
<td>Inhibits</td>
<td>Inhibits</td>
</tr>
<tr>
<td>Arsenite</td>
<td>No effect</td>
<td>Inhibits</td>
</tr>
</tbody>
</table>

DIB also behaved differently than NH₄Cl in the light-activated hydrolysis. It was almost as effective an inhibitor when it was present during the dark phase only, compared with its effect when present throughout both phases.

Since reduced lipoic acid exerted such marked effects on both photophosphorylation and photohydrolysis, the possible involvement of a dithiol enzyme was considered. For this reason, the effect of arsenite on both reactions was examined. No evidence was found for an effect of arsenite on photophosphorylation. However, ATP hydrolysis in continuous light or in the light stage of hydrolysis carried out in two stages was completely inhibited by 1.85 mM arsenite, even in the presence of 14.8 mM reduced lipoic acid. This indicated that the inhibition was not caused by decreasing the effective concentration of reduced lipoic acid but rather suggests that an enzyme dithiol participates in the light phase of ATP hydrolysis, but not in photophosphorylation. However, arsenite did not overcome the inhibition of photophosphorylation by reduced lipoic acid. The concentration of arsenite required to inhibit photophosphorylation is high compared with other dithiol enzyme reactions, probably because a large part of the arsenite is bound by the added reduced lipoic acid. The effective arsenite concentration may be many-fold lower.

The same concentration of arsenite was without effect when added in the dark phase of hydrolysis. However, arsenite inhibited the dark phase at concentrations which approached that of reduced lipoic acid, suggesting that reduced lipoic acid participates both in the light and dark phase of hydrolysis.

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**DISCUSSION**

Photophosphorylation and photohydrolysis appear to be closely interrelated. Both activities are catalyzed by purified chloroplasts, require light, Mg²⁺, and an electron carrier, and both are inhibited by ammonium ions, oxidized lipoic acid, n-butyl 3,5-diiodo-4-hydroxybenzoate, and derivatives of carbonyl cyanide phenylhydrazone. There are two essential differences between the two processes. First, reduced lipoic acid is required for photohydrolysis but inhibits photophosphorylation. In the absence of reduced lipoic acid, however, none of the inhibitors induced any ATPase activity. Thus, complete inhibition of photophosphorylation is itself insufficient to elicit hydrolysis and indicates a more specific function for the sulphydryl derivative in the mechanism of hydrolysis. Second, ATP hydrolysis requires light only to initiate the reaction whereas ATP synthesis is dependent on a continuous source of light energy. In the hydrolysis, PMS, Mg²⁺, and oxidized lipoic acid are required for the light phase. ATP added in the dark within 50 seconds after the light is extinguished is hydrolyzed and hydrolysis continues for at least 30 minutes. These observations suggest that an intermediate is generated in the light phase which supports ATP hydrolysis in the dark. Since hydrolysis continues in the dark, it appears that the intermediate is regenerated during hydrolysis.

Photophosphorylation has also been separated into a light and dark stage by Hind and Jagendorf (16) and by Shen and Shen (17). Their experiments demonstrated that spinach chloroplasts illuminated in the presence of an electron carrier and then added to ADP in the dark still supported ATP synthesis. In contrast to photohydrolysis the dark reaction of photophosphorylation was complete in 5 seconds. Both groups concluded that a nonphosphorylated energy-rich intermediate was generated in the light which transferred its energy to ATP in the dark. The intermediate was consumed in the process and ATP synthesis stopped.

A working hypothesis is suggested to explain the interrelationship between ATP synthesis and hydrolysis in spinach chloroplasts. The mechanism, generally considered by many investigators for both oxidative and photophosphorylation, was modified to accommodate the observations described under "Results."

\[
\begin{align*}
X' + C & \xrightarrow{\text{light}, \text{PMS}} X + C \\
X & \sim C + ADP + Pi \Rightarrow ATP + X + C \\
X & \rightarrow X' \\
X & \sim C \xrightarrow{\text{SH}} X + C
\end{align*}
\]

Step 1 includes the light-induced electron transport, which is coupled at the site X' — X to generate a nonphosphorylated energy-rich intermediate between the carrier X and the coupling factor C. X' — X represents the oxidized and reduced form of a carrier in the electron transport chain. However, there is no evidence to suggest whether the energy-rich intermediate X ~ C involves the oxidized or reduced form of the carrier.

In Step 2, the energy transfer to ATP is catalyzed via a phosphorylated intermediate. Step 3 completes the cycle for photophosphorylation by returning the carrier X to its original state.

ATP hydrolysis, per se, is considered to be a reversal of Step 2. X is required for the reversal, and it must be generated from X' via a reaction involving electron transport. Step 1, the light-induced electron transport through the formation of X ~ C, is considered to be common to both ATP synthesis and hydrolysis.

This suggestion is based on the observation that both photophosphorylation and the light phase of ATP hydrolysis require an electron carrier and Mg²⁺ and both photophosphorylation and the light phase of hydrolysis are inhibited by NH₄Cl, oxidized lipoic acid, and derivatives of CCP. Reduced lipoic acid
uncouples according to Reaction 4 releasing X. However, X may be highly unstable and any free X would immediately revert to its original state $X'$ according to Reaction 3. Thus, it is necessary to stabilize $X$ in order to elicit ATP hydrolysis.

Arsenite did not inhibit photophosphorylation but it completely inhibited the light phase of hydrolysis, suggesting that an enzyme dithiol participates in hydrolysis but not in synthesis. The function attributed to the enzyme dithiol is to bind $X$ and prevent it from reverting to $X'$. ATP is then hydrolyzed via a reversal of Step 2 but utilizing enzyme-bound $X$. $X \sim C$ is regenerated during the hydrolysis step, but it remains enzyme-bound. The cycle of uncoupling by reduced lipoic acid followed by a reversal of Step 2 while $X$ remains enzyme-bound may then continue. This cyclic process explains the observation that ATP continues to be hydrolyzed in the dark following a short period of illumination.

$$X \sim C + L(SH)_2 \rightarrow L-SH-S-X + C \quad (5)$$

$$L-SH-S-X + Enz(SH)_2 \rightarrow Enz-SH-S-X + L(SH)_2 \quad (6)$$

Reactions 5 and 6 describe the mechanism for the uncoupling activity of reduced lipoic acid ($L(SH)_2$). The unique function attributed to reduced lipoic acid is that it couples it acts as a carrier of $X$ to the enzyme dithiol ($Enz(SH)_2$). Support for this sequence is the observation that the inhibition of photophosphorylation by reduced lipoic acid is not overcome by arsenite, suggesting that the reaction involving reduced lipoic acid precedes the one involving the enzyme dithiol. Thus, uncoupling in itself is insufficient to elicit ATP hydrolysis and explains the more specific requirement for reduced lipoic acid.

The ATPase of chloroplasts is considered to involve a light-induced electron flow to generate the "energy-rich" form of the carrier which is required for the reversal of ATP synthesis. This suggestion is consistent with the observations by Wadkins and Lehninger (18) that the rate of mitochondrial ATPase is maximal when the respiratory carriers are in the oxidized state. The effects observed with several inhibitors may be explained by the proposed mechanism. The same concentration of NH$_4$Cl, oxidized lipoic acid, and derivatives of CCP which completely inhibited photophosphorylation also completely inhibited the light phase of hydrolysis, suggesting that these compounds inhibit Step 1. However, at concentrations which partially inhibit photophosphorylation, all three compounds were found to stimulate hydrolysis measured with continuous light and sub-optimal levels of reduced lipoic acid.

Since almost maximum rates of hydrolysis were obtained following only 2 minutes of illumination, it appears that, in continuous light, Step 1 is not rate-limiting for hydrolysis. Consequently, the level of $X \sim C$ may be decreased to that which can be accommodated by Reaction 4 without effect on the actual rate of hydrolysis. However, the cycle of photophosphorylation proceeds in continuous light utilizing the fraction of $X \sim C$ which is not uncoupled by reduced lipoic acid. A decrease in $X \sim C$ to a point where it can be completely uncoupled by reduced lipoic acid would eliminate any contribution via synthesis and thus produce a maximum resultant in the direction of hydrolysis. In contrast, when hydrolysis is carried out in the dark following 2 minutes of illumination, the contribution to the over-all change in $P_i$ by photophosphorylation is negligible. In addition, under these conditions Step 1 is close to being rate-limiting, even for hydrolysis, and consequently any decrease in $X \sim C$ would inhibit hydrolysis.

CCP was unique in that the dark stage of hydrolysis was inhibited at lower levels of CCP than was either photophosphorylation or hydrolysis in continuous light. Thus, it is necessary to attribute a second activity to CCP. It appears to destroy a component which is required for the dark cycle of hydrolysis but which had been generated during the light phase. A possible explanation is that CCP liberates $X$ from the enzyme dithiol. Free $X$ would immediately revert to $X'$ (Reaction 3) and hydrolysis in the dark would stop. This suggestion is supported by the finding that the CCP derivatives react with vicinal dithiols.

DBI inhibited both photophosphorylation and photohydrolysis. It did not stimulate hydrolysis in continuous light as did NH$_4$Cl, and further, it was just as inhibitory when it was added in the dark stage of hydrolysis where the actual cleavage of ATP occurs. Thus, it appears to inhibit Step 2.

According to the proposed mechanism, photohydrolysis interrupts the electron transport pathway at the site where it is coupled to phosphorylation. Consequently, it may be anticipated that further studies with the use of inhibitors of electron transport may localize the site at which coupling occurs during photophosphorylation.

The ATPase activity described above differs from that found in Swiss chard chloroplasts by Avron (19) in that the latter system requires Ca$^{2+}$, is inhibited by Mg$^{2+}$, and is unaffected by sulfhydryl compounds. We have observed a light-dependent ATPase activity in spinach chloroplasts under the conditions described by Avron but the activity was never more than 15% of that found under our conditions. The relationship between the two systems is unclear.

As mentioned above, both Hoch and Martin (12) and Marchant and Packer (19) have confirmed the sulfhydryl-induced ATPase in chloroplasts and have also separated the reaction into light and dark stages. Marchant and Packer (13) have correlated light-induced ATPase with light-scattering changes which appear to reflect structural alterations in the chloroplast membrane. The authors suggest that these changes may be caused by some high energy intermediate which was generated during the light phase of hydrolysis by a mechanism involving electron transport. It is evident that uncoupling by reduced lipoic acid (Step 4 of the proposed mechanism) would release energy which originates either from the light-induced electron flow or from ATP during the dark stage of hydrolysis. This energy could be utilized to support the structural changes.

**SUMMARY**

Photophosphorylation and photohydrolysis appear to be closely interrelated. Both activities are catalyzed by purified chloroplasts, require light, Mg$^{2+}$, and an electron carrier, and both are inhibited by ammonium ions, oxidized lipoic acid, n-butyl 3,5-diiodo-4-hydroxybenzoate, and derivatives of carboxyl cyanide phenylhydrazone. There are two essential differences between the two processes. First, reduced lipoic acid is required for photohydrolysis but inhibits photophosphorylation. Second, ATP hydrolysis requires light only to initiate the reaction whereas ATP synthesis is dependent on a continuous source of light energy. An electron carrier, Mg$^{2+}$, and reduced lipoic acid are required for the light phase of hydrolysis. ATP added in the dark, within 50 seconds after the light is extin-
guished, is hydrolyzed, and hydrolysis continues for at least 30 minutes. These observations suggest that an intermediate is generated in the light phase which supports ATP hydrolysis in the dark. Since hydrolysis continues in the dark, it appears that the intermediate is regenerated during hydrolysis.

Ammonium chloride and oxidized lipoic acid exert their inhibitory effect in the light phase of hydrolysis; n-butyl 3,5-diiodo-4-hydroxybenzoate inhibits the dark phase. Maximum rates of hydrolysis in continuous light are obtained only when photophosphorylation is inhibited 100%. However, complete inhibition of photophosphorylation is, itself, insufficient to elicit ATP hydrolysis; reduced lipoic acid is still required, indicating a more specific function for the sulphydryl derivative in the mechanism of ATP hydrolysis. Arsenite does not affect photophosphorylation, but it inhibits photohydrolysis, in the light phase, at concentrations which suggest the participation of an enzyme dithiol for this activity.

A working hypothesis is suggested to interrelate ATP synthesis and hydrolysis in chloroplasts.

Acknowledgments I would like to express my gratitude to Dr. Fritz Lipmann, in whose laboratory this study was initiated, for creating a most inspiring and stimulating environment (B. P.). We would like to express our appreciation to Dr. Ephraim Racker for considerable help and many valuable discussions during the preparation of this paper. We would also like to thank Dr. Harris H. Tallan and Dr. Paul Greengard for critically reading the paper.

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