Summary

Illumination of chloroplasts in the presence of sulfate, ADP, and Mg2+ causes 35 to 50% inhibition of a subsequent phosphorylation reaction; ADP cannot be replaced by other nucleotides. Compounds that normally inhibit photophosphorylation prevent the appearance of the sulfate-induced inhibition. No evidence was found for phosphorylation site specificity of the sulfate inhibition.

In chloroplasts inhibited by preliminary illumination with sulfate, the light-induced pH rise and the P/Δψ ratio in a phosphorylating Hill reaction are inhibited as much as photophosphorylation is. The pH rise can be restored by dicyclohexylcarbodiimide.

Both the trypsin- and dithiothreitol-activated Ca++-dependent ATPase activity associated with the chloroplast coupling factor (CF1) are decreased in sulfate-inhibited chloroplasts. Removal of CF1 from the membranes followed by recombination with CF1 from noninhibited chloroplasts restores the pH rise completely and phosphorylation to a large extent. From these and other experimental results it is concluded that sulfate, in the presence of ADP and Mg2+, interacts with CF1 on chloroplast membranes under conditions of coupled electron flow so as to cause a modification of CF1 structure.

METHODS

Chloroplast Preparation—Greenhouse-grown spinach leaves were homogenized in a Waring Blendor for 10 sec with a medium containing 0.8 M sucrose-20 mM Tricine-NaOH (pH 7.8)-10 mM NaCl 5 mM ascorbate. After filtering through Miracloth (Chicopee Mills, Inc., New York), the homogenate was centrifuged at 1500 × g for 7 min. Chlorophyll was determined by the method of Arnon (10).

5 mM Tricine-NaOH containing 2 × 10^5 cpm of 32Pi, 50 mM MgCl2, 2.5 mM ADP, 10 mM K2SO4, and 50 μM pyocyanine. A light intensity of 3 × 10^6 ergs per cm² per sec was provided by tungsten photoiod lamps, with the beam filtered through 7 cm of water. K2SO4 was omitted from the controls. After 60 sec the chloroplasts were diluted in 20 ml of sucrose-Tricine-NaOH-NaCl; chlorophyll was determined by the method of Arnon (10).

Sulfate Inhibition—In a final volume of 2.0 ml, chloroplasts (200 μg of chlorophyll) were illuminated at 23° in a reaction mixture containing 50 mM Tricine-NaOH (pH 8.5), 5 mM MgCl2, 2.5 mM ADP, 10 mM K2SO4, and 50 μM pyocyanine. A light intensity of 3 × 10^6 ergs per cm² per sec was provided by tungsten photoiod lamps, with the beam filtered through 7 cm of water. K2SO4 was omitted from the controls. After 60 sec the chloroplasts were diluted in 20 ml of sucrose-Tricine-NaOH-NaCl ascorbate, collected by centrifugation, and resuspended in 1 ml of sucrose-Tricine-NaOH-NaCl.

Phosphorylation by these previously illuminated chloroplasts was carried out at the same temperature and light intensity in a reaction mixture which contained, in 2.0 ml, 50 mM Tricine-NaOH (pH 8.5), 5 mM MgCl2, 2.5 mM ADP, 5 mM K2HPO4 containing 2 × 10^6 cpm of 32Pi, 80 μM pyocyanine, and either with heat, sulfhydryl reagents, or trypsin was shown to be associated with CF1 (2-4).

An approach to elucidating the molecular mechanism of ATP formation in chloroplasts has been made with analogues of the phosphate anion. Thus arsenate seems to replace phosphate in phosphorylation, serving as a competitive inhibitor to phosphate (6). Both on the basis of its chemical similarity and its known chemistry, it is presumed that chloroplasts form an unstable adenosine 5'-diphosphosarsenate, which is rapidly hydrolyzed and therefore has not been detected. Sulfate was recently shown to inhibit photophosphorylation (7) and is competitive with phosphate both in this function (8) and in inhibition of carbon dioxide fixation (9). The formation of adenosine 5'-diphosphosulfate was suggested (8) but not shown. We now report, however, that sulfate has a very different effect on chloroplasts than arsenate does. In the presence of ADP and Mg2+ and under conditions of coupled electron flow, sulfate causes loss of enzymatic activity of CF1, which is so far irreversible.

Inhibition of Photophosphorylation in Spinach Chloroplasts by Inorganic Sulfate*
TABLE I

Inhibition of photophosphorylation caused by sulfate

Chloroplasts (100 µg of chlorophyll per ml) were treated either in the light or in the dark for 60 sec with 5 mM K2HPO4, 5 mM KH2AsO4, or 10 mM K2SO4, in the presence of ADP and Mg++. The chloroplasts were then washed in sucrose-Tricine-NaOH-NaCl-ascorbate, and phosphorylation was measured in a pyocyanine-dependent reaction. Details of these procedures are given in the text.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Illumination</th>
<th>Subsequent phosphorylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>K2SO4 + ADP + Mg++</td>
<td>-</td>
<td>171 µoles ATP/mg chlorophyll/hr</td>
</tr>
<tr>
<td>K2HPO4 + ADP + Mg++</td>
<td>+</td>
<td>83</td>
</tr>
<tr>
<td>KH2AsO4 + ADP + Mg++</td>
<td>+</td>
<td>170</td>
</tr>
<tr>
<td>K2SO4 + ADP + Mg++</td>
<td>-</td>
<td>175</td>
</tr>
<tr>
<td>K2SO4 + ADP + Mg++</td>
<td>+</td>
<td>197</td>
</tr>
</tbody>
</table>

100 µg of chlorophyll. After 2 min in the light the reaction was stopped by adding trichloroacetic acid to a final concentration of 3%. ATP was measured in the supernatant fluid according to Avron (11); radioactivity was determined with a Nuclear-Chicago gas flow counter. Light intensities were measured with a YS1-Kettering radiometer, model G5.

Light-induced pH Rise—Chloroplasts were resuspended in 50 mM NaCl, 2.5 mM MgCl2, and 25 µM pyocyanine to a final concentration of 50 µg of chlorophyll per ml and equilibrated to 15°C. The light-induced pH rise was measured as previously described (12).

Analytical Methods—Ferricyanide reduction was calculated from the decline in extinction at 420 nm in the denatured reaction mixture supernatant solution (13). Protein (14) and Pi (15) were estimated colorimetrically.

Reagents—Phenazine methosulfate, trypsin, dithiothreitol, and phlorizin were purchased from Nutritional Biochemicals, methylviologen from British Drug Houses, and N,N'-dicyclohexylcarbodiimide from Sigma. GDP, CDP, and UDP were from P-L Laboratories and adenosine 5'-methylene diphosphonate was a product of Miles Laboratories. Tricine (16), pyocyanine (17), and diaminodurene (2,3,5,6-tetramethylphenylenediamine) (18) were prepared by methods already described. "Pi was purchased from New England Nuclear.

RESULTS

Illumination of chloroplasts in the presence of sulfate, ADP, and Mg++ results in marked inhibition of a subsequent phosphorylation reaction, in spite of a washing step between the two illuminations (Table I). No significant inhibition was observed when the experiment was repeated with phosphate or arsenate in place of sulfate.

Fig. 1 shows the effect of sulfate concentration on the formation of inhibition. Phosphorylation was about 50% inhibited by prior illumination in the presence of ADP, Mg++, and 10 mM K2SO4; higher concentrations of sulfate were not further effective. Impairment of the phosphorylation mechanism occurs rapidly under these conditions, with a half-time on the order of 5 to 10 sec (Fig. 2).

Requirements for Formation of Sulfate Inhibition—As shown in Table I, inhibition by sulfate was dependent on both ADP (Experiment 1) and Mg++, and on the electron carrier pyocyanine (Experiment 3). Either phosphate or arsenate, when present together with sulfate, completely protected against inhibition (Experiment 1). ATP not only failed to replace ADP, but partially protected against the inhibition when present with ADP (Experiment 4). Inasmuch as these observations suggest an interaction between sulfate and the phos-
phosphorylation site in chloroplasts, the effects of the uncoupler NaNCl and the energy transfer inhibitor phlorizin were also investigated. Both compounds prevented inhibition by sulfate (Experiments 5 and 6).

ADP was not replaced by AMP, ATP, adenosine 5'-methylene diphosphonate, or other nucleoside diphosphates. That GDP failed to replace ADP was surprising, since both are phosphorylated in chloroplasts with comparable efficiency (19, 20).

Sulfate Inhibition of Phosphorylation Supported by Different Electron Flow Patterns—Inhibition of chloroplasts by sulfate results in a 35 to 50% decline in pyocyanine-dependent phosphorylation (Tables I and II). Since cyclic phosphorylation might involve two sites of ATP formation (21-23), the possibility was considered that sulfate might affect only one of these. Results shown in Table III make this unlikely. Phosphorylation was inhibited to an almost identical extent, whether supported by noncyclic electron flow through System II (ferrocyanide in place of pyocyanine during the initial exposure to light) or by noncyclic electron flow through System I only (ascorbate-diaminodurene as donor, methylviologen-oxygen as acceptor, in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea) (24). Moreover, 35 to 50% inhibition of pyo-

\[ \text{sulfate inhibition was formed in a reaction mixture containing K}_2\text{SO}_4, \text{ADP, and Mg}^{2+} \text{, as described under "Methods." K}_2\text{SO}_4 \text{ was omitted from controls. The chloroplasts were washed in sucrose-Tricine-NaOH-NaCl-ascorbate and phosphorylation was measured in the various assay systems shown. Each reaction mixture contained, in a final volume of 2.0 ml, 50 mM Tricine (pH 8.5), 5 mM MgCl}_2, 2.5 mM ADP, 5 mM K}_2\text{HPO}_4 (containing 2 \times 10^5 \text{cpm of } ^{32}\text{P}_i), \text{and 100 pg of chlorophyll. Where indicated, reagents were added to give the following concentrations: 1.5 mM K}_3\text{Fe(CN)}_6, 3 mM ascorbic acid, 1 mM 2,3,5,6-tetramethylphenylenediamine (diaminodurene), 0.1 mM methylviologen, 5 \mu \text{M 3-(3,4-dichlorophenyl)-1,1-dimethylurea, 50 \mu M phenazine methosulfate. Reactions were stopped after 2 min with trichloroacetic acid.}

\[ \text{NaCl and finally resuspended in 10 mM NaCl. Light-induced pH shifts were measured under conditions described under "Methods." K}_2\text{SO}_4 \text{ was omitted from controls. The chloroplasts were washed in sucrose-Tricine-NaOH-NaCl-ascorbate and then in 10 mM NaCl and finally resuspended in 10 mM NaCl. Light-induced pH shifts were measured under conditions described under "Methods." Where indicated, N,N'-dicyclohexylicarbodiimide was added to a final concentration of 0.1 mM. A, control chloroplast; B, control chloroplasts plus N,N'-dicyclohexylcarbodiimide; C, sulfate-inhibited chloroplasts; D, sulfate-inhibited chloroplasts plus N,N'-dicyclohexylcarbodiimide.}

\[ \text{Fig. 3. Inhibition of the light-induced pH rise in sulfate-inhibited chloroplasts, and restoration by N,N'-dicyclohexylcarbodiimide (DCCD). Sulfate inhibition was formed by illumination in the presence of K}_2\text{SO}_4, \text{ADP, and Mg}^{2+} \text{, as described under "Methods." K}_2\text{SO}_4 \text{ was omitted from controls. The chloroplasts were washed in sucrose-Tricine-NaOH-NaCl-ascorbate and then in 10 mM NaCl and finally resuspended in 10 mM NaCl. Light-induced pH shifts were measured under conditions described under "Methods." Where indicated, N,N'-dicyclohexylcarbodiimide was added to a final concentration of 0.1 mM. A, control chloroplast; B, control chloroplasts plus N,N'-dicyclohexyldibromide; C, sulfate-inhibited chloroplasts; D, sulfate-inhibited chloroplasts plus N,N'-dicyclohexylcarbodiimide.}

\[ \text{Electron Transport in Sulfate-Inhibited Chloroplasts—Electron transport from water to ferrocyanide was not impaired by sulfate inhibition. Rates of 643 and 661 pmoles of ferrocyanide reduced per mg of chlorophyll per hour in control and sulfate inhibited membranes, respectively, were associated with the phosphoryl-}

\[ \text{I. J. Ryrie and A. T. Jagendorf, unpublished data.} \]
TABLE IV
Effect of sulfate inhibition on trypsin- or dithiothreitol-activated Ca\textsuperscript{2+}-ATPase Activity

Chloroplasts were illuminated in the presence of K\textsubscript{2}SO\textsubscript{4}, ADP, and Mg\textsuperscript{2+}, washed in sucrose-Tricine-NaOH-NaCl-ascorbate, and resuspended in sucrose-Tricine-NaOH-NaCl as described under "Methods." Controls were illuminated in the absence of K\textsubscript{2}SO\textsubscript{4}. Trypsin-activated Ca\textsuperscript{2+}-ATPase activity was assayed according to McCarty (personal communication). In a final volume of 0.9 ml, chloroplasts containing 50 \textmu g of chlorophyll were incubated with 20 mM Tris-HCl (pH 8.0), 2 mM EDTA, 2 mM ATP, and 400 \textmu M of freshly dissolved trypsin. After 10 min at 20°C, 0.1 ml of trypsin inhibitor (10 mg per ml) was added. Aliquots (0.1 ml) were incubated for 20 min at 37°C with 0.9 ml of a solution containing 50 mM Tris-HCl (pH 8.0), 5 mM CaCl\textsubscript{2}, and 5 mM ATP. Reactions were stopped with trichloroacetic acid. Dithiothreitol activation was carried out at 20°C for 45 min in 80 mM Tris-HCl (pH 8.0) and 50 mM dithiothreitol. Assays were carried out as previously described (4). In a final volume of 0.5 ml, chloroplast equivalent to 100 \textmu g of chlorophyll was incubated with a solution containing 40 mM Tris-HCl (pH 8.5) and 50 mM dithiothreitol. After 30 min at 12°C, the chloroplasts were collected by centrifugation and resuspended in 4 ml of 0.75 mM EDTA (pH 7.0) at room temperature. After 5 min, 0.5-ml aliquots were added to reaction mixtures which contained, in a final volume of 1.0 ml, 25 mM Tris- HCl (pH 7.0), 5 mM CaCl\textsubscript{2}, and 5 mM ATP. Reactions were terminated after incubation for 20 min at 37°C. To estimate the Ca\textsuperscript{2+}-ATPase activity of isolated CF\textsubscript{1}, chloroplasts washed in sucrose-Tricine-NaOH-NaCl-ascorbate were again washed in 10 mM NaCl and resuspended in 0.75 mM EDTA (pH 7.0) to a final concentration of 200 \textmu g of chlorophyll per ml. After 5 min at 20°C, the CF\textsubscript{1}-depleted membranes were removed by centrifugation. Assays were carried out as described above except that dithiothreitol activation was carried out at 20°C for 45 min in 80 mM Tris-HCl (pH 8.0) and 100 mM dithiothreitol.

<table>
<thead>
<tr>
<th>Method of activation</th>
<th>ATPase activity of chloroplasts</th>
<th>Inhibition</th>
<th>ATPase activity of isolated CF\textsubscript{1}</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Sulfate-inhibited</td>
<td>Control</td>
<td>Sulfate-inhibited</td>
</tr>
<tr>
<td></td>
<td>\textmu moles Pi released/mg chlorophyll/hr</td>
<td>%</td>
<td>\textmu moles Pi released/mg protein/hr</td>
<td>%</td>
</tr>
<tr>
<td>Whole chloroplasts + trypsin</td>
<td>108</td>
<td>75</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Isolated CF\textsubscript{1} + trypsin</td>
<td>85</td>
<td>71</td>
<td>17</td>
<td>158</td>
</tr>
<tr>
<td>Whole chloroplasts + DTT*</td>
<td>60</td>
<td>36</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td>Isolated CF\textsubscript{1} + DTT*</td>
<td>143</td>
<td>103</td>
<td>28</td>
<td>175</td>
</tr>
</tbody>
</table>

* DTT, dithiothreitol.

The results shown in Table III, Line 1. Thus sulfate inhibition results in partial uncoupling of phosphorylation, with a decline in P/\epsilon from 0.78 to 0.37.

Effect of Sulfate Inhibition on Light-induced pH Rise—As shown in Fig. 3, chloroplasts incubated by sulfate treatment exhibited only about half of the light-induced pH rise of controls. Addition of N,N'-dicyclohexylcarbodiimide completely restored the pH rise in inhibited chloroplasts, however, whereas pH shifts in control membranes were unaffected. In this respect, our results resemble those of McCarty and Racker (25) where N,N'-dicyclohexylcarbodiimide was found to restore a light-induced pH rise in CF\textsubscript{1}-depleted membranes.

TABLE V
Reconstitution of phosphorylation in sulfate-inhibited chloroplasts by replacement of CF\textsubscript{1}

Sulfate inhibition in chloroplasts was formed by illumination in the presence of K\textsubscript{2}SO\textsubscript{4}, ADP, and Mg\textsuperscript{2+} as described under "Methods." K\textsubscript{2}SO\textsubscript{4} was omitted from controls. The chloroplasts were washed in sucrose-Tricine-NaOH-NaCl-ascorbate and then in 10 mM NaCl and resuspended in cold 0.75 mM EDTA, pH 7.0, to a final concentration of 330 \textmu g of chlorophyll per ml. After 5 min at room temperature, the suspension was centrifuged at 27,000 \times g for 10 min and the CF\textsubscript{1}-depleted membranes were gently resuspended in cold 10 mM NaCl to a final concentration of 1.5 mg of chlorophyll per ml. Incubation of EDTA-extracted membranes with CF\textsubscript{1} and phosphorylation assays were carried out as previously described (25).

<table>
<thead>
<tr>
<th>Source of CF\textsubscript{1}</th>
<th>CF\textsubscript{1}-deficient chloroplasts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>\textmu moles ATP/mg chlorophyll/hr</td>
</tr>
<tr>
<td>None added</td>
<td>1.65</td>
</tr>
<tr>
<td>Control chloroplasts</td>
<td>164</td>
</tr>
<tr>
<td>Sulfate-inhibited chloroplasts</td>
<td>123</td>
</tr>
</tbody>
</table>

Fig. 4. Restoration of the light-induced pH rise in sulfate-inhibited chloroplasts by replacement of CF\textsubscript{1}. Sulfate inhibition in chloroplasts was formed by illumination in the presence of K\textsubscript{2}SO\textsubscript{4}, ADP, and Mg\textsuperscript{2+} as described under "Methods." K\textsubscript{2}SO\textsubscript{4} was omitted from controls. The methods of preparing CF\textsubscript{1}-deficient membranes and for reconstituting the resolved membranes with CF\textsubscript{1} are given in Table V. Reconstituted membranes were twice washed in 10 mM NaCl before light-induced pH shifts were measured. C, CF\textsubscript{1}-deficient control chloroplasts; E, CF\textsubscript{1}-deficient sulfate-inhibited chloroplasts; C\textsubscript{CF1}, CF\textsubscript{1} from control chloroplasts; S\textsubscript{CF1}, CF\textsubscript{1} from sulfate-inhibited chloroplasts.

Effect of Sulfate Inhibition on Trypsin- or Dithiothreitol-activated Ca\textsuperscript{2+}-dependent ATPase Activity Results presented thus far suggest that sulfate may inhibit photophosphorylation by interacting with CF\textsubscript{1}. It might be expected therefore that the ATPase activities associated with CF\textsubscript{1} would show similar inhibitions. Chloroplasts, after sulfate exposure, were incubated with trypsin which releases CF\textsubscript{1} from the membrane and activates its Ca\textsuperscript{2+}-dependent ATPase. Other chloroplasts were incubated with EDTA to release CF\textsubscript{1}, and then with dithiothreitol to activate the ATPase. In some experiments the activation was performed before and in other experiments after removal of the green membranes by centrifugation. The latter protocol eliminated any possibility that the inhibited membranes were affecting either activation or assay of the ATPase. Activities, on either a chlorophyll or protein basis, were decreased by values varying between 17 and 45% in CF\textsubscript{1} released from sulfate-inhibited chloroplasts (Table IV).

Restoration of Phosphorylation in Sulfate-inhibited Chloroplasts—The finding that sulfate causes inhibition of CF\textsubscript{1}-de-
FIG. 5. Kinetic analysis of the effects of sulfate inhibition on photophosphorylation. Control and sulfate-inhibited chloroplasts were prepared as described under "Methods." Photophosphorylation was measured in a reaction mixture which contained, in a total volume of 2.0 ml, 50 mM Tricine-NaOH (pH 8.5), 5 mM MgCl₂, and 50 μM pyocyanine together with the following compounds: A, 2.5 mM ADP, variable Pi, and 100 μg of chlorophyll; B, variable ADP, 2 mM Pi, and 10 μg of chlorophyll; C, 2.5 mM ADP, 5 mM Pi, and 100 μg of chlorophyll. Each reaction mixture con-
radioactivity above that of minus ADP controls. The specific activity of the original sulfate solution (10$^6$ cpm per amole) would have allowed detection of 1 atom of sulfur for every 5000 chlorophyll molecules. In addition, CF$_1$ was extracted by means of EDTA from chloroplasts inhibited by radioactive sulfate, chromatographed on Sephadex G-75, and found to contain no radioactivity.

The possibility was also considered that sulfate might catalyze the binding of ADP to chloroplasts during sulfate inhibition. This was ruled out in experiments similar to those described above, but with $^{14}$C-labeled ADP.

**DISCUSSION**

The inhibition of photophosphorylation by sulfate ions, previously shown (8) to be competitive with respect to phosphate, was found in the present study to include a large apparently irreversible component. This fact has permitted us to examine the requirements for the formation of the inhibition separately from its expression in the various chloroplast reactions, carried on in a second light reaction. We are able to conclude that the inhibition is primarily, if not entirely, due to an alteration in CF$_3$, and that formation of the inhibited state requires those conditions of coupled electron transport in which CF$_1$ is essentially prepared to catalyze anhydride bond formation.

Asada, Deura, and Kasai (7) concluded that inhibition by sulfate was reversible on washing. In their experiments, however, washing of chloroplasts was attempted only after illumination with a mixture of phosphate and sulfate, in a phosphorylation reaction mixture. We have found that less phosphate is required to prevent the irreversible inhibition from occurring than is needed to compete with sulfate directly in a phosphorylation reaction. Thus in the earlier experiments the sulfate to phosphate ratio was not large enough to bring on the irreversible inhibition described here, even though it was sufficient to cause some direct inhibition of photophosphorylation.

The protection by phosphate or arsenate (Table II) indicates a common binding site with sulfate, perhaps the one involved in photophosphorylation. This finding is in agreement with the observation of competition between phosphate and sulfate when both are present during a single photophosphorylation reaction, or during photosynthetic carbon dioxide fixation (8, 9). The requirements for light and an added electron carrier (pyocyanine or ferriyanide) show that electron transport is needed, and preventing the inhibition by ammonium chloride indicates a requirement for maintenance of the high energy conditions of the membranes.

The further requirement for ADP and Mg$^{2+}$ ions, combined with prevention of sulfate inhibition by phlorizin, a known energy transfer (27) and ATPase (28) inhibitor, indicates that almost all of the steps of phosphorylation except for the final attachment of phosphate to ADP are required for the inhibited state to occur.

The high degree of specificity for ADP in the present experiments is striking in that it was not replaced by AMP, ATP, adenosine 5'-methylene diphosphonate, or other nucleotides. Even GDP, which is reported to be phosphorylated directly by chloroplasts rather than through a nucleotide kinase (19, 20), was without effect. This specificity is reminiscent of but more extreme than that for the inhibition of chloroplast ATPase, in which ADP is considerably more effective than GDP (2, 20).

Previous illumination with sulfate inhibits later photophosphorylation supported by any sort of electron flow pattern (Table III), uncouples phosphorylation from associated ferriyanide reduction as shown by a lower P/0$_2$ ratio, and causes a partial inhibition of the light-induced pH rise (Fig. 3). That the pH rise as well as phosphorylation was restored by removing existing CF$_1$ from these chloroplasts and replacing it with the enzyme from control chloroplasts indicates that all of the effects are caused by damage to CF$_1$. This conclusion is strengthened by the failure of CF$_1$ from inhibited chloroplasts to reconstitute adequately the pH rise or phosphorylation in EDTA-extracted control chloroplasts (Fig. 4, Table V). Also consistent is the restoration of the pH rise to sulfate-treated chloroplasts by $N$,N'-dicyclohexylcarbodiimide (Fig. 3), a reagent previously shown to restore the pH rise in chloroplasts depleted in CF$_1$ (25) or with CF$_1$, damaged by exposure to acid and polyanions (12). As before, restoration of the pH rise by control CF$_1$ is not considered as evidence for a direct participation of the enzyme in the mechanism for ion fluxes. A change in the conformation of CF$_1$ on the sulfate-inhibited membranes might allow passive proton leakage, and thus cause a dissipation of the pH gradient or membrane potential (or both) necessary for ATP formation (29).

Conclusive evidence as to the alteration of CF$_1$ during sulfate inhibition is found in the decrease of Ca$^{2+}$-dependent ATPase activity detectable in trypsin-treated chloroplasts (Table IV) or in EDTA extracts of chloroplasts. This decrease was observed even when both activation and assay of the ATPase were carried out apart from the chloroplast membranes.

The nature of the damage to CF$_1$ is not yet known. No signs were found of binding of sulfate-$^{35}$S or ADP-$^{35}$S either to inhibited chloroplasts or to CF$_1$ extracted from them. (These data, together with the irreversible nature of the change, seem to rule out the previous suggestion (8) of formation of ADP-sulfate as being responsible for inhibition of photophosphorylation.) Further, no change was seen in the elution pattern of CF$_1$ from Bio-Gel P-300, or the banding observed after electrophoresis of treated CF$_1$ on polyacrylamide gel, thus making it unlikely that inhibition resulted from dissociation into subunits.

There is a possibility that light might have two roles in the present inhibition—first to bring on the high energy state and then to cause photodynamic or photooxidative damage to CF$_1$, brought into a sensitive configuration due to the presence of sulfate, ADP, Mg$^{2+}$, and the high energy state of the membranes. Irreversible inactivation of electron transport (30, 37) and of phosphorylation (30, 31) in chloroplasts caused by prolonged illumination at high light intensity was described previously. The present results differ in that 30 sec of moderate light intensity are sufficient when all reagents are present, and in that damage is apparently confined to CF$_1$. Several amino acids in proteins are rapidly photooxidized by illumination in the presence of oxidation-reduction dyes in solution, however (38), and it may be that a particular amino acid might be highly sensitized to photooxidation because of the specific conditions used in the present study. The only evidence opposing this concept as yet is the observation that sulfate inhibition is not prevented by performing the illumination in the absence of O$_2$. Photoinduced inhibition of electron transport close to Photosystem II (27) and the high intensity long term photodestruction of phosphorylation (30, 31) also occur anaerobically.

Further experiments are currently directed toward the identification of the site(s) on CF$_1$ affected by sulfate treatment. If
successful they may provide further insight into the reactivity of CF, with respect to the binding of phosphate or sulfate, and the way in which its conformation or reactivity is affected by conditions which permit anhydro bond formation.

Acknowledgments—We wish to thank Dr. Gunter Hauska for a gift of the diaminodurene, and acknowledge the stimulating effect of discussions with G. Polya, W. Cohen, R. McCarty, and G. Hauska. Mrs. I. Ozols provided technical assistance.

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Inhibition of Photophosphorylation in Spinach Chloroplasts by Inorganic Sulfate
I. J. Ryrie and Andre T. Jagendorf


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