Light-dependent Inhibition of Photophosphorylation by N-Ethylmaleimide*

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SUMMARY

The treatment of chloroplasts with 1 mM N-ethylmaleimide (NEM) in the light, but not in the dark, resulted in a partial, permanent inhibition of photophosphorylation. Noncyclic electron flow coupled to phosphorylation was also inhibited on incubation of chloroplasts with NEM in the light, but this inhibition was reversed by ammonium chloride.

The uncouplers, carbonyl cyanide m-chlorophenylhydrazone and ammonium chloride, largely prevented the inhibition. Adenosine triphosphate and adenosine diphosphate partially protected phosphorylation from inhibition by light and NEM.

Light-induced hydrogen ion uptake was unaffected by NEM except that the uptake stimulated by adenosine triphosphate was inhibited.

The calcium-dependent adenosine triphosphatase of coupling factor 1 was also inhibited in chloroplasts treated with NEM in the light prior to extraction of the coupling factor.

It is suggested that light causes a conformational change in the membrane-bound coupling factor, allowing the reaction of a group (or groups) with NEM. The sensitivity of the inhibition to adenine nucleotides suggests that these nucleotides modify the conformation of coupling factor 1 in the light.

Chloroplasts were isolated from market spinach by a reported procedure (5). Chlorophyll was estimated spectrophotometrically (6). Photophosphorylation was assayed in a reaction mixture (1.0 ml) which contained 50 mM tris(hydroxymethyl)-methylglycine-NaOH (pH 8.0), 50 mM NaCl, 5 mM MgCl2, 2 mM potassium phosphate buffer (pH 8), 3 mM ADP, 0.05 mM pyocyamine, 20 mM MgCl2 equivalent to 1 to 2 × 10−4 M, and chloroplasts equivalent to 10 to 50 μg of chlorophyll. The samples were illuminated at room temperature for 2 min with 2 × 105 ergs per cm2 per s of white light. Phosphorylation coupled to methyl viologen reduction was assayed in the same reaction mixture (1.75 ml), except that pyocyamine was replaced with 0.1 mM methyl viologen, and 1 mM NaN3 was present. Oxygen uptake dependent on methyl viologen reduction with water as the electron donor was followed polarographically with a Clark-type electrode. H+ uptake was determined at pH 7.0 ± 0.01, as described previously (1).

The Ca2+-dependent ATPase of CF1 was activated with either trypsin (7) or with DTT (8). The procedure of Vambutas and Racker (7) was used to assay ATPase activity, except that Pr was determined by the method of Taussky and Shorr (9).

In most experiments, chloroplasts were incubated at 20°C with NEM at a chlorophyll concentration of 0.33 mg per ml in a
stirred reaction mixture containing in 1.5 to 15 ml, 50 mM tris-(hydroxymethyl)methylglycine-NaOH (pH 8), 50 mM NaCl, 5 mM MgCl₂, and 0.05 mM pyocyanine. In some experiments, the MgCl₂ was omitted. After a timed period in the light (2 x 10⁶ ergs per cm² per s) or the dark, an amount of DTT calculated on the basis of its -SH content to be about 10% in excess of the amount of NEM present was added. Aliquots of the mixtures were either assayed directly for photophosphorylation or further treated prior to assay as described in the legends to the figures and tables.

**RESULTS**

Inhibition of Phosphorylation by NEM—Although the illumination of chloroplasts in the absence of NEM for 1 min did not inhibit phosphorylation, illumination in the presence of 5 mM NEM severely inhibited it (Table I). Much less inhibition was observed in chloroplasts treated with NEM in the dark, and this inhibition, but not that observed in the light, was abolished by the addition of DTT to remove the NEM prior to the assay of photophosphorylation. Thus, the inhibition due to NEM treatment in the dark was probably caused by the presence of NEM during the phosphorylation assay. Further, since DTT did not reverse the light-dependent NEM inhibition, it is apparent that the inhibition is irreversible. This was confirmed by washing the chloroplasts. DTT, added to the incubation mixtures before the chloroplasts, prevented the inhibition.

The inhibition of phosphorylation by NEM in the light was partial, ranging from 35 to 70%. As seen in Fig. 1, increasing the NEM concentration above 1 mM did not markedly augment the inhibition. Furthermore, the onset in the light of the NEM inhibition of phosphorylation was fairly rapid, nearly reaching completion in about 60 s.

Phosphorylation coupled to the reduction of methyl viologen with water as the electron donor was also inhibited by the treatment with NEM in the light, but not in the dark (Table II). Light and NEM also attenuated methyl viologen reduction assayed in the presence of ADP and P₇. This inhibition was reversed by NH₄Cl, suggesting the inhibition by NEM and light is of the energy transfer type. However, electron flow assayed in the absence of ADP was stimulated by the incubation of the chloroplasts with NEM in the light. For example, whereas phosphorylating electron flow was inhibited 20% by the light and NEM treatment, electron flow in the absence of ADP was stimulated by 23%. Thus, the NEM inhibition is distinct from that of the energy transfer inhibitors Dio-9 (10) and phlorizin (11), which have little effect on electron transport in the absence of ADP.

Uncoupling concentrations of NH₄Cl and CCP protected chloroplasts from the action of NEM in the light (Table III). These results suggest that the light requirement to form the

**TABLE I**

Light-dependent phosphorylation inhibition by NEM

| Treatment of chloroplasts prior to assay | Phosphorylation with pyocyanine |
|----------------------------------------|-------------------------------|
| Light                                  | 600                           |
| Dark                                   | 624                           |
| Light + NEM                            | 200                           |
| Dark + NEM                             | 414                           |
| Light + NEM; DTT added after illumination | 216                       |
| Dark + NEM; DTT added after incubation  | 648                           |
| Light + NEM; DTT added before chloroplasts | 708                        |
| Dark + NEM; DTT added before chloroplasts | 618                        |

**FIG. 1.** Effect of NEM concentration on the inhibition of phosphorylation. Chloroplasts were treated with the indicated concentrations of NEM as described in Table II and 0.05-ml aliquots assayed for pyocyanine-dependent cyclic phosphorylation. Phosphorylation is expressed as μmoles of P₇ esterified per hour per mg of chlorophyll.

**TABLE II**

Effect of NEM on methyl viologen reduction and associated phosphorylation

Chloroplasts containing 0.5 μg of chlorophyll were treated with 1 mM NEM in the usual reaction mixture (1.5 ml). After 90 s in the light or dark, DTT was added to a final concentration of 0.6 mM. For the controls, labeled "DTT-NEM," DTT was added before the chloroplasts. Methyl viologen reduction and associated phosphorylation were assayed in 0.2-ml aliquots. Rates are given as μmoles of methyl viologen reduced or P₇ esterified per hour per mg of chlorophyll. The NH₄Cl concentration was 2.5 mM.

| Treatment               | Methyl viologen reduction | Phosphorylation | P/2c |
|-------------------------|---------------------------|----------------|------|
|                         | −NH₄Cl                    | +NH₄Cl         | −NH₄Cl| −NH₄Cl |
| NEM, light              | 137                       | 212            | 76   | 0.55   |
| DTT-NEM, light          | 198                       | 227            | 141  | 0.71   |
| NEM, dark               | 163                       | 209            | 118  | 0.71   |
| DTT-NEM, dark           | 166                       | 212            | 116  | 0.70   |
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Partial protection from NEM inhibition by NH₄Cl and carbonyl cyanide m-chlorophenylhydrazone

Chloroplasts equivalent to 1 mg of chlorophyll were exposed to 1 mM NEM for 90 s in 3 ml of the standard incubation mixture, which also contained the indicated concentrations of NH₄Cl or CCP. At the end of the incubation, 0.6 mM DTT was added and the mixtures centrifuged at 10,000 x g for 10 min. The pellets were resuspended in 5 ml of a solution which contained 0.4 M sucrose, 0.02 M tris(hydroxymethyl)methylglycine-NaOH (pH 8), and 0.01 M NaCl. After centrifugation as above, the pellets were resuspended in a small volume of the buffered sucrose solution and pyocyanine-dependent phosphorylation determined. Phosphorylation is given as amoles of P₁ esterified per hour per mg of chlorophyll.

| Addition to NEM incubation | Phosphorylation | Percentage of inhibition by light |
|---------------------------|-----------------|-------------------------------|
|                           | Incubated with NEM in light | Incubated with NEM in dark |
| None                      | 127             | 245                           | 48.2 |
| NH₄Cl, 2.5 mM             | 179             | 250                           | 28.4 |
| NH₄Cl, 5 mM               | 200             | 194                           | 0    |
| CCP, 5 µM                 | 200             | 234                           | 14.5 |
| CCP, 10 µM                | 216             | 223                           | 3    |

NEM inhibition is related to the generation of the high energy state.

Protection of Phosphorylation from NEM Inhibition by Adenine Nucleotides—ATP and ADP reduced the inhibition of phosphorylation elicited by NEM and light, whereas their presence during the incubation of chloroplasts with NEM in the dark had little effect on phosphorylation (Table IV). Under phosphorylating conditions, the inhibition by NEM was minimal. P₁ was shown in other experiments to have no effect on the inhibition.

As little as 5 µM ATP partially prevented the inhibition of phosphorylation by 1 mM NEM and light and saturation was achieved at about 20 µM. For example, 5 µM ATP reduced the inhibition from 64 to 55% and 50 µM ATP reduced the inhibition to 31%. At similar concentrations, ATP stimulates H⁺ uptake (1) and inhibits electron flow (12). UTP, GTP, CTP, and TTP (50 µM) did not diminish the light-dependent phosphorylation inhibition by NEM.

Effects of NEM on H⁺ Uptake—Light-induced H⁺ uptake, assayed at pH 7.6, was little affected by the prior treatment of chloroplasts with NEM in the light. H⁺ uptake in chloroplasts illuminated for 90 s in the presence of 1 mM NEM prior to assay was found in one experiment to be 0.18 µeq per mg of chlorophyll, whereas that in chloroplasts incubated with 1 mM NEM in the dark was 0.16. In contrast, H⁺ uptake assayed in the presence of 20 µM ATP was partially sensitive to the light and NEM treatment. The stimulation by ATP of H⁺ uptake in chloroplasts treated with NEM in the light was only 54%, whereas that in chloroplasts incubated in the dark with NEM was 181%. It would appear, therefore, that the ATP-stimulated H⁺ uptake is preferentially inhibited by incubation of chloroplasts with NEM in the light.

Evidence for Interaction of NEM and Chloroplast Coupling Factor I—The sensitivity of the NEM inhibition to adenine nucleotides suggested that CF₁ may be the site of the NEM inhibition. To test this idea, chloroplasts were treated with NEM in the usual way and the Ca²⁺-dependent ATPase activity of CF₁ determined after extraction of the CF₁ with EDTA. The CF₁ extracted from chloroplasts treated with NEM in the light was less active than that derived either from chloroplasts illuminated in the absence of NEM or from chloroplasts incubated in the dark in the standard reaction mixture (3 ml), except that the MgCl₂ was omitted. To the samples which contained NEM, DTT (0.6 mM) was added at the end of the 2-min incubation in the light or the dark. The mixtures were centrifuged at 10,000 x g for 10 min, the precipitate resuspended in 10 ml of 0.75 M sucrose, and the chloroplasts collected by centrifugation at 10,000 x g for 10 min. The pellets were resuspended in 40 ml of 0.75 mM EDTA (pH 7) at room temperature, and, after 10 min, the mixture was centrifuged at 35,000 x g for 30 min. Aliquots of 0.8 µl were activated with 50 µg of trypsin and Ca²⁺-dependent ATPase activity assayed. In Experiment II, chloroplasts (0.5 mg of chlorophyll) were treated with 5 mM NEM for 2 min in the light or dark, in a final volume of 5 ml. ADP and P₁ were added, where indicated, to final concentrations of 2 mM each. DTT was added after the incubation to a final concentration of 50 mM to activate the Ca²⁺-ATPase. After 1 hour at room temperature, the suspensions were centrifuged at 10,000 x g for 10 min and the pellets resuspended in 10 ml of 0.75 mM EDTA. After centrifugation at 30,000 x g for 30 min, aliquots of the supernatant fluids were assayed directly for Ca²⁺-ATPase.

### Table IV

| Additions to NEM incubation | Phosphorylation | Percentage of inhibition by light |
|----------------------------|-----------------|-------------------------------|
|                            | Incubated with NEM in light | Incubated with NEM in dark |
| None                       | 212             | 435                           | 51.4 |
| ADP                        | 328             | 435                           | 24.7 |
| ADP + P₁                   | 444             | 438                           | 3.2  |
| ATP                        | 312             | 438                           | 25.2 |

### Table V

| Experiment | Conditions of treatment of chloroplasts | ATPase activity μmoles P₁ formed by mg chlorophyll |
|------------|----------------------------------------|-------------------------------------------|
| I          | Light                                  | 255                                      |
|            | Light + NEM                            | 163                                      |
|            | Dark                                   | 265                                      |
|            | Dark + NEM                             | 208                                      |
| II         | Light + NEM                            | 79                                       |
|            | Light + NEM + ADP + P₁                 | 100                                      |
|            | Dark + NEM                             | 110                                      |
|            | Dark + NEM + ADP + P₁                  | 117                                      |
bated in the dark with NEM (Table V, Experiment I). Similar amounts of protein were extracted by EDTA in each case, suggesting that the NEM and light treatment did not merely alter the extractability of the CF₁. Further, ADP and P₃ partially protected the Ca²⁺-ATPase of CF₁ from inhibition by NEM and light (Table V, Experiment II).

**DISCUSSION**

The inhibition of photophosphorylation by NEM may be traced to an irreversible reaction of the NEM with the CF₁ on the thylakoid membranes. This conclusion is supported by several lines of evidence, including the observation that the treatment of chloroplasts with NEM in the light causes an inhibition of the Ca²⁺-ATPase of CF₁. The light requirement for the inhibition may be understood if it is assumed that CF₁ on the thylakoid membranes undergoes a conformational change on illumination. This conformational change could render a group(s) (possibly —SH) accessible to NEM, and, if this group is required for CF₁ activity, its reaction with NEM would inhibit phosphorylation. The light requirement for the inhibition is apparently related in some manner to energy conservation, since the inhibition was abolished by uncouplers and by ADP and P₃. Ryrie and Jagendorf (3) showed that the light-induced conformational change in CF₁, as detected by incorporation of $^3$H₂O, was diminished by uncouplers.

If it is accepted that the light potentiation of the NEM inhibition of phosphorylation is caused by light-dependent changes in CF₁, conformation, then the fact that ATP and ADP reduce the extent of inhibition may be taken as evidence that these nucleotides modify the structure of CF₁ in the light. Such a role for ATP was postulated to explain its stimulation of H⁺ uptake in chloroplasts (1). Similar low concentrations of ATP were required to protect from NEM inhibition and stimulate H⁺ uptake. Moreover, other nucleoside triphosphates were ineffective in either blocking the NEM inhibition or stimulating H⁺ uptake (1).

Other —SH reagents have long been known to inhibit photo-phosphorylation (13, 14), but a light dependence for their inhibitory activity has not been previously reported. Izawa and Good (15) established that p-chloromercuribenzoate, added directly to the reaction mixtures, is an energy transfer inhibitor. Hg²⁺ also inhibited phosphorylation and coupled electron flow, but was found to stimulate electron flow under non-phosphorylating conditions. This stimulation was abolished by low concentrations of ATP. Similar results were obtained in chloroplasts treated with NEM in the light.

The inhibition of phosphorylation by treatment with NEM and light, p-chloromercuribenzoate (15), or light plus ADP and sulfate (16) leveled off at about 50%. It is clear that all three treatments affect in some way the activity of bound CF₁. Extraction of about 50% of the CF₁ from chloroplast membranes can inhibit phosphorylation completely (5). Yet phosphorylation may be partially restored to these depleted particles by diethylene glycol-bismaleimide, indicating that the residual CF₁ in the particles may participate in phosphorylation. It is possible, therefore, that there is an excess of CF₁ in chloroplasts and that only about half of the CF₁ in a population of chloroplasts undergoes conformational changes and participates in photophosphorylation. Thus, since NEM may react only with CF₁, which has altered its conformation in the light, a 50% inhibition would be expected. Murakami (17) calculated that there may be as many as 1 CF₁ molecule for every 100 chlorophyll molecules, a value in excess of most of the components of the electron transport chain.

Preliminary experiments with $[^3]$H]NEM have indicated that more NEM is incorporated into a protein, extractable with EDTA, but not with 10 mM NaCl, in chloroplasts illuminated in the presence of NEM than in those kept in the dark. Since CF₁ is certainly the major protein in EDTA extracts of chloroplasts, it is probable that the $[^3]$H]NEM is associated with CF₁. If it is established that there is a light-dependent and uncoupler-sensitive reaction of NEM with CF₁, it will be of interest to identify the functional group which reacts with NEM and, further, to localize this group within the subunit structure of CF₁.

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