Inhibitor Studies on Carbon Dioxide Fixation, Adenosine Triphosphate Formation, & Triphosphopyridine Nucleotide Reduction by Spinach Chloroplasts [1, 2]

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Several workers (2, 3, 4, 5, 10) have studied the effects of inhibitors in attempts to elucidate various aspects of photosynthesis. Most of these studies, however, have concentrated on one process. In the present work we have attempted to examine the effects of various compounds on TPN reduction and the associated ATP formation, CO_2 fixation, and the so-called "cyclic" photophosphorylation catalyzed by PMS[5]. We have also utilized the system, first described by Vernon and Zaugg (18), in which ascorbate is used in place of water as the ultimate source of electrons, thus probably bypassing one of the two photochemical reactions and the oxygen-evolving mechanism. We also report the effects of various compounds which allow CO_2 fixation to proceed in the presence of substances which inhibit TPNH or ATP formation.

Materials & Methods

Materials. ADP, TPN, glucose-6-P, glyceric acid-3-P, fructose-1,6-diP, and Antimycin A were purchased from the Sigma Chemical Company. Glyceraldehyde-3-P was obtained from the Nutritional Biochemical Corporation as its diethylacetal barium salt. Pi^{32} was purchased from the Squibb Laboratories and treated with N HCl before use (17). CCCP was a gift from Dr. P. G. Heytler, Central Research Department of the E. I. du Pont de Nemours Company, Inc. A 10^{-3} M stock solution was prepared daily in 10^{-2} M NaOH. Crystalline CMU, obtained from Dr. E. Shantz, and Antimycin A were dissolved in 95% ethanol. Dr. J. W. Lightbown kindly provided samples of heptyl-HQNO and nonyl-HQNO. Fresh solutions of these two inhibitors were prepared daily in 10^{-3} M NaOH and the concentrations determined by absorption measurements at 346 mμ (6).

Preparation of Chloroplasts and PPNR. Intact chloroplasts and chloroplast fragments were prepared from spinach leaves as previously described (17). The chloroplasts were used immediately after preparation and the same samples used for examining TPN reduction, ATP formation, and CO_2 fixation in concurrent experiments. Chlorophyll contents were determined by the method of Arnon (1). PPNR was purified from spinach leaves as described by San Pietro (16).

Reaction Mixtures and Methods of Assay. CO_2 fixation was assayed in 15 ml Warburg vessels containing: Tris-HCl buffer, pH 7.5, 68 μmoles; $MnCl_2$, 0.65 μmole; KH_2PO_4-K_2HPO_4, pH 7.5, 0.2 μmole; NaCl, 280 μmoles; $NaHCO_3$, 0.49 μmole (containing 2.14 μc of C^{14}); intact chloroplasts containing between 100 and 150 μg of chlorophyll; total volume, 1.0 ml. The concentration of other components are given in the text. All components were added to the Warburg vessels at 4° in dim light (less than 10 ft-c). The vessels were flushed with N_2 for 5 minutes at 15° in the dark prior to illumination. The light source was a bank of eight Sylvania 150-w-flood lamps which gave approximately 1,500 ft-c at the reaction mixtures, which were shaken slowly. The reactions were terminated after 30 minutes by the addition of 0.3 ml N HCl. Aliquots (0.1 ml) were then evaporated to dryness and assayed for radioactivity (17). Dark CO_2 fixation values, obtained from replicate samples held in the dark, were subtracted from the light values.

TPNH and ATP^{32} formation were measured in the following reaction mixture: Tris-HCl buffer, pH 7.6, 80 μmoles; $MgCl_2$, 4 μmoles; KH_2PO_4-K_2HPO_4, 1.54 μmoles (containing approximately 1 μc of P^{32}); ADP, 2 μmoles; TPN, 1.5 μmoles; PPNR, 3.6 units; chloroplast fragments containing from 50 to 75 μg of chlorophyll; total volume 2.0 ml.

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[5] The following abbreviations are used: PPNR, photosynthetic pyridine nucleotide reductase; DCIP, 2,6-dichlorophenol-indophenol; CMU, 3-(p-chlorophenyl)-1,1-dimethylurea; CCCP, m-chlorocarbonyl cyanide phenylhydrazone; heptyl-HQNO, 2-heptyl-4-hydroxyquinoline-N-oxide; nonyl-HQNO, 2-nonyl-4-hydroxyquinoline-N-oxide; PMS, phenazine methosulphate.

The reaction mixture for studying TPNH and ATP^{32} formation with DCIP and ascorbate as the electron donor contained the same components with the addition of 0.2 μmole of DCIP and 14 μmoles of ascorbate. Photophosphorylation in the presence of PMS was measured in reaction mixtures containing: Tris-HCl buffer, pH 7.6, 40 μmoles; $MgCl_2$, 2 μmoles; KH_2PO_4-K_2HPO_4, 0.77 μmole (containing approximately 0.5 μc P^{32}); ADP, 1 μmole; PMS, 0.1 μmole; chloroplast fragments containing from 25 to 35 μg of chlorophyll; total volume 1.0 ml. Reaction mixtures were illuminated laterally in quartz cuvettes with 3,000 ft-c of white light under aerobic conditions at room temperature (21–23°). After illumination the reaction mixtures were centrifuged at 1,000 × *g* for 10 minutes and TPNH and ATP^{32} determined in the supernatant solutions as previously described (17).

Inhibitors were added in the concentrations stated in the text. When the inhibitors were prepared in ethanol or NaOH, equivalent amounts of these compounds were added to control reaction mixtures. The reaction components were incubated with the inhibitors in the dark for 5 minutes prior to illumination.

Results

Effects of CMU. CMU (fig 1) was a potent in-

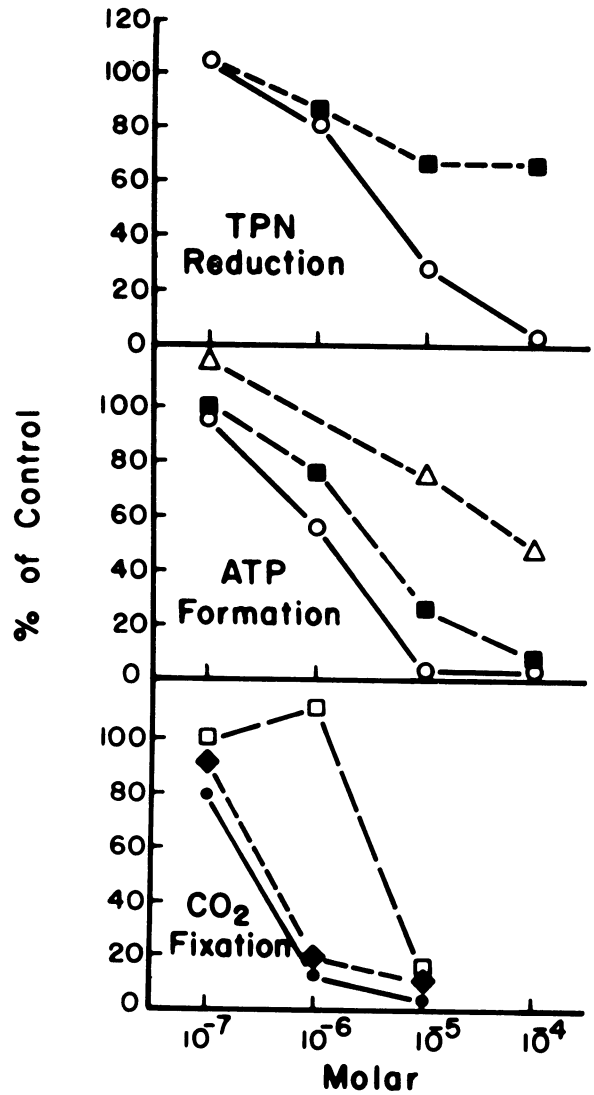


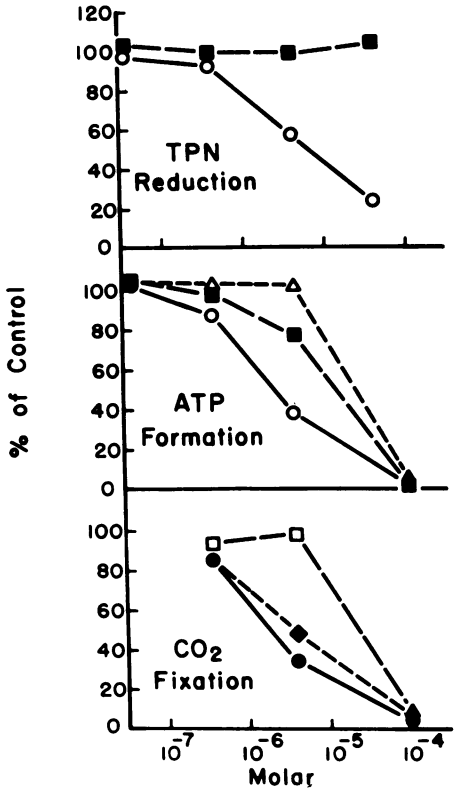
FIG. 1. Inhibition by CMU. The control rates of the various reactions were (μmoles/mg chlorophyll/hr): TPN reduction with water as the donor (○ —— ○), 58; TPN reduction with DCIP/ascorbate (■ —— ■), 35; ATP formation with water (○ —— ○), 31; ATP formation with DCIP/ascorbate (■ —— ■), 29; ATP formation with PMS (△ —— △), 31; CO_2 fixation (● —— ●), 0.25; CO_2 fixation in the presence of 10 μmoles/ml ascorbate (□ —— □), 0.30; CO_2 fixation with 1 μmole/ml fructose-1,6-diP (◆ —— ◆), 0.44.

FIG. 2 (*upper left*). Inhibition by heptyl-HQNO. The control rates of the various reactions were (μmoles/mg chlorophyll/hr): TPN reduction with water as the donor (○ —— ○), 107; TPN reduction with DCIP/ascorbate (■ —— ■), 65; ATP formation with water (○ —— ○), 82; ATP formation with DCIP/ascorbate (■ —— ■), 104; ATP formation with PMS (△ —— △), 54; CO_2 fixation (● —— ●), 1.43; CO_2 fixation in the presence of 10 μmoles/ml ascorbate (□ —— □), 2.0; CO_2 fixation with 1 μmole/ml fructose-1,6-diP (◆ —— ◆), 2.34.

FIG. 3 (*upper right*). Inhibition by nonyl-HQNO. The control rates of the various reactions were (μmoles/mg chlorophyll/hr): TPN reduction with water as the donor (○ —— ○), 87; TPN reduction with DCIP/ascorbate (■ —— ■), 55; ATP formation with water (○ —— ○), 62; ATP formation with DCIP/ascorbate (■ —— ■), 86; ATP formation with PMS (△ —— △), 50; CO_2 fixation (● —— ●), 0.73; CO_2 fixation in the presence of 10 μmoles/ml ascorbate (□ —— □), 0.88; CO_2 fixation with 1 μmole/ml fructose-1,6-diP (◆ —— ◆), 1.50.

FIG. 4 (*lower left*). Inhibition by CCCP. The control rates of the various reactions were (μmoles/mg chlorophyll/hr): TPN reduction with water as the donor (○ —— ○), 51; TPN reduction with DCIP/ascorbate (■ —— ■), 25; ATP formation with water (○ —— ○), 26; ATP formation with DCIP/ascorbate (■ —— ■), 35; ATP formation with PMS (△ —— △), 40; CO_2 fixation (● —— ●), 0.10; CO_2 fixation in the presence of 10 μmoles/ml ascorbate (□ —— □), 0.20; CO_2 fixation with 1 μmole/ml fructose-1,6-diP (◆ —— ◆), 0.33.

FIG. 5 (*lower right*). Inhibition by Antimycin A. The control rates of the various reactions were (μmoles/mg chlorophyll/hr): TPN reduction with water as the donor (○ —— ○), 94; TPN reduction with DCIP/ascorbate (■ —— ■), 62; ATP formation with water (○ —— ○), 66; ATP formation with DCIP/ascorbate (■ —— ■), 65; ATP formation with PMS (△ —— △), 56; CO_2 fixation (● —— ●), 0.83; CO_2 fixation in the presence of 10 μmoles/ml ascorbate (□ —— □), 0.90; CO_2 fixation with 1 μmole/ml fructose-1,6-diP (◆ —— ◆), 1.36.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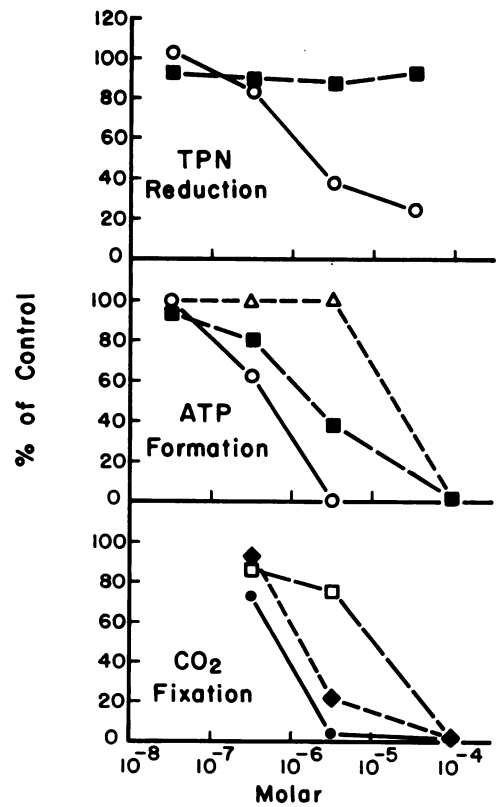
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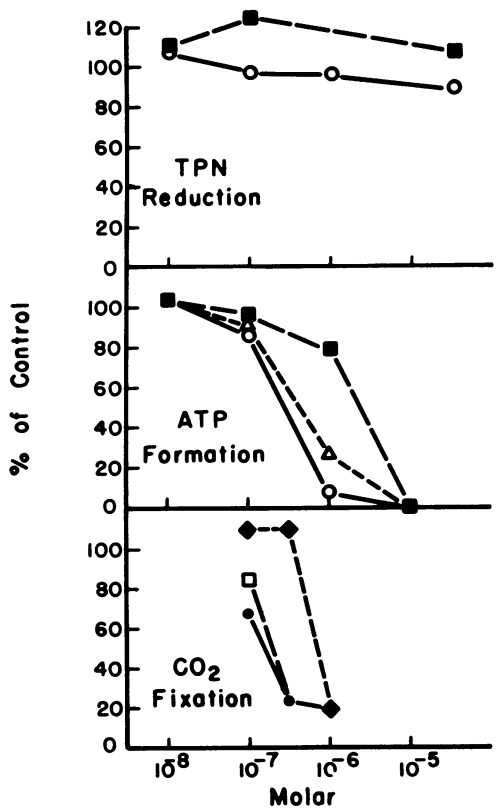
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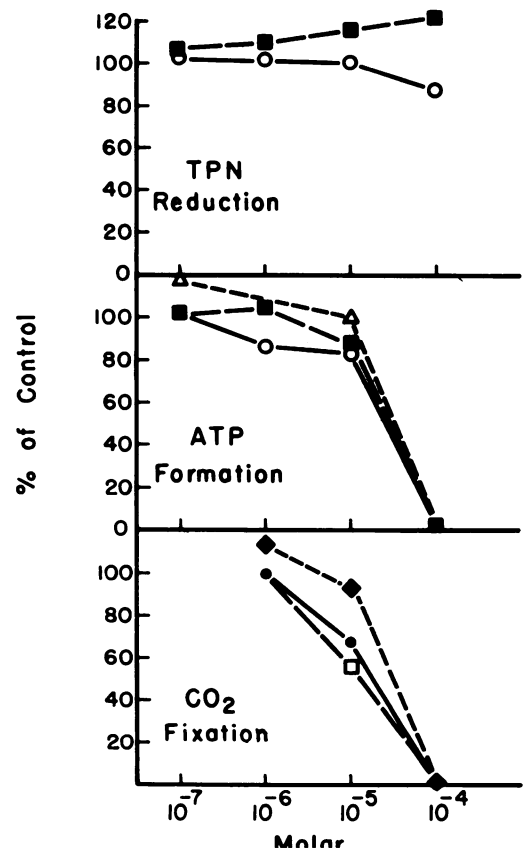
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hibitor of TPN reduction with water as the donor, producing a 72% inhibition at 10^{-5} M. DCIP and ascorbate overcame this effect to a large extent, so that TPN reduction was inhibited by only 32% at a CMU concentration of 10^{-4} M. DCIP and ascorbate had a smaller, but still apparent, sparing effect on the phosphorylation associated with TPN reduction. Thus 10^{-5} M CMU completely prevented ATP formation when water was the donor, but allowed 26% of the control activity in the presence of DCIP and ascorbate. The PMS phosphorylation was even less affected, retaining half its activity even in the presence of 10^{-4} M CMU. CO_2 fixation was very sensitive to this compound: ascorbate, but not fructose-1,6-diP, had a sparing effect. Thus 10^{-6} M CMU depressed CO_2 fixation by about 80% in the presence or absence of fructose-1,6-diP, whereas the same concentration of CMU had no inhibitory effect in the presence of ascorbate.

Effects of Heptyl-HQNO and Nonyl-HQNO. The effects of these two compounds (fig 2 & 3) were very similar to those of CMU. DCIP and ascorbate prevented suppression of TPN reduction. The PMS-induced phosphorylation was inhibited only at relatively high concentrations, i.e., greater than 10^{-5} M. As with CMU, ascorbate but not FDP was able to overcome the inhibition of CO_2 fixation. Nonyl-HQNO was slightly more effective than heptyl-HQNO at equal concentrations.

Effects of CCCP. The compound CCCP (fig 4) was a potent uncoupling agent. It had no effect on TPN reduction at concentrations as high as 10^{-5} M whereas phosphorylation was 50% inhibited at from 1/2 to 1/20 of this level. CO_2 fixation was 88% inhibited at 3×10^{-7} M CCCP but this effect was completely overcome by fructose-1,6-diP. Ascorbate did not alleviate the inhibition.

Effects of Antimycin A. Figure 5 shows that except at very high concentrations, Antimycin A had relatively little effect on any of the processes studied. No significant inhibition of the rate of TPNH formation was ever observed. Phosphorylation was inhibited only at 10^{-4} M. CO_2 fixation showed some inhibition (32%) at 10^{-5} M and this was partially overcome by the addition of fructose-1,6-diP.

Discussion

In the experiments described in this paper, we have examined the action of a number of inhibitors on TPNH and ATP formation and the fixation of CO_2 in an attempt to compare the effects of each compound on the various reactions. The experimental conditions were as uniform as possible; for instance, the effect of each inhibitor was studied on all the processes in concurrent experiments using the same chloroplast preparations. There were inevitable differences in the techniques used to study each process. Fragmented chloroplasts were used in experiments on TPNH and ATP formation. Whole chloroplasts were used to study CO_2 assimilation, since Havir and Gibbs (8) have demonstrated that fragmented chloroplasts are not able to carry out the complete photosynthetic carbon cycle. The cofactor requirements also varied from reaction to reaction. Probably the most important difference was the discrepancy in rates between TPNH and ATP formation and CO_2 fixation. However, by limiting the level of PPNR, TPNH and ATP formation were measured at from 30 to 110 μmoles/mg chlorophyll/hr which is well below the maximum possible (12), but still considerably more than the rate of CO_2 fixation (0.1–2.0 μmoles/mg chlorophyll/hr). This probably explains the greater sensitivity of CO_2 fixation to all the inhibitors. It is considered that DCIP merely accelerates the ascorbate effect during TPN reduction (14) and it was omitted when studying CO_2 fixation because of an inhibitory effect.

In confirmation of results obtained by other workers (3, 11), CMU and both heptyl-HQNO and nonyl-HQNO inhibited TPN reduction. Their effects were bypassed by the use of ascorbate as electron donor. Since DCIP and ascorbate also allowed ATP formation to occur, the site of phosphorylation must be between the natural acceptor from ascorbate [at present unknown (14)] and TPN. CMU is thought to inhibit the oxygen-evolving mechanism (11). Heptyl-HQNO and nonyl-HQNO appear to inhibit the mammalian electron transport sequence between cytochrome b and cytochrome c but other sites may also be involved (13). By analogy it would seem that their point of action in the chloroplast could well be between cytochrome b_6 and cytochrome f. If this is indeed the case, then this is unlikely to be a site of phosphorylation.

Heytler and Pritchard (9) have previously shown that CCCP is a potent inhibitor of the cyclic phosphorylation with PMS. This compound (fig 4) also uncoupled ATP formation from TPN reduction with either water or ascorbate as the electron donor.

In confirmation of results obtained by Arnon (2) and Baltscheffsky (5), Antimycin A had little effect on any of the chloroplast reactions (fig 5). It appeared to act as an uncoupling agent at 10^{-4} M but this effect is obscure since the concentration is many times greater than that normally used in experiments on mammalian electron transport (15). These results are in marked contrast to the susceptibility of bacterial photophosphorylation to Antimycin A (5, 7).

Compounds which inhibited TPN reduction and phosphorylation also prevented CO_2 fixation. These studies confirmed the requirements for both TPNH and ATP (or possibly their precursors) in the photosynthetic carbon cycle. Ascorbate, which allowed TPN reduction in the presence of CMU, heptyl-HQNO, and nonyl-HQNO, also overcame the inhibitory effects of these compounds on CO_2 fixation (fig 1–3). Ascorbate had no effect on CO_2 fixation in the presence of CCCP (fig 4); this is consistent with the fact that this compound uncoupled phosphorylation under all the conditions studied and al-

lowed electron transfer to proceed at an unchanged rate. The CCCP suppression of CO_2 fixation could, however, be overcome by the addition of fructose-1,6-diP. Other phosphorylated compounds such as glyceraldehyde-3-P, glyceric acid-3-P, glucose-6-P, and ribose-5-P had a similar effect in overcoming the CCCP inhibition (unpublished results). The mechanism whereby these compounds can apparently overcome a lack of ATP is at present obscure.

Summary

I. The effects of Antimycin A, 3-(p-chlorophenyl)-1,1-dimethylurea, m-chlorocarbonyl cyanide phenylhydrazone, 2-heptyl-4-hydroxyquinoline-N-oxide, and 2-nonyl-4-hydroxyquinoline-N-oxide have been studied on triphosphopyridine nucleotide reduction, "cyclic" and "non-cyclic" photophosphorylation, and carbon dioxide assimilation by spinach chloroplasts.

II. 3-(p-chlorophenyl)-1,1-dimethylurea and the hydroxyquinoline compounds inhibited triphosphopyridine nucleotide reduction and the associated phosphorylation but the effect could be overcome by the addition of 2,6-dichlorophenol-indophenol and ascorbate. Ascorbate overcame the inhibition of carbon dioxide fixation.

III. m-chlorocarbonyl cyanide phenylhydrazone was a potent uncoupler of phosphorylation from electron transfer. It inhibited CO_2 fixation but this effect could be overcome by the addition of fructose-1,6-diphosphate.

IV. Antimycin A had relatively little effect on any of the processes examined.

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