This investigation was supported in part by Public Health Research Grant GM-10705-02 from the National Institute of General Medical Sciences. One of us (L.A.) was a trainee under a National Institute of Health Training Grant 5T1-GM 961-02, during the course of this work.

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Received August 18th, 1966

*Biochim. Biophys. Acta*, 131 (1967) 198–201

BBA 43153

## 2-Amino-1,1,3-tricyanopropene: a new inhibitor of oxygen evolution in photosynthesis

One of the most fundamental problems of photosynthesis is the mechanism of $O_2$ evolution; despite many studies of this problem, remarkably little is known. Investigations by WESSELS AND VAN DER VEEN[1] showed that some substituted phenylureas (*e.g.* DCMU) act as inhibitors of $O_2$ evolution; further work (GINGRAS AND LEMASSON[2], IZAWA AND GOOD[3], has been carried out with these inhibitors on the mechanism of inhibition which may throw light upon the $O_2$ evolution process itself. Some substituted benzimidazoles and aminotriazines also inhibit the oxygen-evolving system of photosynthesis (BÜCHEL *et al.*[4], BISHOP[5]). The purpose of the present work was to search for other compounds capable of specifically inhibiting $O_2$ evolution. 2-Amino-1,1,3-tricyanopropene (TCAP), previously shown to uncouple phosphorylation in mitochondria (EBERTS[6], PARKER[7]), was found in this study to inhibit selectively $O_2$ evolution of the photosynthetic reactions studied (ARNON *et al.*[8]).

Fresh spinach leaves obtained commercially were used in the preparation of chloroplasts by a modification of the method of WHATLEY AND ARNON[9]. 50 g of sliced,

Abbreviations: TCAP, 2-amino-1,1,3-tricyanopropene; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCIP, 2,6-dichlorophenolindophenol; PMS, phenazine methosulphate.

deribbed leaf blades were ground with 100 ml of sucrose medium (0.4 M sucrose, 0.02 M Tris–HCl, pH 8.0, 0.01 M NaCl) and 50 g of cold sand. The green juice obtained by squeezing the slurry through cheesecloth was centrifuged for 1 min at 200 × *g* to sediment sand, leaf debris and whole cells. The green supernatant liquid was decanted and centrifuged for 7 min at 4000 × *g*. As a result of this centrifugation most of the intact chloroplasts were sedimented: they were resuspended in 50 ml of the sucrose grinding medium and again centrifuged for 7 min at 4000 × *g*. 10 ml of 0.05 M Tris–HCl (pH 8.0) were then added to the sedimented chloroplasts to yield a suspension of broken chloroplasts. All experiments were carried out in 14-ml Warburg flasks using a Braun photosynthetic Warburg bath (Type V185). Reactions were started by tipping the $^{32}P$ and oxidant (where required). Illumination was 1000 ft-candles and $O_2$ evolution was measured manometrically. ATP was determined as organic $^{32}P$ by the method of HAGIHARA AND LARDY[10] and an aliquot counted in a Geiger counter. TPNH and ferricyanide were measured spectrophotometrically at 340 mμ and 400 mμ respectively. Dark controls were measured in all cases. Ferredoxin was prepared by the method of TAGAWA AND ARNON[11]. TCAP was kindly supplied by Upjohn Ltd., Crawley, England. Chlorophyll was estimated by the method of ARNON[12].

TCAP, at low concentrations, inhibits $O_2$ evolution in all non-cyclic systems tested where the evolution of $O_2$ is an integral step of the electron-transport chain (*e.g.* for 50% inhibition of $TPN^+$–$H_2O$, [TCAP] = $1.3 \cdot 10^{-5}$ M; ferricyanide–$H_2O$, $8.0 \cdot 10^{-5}$ M; and ferricyanide–DCIP, $1.1 \cdot 10^{-4}$ M) (see also Table I). In the only non-cyclic system tested where electron transport does not involve the evolution of $O_2$, namely, $TPN^+$–ascorbate *plus* DCMU, the photoreduction of $TPN^+$ and the accompanying photophosphorylation were both unaffected (Table I). During these

TABLE I

EFFECT OF TCAP ON $O_2$ EVOLUTION AND ON PHOSPHORYLATION

The reaction mixture included in a final volume of 3 ml, chloroplast fragments containing 0.2 mg chlorophyll, and the following (in μmoles): Tris–HCl buffer (pH 8.0), 75; $MgSO_4$, 5; ADP, 10; $K_2H^{32}PO_4$, 10; and the following additions (in μmoles): in Expt. A: $K_3Fe(CN)_6$, 15; Expt. B: $K_3Fe(CN)_6$, 15; DCIP, 0.2; Expt. C: $TPN^+$, 4 (*plus* spinach ferredoxin); Expt. D: $TPN^+$, 4 (*plus* spinach ferredoxin); sodium ascorbate, 20; DCIP, 0.2; Expt. E: PMS, 0.1; Expt. F: Vitamin $K_3$, 0.3; sodium ascorbate, 10; DCMU, 0.3 (see refs. 13, 14); Expt. G: Vitamin $K_3$, 0.3; sodium ascorbate, 10. All reactions were carried out at 15° under argon except Expt. G, which was under air. Light intensity was 1000 ft-candles. TCAP was dissolved in 50% methanol in water.

| *Expt.* | *Percentage inhibition by TCAP* ($2 \cdot 10^{-4}$ *M*) | |
|---|---|---|
| | *Electron transport* | *ATP formation* |
| Non-cyclic photophosphorylation | | |
| A. Ferricyanide–$H_2O$ | 83 | 81 |
| B. Ferricyanide–DCIP | 66 | — |
| C. $TPN^+$–$H_2O$ | 100 | 100 |
| D. $TPN^+$–ascorbate | 0 | 0 |
| Cyclic and pseudocyclic photophosphorylation | | |
| E. PMS | — | 0 |
| F. Vitamin $K_3$, anaerobic | — | 26 |
| G. Vitamin $K_3$, aerobic | — | 88 |

studies, it was found that ascorbate reacted with TCAP in the presence of chloroplasts and light to form a substance having significant light-absorbing properties at 340 mμ: therefore appropriate controls were included in all experiments involving the $TPN^+$–ascorbate system thus permitting correction of the TPNH assays. At considerably higher concentrations, TCAP inhibits anaerobic cyclic photophosphorylation, catalysed by vitamin $K_3$ ($8 \cdot 10^{-4}$ M TCAP for 50 % inhibition) and at even higher concentrations, photophosphorylation catalysed by PMS ($2 \cdot 10^{-3}$ M TCAP for 50 % inhibition).

The experimental results (Table I) show that TCAP is a potent inhibitor of $O_2$ evolution and consequently of any of the reactions of photosynthesis dependent upon water as a source of electrons. In this respect, TCAP resembles the action of the substituted phenylureas which also curtail $O_2$ evolution at very low concentrations. The similarities of the effects of these inhibitors extend to cyclic photophosphorylation where inhibition occurs at higher concentration of inhibitors; however, the chemical structures of these inhibitors show few likenesses.

TCAP is much more effective as an inhibitor of $O_2$ evolution in photosynthesis than as an uncoupler of oxidative phosphorylation where a 50 % inhibition demands a concentration of inhibitor of $8 \cdot 10^{-4}$ M which is approximately equivalent to the concentration required for a similar inhibition of anaerobic cyclic photophosphorylation catalysed by vitamin $K_3$.

The authors wish to acknowledge the skilled technical assistance of Mrs. SONIA DRUERY, and one of us (A.A.H.) wishes to thank Professor F. R. WHATLEY for generously providing the excellent facilities which permitted these studies to be made.

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Received September 29th, 1966