FERREDOXIN AND THE DARK AND LIGHT REDUCTION OF DINITROPHENOL*

F.F. del Campo, J.M. Ramírez, A. Paneque, and M. Losada

Sección de Fisiología Celular
Instituto de Biología Celular, C.S.I.C., Madrid, Spain

Received January 28, 1966

It has been shown by Arnon's and Jagendorf's groups that, at low concentration, DNP**, which is an effective uncoupler of oxidative phosphorylation, does not affect photosynthetic phosphorylation at all, either cyclic or noncyclic. At high concentration, however, they found that it inhibits the noncyclic electron flow from water to NADP or ferricyanide (cf. review by Losada and Arnon, 1963). Wessels (1959, 1960, 1963) found later unexpectedly that, at an intermediate concentration of 0.2 mM, DNP could serve as a catalyst of pseudocyclic photophosphorylation and as a Hill oxidant, both reactions being inhibited by poisons which block the photolytic splitting of water. The photoreduction of DNP by chloroplasts proceeded up to the level of ANP, and was accompanied by the evolution of stoichiometric amounts of oxygen and presumably by the formation of ATP (see also Wessels, 1965).

The observations by Kessler (1959) and Hattori (1962) that, in algae, the dark reduction of nitrite under aerobic conditions or with molecular hydrogen was strongly inhibited by DNP, where-

* Aided by grant AM 06848-03 from the National Institutes of Health, United States Public Health Service.

** Abbreviations: ATP, adenosine triphosphate; NADP and $NADPH_2$, oxidized and reduced nicotinamide-adenin dinucleotide phosphate, respectively; DNP, 2,4-dinitrophenol; ANP, 2-amino-4-nitrophenol; DPIP, 2,6-dichlorophenol indophenol; Fd, ferredoxin.

as its photochemical reduction was much less sesitive to this poison, led these authors to the conclusion that ATP was required for nitrite reduction. They also concluded that, since the reduction of nitrate to nitrite was much less sesitive to DNP than the further reduction of nitrite, high-energy phosphate was not necessary for the first reductive step.

In recent work from our laboratory (Losada et al., 1965), it was shown that, in spinach, neither the reduction of nitrate to nitrite nor the reduction of nitrite to ammonia depended on ATP. Each step was catalyzed by a different chloroplast enzyme and specifically required reduced flavin nucleotides and ferredoxin, respectively, as electron donors. As source of reducing power, the three following systems were effective in keeping the cofactors in the reduced state: 1) illuminated spinach grana, 2) $NADPH_2$-spinach NADPreductase, and 3) H_2-Clostridium pasteurianum hydrogenase. When the first system was used, the photochemical reduction of nitrate and nitrite were coupled to the formation of stoichiometric amounts of ATP.

The results presented here show that DNP can be reduced under the appropiate experimental conditions in the dark by H_2 and in the light by chloroplasts, and shed new light on the interpretation of the behaviour of this nitroderivative in the reduction of inorganic nitrogen compounds as well as in cyclic and noncyclic photophosphorylation.

RESULTS AND DISCUSSION - Fig. 1 shows that the dark reduction of DNP by molecular hydrogen in the presence of a crude C. pasteurianum hydrogenase required ferredoxin from the same organism as electron carrier. Boiled ferredoxin was much less active. In the absence of the bacterial hydrogenase, the cofactor or the electron acceptor, no hydrogen uptake took place. Spinach ferredoxin, flavin mononucleotide and benzyl viologen were also effective in mediating the transfer of electrons from the hydrogen-hydrogenase system to DNP. According to the data presented

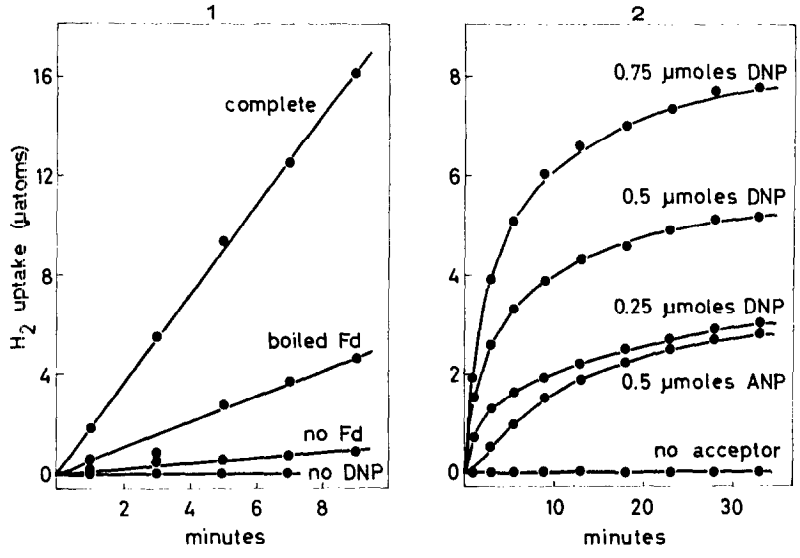

Figure 1. (left) Ferredoxin as electron carrier in the dark reduction of DNP with hydrogen gas as the electron donor. The complete reaction mixture contained, in a final volume of 3 ml, ferredoxin-free C. pasteurianum hydrogenase, 5.6 mg; C. pasteurianum ferredoxin, 0.18 mg; and the following in umoles: Tris buffer, pH 8.0, 200; cysteine, 10; DNP, 4.5. The reactions were carried out at 30° in Warburg manometer flasks under hydrogen. Other conditions were the same as those previously described (Paneque et al., 1964).

Figure 2. (right) H_2 uptake as a function of added DNP or ANP. Experimental conditions as in Fig. 1, except that DNP and ANP were added as indicated, and 0.36 mg of ferredoxin were used.

---

in Fig. 2, the reduction of DNP proceeded up to the level of diaminophenol, i.e., 6 moles of H_2 per mol of DNP, this being in agreement with the fact shown in the same figure that ANP could also be reduced to that stage under the same conditions. No hydrogen uptake occurred with ANP as electron acceptor in the absence of ferredoxin.

The effect of spinach ferredoxin on the light reduction of DNP and ANP by heated spinach chloroplast fragments with ascorbate-DPIP as electron donor and under strict anaerobic conditions is shown in Table I. Since, in the absence of added spinach ferredoxin, the photoreduction of NADP was insignificant, it

TABLE I

COMPARATIVE EFFECT OF FERREDOXIN ON THE PHOTOREDUCTION OF NADP, DNP AND ANP BY CHLOROPLASTS WITH ASCORBATE AS ELECTRON DONOR

Addition	Ascorbate disappeared (micromoles)	$NADPH_2$ formed (micromoles)	ANP present (micromoles)
NADP	0.3	0.3	—
NADP, Fd	6.4	6.5	—
DNP	7.3	—	1.8
DNP, Fd	6.4	—	2.0
ANP	5.4	—	4.3
ANP, Fd	5.3	—	4.2

The reaction mixture included, in a final volume of 3 ml, heated once-washed chloroplast fragments containing 0.4 mg of chlorophyll and the following in umoles: Tris-HCl, pH 8.0, 200; sodium ascorbate, 20; DPIP, 0.2. Where indicated, 6.5 umoles of NADP, 4.5 umoles of DNP, 4.5 umoles of ANP, and 0.8 mg of spinach ferredoxin were added. The reactions were carried out at 17° for 12 min. in Warburg manometric flasks under argon purified by passing through alkaline pyrogallol, and methylene blue reduced by Zn powder. At the end of the experiment, $NADPH_2$ and ANP were estimated spectrophotometrically at 340 mu and 485 mu, respectively, and ascorbate was titrated with DPIP. Other conditions were the same as those previously described (Losada et al., 1965).

---

could be concluded that the once-washed chloroplast fragments contained negligible amounts of the cofactor. Using the same chloroplast preparation, the light-dependent oxidation of ascorbate proceeded at similar rates when NADP was replaced as electron acceptor by either DNP or ANP, ferredoxin being not required in these cases. No significant disappearance of ascorbate occurred in the absence of a terminal electron acceptor even if ferredoxin was present.

If the photoreduction of DNP was carried out to completion, this compound was quantitatively converted into ANP (identified as such by its absorption spectrum in an aliquot of the reaction mixture in a recording spectrophotometer), but more ascorbate disappeared than was needed to reduce DNP to ANP. This anomaly became even more apparent using, instead of DNP, ANP itself, for this aminonitroderivative was practically recovered as such at the end of the experiment in spite of substantial amounts of ascorbate being consumed during the reaction (see also Table I). Although the cause of this phenomenon remains unknown, it should be underlined that no gas absorption was observed during the experiments, performed under strict anaerobic conditions, thus excluding the participation of oxygen.

When DNP was added to the system containing NADP, the electron flow remained essentially unchanged (as measured by the oxidation of ascorbate) but the reduction of NADP (estimated fluorometrically) decreased appreciably, DNP being simultaneously reduced. These results show that NADP and DNP can compete for the electrons produced in the photochemical reaction. Since DNP can act as a good electron acceptor before and after ferredoxin in the photosynthetic system, it might well inhibit, even at low concentration, cyclic photophosphorylation by trapping electrons and interrupting the flow in the closed circuit, in a manner analogous to that of ferricyanide (Arnon, 1961). Removal of electrons and not uncoupling may thus be the cause of the inhibition by DNP of the ferredoxin-dependent cyclic photophosphorylation recently reported by Arnon (1965).

The unexpected finding that, at the high concentration of DNP used (1.5 mM), the noncyclic electron flow from ascorbate was not inhibited in heated chloroplasts prompted us to compare the effect of ascorbate and water as electron donors using a fresh chloroplast system. In agreement with previous results from other authors (see Introduction), DNP at high concentration completely blocked the electron flow from water to NADP or ferricyanide.

Besides, it was found that although no reduction of DNP by water (as measured by oxygen evolution or ANP formation) could occur at such high concentration, DNP could be reduced if ascorbate--DPIP was added as electron donor to the system (Table II). These results may explain why cyclic photophosphorylation catalyzed by phenazine methosulfate and menadione is resistant to inhibition by DNP whereas pseudocyclic photophosphorylation catalyzed by flavin mononucleotide is not (Whatley et al., 1959).

The results presented in this communication complement and partly challenge those just published by Wessels (1965) and

TABLE II

INHIBITION BY DNP OF ITS OWN PHOTOREDUCTION BY CHLOROPLASTS WHEN WATER IS THE ELECTRON DONOR

System	O_2 evolved	Ascorbate oxidized (micromoles)	ANP formed (micromoles)
Complete	0	2.2	0.5
Ascorbate-DPIP omitted	0	—	0

Experimental conditions as in the DNP system of Table I, except that unwashed nonheated chloroplast fragments were used.

Hertogs and Wessels (1965).

REFERENCES

Arnon, D.I., In A Symposium on Light and Life, edited by W. McElroy and B. Glass, The Johns Hopkins Press, 1961, p. 489.

Arnon, D.I., Science 149, 1460 (1965).
Hattori, A., Plant Cell Physiol. 3, 355 (1962).
Hertogs, M., and J.S.C. Wessels, Biochim. Biophys. Acta 109, 610 (1965).
Kessler, E., Symp. Soc. Exptl. Biol. 13, 87 (1959).
Losada, M., and D.I. Arnon, in Metabolic Inhibitors vol. II, edited by M. Hochster and J. H. Quastel, Academic Press, 1963, p. 585.
Losada, M., A. Paneque, J. M. Ramírez, and F. F. del Campo, in A Symposium on Non-heme Iron Proteins: Role in Energy Conversion, edited by A. San Pietro, Antioch Press, 1965, p. 211.
Losada, M., J.M. Ramírez, A. Paneque, and F.F. del Campo, Biochim. Biophys. Acta 109, 86 (1965).
Paneque, A., J.M. Ramírez, F.F. del Campo, and M. Losada, J. Biol. Chem. 239, 1737 (1964).
Wessels, J.S.C., Biochim. Biophys. Acta 36, 264 (1959).
Wessels, J.S.C., Biochim. Biophys. Acta 38, 195 (1960).
Wessels, J.S.C., in Mechanism of Photosynthesis, edited by H. Tamiya, Pergamon Press, 1963, p. 263.
Wessels, J.S.C., Biochim. Biophys. Acta 109, 357 (1965).