

Energy Transfer Inhibition Induced by Nitrofen

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The herbicide nitrofen was shown to act as an energy transfer inhibitor. The results proved nitrofen to act by inhibiting nucleotide exchange on the chloroplast coupling factor. A strong correlation was found between the inhibition of phosphorylation, ATPase activity, and nucleotide exchange. These results are discussed in terms of a regulatory effect of tightly bound ADP on the enzymatic activity of the chloroplast coupling factor.

Introduction

Nitrofen is used to control annual weeds in cultures of parsley, onion, carrot, celery, and rice [1]. Until now its mode of action is a controversial question. The main herbicidal effect of nitrofen may probably be located in the photosynthetic apparatus [2]. Besides chlorophyll bleaching [3–5] and electron transport inhibition [2, 4] nitrofen causes energy transfer inhibition [4, 6]. From their own results Lambert *et al.* [6] concluded that nitrofen must be competitive to ADP but not to phosphate in the process of photophosphorylation. This result is rather surprising and therefore particularly interesting because of the lack of structural similarity between the substrate ADP and the inhibitor nitrofen. However, a competitive type of inhibition could also be caused by other effects than substrate displacement from the catalytic site [7]. In this paper, the mechanism of energy transfer inhibition by nitrofen is studied in greater detail.

The terminal process of photophosphorylation may be subdivided into two partial reactions, I) H^+ translocation through the CF_0 -part of the ATPase complex, and II) the ATP synthase reaction catalysed by CF_1 . Of the known energy transfer inhibi-

tors, covalent binding of the carbodiimide DCCD to CF_0 [8–10] and interaction with tributyltin chloride [11, 12] inhibit the former reaction, while others affect CF_1 , e.g. phlorizin [13–16], Dio-9 [17–19], tentoxin [20–27], and a number of 3'-esters of ADP [28].

Chloroplast ATPase is a latent enzyme which needs activation in order to display its catalytic activity. Physiological activation results from membrane energization [29, 30]. Recently it was shown that activation is paralleled by energy dependent release of tightly bound ADP from CF_1 and deactivation to the reverse reaction [30–32]. Probably the rate of photophosphorylation is usually controlled by enzyme activation rather than by fatigue number of the enzyme [29, 33]. Inhibition of ATPase activation as well as ATPase reaction would of course affect the rate of photophosphorylation and produce the characteristics of energy transfer inhibition (*i.e.* inhibition of coupled electron transport).

The goal of the present study was to localize the effect of nitrofen with regard to the partial reactions mentioned earlier.

Methods

Chloroplast isolation from spinach leaves was carried out as described by Strotmann *et al.* [34]. For chlorophyll determination Arnon's method [35] was employed.

Non-cyclic electron transport from water to ferricyanide was measured by following the decrease in 420 nm absorbance in a spectrophotometer (PMQ II, Zeiss) with cross illumination equipment for one minute (continuous registration). The cross illu-

Abbreviations: CF_1 , chloroplast coupling factor; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCPIP, 2,6-dichlorophenol-indophenol; Fecy, $K_3[Fe(CN)_6]$; I_{50} , inhibitor concentration causing 50% of maximum effect; MV, methylviologen, paraquat, N,N' -dimethyl-4,4'-bipyridinium dichloride; nitrofen, 2,4-dichlorophenyl-4'-nitrophenylether = Tok®; PMS, phenazine methosulfate; tricine, N-tris(hydroxymethyl)methylglycine.

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mination equipment contains a 2 mm heat filter (Schott) within a cooled lens system. The red actinic light (filter RG 630, Schott) was 870 W/m², measured within the reaction cuvette, which was kept at a temperature of 20 °C. The reaction medium contained 25 mM tricine buffer, pH 8.0, 50 mM NaCl, 5 mM MgCl₂, 5 mM Pi, pH 8.0, 0.5 mM ADP, 1 mM K₃[Fe(CN)₆], and 12 to 20 µg chlorophyll in a total volume of 2 ml.

For the measurement of non-cyclic phosphorylation the medium contained 5 mM ³²P-labeled phosphate (1.8 MBq/ml, Amersham Buchler). The reaction was stopped after one minute by addition of HClO₄ at a final concentration of 0.3 M. After centrifugation (2 minutes at 10 000 × g) an aliquot was analyzed for organic phosphate employing the method of Strotmann [36].

For measurement of uncoupled electron transport, CF₁ stripped chloroplasts were prepared as described by Tischer and Strotmann [37]. Cyclic phosphorylation was measured with 50 µM PMS instead of Fecy. Photosystem I dependent phosphorylation was followed in a system containing 20 µM DCMU, 5 mM ascorbate, 0.2 mM DCPIP, and 0.2 mM MV instead of Fecy. In the same system, electron transport was followed by measuring the consumption of O₂ with a Clark type electrode.

Trypsin activated ATPase activity of isolated CF₁ was measured as described by Strotmann *et al.* [38] and Hesse *et al.* [39]. Light triggered ATPase was measured as described by Schumann and Strotmann [31]. Chloroplasts were illuminated for one minute in a water bath at 20 °C with white light (1200 W/m²)

of a commercial lantern-slide projector. The reaction medium contained 25 mM tricine buffer, pH 8.0, 5 mM MgCl₂, 50 µM PMS, 20 µM DCMU, and 0.13 to 0.15 mg chlorophyll per ml. After 5 seconds in the dark, a sample of the activated chloroplasts was injected into the ATPase test medium, containing no PMS but 0.5 mM ATP labelled by γ-[³²P]ATP (3–4 KBq/ml).

Kinetic measurements of light induced exchange of ADP on membrane bound CF₁ was measured using the method of Strotmann *et al.* [40]. Light dependent proton transport across the thylakoid membranes was measured with a glass electrode at 20 °C in a medium containing 50 mM NaCl, 5 mM MgCl₂, 50 µM PMS, and 60 to 80 µg chlorophyll in a final volume of 3 ml. Illumination was performed employing a commercial lantern-slide projector. The red actinic light (filter RG 630, Schott) was 935 W/m², measured inside the temperature controlled (20 °C) glass reaction chamber.

Results

1. Nitrofen as an inhibitor of photosynthetic electron transport

Energy transfer inhibition by nitrofen is superimposed by direct inhibition of electron transport [2, 41]. The latter effect can be isolated by following uncoupled electron transport from water to Fecy. Half maximal inhibition is achieved by 10 µM nitrofen. Congruent to earlier results [2] no inhibition of uncoupled photosystem I electron transport from

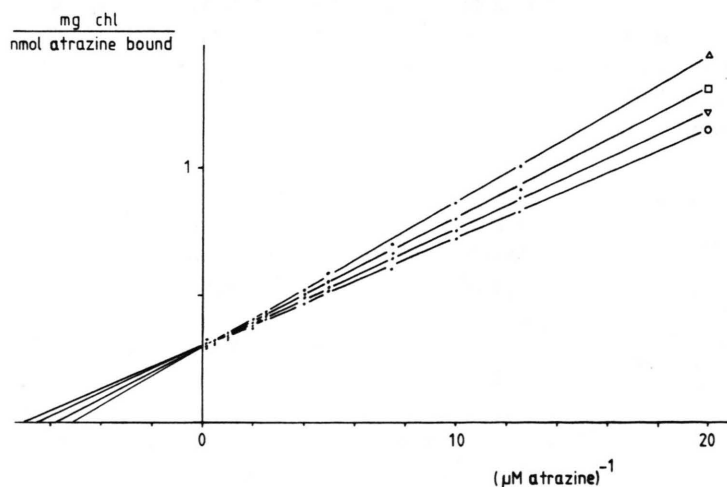


Fig. 1. Double-reciprocal plot of atrazine binding to CF₁-stripped chloroplasts competing with 4 µM (Δ), 2 µM (□), and 1 µM (▽) nitrofen or without addition of nitrofen (○). Chlorophyll content was 27 µg/ml. Experimental conditions corresponded those of uncoupled electron transport measurement with the exception of the electron acceptor being omitted.

Table I: Inhibition of electron transport through photosystem I. The reaction medium contained 25 mM tricine buffer, pH 8.0, 50 mM NaCl, 5 mM MgCl_2 , 5 mM ascorbate, pH 8.0, 0.2 mM MV, 0.2 mM DCPIP, 15 $\mu\text{g/ml}$ chlorophyll, and 0.2 mM ADP and 5 mM phosphate, pH 8.0, when measuring phosphorylation. As the herbicide and DCPIP were dissolved in methanol, all samples contained 5% (v/v) methanol. Uncoupled chloroplasts were prepared as described by Tischer and Strotmann [37] from the same leaves in parallel. Electron transport was measured by following O_2 consumption in Clark type O_2 electrode.

Conditions	$\frac{\mu\text{mol O}_2 \text{ consumed}}{\text{mg Chl} \cdot \text{h}}$		
	Non-phosphorylating	Phosphorylating	Uncoupled
40 μM nitrofen	37.4	76.5	497.0
control	37.6	162.3	496.4
inhibition	—	52.9%	—

$\frac{\text{mg chl} \cdot \text{h}}{\mu\text{mol ATP}}$

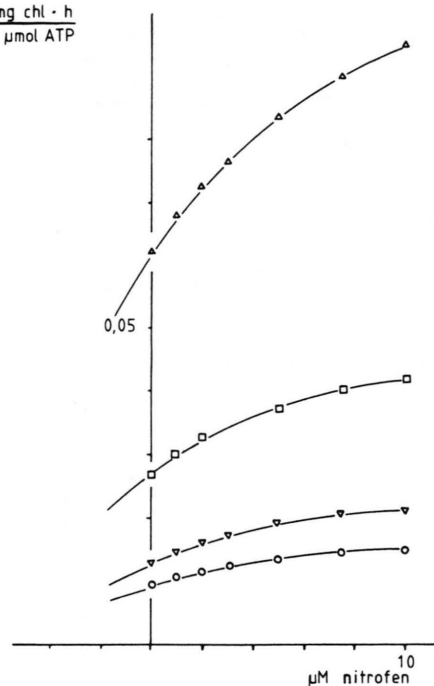


Fig. 2. Dixon plot of a phosphorylation experiment. The reaction medium contained 25 mM tricine buffer, pH 8.0, 50 mM NaCl, 5 mM MgCl_2 , 5 mM phosphate, pH 8.0, 20 μM DCMU, 50 μM PMS, 20 $\mu\text{g/ml}$ chlorophyll, 10 mM glucose, 20 units/ml hexokinase, and nitrofen as indicated. Methanol content was constant 2% (v/v). ADP concentrations were 2 μM (Δ), 5 μM ($\square$), 10 μM (∇), and 15 μM ($\circ$) respectively.

DCPIP to MV was obtained (Table I), indicating that the site of nitrofen inhibition is around photosystem II. Fig. 1 shows that nitrofen is a competitive inhibitor in the binding of ^{14}C -labelled atrazine. The calculated K_i is 11 μM . It appears therefore that the site of electron transport inhibition by nitrofen is identical with the DCMU binding site.

2. Nitrofen acting as an energy transfer inhibitor

In contrast to the uncoupled system, photosystem-I dependent electron transfer in the presence of P_i and ADP is diminished by the herbicide (Table I) with an I_{50} of 1.5 μM . The same value is obtained all the same whether PMS mediated photophosphorylation or non-cyclic electron transport mediated phosphorylation in a water-ferricyanide system are measured. Accordingly, this effect of nitrofen may be ascribed to energy transfer inhibition. Lambert *et al.* [6] found that inhibition of phosphorylation by nitrofen is competitive to ADP. This result is confirmed by double-reciprocal plots of our own experiments. But a Dixon-plot of these experiments indicates partial competitive inhibition with respect to ADP interacting with CF_1 (Fig. 2). As a working hypothesis derived from the Briggs-Haldane form of an enzyme reaction, the occurrence of a CF_1 -ADP complex during ATP-synthesis is postulated. This is in good agreement with models based on experimental results of Boyer *et al.* [42] and Bickel-Sandkötter and Strotmann [43]. On the basis of this

assumption, the shown results indicate that nitrofen binds as well to CF_1 as to CF_1 -ADP-complex without forming a ternary dead-end-complex but reducing turnover rate. On the other hand, the inhibition kinetics are complicated with regard to the substrate P_i . At P_i concentrations less than 150 μM the effect of nitrofen is not manifest any more, indicating that phosphate binding and even phosphoryl transfer may be unaffected. The observed phosphorylation rates of 161 $\mu\text{mol ATP}$ per mg chl and hour (150 μM P_i) down to 43 $\mu\text{mol ATP}$ per mg Chl and hour (20 μM P_i) were not inhibited by addition of 12 μM nitrofen any more. Therefore our further interest was focused on nitrofen interactions with nucleotide dependent reactions.

3. Effect of nitrofen on exchange of tightly bound adenine nucleotides and on ATP hydrolysis

In addition to the catalytic ADP binding site, CF_1 contains a regulatory site which binds ADP and

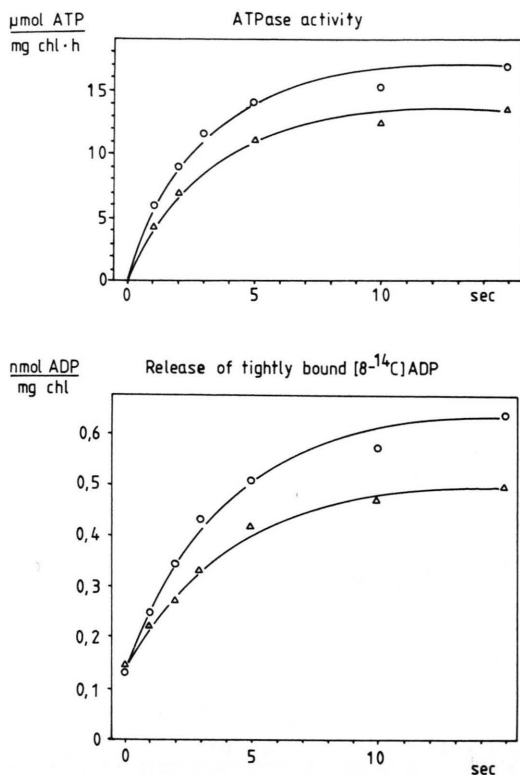


Fig. 3. Correlation between the activation of ATPase activity (upper part) and liberation of tightly bound nucleotides (lower part) with (Δ) and without ($\circ$) addition of 12 μM nitrofen, respectively, during the activation step. The reaction medium contained 25 mM tricine buffer, pH 8.0, 5 mM MgCl_2 , 50 μM PMS, 20 μM DCMU, and 28 $\mu\text{g}/\text{ml}$ chlorophyll. The chloroplasts had been pre-loaded with $[8-^{14}\text{C}] \text{ADP}$ as described by Schumann and Strotmann [31]. Employing their method, each sample was split into two portions. One was applied in an ATPase experiment, the other one was analysed for liberated ADP.

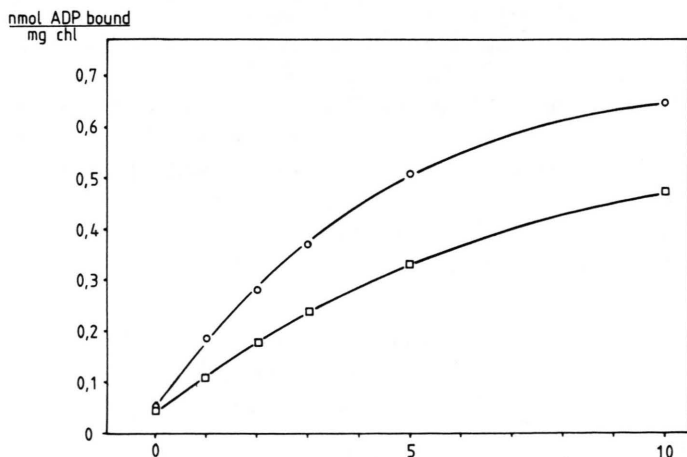


Fig. 4. Binding of $[8-^{14}\text{C}] \text{ADP}$ to ADP depleted membranes. Chloroplasts had been illuminated in the absence of added nucleotides for one minute. When light was switched off, FCCP at a final concentration of 30 μM was added. 5 seconds later ADP was added at a final concentration of 23.1 μM . This instant corresponds to zero on the time scale. At indicated incubation intervals samples were analysed for bound nucleotides as described elsewhere [34]. The control experiments ($\circ$) contained methanol without addition of nitrofen, in the parallel experiment nitrofen at a final concentration of 5 μM was added ($\square$).

probably ATP too [34, 42–45]. This site changes its affinity to the ligand in energized and de-energized membranes. Tightly bound nucleotides present in the de-energized state are liberated or exchanged, respectively, when the thylakoid membranes are energized by light [34, 46], pH jump [34, 47] or external electrical fields [29]. It has been shown that energy induced release of bound ADP is related to the induction of ATPase activity ("light-triggered ATPase") and re-binding of ADP causes deactivation of the enzyme [31, 32, 48]. Probably the same mechanism is also involved in the control of phosphorylation [29, 49].

Fig. 3 shows the effect of nitrofen on the kinetics of the release of tightly bound $[^{14}\text{C}] \text{ADP}$ and concomitant activation of ATPase activity. Both reactions are inhibited in parallel. Under the employed conditions (PMS mediated electron transport) an inhibition of electron transport can be excluded (s. Table I) so that the observed effect must be attributed to a reaction related to the ATPase complex itself.

A nucleotide binding site of CF_1 which has been depleted by membrane energization, can be re-filled with ADP yielding a tightly bound nucleotide again [40]. This reaction is independent of energy input and virtually irreversible in non-energized conditions [44–46]. Fig 4 shows that the rate of re-binding of $[^{14}\text{C}] \text{ADP}$ is inhibited by nitrofen, too.

In Fig. 5 the effect of nitrofen on the rate of ATP hydrolysis induced by pre-illumination is shown. The herbicide is added after the light pre-treatment in order to exclude superposition of the nitrofen

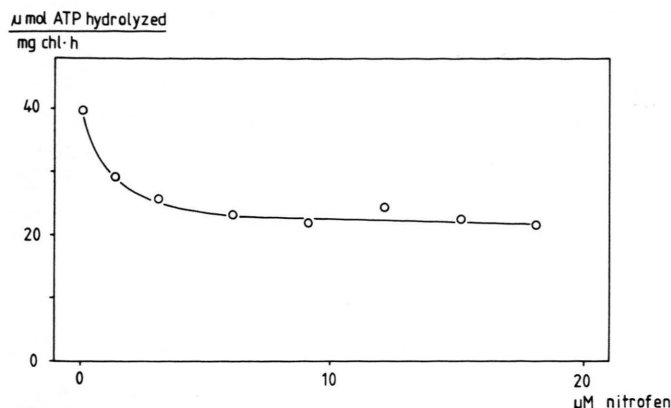


Fig. 5. Inhibition of light triggered ATPase by addition of nitrofen. ATPase was activated in the absence of herbicide as described by Schumann and Strotmann [31]. Afterwards activated chloroplasts were injected into an ATPase test medium containing 25 mM tricine buffer, pH 8.0, 5 mM $MgCl_2$, 1 mM ATP, 87 $\mu g/ml$ chlorophyll, and nitrofen as indicated. Methanol content was kept constant 2% (v/v).

effect on ATPase activation. Nevertheless inhibition of ATP hydrolysis is apparent under these conditions. However, maximum inhibition is not more than 50%.

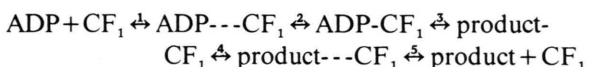
Discussion

The presented results show nitrofen to inhibit the overall reaction of photophosphorylation at two sites:

- I) nitrofen inhibits electron transport and shows typical reactions of a photosystem II herbicide;
- II) nitrofen acts like an energy transfer inhibitor.

The experiments should yield some more insight into the mode of action resulting the latter effect. Nitrofen did not only cause an inhibition of photophosphorylation but also of light triggered ATPase activity and nucleotide exchange. Discussing the results a central reaction inhibited by nitrofen should be found.

The first experiments showed a partial competitive effect of nitrofen with respect to ADP taking part in photophosphorylation. The participation of phosphate in this reaction was not at all affected. To get an idea of the mechanism of inhibition, the following very simplified sequence of reactions leading to phosphorylation of ADP is introduced:



Remembering the theoretical considerations concerning a partial competitive inhibition, one can demand nitrofen affecting the steps 1, 2 or 3. But if

nitrofen should affect step 3, competition like Lineweaver-Burk plots would not have been found. This means nitrofen inhibits step 1 or 2. Employing our methods, it is impossible to differentiate between these two steps. One only can see the overall reaction of these two steps in terms of nucleotide exchange on CF_1 .

From our experience with phosphorylation experiments one would rather say nitrofen affects affinity of CF_1 to nucleotides (or the binding constants) than the maximal phosphorylation rate. But, due to the mechanism of partial competitive inhibition, another possibility has to be mentioned: Retarded activation of a portion of the CF_1 population of each thylakoid would pretend a reduced affinity. The experiments reported by Strotmann *et al.* [49] proved the necessity of an activation of CF_1 before ATP synthesis or hydrolysis, respectively, can be catalysed. As shown by the results presented, the postulated activation steps of both reactions are inhibited by addition of nitrofen. The activation of the enzyme is paralleled by a liberation of, up to that moment, tightly bound ADP. From these connections it seems reasonable that the I_{50} were determined to be in the range of 2 μM for inhibition of ATP synthesis, ATP hydrolysis, and nucleotide exchange respectively.

Another effect of nitrofen is the deletion of the regulatory effect of ADP on the size of the transmembrane proton gradient. As the experiments concerning measurement of proton gradient were carried out employing non-phosphorylating conditions, one can only speculate about the possible connections between enhancement of proton gradient and regulation of the enzymatic activity of CF_1 . As a matter of fact, nitrofen concentrations in the range of

2 μM were found to result 50% deletion of ADP-effect. This can be understood as a reference to a correlation between these effects. Further experiments concerning this problem will be carried out.

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