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Michel Tissut, Georges Merlina, Eugène Genies, Jean-Pierre Calmon. p-Nitroacetophenoxime N-methylcarbamate, a new electron acceptor in isolated thylakoids.. Plant Science, 1985, 38 (3), pp.155-161. <10.1016/0168-9452(85)90033-0>. <hal-03487148>

HAL Id: hal-03487148

<https://hal.science/hal-03487148v1>

Submitted on 17 Dec 2021

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***p*-NITROACETOPHENOXIME *N*-METHYLCARBAMATE, A NEW ELECTRON ACCEPTOR IN ISOLATED THYLAKOIDS**

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(Received June 29th, 1984)

(Revision received January 17th, 1985)

(Accepted January 17th, 1985)

p-Nitroacetophenoxime *N*-methylcarbamate (MCPNA) is a rather potent inhibitor of the electron transfer in spinach class A chloroplasts. In isolated thylakoids, MCPNA is an electron acceptor at the level of photosystem I (PS I). It inhibits O₂ evolution in the presence of NADP and ferredoxin but not the reduction of ferricyanide. MCPNA is active as an acceptor between 3 μM and 100 μM. At concentrations higher than 300 μM, inhibition of photosystem II (PS II) occurs. MCPNA has no uncoupling effect on photophosphorylation. Reduction of MCPNA by thylakoids in the presence of light is in accordance with the *E*'₀ of this compound (>−0.57 V) and is followed by an electron transfer to O₂. This reaction probably explains the inhibitory effect of MCPNA on class A chloroplasts.

Key words: *N*-methylcarbamate of *p*-nitroacetophenoxime; chloroplasts; electron transfer; methylviologen; thylakoids; spinach

Introduction

The aim of this investigation was to study the action of MCPNA on isolated thylakoids. An oxime function is present in this compound as in the well-known insecticide aldicarb. MCPNA is related to phenylcarbamate and phenylurea herbicides (Scheme II). The synthesis of MCPNA has already been described [1].

Abbreviations: DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; Diuron (DCMU), 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DPIP, dichlorophenolindophenol; I₅₀, concentration of the product giving a 50% inhibition; MCPNA, *p*-nitroacetophenoxime *N*-methylcarbamate; MOPS, morpholinopropane sulfonic acid; MV, methylviologen; PS I, photosystem I; PS II, photosystem II; TMPD, *N*-tetramethyl-*p*-phenylenediamine.

Materials and Methods

Isolation and purification of chloroplasts

Spinach leaves were cut into small pieces directly within the ice-cold chloroplast extraction medium (330 mM mannitol, 2 mM EDTA, 0.2 g · l^{−1} bovine serum albumin, 30 mM sodium pyrophosphate (pH 7.9); tissue/medium ratio 1:3.3). Leaves were homogenized using a Waring Blendor for 3 × 2 s. The brei was filtered through 8 layers of muslin and intact chloroplasts were prepared by differential centrifugation according to Nakatani and Barber [2]. The chloroplasts thus obtained (chlorophyll concentration: 7 to 8 mg · ml^{−1}) were purified by isopycnic centrifugation in Percoll, a non-toxic silica sol gradient (Pharmacia); isoosmotic conditions were thus maintained throughout the isolation procedure [3]. The intact purified

chloroplasts (class A chloroplasts) were diluted with the extraction medium (1:10, v/v) and recovered as a pellet after centrifugation ($5500 \times g$, 90 s, SS 34 rotor, Sorvall RC5).

Chlorophyll concentration

Chlorophyll concentration was measured according to Arnon [4].

Photosynthesis assay

O₂ evolution was followed polarographically at 25°C using a Clark-type electrode system. The reaction medium contained: 330 mM mannitol, 50 mM morpholinopropane sulfonic acid (MOPS)—NaOH (pH 7.6), 2 mM EDTA, 5 mM NaHCO₃, 100 μM Na₂HPO₄ and chloroplasts in an amount equivalent to 70–80 μg of chlorophyll in a total volume of 1 ml. The reaction medium was gassed with argon before chloroplast addition and equilibrated with the O₂ electrode at 25°C. The light source was a 150 W xenon arc lamp giving an irradiance of 1700 W · m⁻² at the surface of the vessel.

Percentage of broken chloroplasts

Broken chloroplasts were determined from ferricyanide-dependent O₂ evolution in the presence of NH₄Cl (2 mM) before and after an osmotic shock [5] and under reduced irradiance (700 W · m⁻²). Thylakoids (class C chloroplasts) were obtained by an osmotic shock (10 s in distilled water) and diluted in the reaction medium. Reduction of NADP was followed by O₂ evolution at pH 7.9 in a medium containing 1 mM NADP, 10 μM ferredoxin.

Determination of E'_o . Cyclic voltammetry

Electrochemical measurements were made using a Princeton Applied Research (PARC) Model 173D-179 potentiostat/galvanostat coupled to a PARC Model 175 universal programmer. Voltamograms were recorded on a Hewlett Packard recorder 7045A. The working electrode was made of glass coated with indium oxide. Such an electrode allows the

electrochemical study of many compounds on a large range of negative potentials. The reference electrode was an Ag/AgCl electrode in saturated potassium chloride solution. The counter electrode was a simple platinum wire [6,7].

Results

MCPNA is a rather potent inhibitor of O₂ evolution by isolated class A chloroplasts in the presence of NaHCO₃, Na₂HPO₄ and light. A 50% inhibition of O₂ evolution was observed in the presence of 1 mM sodium bicarbonate and 100 μM phosphate for a concentration of MCPNA between 2 μM and 4 μM, when the chloroplast preparation was composed of 80% class A and 20% class C chloroplasts (chlorophyll concentration $\leq 10^{-2}$ mg · ml⁻¹) (Fig. 1). When chlorophyll

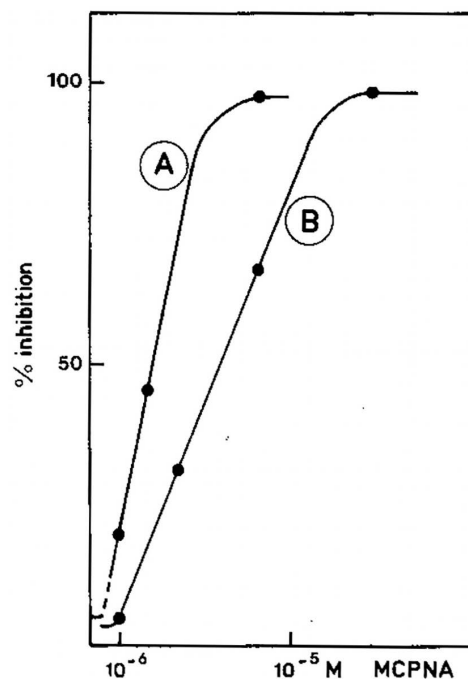


Fig. 1. Effect of MCPNA on light-dependent O₂ evolution by Spinach class A chloroplasts in the presence of NaHCO₃, 1 mM, Na₂HPO₄, 0.1 mM. (A) chlorophyll concentration reaches 10 μg · ml⁻¹ in the reaction medium; (B) chlorophyll concentration is 40 μg · ml⁻¹.

concentration increased from 10^{-2} to 4.10^{-2} $\text{mg} \cdot \text{ml}^{-1}$, the I_{50} for MCPNA increased only from 4 μM to 6 μM . With more purified class A chloroplast suspensions (95% class A obtained on Percoll gradients) O_2 evolution was not as sensitive to MCPNA and 20 μM were needed to induce a 50% inhibition at a 10^{-2} $\text{mg} \cdot \text{ml}^{-1}$ chlorophyll concentration. The chloroplasts were insensitive to the presence of small amounts of Percoll which originated from the gradient: up to 2%, Percoll had no effect on the electron transfer in thylakoids and on the inhibitory action of MCPNA.

PS I electron transfer was studied using ascorbate-reduced *N*-tetramethyl-*p*-phenylenediamine (TMPD) as electron donor and methylviologen (MV) as electron acceptor. Although 300 μM MCPNA markedly inhibited the electron transfer between H_2O and MV, no major inhibition appeared at the level of

PS I: 1 mM MCPNA only induced a 14% decrease in the electron flow in PS I (Table I). PS II electron transfer was then investigated between H_2O and 100 μM *p*-phenylenediamine in the presence of 5 mM potassium ferricyanide and after addition of 3 μM 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB) as a plastoquinone antagonist [8–10]. Under these conditions, 500 μM MCPNA gave rise to an 80% inhibition of the electron flow (Table I). Then, the electron transfer was studied between reduced duroquinone [11] and MV, this test allowing the analysis of inhibitions located in the plastoquinone region. MCPNA was completely ineffective in this test, even at a 200 μM concentration.

An electron transfer between H_2O as donor and NADP as acceptor was then studied in the presence of ferredoxin. This transfer took place in the light with freshly

Table I. Intensity of the photosynthetic electron flow in class C chloroplasts with different electron donors and acceptors when increasing MCPNA concentration. Columns 1–6: percentage of the value obtained without MCPNA. Columns 7–8: percentage of the maximum value of the oxygen consumption when MCPNA was electron acceptor.

Experimental conditions: (1) $\text{H}_2\text{O} \rightarrow \text{NADP}$: oxygen evolution measured in the presence of 1 mM NADP and 10 μM Fd with freshly broken chloroplasts (class C). (2) $\text{H}_2\text{O} \rightarrow \text{MV}$: oxygen consumption measured in the presence of 100 μM MV and 10 mM NH_4Cl . (3) Asc + DPIP \rightarrow MV: oxygen consumption measured in the presence of 100 μM MV, 10 mM NH_4Cl , 10 μM DCMU, 500 μM ascorbate and 100 μM DPIP. (4) Dq \rightarrow MV: oxygen consumption measured in the presence of 100 μM reduced duroquinone, 100 μM MV and 10 mM NH_4Cl . (5) $\text{H}_2\text{O} \rightarrow \text{FeCN}_6^{3-}$: oxygen evolution measured in the presence of 5 mM FeCN_6^{3-} and 10 mM NH_4Cl . (6) $\text{H}_2\text{O} \rightarrow \text{PD}$: oxygen evolution measured in the presence of 5 mM FeCN_6^{3-} , 3 μM DBMIB, 100 μM PD and 10 mM NH_4Cl . (7) $\text{H}_2\text{O} \rightarrow \text{MCPNA}$: oxygen consumption measured in the presence of MCPNA as electron acceptor, with 10 mM NH_4Cl . (8) Asc + DPIP \rightarrow MCPNA: oxygen consumption measured in the presence of 10 mM NH_4Cl , 10 μM DCMU, 500 μM ascorbate, 100 μM DPIP and MCPNA. For tests 1–6 see Refs. 12,13. Asc, ascorbate; Dq, reduced duroquinone; FeCN_6^{3-} , potassium ferricyanide; PD, *p*-phenylene-diamine.

MCPNA	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
	$\text{H}_2\text{O} \rightarrow$ NADP %		Asc + DPIP \rightarrow MV %	Dq \rightarrow MV %	$\text{H}_2\text{O} \rightarrow$ FeCN_6^{3-} %	$\text{H}_2\text{O} \rightarrow$ PD %	$\text{H}_2\text{O} \rightarrow$ MCPNA %	Asc + DPIP \rightarrow MCPNA %
10 μM	48	100	100	100	100	100	100	100
20 μM	12	100	100	100	100	100	100	100
100 μM	0	100	100	100	97	90	100	100
500 μM	0	32	92	90	22	20	20	88
1 mM	0	0	86	81	0	0	0	80

broken chloroplasts (Table I). MCPNA at a concentration close to $25 \mu\text{M}$ fully inhibited O_2 evolution in these conditions. Furthermore, $100 \mu\text{M}$ MCPNA induced a very marked decrease of the O_2 concentration in the reaction vessel, showing a significant light-dependent O_2 consumption which was never seen in the presence of classic inhibitors such as DBMIB or DCMU. Addition at this stage of small amounts of ferricyanide ($500 \mu\text{M}$) was followed by a transient O_2 evolution which stopped when ferricyanide was fully reduced.

As suggested from the preceding experiment, MCPNA alone ($10 \mu\text{M}$) induced an intense O_2 consumption ($150 \mu\text{mol O}_2 \cdot \text{h}^{-1} \cdot \text{mg}^{-1}$ chlorophyll) by class C chloroplasts in the presence of light (Table I). This consumption was inhibited by $5 \mu\text{M}$ 3-(3,4-dichlorophenyl)-1,1-dimethylurea (Diuron, DCMU) and was thereafter restored by addition of ascorbate reduced TMPD or dichloropheno-

lindophenol (DPIP). The O_2 consumption appeared when MCPNA concentration reached $2 \mu\text{M}$ and its intensity increased thereafter for concentrations between $2 \mu\text{M}$ and $10 \mu\text{M}$. Between $10 \mu\text{M}$ and $100 \mu\text{M}$, a constant value of $150 \mu\text{M O}_2 \cdot \text{h}^{-1} \cdot \text{mg}^{-1}$ chlorophyll was obtained. At concentrations greater than $150 \mu\text{M}$, the intensity of the electron transfer gradually decreased (Fig. 2).

A comparative study of the redox systems of MCPNA and MV has been carried out through cyclic voltametry (Fig. 3). The voltamogram of MV (curve A, Fig. 3) indicates that it is a perfect monoelectronic, fast and reversible electrochemical system [14]. In this case, the potential difference ΔE_p between the cathodic and anodic peaks at $20 \text{ mV} \cdot \text{s}^{-1}$ is almost 60 mV and the E'_o of MV is $-0.53 \pm 0.01 \text{ V}$. For MCPNA (curve B, Fig. 3) the electrochemical reaction is much more complex. The cathodic and corresponding anodic peaks of the voltamo-

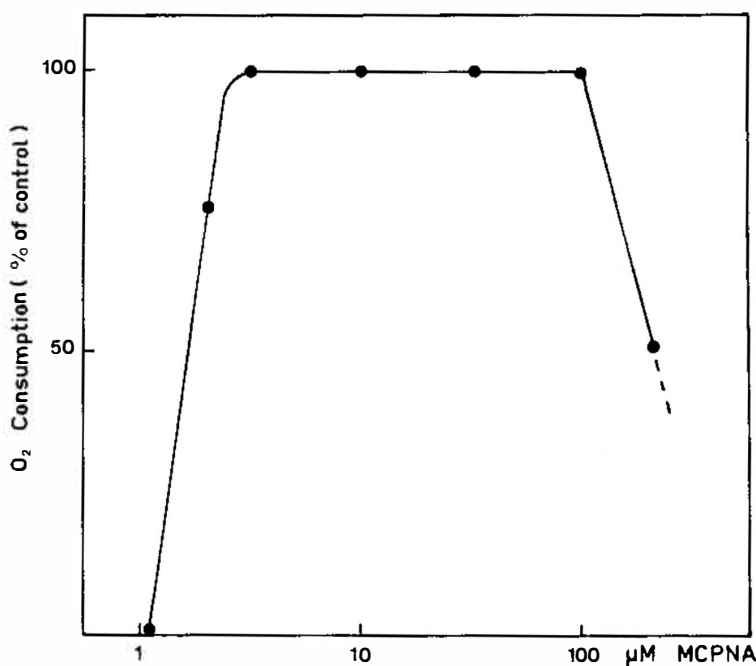


Fig. 2. Relation between the oxygen consumption rate with MCPNA as electron acceptor and MCPNA concentration. One hundred percent refers to $160 \mu\text{mol O}_2$ consumed $\cdot \text{h}^{-1} \cdot \text{mg}^{-1}$ chlorophyll.

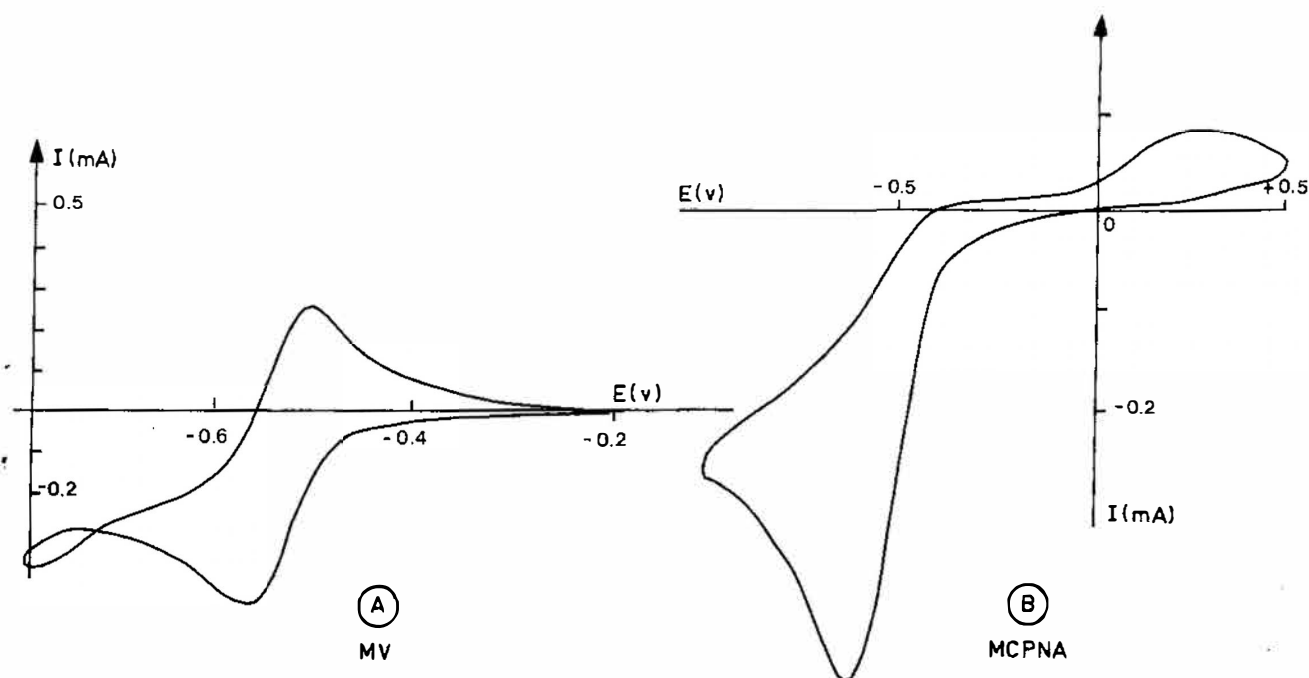


Fig. 3. Cyclic voltammetry in water with 0.2 M MOPS of (A) methylviologen 3 mM at $20 \text{ mV} \cdot \text{s}^{-1}$ and (B) MCPNA 2 mM at $200 \text{ mV} \cdot \text{s}^{-1}$. Presence of residual O_2 in the solution can explain the low value of the anodic peak at the oxidized state.

gram are of -0.62 and $+0.27 \text{ V}$ at $200 \text{ mV} \cdot \text{s}^{-1}$, respectively. The ΔE_p is 890 mV . This strongly suggests a slow overall transfer of several particles. According to the structure, it must be $2e^-$ and 2H^+ . In such a case, it is probably an ECEC mechanism [14] where a proton transfer is a determining step. In this situation, it is not possible to give a quasi thermodynamic standard potential value E'_0 for MCPNA. We can give an E''_0 -value for the reduction of MCPNA ox to MCPNA red which is a maximum value: $E''_0 = -0.57 \text{ V} \pm 0.03$ ox \rightarrow red. In homogeneous oxido-reduction transfer we probably have to take into account a lower value. In the inverse direction red \rightarrow ox, the potential E''_0 is about $+0.20 \pm 0.03 \text{ V}$. In homogeneous phase with different intermediates, owing to proton acceptor or to donor effect, the potential that we have to take into account can be between -0.57 and $+0.2 \text{ V}$.

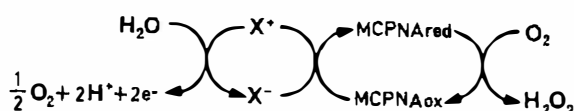
The E''_0 for MCPNA ox \rightarrow MCPNA red is in accordance with the acceptance of e^- generated by PS I.

Discussion

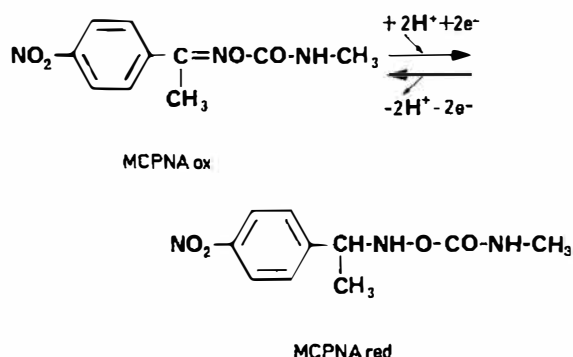
The O_2 uptake induced by MCPNA indicates that MCPNA from 2 to $100 \mu\text{M}$ is able to accept electrons emitted by PS I in the light: when the two photosystems are operating, the electron transfer to MCPNA is inhibited by DCMU and restored by addition of ascorbate-DPIP. As can be predicted when considering the E'_0 of MCPNA, ascorbate and DPIP, these two latter compounds are not able to transfer e^- directly to MCPNA without thylakoids acting in the presence of light.

Addition of less than $10 \mu\text{M}$ MCPNA is sufficient to obtain complete oxygen consumption in our conditions. This suggests that MCPNA does not accumulate in the

reduced form. The fact that the e^- transfer to this compound is followed by an oxygen consumption, and that MCPNA does not accumulate in the reduced form during the course of water photolysis in the thylakoids, shows that an electron transfer takes place between the reduced form of MCPNA and O_2 , in a way which can probably be compared to that affecting γ, γ' dipyridyl salts and low potential quinones such as anthraquinone sulfonates [13] (Scheme I).



The oxido-reduction activity of MCPNA appears to be associated with the presence of a =NO-structure, as suggested by Scheme II.



We have studied elsewhere the effects of amides and phenylcarbamates [15,16] on photosynthesis. Such compounds, the structures of which are close to that of MCPNA, are unable to transfer e^- to O_2 in the presence of thylakoids.

In the case of MCPNA itself, the addition of an electron withdrawing region on the phenyl ring, which is represented by a p - NO_2 , seems to increase the reactivity of the oxime function.

At concentrations higher than 300 μM , MCPNA inhibits the electron transfer in the

thylakoids. This inhibition can be seen with the PS II acting alone, with PD and $FeCN_3^{3-}$ as acceptors, after addition of DBMIB. PS II inhibition by MCPNA could be related to the occurrence of the carbaomyl function $-CO-NH-CH_3$ and could thus be compared to that by DCMU [17]. An s -triazine-like structure might also account for the effect of MCPNA on PS II. It should be noted that the inhibitory effect of MCPNA on PS II is low, as in the case of comparable structures of the phenylamide or phenylcarbamate families [15,16]. For this action, p -substitution by NO_2 on the phenyl ring is not favourable.

Addition of MCPNA to thylakoids does not prevent the acceleration of the electron transfer induced by NH_4Cl (result not shown). MCPNA is therefore devoid of uncoupling activity. The inhibitory effect of MCPNA at low concentration, on the whole photosynthetic mechanism in class A chloroplasts can be explained neither by the inhibition of the electron transfer in the thylakoids, nor by an uncoupling effect. On the other hand, the MCPNA concentration needed for electron transfer to O_2 and for the inhibitory action on class A chloroplasts are almost the same. This suggests that MCPNA is able to enter into the class A chloroplasts and compete with electron transfer from PS I to NADP.

Nevertheless, we have to note that the presence of small amounts of class C chloroplasts in class A suspensions increases the O_2 consumption associated with MCPNA action and leads to a reduction of the oxygen concentration in the medium. This explains why the apparent inhibitory effect of MCPNA is greater on preparations of A plastids containing a small amount of thylakoids than on very pure class A preparations.

The demonstration of the redox properties of the $-C=N-O-CO-NH-CH_3$ chain of MCPNA and of its correlative effect on photosynthesis leads to further studies in the chemical family of the systemic insecticide aldicarb in which the same chain is also present.

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