

Interference by Nickel(II) Salts and Their 5-Methylimidazole-4-carboxylate Coordination Compounds on the Chloroplast Redox Chain

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Nickel(II) salts and their coordination compounds with ethyl 5-methylimidazole-4-carboxylate (emizco), [Ni(emizco)₂Cl₂], [Ni(emizco)₂Br₂], [Ni(emizco)₂(H₂O)₂](NO₃)₂·H₂O, Ni(NO₃)₂, inhibit photosynthetic electron flow (basal, phosphorylating and uncoupled) and ATP-synthesis, therefore behave as Hill reaction inhibitors. Coordination compounds are more potent inhibitors than the salts. It was found that the target for NiCl₂, NiBr₂ and Ni(NO₃)₂ is at the b₆f level. On the other hand, the complexes [Ni(Emizco)₂Cl₂], [Ni(Emizco)₂Br₂] and [Ni(emizco)₂(H₂O)₂](NO₃)₂·H₂O binding sites are located at Q_B(D1)-protein and b₆f level. Therefore, they have a common inhibition site located at b₆f avoiding the PQH₂ oxidation. The Q_B inhibition site was corroborated by variable chlorophyll *a* fluorescence yield [V(j)]. The emizco ligand has no activity on photosynthetic electron flow.

Introduction

Continuing our earlier work related with the study of the effect of transition metals ions coordination compounds on different photosynthetic activities (Barba-Behrens *et al.*, 1993; Fernández-Vargas *et al.*, 1995), we report the behaviour of

nickel coordination compounds with the ligand ethyl 4-methyl-imidazolecarboxylate (emizco) on photosynthesis. Their effect was compared with that of the ligand and nickel(II) salts. Recently, we found that quinic acid uncouples photophosphorylation from photosynthetic electron flow, while Co(II) coordination compounds enhance this activity (Barba-Behrens *et al.*, 1991). Emizco was chosen as a simple imidazolic derivative that may act as a chelate towards metal ions, similarly to 2-methyl-5-nitroimidazole (Barba-Behrens *et al.*, 1991). It is known that imidazole derivatives have biocidal properties, they are extensively used in the pharmaceutical (Bennett, 1990) and agrochemical industries (Garaboyes, 1982; Parsons *et al.*, 1990). Emizco derivatives present antiviral (Alonso *et al.*, 1985) and herbicidal (Beck *et al.*, 1979) activities. Nickel(II) is an essential micronutrient for legumes and suggested possible essentiality for all higher plants (Fargo *et al.*, 1988). There is evidence that nickel is required in microbial urea-utilising plants (Fargo *et al.*, 1988). It is reported to be mutagenic and carcinogenic causing chromosome aberrations and micronucleus formation (Leonard *et al.*, 1981) and it is toxic at 0.1–

Abbreviations: Chl, chlorophyll; PSII, Photosystem II, PS I, Photosystem I, Q_A, primary quinone electron acceptor of PSII; Q_A⁻, reduced Q_A; Q_B, secondary quinone electron acceptor of PSII; V(j), relative quantum yield of the transient phase J; Fm, maximum yield of Chl-*a* fluorescence when the PSII reaction centres are closed; Fo, minimum yield of Chl-*a* fluorescence when the PSII reaction centres are open; F(v), Fm-Fo; PQ, plastoquinone; emizco, ethyl 5-methylimidazole-4-carboxylate; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCP, 2,6-dichlorophenolindophenol; DCBQ, dichloro-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PMS, phenazine methosulfate; TMQH₂, tetramethyl-*p*-benzohydroquinone, HEPES, (N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]); tricine, [N-[tris(hydroxymethyl)methyl]glycine]; MV, methylviologen; SiMo, silicomolybdic acid hydrate.

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1 M concentrations, which inhibited seed germination of *Helianthus annuus* (Chakravarty *et al.*, 1992). Photosynthesis and respiration of lichens have been shown to be affected by metals, it was found that nickel(II) is the less toxic one (Farago *et al.*, 1988). In this work we studied the effect of nickel(II) salts, emizco and their coordination compounds at lower concentrations (0–500 μM). Nickel(II) salts and emizco were used as control experiment in order to see if the ligand modified the potency of the salt. As far as we know the effects of these compounds on photosynthesis in chloroplasts in higher plants have not been investigated.

Materials and Methods

Freshly lysed chloroplasts isolation and chlorophyll determination

Intact chloroplasts were isolated from spinach leaves obtained from local markets as described earlier (Calera *et al.*, 1995; Mill *et al.*, 1980; Saha *et al.*, 1971). Chloroplasts were suspended in the following medium: 400 mM sucrose, 10 mM KCl, 5 mM MgCl_2 and 30 mM tricine buffer (pH 8 with the addition of KOH). They were stored as a concentrated suspension in the dark for 1 hour at 0 °C. Intact chloroplasts were efficiently lysed to yield free thylakoids previous to each experiment by incubating them in the following medium: 100 mM sorbitol, 10 mM KCl, 5 mM MgCl_2 and 30 mM tricine buffer (pH 8 with the addition of KOH) for electron transport measurements. For ATP synthesis determination the same medium was used but tricine buffer concentration was 1 mM (Dilley, 1972). Chlorophyll was determined according to the method of Arnon (1949).

Measurement of electron transport and ATP synthesis

ATP-synthesis was determined titrimetrically using a microelectrode Orion Mod. 8103 Ross connected to a Corning potentiometer Model 12, with expanded scale as reported by Dilley (Dilley, 1972). The pH changes were registered using a Gilson recorder. The ATP-synthesis reaction medium used contained 100 mM sorbitol, 10 mM KCl, 5 mM MgCl_2 , 0.5 mM KCN, 50 μM MV, 1 mM

HEPES-KOH (pH 8.0) where the intact chloroplasts were freshly lysed.

Photosynthetic non-cyclic electron transport in the presence of methylviologen was monitored with YSI (Yellow Springs Instrument C) model 5300 oxygen monitor using a Clark electrode in a temperature regulated flask at 20 °C. The reaction medium contained 100 mM sorbitol, 10 mM KCl, 5 mM MgCl_2 , 50 μM MV, 0.5 mM KCN, 15 mM HEPES-KOH, (pH 8.0), chloroplasts were added to give a Chl concentration of 20 $\mu\text{g}/\text{ml}$. The sample was illuminated for 1 minute in presence or absence of 6 mM NH_4Cl (Calera *et al.*, 1995; Saha *et al.*, 1971).

PSII was measured by photoreduction of DCPIP supported O_2 evolution monitored polarographically. The reaction medium for assaying PSII activity contained the same whole-chain electron transport medium ($\text{H}_2\text{O} \rightarrow \text{MV}$) above mentioned without methylviologen, but in the presence of 1 μM DBMIB, 100 μM DCPIP, 300 μM $[\text{Fe}(\text{CN})_6]$ and 6 mM NH_4Cl . Uncoupled electron transport from water to DCBQ (Yruela *et al.*, 1991) was measured with a reaction mixture as in photosystem II, with addition of 100 μM DCBQ, 1 μM DBMIB and 6 mM NH_4Cl without DCPIP and $[\text{Fe}(\text{CN})_6]$.

Photosystem I electron transport was determined in a similar form to non-cyclic electron transport. The following reagents were added: 100 μM DCPIP, 300 μM ascorbate, 10 μM DCMU and 6 mM NH_4Cl (Allen *et al.*, 1974).

PS I electron transport from PMS/ASC to MV was measured using KCN-poisoned chloroplasts with 500 μM PMSred/1000 μM ascorbate as the electron donor to P700, MV as PS I electron acceptor, as well as 10 μM DCMU, 6 mM NH_4Cl and 1 μM DBMIB to fully inhibit any electron flow prior to PC. Cyanide-treated chloroplasts were prepared by incubating chloroplasts for 30 min at 0 °C in a 30 mM KCN and then centrifuged at 8000 $\times g$ (Sorvall Super T21) for 1 min and resuspended in the reaction medium (Ouitrakul *et al.*, 1973). Moreover, EPR spectroscopy confirmed the ability of reduced PMS to interact directly with P₇₀₀ (Izawa *et al.*, 1973).

Chl a fluorescence measurements

Freshly lysed chloroplasts aliquots containing 15 μg of Chl were suspended in electron transport

medium and transferred by gravity onto filter paper with a dot-blot apparatus (Bio-Rad USA) to ensure an homogeneous and reproducible distribution of thylakoids in the filter paper. Thylakoids blots were transferred immediately to vials containing 3 ml of different solutions of the tested compounds and incubated for 5 min in the dark. Chl *a* fluorescence induction curves were measured at room temperature with a Plant Efficiency Analyser (PEA)(Hansatech UK), as described (Strasser *et al.*, 1995).

Kinetic analyses of the relative variable fluorescence $[V(t)=(F_t-F_0)/F_m-F_0]$ were performed by deconvolution of normalised induction curves, employing a non-linear fitting procedure.

Preparation of Ni-emizco compounds

$[\text{Ni}(\text{emizco})_2\text{Cl}_2]$, $[\text{Ni}(\text{emizco})_2\text{Br}_2]$ and $[\text{Ni}(\text{emizco})_2(\text{H}_2\text{O})_2](\text{NO}_3)_2 \cdot \text{H}_2\text{O}$ were prepared as described below.

The nickel(II) coordination compounds were prepared using methanol as solvent and 1:2 ligand:Ni molar ratio was employed (mmol scale). The metal salts, $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, $\text{NiBr}_2 \cdot 3\text{H}_2\text{O}$, $\text{Ni}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ (Baker) were dissolved in 15 cm^3 of hot methanol and added to a solution of emizco (Aldrich) in 15 cm^3 of hot methanol. The reaction mixture was refluxed for ca 5 h and then allowed

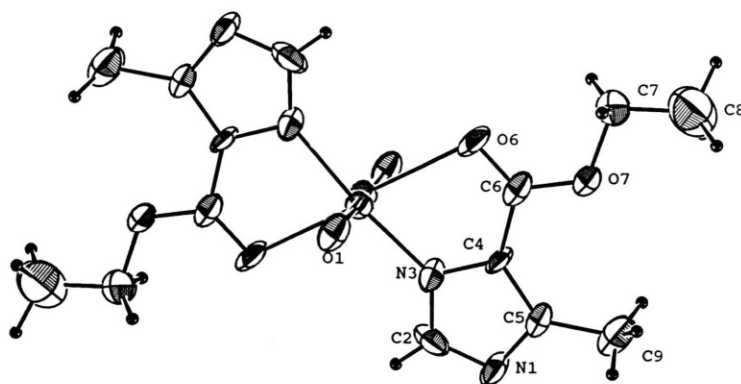
to stand at room temperature for 3 weeks. The resulting precipitates were filtered, washed and dried. Nickel(II) coordination compounds were characterised by elemental analyses, magnetic susceptibility, IR and UV-Vis spectroscopy.

Results and Discussion

Geometry and stability of nickel(II) coordination compounds with emizco

These coordination compounds have octahedral geometry in solid state and in aqueous solution as shown in Fig. 1. The emizco ligand is coordinated to nickel(II) through the imidazolic nitrogen and the oxygen from the ester group, behaving as a chelating ligand, therefore yielding a very stable compound. Consequently, the ligand is not substituted from the complexes; however the initially coordinated chloride and bromide anions were exchanged by water molecules as can be inferred from the UV-visible absorption spectra in solution and in solid state (diffuse reflectance). In the compound $[\text{Ni}(\text{emizco})_2(\text{H}_2\text{O})_2](\text{NO}_3)_2 \cdot \text{H}_2\text{O}$ the nitrate groups were not coordinated from the beginning.

It was shown that the buffer did not substitute any ligand from the nickel(II) coordination sphere. Experiments using different buffering sub-



$[\text{Ni}(\text{emizco})_2(\text{H}_2\text{O})_2](\text{NO}_3)_2$

Fig. 1. Structure of octahedral nickel(II) coordination compounds.

stances: HEPES, tricine and Tris at various concentrations were carried out. The concentration of the buffer was varied from 20–40 mM, for two different pH, 7.0 and 8.0. The UV-visible spectra remained unchanged for solutions of increasing buffer concentration. Therefore, it is assumed that in the photosynthetic experiments the nickel(II) complexes are stable in aqueous media at least for two days, as determined from kinetic studies.

ATP formation and whole chain electron transports

The degree of inhibition of the entire electron transport chain rate of spinach thylakoids was measured with emizco, nickel(II) salts and their coordination compounds. Emizco lacks any effect on photosynthetic activities. Figure 2A shows the inhibiting effect of increasing concentration of NiCl_2 on methylviologen photoreduction with water as electron donor. Methylviologen photoreduction and its auto-oxidation with oxygen in the medium that results in O_2 uptake by isolated freshly lysed intact chloroplasts, was inhibited. The results obtained indicate that NiCl_2 act as Hill reaction inhibitors, since it inhibited basal, phosphorylating and uncoupled conditions. In order to know if emizco modifies the activity of the

nickel(II) ion in the coordination compound, the effect of its complexes on electron flow was tested. Figure 2B, shows the effect of increasing concentration of the $[\text{Ni}(\text{emizco})_2\text{Cl}_2]$ on basal, phosphorylating and uncoupled electron flow. Electron flow under all conditions was partially inhibited, and the extent of inhibition (up to 50%) increased with concentration from 0 to 500 μM . The extent of inhibition by the coordination compounds suggests that these compounds do not interact at the Q_B site of the D_1 protein interacting with another site of D_1 .

Electron flow is coupled to ATP-synthesis and the energy transduction theory of Mitchell (Mitchell, 1961) has been proposed to account for the mechanism of coupled electron transport and ATP-synthesis, therefore any chemical that inhibits electron flow will inhibit photophosphorylation, as is the case of the nickel(II) salts and their complexes. The ATP-synthesis inhibition order is as follows: 46% and 17% at 500 μM for $[\text{Ni}(\text{emizco})_2\text{Cl}_2]$ and for NiCl_2 respectively. It is possible that coordination compounds are more hydrophobic than the salts therefore allowing them to reach the target more easily. In order to localise the inhibition site of NiCl_2 , NiBr_2 , $\text{Ni}(\text{NO}_3)_2$, $[\text{Ni}(\text{emizco})_2\text{Cl}_2]$, $[\text{Ni}(\text{emizco})_2\text{Br}_2]$, $[\text{Ni}(\text{emizco})_2\text{Cl}_2]$.

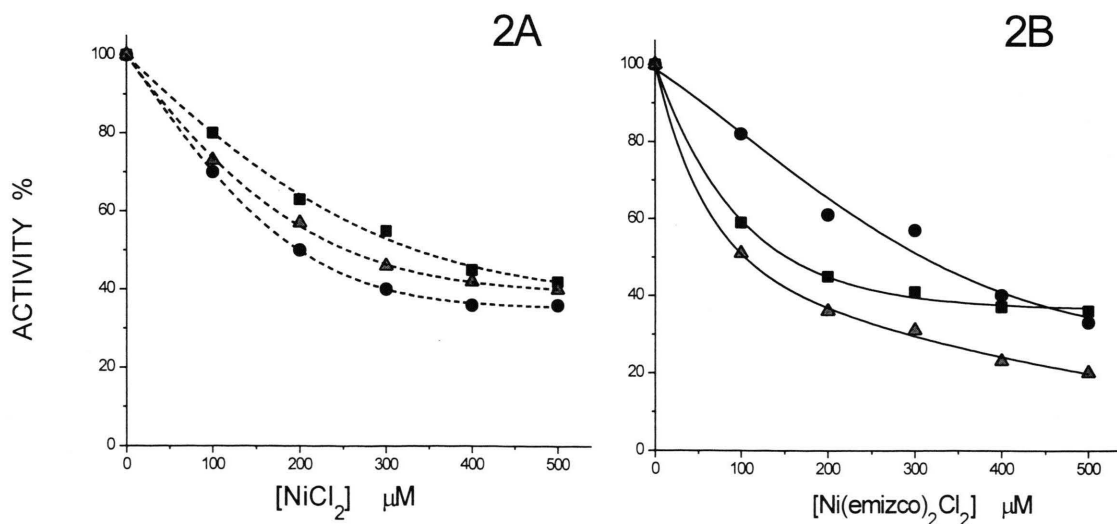


Fig. 2. Photosynthetic electron transport from water to MV on freshly lysed spinach chloroplasts: basal (■), phosphorylating (●) and uncoupled (▲) electron transport rates. In the presence of (A) NiCl_2 and (B) $[\text{Ni}(\text{emizco})_2\text{Cl}_2]$. Control average rate values are 311, 640, 1018 $\mu\text{equiv. e}^-/\text{mg chl per h.}$, for basal, phosphorylating and uncoupled electron flows, respectively. Other conditions as described in Material and Methods. Each curve is the average of three replicates.

(H₂O)₂](NO₃)₂·H₂O and Ni(NO₃)₂ on the electron transport chain, their effect on photosystem I and II were studied.

PSII-supported electron transport

[Ni(emizco)₂Cl₂], partially inhibited uncoupled PSII electron flow from water to DCPIP or DCBQ (Table I). The PSII inhibition was 50% at 500 μM (178 μequiv. e⁻ h⁻¹ mg Chl⁻¹, as compared with 356 μequiv. e⁻ h⁻¹ mg Chl⁻¹ for the control). Noteworthy, nickel(II) salts do not affect this span of the PSII electron transport chain, as the rate of electron transport from H₂O to DCBQ is the same in the presence or absence of the salts, *i.e.* 356 μequiv. e⁻ h⁻¹ mg Chl⁻¹.

To further localise the inhibition site of nickel complexes on PSII, electron transport from water to SiMo in presence of 10 μM DCMU, was measured. Results show that these complexes did not inhibit this span of the electron transport chain (Table I). It is known that SiMo accepts electrons through Q_A level, therefore, it is concluded that one of the targets for [Ni(emizco)₂Cl₂], [Ni(emizco)₂Br₂], [Ni(emizco)₂(H₂O)₂](NO₃)₂·H₂O and Ni(NO₃)₂ is located at the Q_B-protein or D1-protein level.

PSI-supported electron transport

To determine the target of NiCl₂ and [Ni(emizco)₂Cl₂] beyond Q_B their effect on uncoupled PSI activity from reduced DCPIP to MV (plus 10 μM DCMU) were determined. PSI electron transport was partially inhibited, *i.e.* 38% at 500 μM by NiCl₂ suggesting that the inhibition site for these salts is on PSI, as well as in [Ni(emizco)₂Cl₂] (Table I).

Due to the fact that nickel(II) salts do not affect PSII activity and partially inhibit PSI on one hand, on the other, their emizco coordination compounds show only partial inhibition on PSII, their behaviour from PQ pool to F_x were tested. This was done adding TMQH₂ as electron donor. The results show that this activity was inhibited by the salts and their coordination compounds *i.e.* 50%, 500 μM which is the same inhibition percentage of electron flow from reduced DCPIP to MV, thus indicating that the interaction target is located at b₆f level.

The span of PSI electron transport from P₇₀₀ to MV (adding PMS⁻ as electron donor) was studied for all compounds. The results show that this span of electron flow was not affected by the coordination compound or nickel(II) salt. Since PSI activity from DCPIPred to MV was inhibited by nickel(II) salts and coordination compounds, suggesting that their target is located at b₆f complex. Therefore, we may conclude that NiCl₂ have only one target. On the other hand, the [Ni(emizco)₂Cl₂] binding sites are located at Q_B-protein and b₆f level.

Effect of Ni²⁺ and their coordination compound on Chl fluorescence

Chl *a* fluorescence induction curves of thylakoids show the polyphasic sequence of transients (*OJIP*) described for plants, green algae and cyanobacteria (Iglesias-Prieto, 1995; Govindjee, 1995). This series of transients reflects the sequential reduction of the electron acceptor pool of PSII (Strasser *et al.*, 1995). Addition of 50 μM DCMU results in transformation of the regular *OJIP* sequence into an *OJ* sequence. Inhibition of PSII electron transport at the Q_B-protein site by

Table I. Treatment of nickel salts and their coordination compounds at 500 μM on electron transport rate uncoupled, PSII, PSI and partial reactions. Left in μequiv. e⁻/mg Chl per h. Right in per cent of inhibition.

	Uncoupled		PSII		PSI		TMQH ₂ → MV		H ₂ O → DCBQ		PMS → MV	
Control	1018	0%	467	0%	1800	0%	267	0%	356	0%	640	0%
NiCl ₂	407	60%	467	0%	1116	38%	134	50%	—	—	646	0%
[Ni(EMIzCO) ₂ Cl ₂]	153	85%	234	50%	1800	0%	174	35%	178	50%	631	0%
NiBr ₂	407	60%	467	0%	1224	32%	134	50%	—	—	640	0%
[Ni(EMIzCO) ₂ Br ₂]	163	84%	248	47%	1800	0%	166	38%	200	44%	633	0%
Ni(NO ₃) ₂	407	60%	467	0%	1350	25%	134	50%	—	—	640	0%
[Ni(emizco) ₂ (H ₂ O) ₂](NO ₃) ₂ ·H ₂ O	204	80%	272	42%	1800	0%	187	30%	164	54%	633	0%
Emizco	1025	0%	467	0%	1800	0%	266	100%	356	0%	633	0%

DCMU results in the rapid accumulation of Q_A^- during the first 2 ms of the induction curve. In contrast, control thylakoids require approximately 900 ms to completely close all PSII reaction centres. Increases in the relative variable fluorescence yield at the transient $J[V(J)]$ as a function of $[\text{Ni}(\text{emizco})_2\text{Cl}_2]$ concentration are indicative of a loss in Q_A^- re-oxidation capacity similar to that observed in DCMU-treated thylakoids (Fig. 3). Consistent with the electron transport determinations, thylakoids exposed to different concentra-

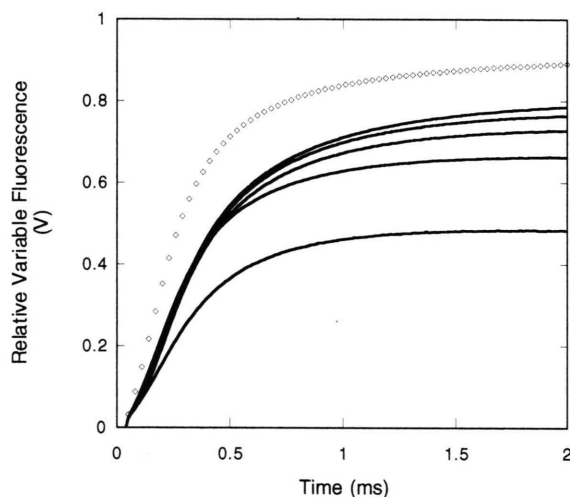


Fig. 3. Normalized relative variable fluorescence rise of the photochemical phase. The curve with the lowest fluorescence yield represent control thylakoids. Symbols represent controls with acceptor side impairment after infiltration with DCMU. The intermediate curves represent thylakoids exposed sequentially to 100, 250, 500 and 1500 μM $[\text{Ni}(\text{Emizco})_2\text{Cl}_2]$. Results are average of 4 replicates.

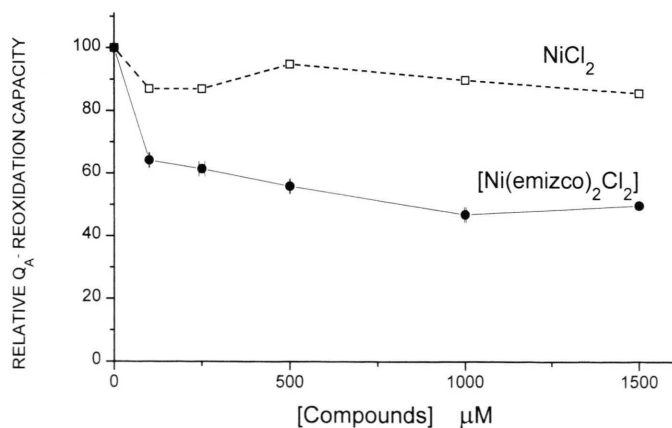


Fig. 4. Relative variable fluorescence, $F(V)$, corresponding to the electron transfer from Q_A^- to Q_B as a function of increased concentrations of NiCl_2 , or $[\text{Ni}(\text{Emizco})_2\text{Cl}_2]$ treatment. Concentrations of Nickel(II) chloride in open squares and coordination compound in solid circles.

tions of $[\text{Ni}(\text{emizco})_2\text{Cl}_2]$ showed a significant concentration-dependent reduction in their relative Q_A^- re-oxidation capacity (Fig. 4). Polarographic determinations of the inhibition of PSII electron transport activity from water to DCBQ as a function of $[\text{Ni}(\text{emizco})_2\text{Cl}_2]$ (Table I), coordination compound concentration (up to 500 μM) is correlative with the accumulation of Q_A^- shown by the Chl *a* fluorescence analyses (Fig. 4). On the other hand, addition of various concentrations of metal salts did not result in any detectable variations in the fluorescence characteristics of the thylakoids. These observations strongly suggest that the target site of the coordination compound is located at the acceptor side of PSII at the Q_B -protein.

Further support for this interpretation was obtained by the kinetic analyses of Chl *a* fluorescence induction curves. The rise of V during the first 2 ms of the induction curve requires two components to be accurately described (Fig. 5A and 5B). An exponential component with rate constants between 0.500 and 0.730 ms^{-1} and a sigmoidal one with time constants close to 0.09 ms^{-1} (Table II). Kinetic analyses indicate that the increase in the fluorescence yield during the J transient after DCMU infiltration, results from increments in the relative amplitude of both components with major changes in their rate constants (Table II).

Analogous responses were obtained for $[\text{Ni}(\text{emizco})_2\text{Cl}_2]$, $[\text{Ni}(\text{emizco})_2\text{Br}_2]$ and $[\text{Ni}(\text{emizco})_2(\text{H}_2\text{O})_2](\text{NO}_3)_2 \cdot \text{H}_2\text{O}$ infiltrated thylakoids. Although small variations in the rate constants were also detected, the main characteristics of the fluorescence raise during the J event are the dif-

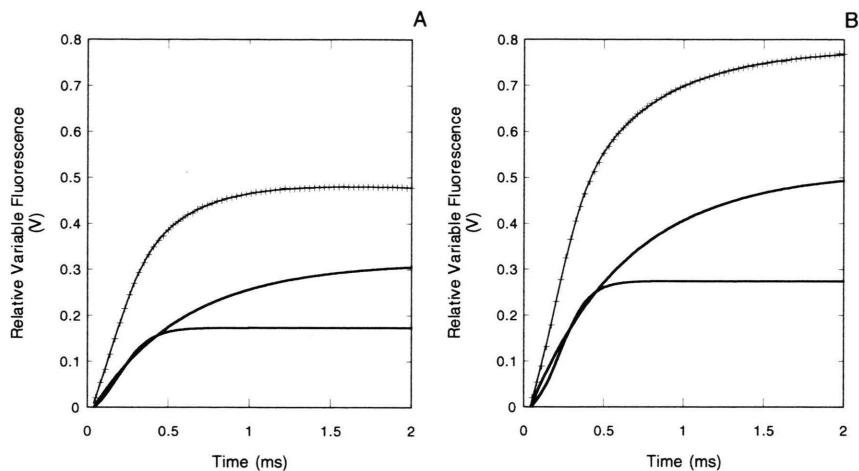


Fig. 5. Deconvolution of the photochemical phase of the induction curve of spinach thylakoids showing the two components (one sigmoidal and one exponential, solid line) needed to describe their fluorescence amplitude rise, crosses For simplicity only one third of the data are presented. (A) Control thylakoids. (B) Thylakoids infiltrated with 1500 μM $[\text{Ni}(\text{Emizco})_2\text{Cl}_2]$.

Table II. Kinetic analyses of the relative variable fluorescence (V) yields during the first 2 ms of the induction curve. Values are means of 4 replicates. Values were obtained by numeric deconvolution. Residuals vary from -0.002 to 0.39.

	Sigmoidal	Component	Exponential	Component
	Amplitude (relative)	Time constant (ms^{-1})	Amplitude (relative)	Time constant (ms^{-1})
$[\text{Ni}(\text{Emizco})_2\text{Cl}_2]$				
Control	0.201	0.084	0.272	0.516
100 μM	0.251	0.084	0.345	0.504
250 μM	0.276	0.080	0.403	0.567
500 μM	0.280	0.088	0.433	0.615
DCMU (50 μM)	0.446	0.103	0.474	0.573
$[\text{Ni}(\text{Emizco})_2\text{Br}_2]$				
Control	0.206	0.098	0.264	0.592
100 μM	0.240	0.096	0.375	0.702
250 μM	0.252	0.096	0.391	0.725
500 μM	0.255	0.093	0.412	0.729
DCMU (50 μM)	0.446	0.099	0.508	0.509

ferential amplitude increases of both components. The concentration-dependent increases in the amplitude of the sigmoidal components are smaller than those observed for the exponential components. They appear to be saturated at concentrations above 100 μM . Exposure of thylakoids to solutions of the coordination compounds (concentrations higher than 700 μM) resulted in increments in the amplitude of the exponential components. The amplitudes are comparable to those observed for DCMU-infiltrated thylakoids. PSII heterogeneity has been interpreted as the result

of the presence of two populations of PSII with different optical cross-section and connectivities (Melis *et al.*, 1983), these two populations also have different distributions in the thylakoid membranes. The differential sensitivity of both types of PSII to Ni(II)-coordination compounds, suggest that the complexes can not infiltrate efficiently the regions of the thylakoids where the PSII with sigmoidal kinetics are located.

Apparently, neither the anions (Cl^- , Br^- , NO_3^-) nor emizco by themselves are important for the inhibition of electron transport (Table I), however

when the ligand is bound to nickel(II) the coordination compounds inhibit electron transport. The coordination compounds tested are more potent inhibitors on the photosynthetic electron flow than the salts, Table I.

Acknowledgements

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