Inhibition potencies (I_{50}-values, M) of DEMO and DIPMO as compared with an organophosphate and a carbamate insecticide

	ChE Human plasma	AChE Bovine	Eel	Housefly	*Spodoptera*
DEMO	1.1×10^{-6}	7.2×10^{-6}	1.5×10^{-4}	$\sim10^{-5}$	6.0×10^{-4}
DIPMO	1.9×10^{-6}	6.6×10^{-6}	1.3×10^{-4}	$\sim10^{-4}$	$>10^{-3}$
Monocrotophos	1.4×10^{-6}	2.5×10^{-5}	1.4×10^{-5}	1.3×10^{-6}	4.0×10^{-6}
Dioxacarb	1.4×10^{-5}	5.4×10^{-6}	2.7×10^{-6}	6.7×10^{-7}	3.4×10^{-6}

caged in a 9-cm Petri dish, evenly coated with 20 mg DEMO, however, showed severe intoxication symptoms, and 100% mortality after 24 h. The acute LD_{50} for DEMO in mice was 160 mg/kg after i.v. injection. Intoxication symptoms were comparable to those observed after organophosphate poisoning.

In addition to cholinesterases, rabbit plasma A-esterase was also tested for its sensitivity towards DEMO and DIPMO, the substrate being paraoxon. Both were found to be reversible, competitive inhibitors, the inhibition constants K_I being 0.56 mM for DEMO and 0.04 mM for DIPMO.

Discussion. In contrast to esters of α-keto acids, which are known to be reversible inhibitors of cholinesterases[3], diesters of mesoxalic acid hydrate represent a new type of esterase inhibitors. Mammalian cholinesterases appear to be more sensitive than the corresponding insect enzymes. The insensitivity of the insect enzymes may explain the lack of sufficient insect toxicity. In addition, due to its high polarity, DEMO may not be able to penetrate the insect cuticle to a satisfactory extent.

At this time the mode of the irreversible inhibition of cholinesterases by DEMO and DIPMO is still unknown. However, the fact that cholinesterases and other B-esterases are irreversibly, A-esterases on the contrary reversibly inhibited, the I_{50}-values and the reactivation rates make them look like a carbamate or an organophosphate inhibitor. Therefore we suggest that DEMO and DIPMO acylate the cholinesterases, and that the slow reactivation, i.e. deacylation rates, are due to the gem. diol of the hydrates which bind to the active site as analogues of the tetrahedral transition state of the deacetylation reaction, as shown in the figure.

This hypothesis is only of speculative nature but is supported by the recent finding that trifluoroacetophenone hydrate and other stable hydrates are inhibitors of cholinesterases[4].

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Reversible inactivation of the nitrate reductase of rice plants

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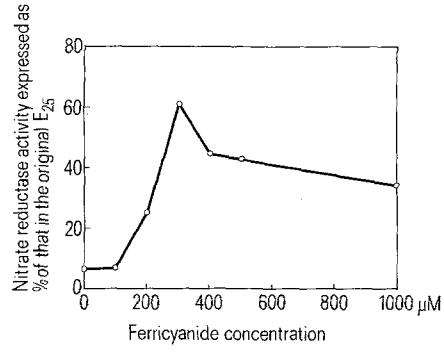
Summary. Studies of the inactivation of the rice nitrate reductase showed that the nitrate-reducing moiety and not the diaphorase moiety was reversibly inactivated by NADH and cyanide. Ferricyanide could reverse the inactivation, and nitrate could protect the enzyme against inactivation. Although the general characteristics of the reversible inactivation of rice nitrate reductase appeared similar to those of the algal nitrate reductase, it was found that the rice enzyme was automatically reactivated when NADH and cyanide were removed. Attempts to isolate inactivated nitrate reductase from ammonium-treated tissue were unsuccessful.

In the extracted state, nitrate reductase of algae exists in 2 forms, an active oxidized form and an inactive reduced form[1-5]. The enzyme was changed by reduced pyridine nucleotides and cyanide to the inactive form, which could be fully reactivated after reoxidation with ferricyanide[3,4]. Losada et al.[6] reported that reversible inactivation of nitrate reductase was induced in *Chlorella* cells by ammonium. Since Shen[7] has demonstrated the preferential assimilation of ammonium by rice seedlings, the purpose of this work was to see whether rice nitrate reductase is regulated by the same mechanism.

Materials and methods. Rice seedlings (*Oryza sativa*, L.var. IR8) were grown in culture solution[7]. Leaves of 12-day-old plants were harvested in a chilled medium containing 0.1 M phosphate buffer, pH 7.5, 1 mM cysteine hydrochloride and 5 µM FAD. The homogenate was filtered through cheesecloth and centrifuged at 20,000 × g for 20 min in a refrigerated centrifuge. The supernatant was passed over a Sephadex G-25 column. The eluate containing nitrate reductase was designated as E_{25}.



Reactivation of NADH-cyanide inactivated nitrate reductase by ferricyanide. E_{25} was inactivated by incubation with 200 µM-NADH and 10 µM-KCN in phosphate buffer (0.1 M, pH 7.5) at 24 °C for 10 min. Then the enzyme was incubated with ferricyanide of appropriate concentration at 24 °C for 10 min before nitrate reductase assay.

Table 1. Effects of NADH and cyanide on NADH:nitrate reductase and NADH:cytochrome c reductase activities

	Pretreatment NADH (μM)	Pretreatment KCN (μM)	Enzyme assay NADH (μM)	Enzyme assay KCN (μM)	NADH:nitrate reductase activity, nmoles NO_2^- produced/h/ml	NADH:cytochrome c reductase activity, nmoles cytochrome c reduced/h/ml
1	0	0	100	0	3120	2960
2	200	10	100	3	320	3740
3	0	10	100	3	1660	2950
4	200	0	100	0	2970	3610
5	200	0	100	3	1900	-
6	0	0	100	3	1900	-

Rice leaf nitrate reductase was pretreated by incubating E_{25} at 24 °C for 10 min with 200 μM-NADH or 10 μM-KCN or both. For the control E_{25} was preincubated with phosphate buffer alone. At the end of the pretreatment, aliquots of the pretreatment mixture were taken for NADH:nitrate reductase or NADH:cytochrome c reductase assays. NADH and KCN concentrations in each individual assay mixture were adjusted as indicated.

Table 2. Reactivation of NADH-cyanide inactivated nitrate reductase by passage over a Sephadex G-25 column

Fraction	Total NADH:nitrate reductase activity, nmoles NO_2^- produced/h
E_{25}	6400
E_i	180
E'_{25}	1700

E_{25} was inactivated by incubation with 1 mM-NADH and 100 μM-KCN at 24 °C for 10 min. The inactivated nitrate reductase (E_i) was passed over a Sephadex G-25 column and the eluate (E'_{25}) was collected.

NADH:nitrate reductase and NADH:cytochrome c reductase were assayed according to the methods of Wray and Filner[8]. The incubation mixture contained 0.1 mM NADH. However, when ferricyanide was present in the incubation mixture, 0.6 mM NADH was used to ensure sufficient reductant for nitrate reduction. Excess NADH was destroyed with lactate dehydrogenase (rabbit muscle, Sigma) after the incubation period.

Results and discussion. Nitrate reductase of rice in the crude extract and in E_{25} preparation was in the active form. It could not be activated with ferricyanide. The effects of NADH and cyanide on nitrate reductase are shown in table 1. NADH and cyanide, when acting together, inactivated the NADH:nitrate reductase by 90% of its original activity. Cyanide alone inhibited the NADH:nitrate reductase by about 50%. However, using treatment 6 for comparison, it is seen that pretreatment with cyanide alone may not have significant effect on the inactivation of the enzyme. E_{25} was not significantly inactivated by NADH alone. Comparison of NADH:nitrate reductase activities in treatments 3, 5 or 6 with treatment 2 showed that the presence of nitrate during the incubation period protected NADH:nitrate reductase from the inactivation caused by the combined effect of NADH and cyanide. NADH:cytochrome c reductase was not inactivated by the combination of NADH and cyanide, suggesting that the site of control of reversible inactivation was the molybdo-protein moiety. The above results indicate that rice nitrate reductase in the extracted form can be inactivated by a process identical to that of the algal nitrate reductase[4,5].

The NADH-cyanide inactivated enzyme was reactivated by ferricyanide. Potassium ferricyanide at optimum concentration (300 μM) reactivated the enzyme to 61% of the activity of the original active enzyme (figure). This suggests that ferricyanide may have fully reactivated the enzyme, but could not nullify the effect of cyanide. Higher concentrations of ferricyanide brought about a decrease in the level of reactivation. This is in agreement with the observation of Jetschmann et al.[9] for the *Chlorella vulgaris* nitrate reductase.

The results in table 2 show that NADH-cyanide inactivated rice nitrate reductase could be reactivated by passage over a Sephadex G-25 column. In spite of some loss of nitrate reductase activity during chromatography, about 30% of the activity of the original active enzyme was recovered. This G-25 eluate (E'_{25}) could not be further activated by ferricyanide addition. This result appears to contradict the observation that the inactivated nitrate reductase of *Chlorella* remained in the inactive state after passage over a Sephadex G-25 column as reported by Solomonson[3]. He proposed that a substance such as cyanide could complex with the molybdo-moiety to alter the molybdocentre such that it could be reduced by electron donors to a stable Mo(III) state which cannot reduce nitrate. It appears that this is not true for the rice nitrate reductase. The reduced nitrate reductase of rice plant was unstable in the absence of reducing power and tended to be activated, as with the nitrate reductase of *Neurospora*[10]. Results suggest that the stability of the reduced enzyme molecule may depend to a great extent on the enzyme structure which varies according to its source rather than purely on the chemical nature of molybdenum.

Rapid in vivo inactivation of nitrate reductase of *Chlorella fusca*[6] and *Chlamydomonas reinhardi*[11] was brought about by addition of ammonium to cultures grown in nitrate medium. Though preferential assimilation of ammonium was observed in rice seedlings[7], attempts to extract inactive nitrate reductase from rice seedlings after ammonium treatment were unsuccessful. However, this does not rule out the possibility that an inactive state of nitrate reductase was present in the rice tissue after ammonium treatment, but it was spontaneously reactivated during the extraction process.

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