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The Reactivation of Nitrate Reductase from Spinach (*Spinacia oleracea* L.) Inactivated by NADH and Cyanide, Using Trivalent Manganese Either Generated by Illuminated Chloroplasts or as Manganipyrophosphate

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Abstract. Nitrate reductase of spinach (*Spinacia oleracea* L.) leaves which had been inactivated in vitro by treatment with NADH and cyanide, was reactivated by incubation with oxidant systems and measured as $FMNH_2$-dependent activity. Reactivation was produced with trivalent manganese compounds represented either by manganipyrophosphate or produced by oxidation of Mn^{2+} ions in the presence of illuminated chloroplasts and compared with reactivation obtained with ferricyanide. Reactivation in the chloroplast system was equivalent to that with ferricyanide when orthophosphate was used but was variable and weak in the presence of pyrophosphate, although manganipyrophosphate was formed freely. Reactivation by manganipyrophosphate in dark reaction conditions was less effective than with ferricyanide but was not inhibited by the addition of pyrophosphate. Reactivation with illuminated unheated chloroplasts was dependent on added manganese and oxidation of manganese in the presence of pyrophosphate was abolished by boiling the chloroplasts. In the presence of orthophosphate however, boiled, illuminated chloroplasts reactivated the enzyme in the absence of added manganese. Reactivation occurred spontaneously in air, more slowly than with the other oxidants, but to a similar extent to that produced by manganipyrophosphate. The results provide a possible model for physiological reactivation mechanisms.

Key words: Chloroplasts – Manganese oxidation – Nitrate reductase reactivation – *Spinacia*.

Introduction

Higher plant nitrate reductase catalyses the reduction of nitrate by NADH and additionally displays two partial functions; the NADH mediated reduction of either cytochrome *c* or 2,6-dichlorophenolindophenol or ferricyanide (dehydrogenase function), and the reduction of nitrate by either reduced FMN or benzyl-(methyl) viologen (see Hewitt et al. 1976).

The nitrate reducing capacity of the enzyme can exist in vivo and in vitro in both active and inactive forms, conversion to the inactive form being accomplished in vitro by incubation with NADH and cyanide (Relimpio et al. 1971). The dehydrogenase function of the inactivated nitrate reductase is unaffected (Palacian et al. 1974). Reactivation of the inactivated enzyme can be achieved in vitro either completely and rapidly with ferricyanide (Palacian et al. 1974), acting at a site believed to be different from that of the dehydrogenase acceptor (Rosa et al. 1976) or, less efficiently (as shown with the *Chlorella* enzyme) with Mn^{3+} pyrophosphate (Funkhouser and Ackermann 1976). Reactivation of the NADH and cyanide treated enzyme also occurs in the presence of flavin nucleotides in a few minutes in blue or white light and more slowly in darkness (Aparicio et al. 1976; Roldan et al. 1978).

Manganese has a key role in oxygen evolution by chloroplasts (Cheniae 1970), being closely linked to the oxygen evolution reaction and involving a very positive photochemically generated redox potential (Gerretsen 1950). In the presence of illuminated chloroplast preparation Mn^{2+} ions are oxidised to Mn^{3+} ions and can be stabilised by pyrophosphate (Kenten and Mann 1955). This reaction is probably mediated by superoxide ions and protons (Kono et al. 1976).

We therefore sought to test whether nitrate reductase, previously inactivated by reduction in the presence of cyanide, could be reactivated by photochemical means using manganese and chloroplast preparations and thus provide a possible model for a physiological system involving manganese in the regulation of nitrate reductase, as distinct from the alternative

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possibility of inhibition of nitrate assimilation resulting from decreased photosynthesis.

Materials and Methods

Plant Material and Enzyme Assay. Spinach (*Spinacia oleracea* L. cv. Noorman) plants were grown in a glasshouse using a nutrient film technique with Long Ashton type solutions (Hewitt 1966) and leaves were sampled after 5–6 weeks growth.

Nitrate reductase was extracted in three vol/wt ratio of 0.1 M potassium phosphate buffer pH 7.5, with 1 mM EDTA and partially purified using streptomycin sulphate, ammonium sulphate and hydroxylapatite (Notton et al. 1977). Protein was precipitated with 50% saturation ammonium sulphate, dissolved in extracting buffer and stored at −40° C in separate small samples. The specific activity of the preparation (0.2 units/mg protein) remained unchanged after several months of storage under these conditions.

$FMNH_2$-nitrate reductase activity was determined by measuring nitrite formed (Paneque et al. 1965). The assay system in 1 ml contained 50 mM phosphate buffer pH 7.5, 1 mM EDTA, 10 mM potassium nitrate, the enzyme preparation and 0.2 mM FMN reduced by 0.1 ml $S_2O_2^{2-}$ (8 mg ml^{-1} in 50 mM $NaHCO_3$) as electron donor. After 10 min incubation at 27° C, the reaction was stopped by vigorous shaking in a rotamixer until the reduced FMN was reoxidised and nitrite was measured by addition of 1 ml of sulphanilamide reagent followed by 1 ml of *N*-(1-naphthyl) ethylenediamide reagent. Activity was expressed as n moles of nitrite formed per minute and per ml of preincubation mixture. Protein concentration was calculated from light absorbance at 280 nm and 260 nm (Warburg and Christian 1941).

Nitrate Reductase Inactivation. To prepare in vitro inactivated nitrate reductase, the enzyme was incubated for 30 min at room temperature with 0.1 M potassium phosphate pH 7.5, 2.0 mM NADH and 0.5 mM KCN. To eliminate excess of NADH and cyanide the preparation was passed through a G-25 Sephadex column equilibrated with 0.1 M potassium phosphate at the pH which was used later for each particular experiment.

Chloroplast Preparation. Chloroplasts were prepared from young leaves of spinach according to the method of Asada and Takahashi (1971) with some modifications. Deveined leaves were cut into small pieces and ground with three vol/wt ratio of 0.4 M sucrose containing 50 mM tricine-KOH buffer pH 8.0 and 10 mM NaCl in a Polytron for 4 s at full speed. The homogenate was filtered through 4 layers of gauze and the filtrate was centrifuged at 2,000 *g* for 10 min. The supernatant was discarded and the pellet resuspended in one vol/wt ratio of the grinding medium and centrifuged again under the same conditions. The resultant pellet was finally resuspended in the grinding medium to give a concentration of 2–4 mg of chlorophyll per ml. An equal volume of cold glycerol was then added, mixed well and the suspension stored at −20° C. Under these conditions the chloroplasts, hereafter designated unheated, retained full Hill reaction properties and the photoxidation activity of Mn^{2+} for more than a month (Kono et al. 1976). Chlorophyll was determined according to Arnon (1949).

Assay of Manganese Oxidation. Unless otherwise indicated the reaction conditions for the oxidation of manganese by chloroplasts were: 50 mM sodium pyrophosphate or orthophosphate pH 7.8, 10 mM NaCl, 1 mM $MnCl_2$ and chloroplasts equivalent to 180 µg of chlorophyll in a total volume of 2 ml. The mixture was incubated for 1 h at 20° C during illumination by a focussed 500 W projector lamp and frequently shaken. Experiments were terminated by switching off the light and centrifuging the contents of each reaction mixture for 20 min at 700 *g*. The amount of Mn^{3+} pyrophosphate formed was determined in the clear supernatant solution from its absorbance at 258 nm and assuming an extinction coefficient for the complex of $6.2 \cdot 10^3$ l mol^{-1} cm^{-1} (Kenten and Mann 1955). The supernatant from a mixture incubated in darkness without added $MnCl_2$ was used in the blank cell.

Chemicals and Materials. FMN was obtained from Sigma (London) Chemical Corp.; NADH from Boehringer Corp. (Mannheim); hydroxylapatite (Biogel HTP) from Biorad Labs.; Sephadex G-25 from Pharmacia. All other chemicals were of analytical reagent grade. Mn^{3+} pyrophosphate was prepared according to Funkhouser and Ackermann (1976).

Results

Dark (Chemical) Reactivation. The effect of mangani (Mn^{3+}) pyrophosphate (0.5, 1.0 and 2.5 mM), prepared chemically from mangani acetate and excess of pyrophosphate, were first tested in a dark system to compare them with effects of ferricyanide (Fig. 1). Reactivation by ferricyanide was maximal in 5 min but activity then decreased slowly during 35 min tested. Reactivation by Mn^{3+} pyrophosphate increased more slowly up to 15 min and then remained almost constant. The extent of reactivation was the same with added Mn^{3+} between 0.5 and 2.5 mM. However the activity produced was only 60% of the maximum obtained with ferricyanide. In this particular experiment no spontaneous reactivation was observed in the absence of either oxidant, although this sometimes occurred slowly (see e.g. Fig. 2). Under the reaction conditions used, with Mn^{3+} pyrophosphate prepared as described by Funkhouser and Ackermann (1976) there was a 4:1 ratio of pyrophosphate to Mn^{3+}

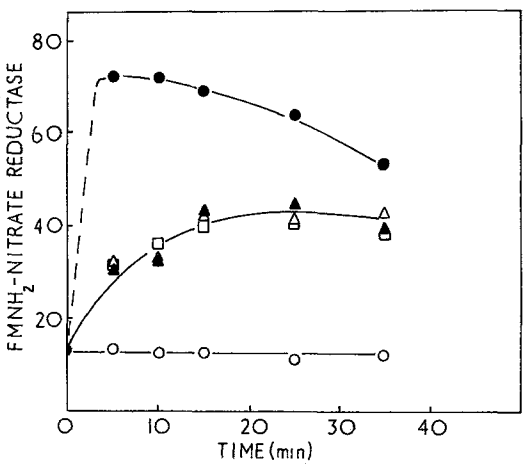


Fig. 1. Reactivation of nitrate reductase by Mn^{3+}-pyrophosphate. The enzyme was incubated, at room temperature in a final volume of 1.0 ml with 30 µmol phosphate buffer pH 6.4, either alone (○) or with the following additions: (●) 0.5 µmol potassium ferricyanide; (△) 2.5; (▲) 1.0; (□) 0.5 µmol Mn^{3+}-pyrophosphate respectively. Molarities of Mn^{3+}-pyrophosphate are expressed in terms of manganese content. Nitrate reductase activity was assayed on aliquots taken from preincubation mixtures at the times indicated

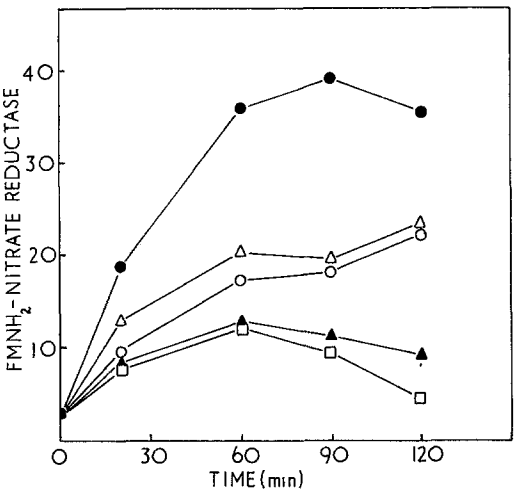


Fig. 2. Effect of Mn^{2+} on the nitrate reductase reactivation by Mn^{3+}-pyrophosphate. The enzyme was incubated with 50 µmol phosphate buffer pH 6.4, either alone (○) or with 1.0 µmol Mn^{3+}-pyrophosphate (●) plus the following additions: (△) 1.0; (▲) 5.0; (□) 10 µmol Mn^{2+} respectively. Mn^{2+} was added as $MnCl_2$. Other experimental conditions as in Fig. 1

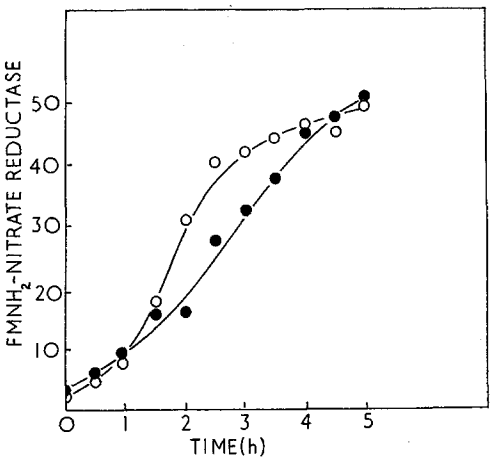


Fig. 3. Spontaneous reactivation with time of nitrate reductase in the absence and in the presence of nitrate. The inactive enzyme was incubated, in a final volume of 2.0 ml, with 60 µmol of phosphate buffer pH 6.4, either alone (○) or in the presence of 40 µmol potassium nitrate (●)

giving a maximum total pyrophosphate concentration of about 30 mM in their system and 10 mM in ours. The effects of adding more Mn^{2+} (1, 5 or 10 mM) to provide between 2:1 and about 1:1 ratios of pyrophosphate to total manganese were therefore tested. As shown in Fig. 2, additional Mn^{2+} ions were found to be severely inhibitory to the dark (chemical) reactivation system.

The progress of spontaneous reactivation with time in darkness and in air was examined over 5 h in the absence or presence of nitrate (20 mM) (Fig. 3). Reactivation occurred slowly to a final extent which

Table 1. Photoxidation of Mn^{2+} by spinach chloroplasts

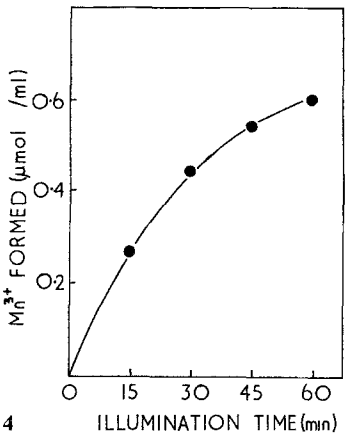
System	Mn^{3+}-pyrophosphate formed (µmol ml^{-1})
Complete, light	0.570
minus added Mn^{2+}	0.019
minus chloroplasts	0.004
chloroplasts boiled for 10 min	0.062
Complete, dark	0.000

was comparable to, or perhaps slightly more than, that obtained much more rapidly with Mn^{3+} pyrophosphate in different experiments (Figs. 1 and 2). Nitrate did not influence the extent of spontaneous reactivation but appeared to alter the kinetics from sigmoidal to more nearly linear with respect to time.

Light (Photochemical) Reactivation. The photochemical oxidation of Mn^{2+} to Mn^{3+}, by illuminated chloroplast in the presence of pyrophosphate was next tested as described in the Methods. Oxidation was dependent on light or presence of chloroplasts and was decreased 90% using boiled-chloroplasts (Table 1). Endogenous manganese found in chloroplast systems (Kenten and Mann 1955) was similar in our system, namely about 1.5% of that added (1 mM) as calculated from the change in absorbance observed without added manganese.

As shown in Table 1 only 55% to 60% of added manganese was stabilized as Mn^{3+} and detected thus in the presence of 50 mM pyrophosphate. As shown in Fig. 4 this yield appears to approach a steady state with time, after 1 h, when chloroplasts equal to 90 µg chlorophyll ml^{-1} were used. However, as shown in Fig. 5, the yield of Mn^{3+} pyrophosphate was increased to nearly theoretical (1 mM) when chloroplasts were increased to 300 µg chlorophyll ml^{-1}, with a similar illumination period of 1 h. These conditions were later adopted as optimal for the photochemical reactivating systems when orthophosphate was substituted for pyrophosphate (see below).

When the unheated chloroplast system (above), containing 90 µg chlorophyll ml^{-1}, was used for the reactivation of the enzyme during 1 h illumination, there was little success, as shown in Table 2, when the medium contained pyrophosphate (25 mM); the reactivation being less than that occurring spontaneously. In several tests under similar conditions, reactivation by this system in the presence of pyrophosphate was variable, and ranged from less than the spontaneous change to not more than 25% of that obtained with ferricyanide over the period of 1 h. Nevertheless the concentration of Mn^{3+} pyrophosphate produced (0.5 to 0.6 mM) in 1 h using



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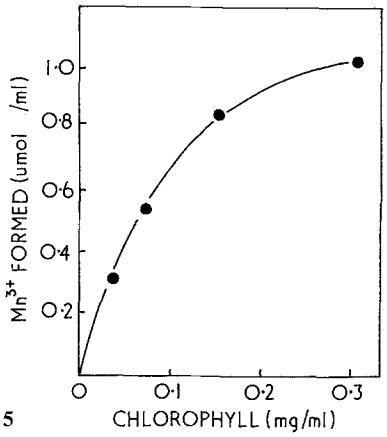

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Fig. 4. Time course of Mn^{2+} oxidation by illuminated chloroplasts. Experimental conditions were as described in Materials and Methods except for the illumination time

Fig. 5. Effect of the concentration of chloroplast on the Mn^{2+} photoxidation. Experimental conditions were as described in Materials and Methods except that chloroplasts were added as indicated

Table 2. Effect of orthophosphate and pyrophosphate on nitrate reductase reactivation by illuminated chloroplasts and manganese

System	$FMNH_2$-nitrate reductase (nmol NO_2^- produced min ml^{-1})
Inactivated enzyme *plus*	
Mn^{2+} photoxidising system in	
25 mM orthophosphate, pH 7.5	37.5
25 mM pyrophosphate, pH 7.5	4.5
25 mM orthophosphate *plus*	
25 mM pyrophosphate, pH 7.5	8.0
Inactive enzyme, dark	5.7

The inactive enzyme was incubated in the light with the Mn^{2+}-photoxidising system described in Materials and Methods, except that the systems were buffered as shown above. Other experimental conditions as in Fig. 6. The amount of $Mn^{3\pm}$pyrophosphate formed was the same either in the presence or in the absence of orthophosphate

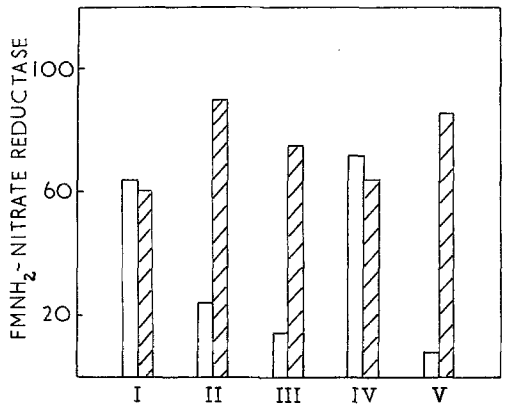

Fig. 6. Reactivation of nitrate reductase by illuminated chloroplasts in the presence of mangenese. The complete Mn^{2+}-photoxidising system was as described in Materials and Methods except that orthophosphate was used in place of pyrophosphate as buffer and inactive nitrate reductase was incubated with the system. After 1 h the reactions were terminated as described in Materials and Methods and 0.1 ml aliquots from the supernatants were assayed for $FMNH_2$-nitrate reductase before (plain) and after (bars) incubating with 0.3 mM ferricyanide for 3 min. (I) Complete system, light; (II) chloroplasts omitted; (III) Mn^{2+} omitted; (IV) with chloroplasts previously boiled for 10 min; (V) complete system, dark

90 µg chlorophyll ml^{-1} was about the same, as shown in Fig. 1, (0.5 mM) which produced substantial reactivation in the dark (chemical) system and which approached (but more slowly) that obtained finally with ferricyanide. However when orthophosphate was used in the place of pyrophosphate in the chloroplast system with 90 µg chlorophyll ml^{-1}, reactivation of the enzyme was substantial (Table 2) and equal to that produced in the dark (chemical) system by Mn^{3+} pyrophosphate (Figs. 1 and 2). The presence of orthophosphate did not influence the rate of Mn^{3+} pyrophosphate formation (not shown) but it appeared to lessen slightly the inhibitory effect of pyrophosphate on reactivation (Table 2). In the chloroplast system with pyrophosphate, increasing concentrations of Mn^{2+} between 0.5 and 5 mM produced no reactivation. The yields of Mn^{3+} measured as pyrophosphate were between 500 and 670 µM over this range, i.e. between 100% and 13.4% respectively.

We finally adopted the optimum conditions described above for photochemical oxidation of manganese by illuminated chloroplasts as measured in the presence of pyrophosphate (Figs. 4 and 5), namely using 300 µg chlorophyll ml^{-1} for 1 h illumination, to test the reactivation of the enzyme in the system where orthophosphate now replaced pyrophosphate. As shown in Fig. 6, reactivation was now practically complete and equivalent to that obtained with ferricyanide in a dark (chemical) system. The reactivated enzyme was not additionally reactivated by further

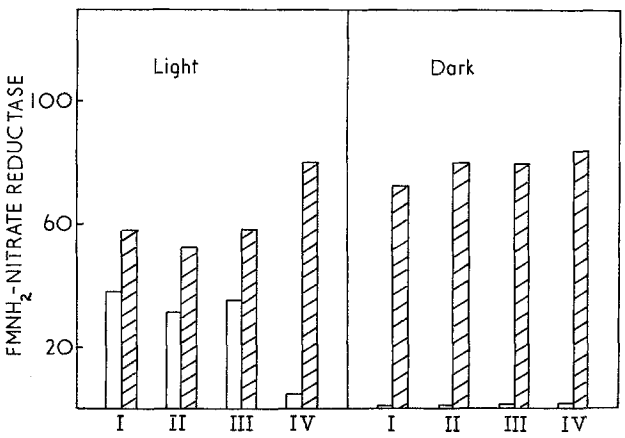

Fig. 7. Reactivation of nitrate reductase using boiled chloroplasts. Inactive nitrate reductase was preincubated with the Mn^{2+} photoxidising system either in the light or in the dark. Other experimental conditions as described in Fig. 6. Nitrate reductase activity before (plain) and after (bars) incubation with ferricyanide. (I) Complete system; (II) with boiled chloroplasts; (III) with boiled chloroplasts but Mn^{2+} omitted; (IV) chloroplasts and Mn^{2+} omitted

treatment with ferricyanide after the illumination period. Reactivation was dependent on added manganese when unheated chloroplasts, prepared as described, were used in the orthophosphate medium (Fig. 6).

In this system however, where orthophosphate replaced pyrophosphate, boiled illuminated chloroplasts were now as effective as unheated chloroplasts for the complete reactivation of the enzyme (Figs. 6 and 7). Furthermore, as shown in Fig. 7, reactivation by boiled (10 min) chloroplasts in the orthophosphate system was no longer dependent on added manganese but was still dependent on light. In the pyrophosphate system, by contrast, photochemical oxidation of manganese was prevented by heating the chloroplasts (Table 1) and the small variable extent of reactivation described above was dependent on added manganese.

Discussion

Our experiments confirm, for a higher plant nitrate reductase, that Mn^{3+} pyrophosphate in the dark is able to reactivate the NADH-reduced and cyanide-bound state of the enzyme, as originally found by Funkhouser and Ackermann (1976) for the enzyme from *Chlorella vulgaris*. In the enzyme from both species, the reactivation was similar in extent namely about two-thirds that produced by ferricyanide, but slower. The reaction was similarly rather insensitive to effects of Mn^{3+} pyrophosphate concentrations in the range of 1–3 mM.

The reversible inactivation of nitrate reductases by 'over-reduction' especially in the presence of cyanide has been proposed as an important regulatory mechanism, (see Hewitt et al. 1979). If such a mechanism is effective in vivo, suitable metabolically-generated oxidants must be produced, which can substitute for the very efficient behaviour of ferricyanide in reactivating the enzyme in vitro. The effects of Mn^{3+} pyrophosphate suggest that manganese may be involved as a potential source of oxidant for the in vivo regulation of the enzyme.

Our experiments with chloroplasts prepared and used under conditions which preserve the capacity for oxygen evolution and Hill reaction activity (Asada and Takahashi 1971; Kono et al. 1976) support our idea (Hewitt et al. 1979) that manganese may be involved in a physiological redox mechanism for regulation of nitrate reductase activity and that illuminated chloroplasts may constitute one possible oxidation system. This mechanism, as tested here, is not dependent on the existence of pyrophosphate in the cells and it is actually inhibitory. The concentrations of manganese estimated by Kono et al. (1976) in chloroplasts (about 0.5 mM) would be consistent with that found adequate for reactivating the enzyme in our experiments.

We would suppose the life of free Mn^{3+} ions to be very brief in the absence of a stabilizing agent such as pyrophosphate and would rapidly generate hydrogen peroxide:

$$2\ Mn^{3+} + 2\ H_2O \rightleftharpoons 2\ Mn^{2+} + H_2O_2 + 2\ H^+.$$

However orthophosphate can replace pyrophosphate in the formation of similar amounts of quite stable higher valency states of manganese (Kenten and Mann 1955), but the products tend to be insoluble and in living cells may precipitate as MnO_2 (Kenten and Mann 1957; Stenuit and Piot 1960). Presumably some form of fairly stable higher valency manganese compound was formed in our experiments with chloroplasts and orthophosphate, which could reactivate the enzyme in a manner similar to that produced by Mn^{3+} pyrophosphate.

In the presence of excess Mn^{2+} ions and a suitable stabilizing reagent (Kenten and Mann 1955) some Mn^{3+} would be formed according to the reversible disproportionation:

$$Mn^{2+} + Mn^{4+}(MnO_2) \rightleftharpoons 2\ Mn^{3+}.$$

The equilibrium reached in the photoxidation of Mn^{2+} and the yield of Mn^{3+} pyrophosphate clearly depends on experimental conditions, as shown here with respect to chlorophyll concentration and in many other ways in previous experiments (Kenten and Mann 1955; Homann 1965; Bachofen 1966). These observations may be explained by the facts that Mn^{3+} pyrophosphate is a reducible Hill reagent (Swenson

and Vernon 1969) and that photochemical manganese oxidation depends both on oxygen evolution by the chloroplasts (Homann 1965; McKenna and Bishop 1967; Habermann et al. 1968) and on the presence of oxygen in the system (Bachofen 1966; McKenna and Bishop 1967; Kono et al. 1976). This oxidation is variously stated either to be independent of hydrogen peroxide though still closely correlated with a capacity for the Hill reaction (Bachofen 1966), or to depend on peroxide formed by the Mehler reaction as the oxidant for manganese (McKenna and Bishop 1967). Alternatively the superoxide anion (O_2^-), which is generated by autoxidation of ferredoxin or $FMNH_2$ and normally behaves as a reductant, is believed to oxidise Mn^{2+} directly (Asada et al. 1974; Kono et al. 1976), though probably via the hydrated form $Mn(OH)^+$ for kinetic reasons (Homann 1965).

$$Mn(OH)^+ + O_2^- + 3\ H^+ \rightleftharpoons Mn^{3+} + H_2O_2 + H_2O.$$

The superoxide dependent oxidation is severely inhibited in the illuminated chloroplast system by superoxide dismutase. In our experiments (not shown) the addition of either superoxide dismutase or catalase to the chloroplast system with orthophosphate had no effect on the extent of reactivation of the enzyme. In the presence of pyrophosphate the formation of Mn^{3+} was not greatly influenced (only +20% or −15%) by the respective enzymes.

Evidence at present indicates that nitrate reductase is outside chloroplast limiting membranes (see Hewitt et al. 1976). The extent to which Mn^{3+} compounds would be available for the photochemical reactivation of nitrate reductase outside the chloroplasts in vivo would therefore depend on complex interaction of several factors. We must also suppose that if intact chloroplasts in vivo provide a manganese based oxidant, either it can diffuse out of the chloroplasts in a stabilized state or it produces a secondary oxidant such as hydrogen peroxide, which may function alone or in a manganese-catalysed reaction to reactivate the enzyme in a cytoplasmic location. Alternatively the photochemical system used in vitro may provide only a model for manganese oxidation which is perhaps achieved in other parts of the cell by peroxidase systems. We shall describe work bearing on some of these points in a separate paper.

Our experiments using illuminated boiled-chloroplasts indicated that reactivation of nitrate reductase can be achieved by other means than a manganese-dependent oxidation. The mechanism involved was probably similar to that for the oxidation of diketogulonic acid mediated by photosensitive pigments such as FMN (Homann 1965; Habermann et al. 1968) and was presumably dependent on a denatured protein-chlorophyll complex (Homann 1965). The reactivation of nitrate reductase differed however in at least one important respect from photochemical oxidation of diketogulonic acid, because the latter was still essentially dependent on manganese regardless of whether undenatured, or heated chloroplasts, or FMN were used. Moreover the rapid photochemical reactivation of nitrate reductase which is produced in vitro by illumination in the presence only of FAD or FMN does not appear to require the addition of manganese (Rosa et al. 1976).

As shown for nitrate reductase reactivation, the oxidation of diketogulonic acid can be achieved by Mn^{3+} pyrophosphate in a dark reaction (Habermann et al. 1968) and additionally, pyrophosphate alone was reported to inhibit the photochemical oxidation of diketogulonic acid by FMN (McKenna and Bishop 1967). Although in the dark system Mn^{3+} pyrophosphate, in the presence of as much as 7.5 mM excess pyrophosphate in our experiments, or up to 22.5 mM in those of Funkhouser and Ackermann (1976), effectively reactivated the enzyme in about 30 min, a similar initial concentration of free pyrophosphate (25 mM) in the chloroplast system was variably, but generally severely, inhibitory. The presence of pyrophosphate in the chloroplast system did not, however, prevent (within an appropriate time interval) the accumulation of Mn^{3+} pyrophosphate in amounts similar to those which produced reactivation in the dark systems used by ourselves and Funkhouser and Ackermann (1976). We cannot suggest any obvious explanation for this curious paradox, except to note the possibly analogous results of McKenna and Bishop (1967) above.

The enzymic site for the oxidative reactivation of nitrate reductase is not known. Manganese catalysed oxidations are considered to be essentially one-electron type reactions (see Homann 1965) and this would apply also to the action of ferricyanide. The haem group of nitrate reductase is readily autoxidizable (in seconds) (R.J. Fido, E.J. Hewitt and B.A. Notton, unpublished work) and is equally rapidly oxidised by 2,6-dichlorophenolindophenol after reduction by NADH (Notton et al. 1977), but spontaneous reactivation in air takes several hours (this work; Jetschmann et al. 1972, Moreno et al. 1972), compared with only several minutes or seconds using Mn^{3+} pyrophosphate or ferricyanide respectively. We therefore believe these oxidants must reactivate the enzyme at a site other than the haem group. We have evidence to suggest that the dehydrogenase acceptors, ferricyanide and 2,6-dichlorophenolindophenol can react at different sites (Maldonado et al. 1978). Thus ferrocyanide (Fe^{2+} state) inhibits the enzyme competitively with nitrate but reduced indophenol has no effect on activity. Oxidised indophenol

and oxygen only very slowly and inefficiently reactivate the enzyme, unlike ferricyanide or Mn^{3+} pyrophosphate. These observations are consistent with the conclusion that Mn^{3+} (or ferricyanide) reactivate the 'over-reduced' enzyme by direct oxidation of molybdenum from Mo^{IV} to Mo^{VI} by successive external one-electron transfers from the very stable cyanide-Mo^{IV} co-ordinated state, to the unstable cyanide-Mo^{VI} co-ordinated state, from which cyanide dissociates spontaneously and easily (Notton and Hewitt 1971 b). In the absence of added oxidants reactivation probably occurs by very slow internal electron transport from Mo^{IV} to the readily autoxidisable haem centre and a similar slow reaction may occur with 2,6-dichlorophenolindophenol.

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