BBA 46329

PROPERTIES OF A NITRATE REDUCTASE OF *CHLORELLA*

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(Received December 20th, 1971)

SUMMARY

1. An NADH–nitrate oxidoreductase (EC 1.6.6.1) of *Chlorella* has the unusual property of existing in cell-free extracts mainly in the form of an inactive precursor which can be activated by a variety of procedures. This enzyme is associated with a cytochrome of the *b* type.

2. The inhibitors, azide, cyanate, thiocyanate and nitrite, react rapidly with the enzyme, with kinetics which show that they are competitive with nitrate.

3. The inhibitors, cyanide and hydroxylamine, react slowly with the reduced form of the enzyme to give an inactive product which can slowly be reactivated in the presence of nitrate. There is at least a superficial similarity between the reactivation of the inhibited enzyme and the activation of the enzyme precursor in fresh extracts.

4. Mammalian cytochrome *c*, dichlorophenolindophenol and ferricyanide can substitute for nitrate as oxidants for NADH in the presence of the enzyme. This "diaphorase" reaction does not require activation, but is fully active in fresh extracts. It is not inhibited by cyanide, hydroxylamine, azide, cyanate, thiocyanate, or by the substrate, nitrate. Oxidized cytochrome *c*, on the other hand, inhibits the reduction of nitrate by NADH in the presence of the enzyme.

5. Pyridoxal phosphate inhibits both nitrate reductase and cytochrome *c* reductase to about the same extent.

INTRODUCTION

The partial purification of an NADH–nitrate oxidoreductase from *Chlorella* has been previously described[1]. The strain of *Chlorella* used has long been called *pyrenoidosa*. Pending reclassification, it will here be referred to as the Berlin strain[2]. As already pointed out[2], the nitrate reductase from this strain differs in some respects from the nitrate reductase of *Chlorella fusca*, which has been extensively investigated by Losada and his associates[3-7]. The present paper describes further studies of the nitrate reductase of the Berlin strain. The cytochrome associated with the enzyme will be characterized as a *b*-type cytochrome, (cytochrome b_{557} *Chlorella*) further corroborating the similarity of the enzyme to the nitrate reductase of *Neurospora crassa*, described by Garrett and Nason[8,9].

We will also show that this enzyme complex, like other nitrate reductases, exhibits a cytochrome *c* reductase (diaphorase) activity, which is relatively independent of the activity of the nitrate-activating component of the enzyme, even though the

two activities are closely associated. Thus, a group of inhibitors which compete with nitrate have no inhibitory effect on the cytochrome *c* reductase activity of the complex. Furthermore, the cytochrome *c* reductase moiety is fully active in fresh extracts, though the nitrate reductase moiety is almost inactive.

EXPERIMENTAL

Preparation of nitrate reductase

The Berlin strain[2] of *Chlorella* was grown autotrophically in continuous light with nitrate as the nitrogen source, as previously described[1]. The cells were harvested by gentle centrifugation, washed once with distilled water (about 0.1 vol. of original cell suspension), and suspended in sufficient distilled water to give a suspension containing 250 μl cells per ml. The cells were broken by sonication. An MSE 100-W ultrasonic disintegrator (Measuring and Scientific Equipment Ltd, London) was used, with the large probe at maximum amplitude. Aliquots of not more than 40 ml cell suspension in a 100-ml beaker cooled in an ice bath were sonicated for two 5-min periods, interrupted by 5 min of cooling. The suspensions were freed of whole cells and larger particles by centrifugation for 10 min at 3000 $\times$ *g*. To maximize the enzyme yield, the sediment was resuspended in water to one-half the original volume, and the sonication and centrifugation were repeated. The combined extracts were stored frozen at -20 °C, and worked up in batches of approximately 1 l by the procedure previously described[1], with minor modifications, as follows.

The $(NH_4)_2SO_4$ precipitations were carried out by addition of a solution of $(NH_4)_2SO_4$ saturated at 0 °C, with a pH of 7.6, adjusted by addition of 1.7 ml of 5.6 M NH_4OH to 1 l. For the first $(NH_4)_2SO_4$ precipitation, the protein fraction which was precipitated between 0.27 and 0.47 saturation was collected, and dissolved in 0.1 the original volume of water. After dialysis against $1 \cdot 10^{-3}$ M NH_4OH for about 18 h, the fractional precipitation with protamine sulfate was carried out as previously described. The specific activity of the final preparation is dependent on the care taken with this step, which is controlled by assays of the activity remaining in solution. The small, sticky, brown precipitate containing the enzyme was extracted with 0.1 M Tris buffer (pH 8.0) as described[1]. In the second $(NH_4)_2SO_4$ fractionation, fractions were collected at 0–0.27, 0.27–0.37, and 0.37–0.5 satn with $(NH_4)_2SO_4$. The middle fraction always contained the product of highest specific activity, generally about 4–5 units of enzyme per mg protein.

Measurement of nitrate reductase

The nitrate reductase activity was assayed by measuring the rate of oxidation of NADH by added nitrate. One unit of enzyme is defined as that amount which catalyzes the oxidation of 1 μmole of NADH per min under the specified conditions of the assay[1].

Measurements with alternate substrates

The catalysis of the oxidation of NADH by cytochrome *c*, dichlorophenolindophenol or ferricyanide were all measured under the conditions of the standard assay system, but with the alternate oxidant substituted for the nitrate.

With cytochrome *c*, the reaction was followed by measurement with a Zeiss spectrophotometer, of the increase in absorbance at 550 nm, due to reduction of cytochrome *c*. Unless otherwise noted, 1.5 mg cytochrome *c* was added per 3.0 ml of

reaction mixture. A $\Delta\varepsilon_{mM}$ of 21 was used to calculate the amount of cytochrome *c* reduced[10]. After measurement of the initial reaction rate, the total concentration of cytochrome *c* was determined by measuring the absorbance at 550 nm after reduction by $Na_2S_2O_4$. The measured reaction velocity was corrected to maximum velocity at a saturating concentration of cytochrome *c* from a standard plot of *V/v versus* cytochrome concentration. One unit of cytochrome *c* reductase is the amount of enzyme which catalyzes the reduction of one μmole of cytochrome *c* by NADH per min, under the specified conditions but with a saturating concentration of cytochrome *c*.

The reduction of dichlorophenolindophenol by NADH was measured by determining the initial rate of decrease of absorbance at 600 nm. An ε_{mM} of 21 (ref. 11) was used to calculate the amount of dichlorophenolindophenol reduced. A blank containing all reagents except enzyme was run at the same time and the blank rate was subtracted from the reaction rate in the presence of enzyme. The reduction of ferricyanide by NADH was measured in analogous fashion by determining the initial rate of decrease in absorbance at 420 nm, using $\varepsilon_{mM} = 1.02$ for ferricyanide[12].

Activation of the enzyme

As previously described[1], the nitrate reductase of the Berlin strain of *Chlorella* is present in crude extracts mainly in the form of an inactive precursor, which is fractionated together with the active enzyme, and undergoes activation during the fractionation procedure. If the fractionation is conducted as rapidly as possible, the final product obtained after the second $(NH_4)_2SO_4$ fractionation can still be activated 2- or 3-fold. To determine the maximum activity at this stage of purification, the enzyme was incubated in the ice box at 4 °C with 10^{-1} M phosphate buffer (pH 6.7), $5 \cdot 10^{-2}$ M KNO_3, $1 \cdot 10^{-3}$ M dithioerythreitol and $5 \cdot 10^{-4}$ M EDTA. Maximum activity was generally reached in about 3 or 4 weeks (see Fig. 7). The solutions remained clear, and the protein content did not change during the incubation. The dithioerythreitol and EDTA had no effect on the initial rate of activation, but stabilized the active enzyme. The above activation medium does not give maximal activation in unfractionated extracts. It is recommended only for the fractions obtained subsequent to the first $(NH_4)_2SO_4$ fractionation.

Protein was determined by a modification of the method of Lowry *et al.*[13], or from absorbance measurements at 280 and 260 nm (ref. 14). Nitrite was measured as previously described[1].

Reagents

Protamine sulfate was obtained from Nordisk Insulin Laboratorium, Copenhagen; horseheart cytochrome *c* and β-NADH (Grade I and II) from Boehringer; 2,6-dichlorophenolindophenol from Schuchart; Antimycin A from Serva; 2-heptyl-4-hydroxyquinoline-*N*-oxide from Sigma; hemin chloride from Calbiochem; pyridine (for spectroscopy) from Merck. All other reagents used were of the purest commercial grade obtainable.

RESULTS

The cytochrome associated with nitrate reductase

The cytochrome associated with the nitrate reductase can provisionally be classified in the *b* group, since the prosthetic group is easily extracted with acetone

and is probably protohaem[15]. If salts were removed by dialysis against $5 \cdot 10^{-4}$ M phosphate buffer (pH 7.1), it was not necessary to add acid to the acetone. 3 ml of a well-dialyzed enzyme solution, containing 10–15 units of nitrate reductase per ml, were mixed with 12 ml acetone cooled to −20 °C. The precipitate was removed by centrifugation and the extraction was repeated. The combined acetone extracts were evaporated to dryness under reduced pressure. A diethyl ether-soluble pigment was obtained, which gave the absorption spectrum of a pyridine hemochromogen when taken up in pyridine–KOH solution and reduced with a few grains of $Na_2S_2O_4$. If the dialysis of the enzyme solution was omitted, it was necessary to add acid to the acetone (0.02 vol. of 2.5 M HCl) in order to extract the pigment. The residue from the acid acetone extraction gave no pyridine hemochromogen.

The spectrum of the pyridine hemochromogen prepared from the enzyme was similar to that obtained from reference samples of hemin chloride treated similarly, giving peaks in the reduced form at 555, 524 and 418 nm. There were anomalies, however, in the relative magnitude of the absorbance at different wavelengths, suggesting the presence of another pigment. Three separate determinations of the amount of pyridine hemochromogen recovered from acetone or acid acetone extracts gave an average recovery of 80 %, when ($A_{555\,nm}$ *minus* $A_{540\,nm}$) was used as a basis for the calculation, as described in the legend for Fig. 1.

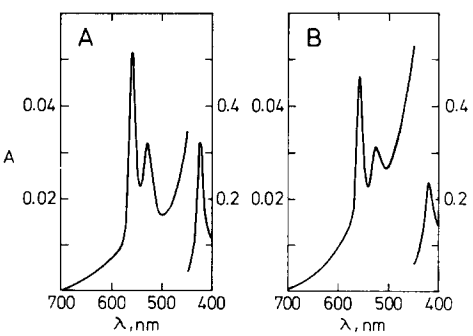


Fig. 1. Absorption spectra of nitrate reductase, and of its pyridine hemochromogen. A. Nitrate reductase in 0.1 M phosphate buffer (pH 7.6). The solution contained 1.9 mg protein, of spec. act. 6.4, per ml. Blank, water. B. Pyridine hemochromogen, in 25 % (v/v) pyridine, 0.1 M KOH. The solution contained the same concentration of protein as A. The sample was incubated with pyridine–KOH at room temperature for 30 min prior to measurement of the spectrum. Blank, 25 % pyridine, 0.1 M KOH. In both A and B, the samples were reduced with $Na_2S_2O_4$ immediately before the measurements were recorded with a Zeiss recording spectrophotometer DMR 21 at 20 °C, optical path, d = 1 cm. The difference between the absorbance of the 555-nm maximum and the adjacent 540-nm minimum of the pyridine hemochromogen preparation was used for calculating the amount of haem present. For this calculation, a reference extinction coefficient of (ε_{555} *minus* ε_{540}) = 23.7 $mM^{-1} \cdot cm^{-1}$ was determined on a fresh solution of haem at 20 °C in 25 % (v/v) pyridine, 0.1 M KOH, reduced with a few grains of $Na_2S_2O_4$. At the same time, a value of ε_{555} = 33.2 $mM^{-1} \cdot cm^{-1}$ was determined, which is in good agreement with published values (*cf.* refs 16 and 17). From the data shown, *plus* the value of $A_{412\,nm}$ for the oxidized enzyme (not shown), the following extinction coefficients were calculated:

λ (*nm*):	*412 (ox)*	*423*	*527*	*557*	*557–540*
ε_{mM}	238	305	30.2	48.5	27.8

Fig. 1A shows the spectrum of an enzyme preparation after reduction with dithionite. Fig. 1B shows the spectrum of the reduced pyridine hemochromogen prepared directly from the same enzyme. The legend for the figure gives ε_{mM} for the enzyme preparation calculated from the data shown. These values are provisional, since the enzyme is not pure, and different preparations gave rather different extinction coefficients. A reasonable constancy was obtained, however, for the ratio of the ($A_{557\ nm}$ *minus* $A_{541\ nm}$) value to the amount of nitrate reductase present after maximum activation. We have arbitrarily selected ε_{557} *minus* $\varepsilon_{541} = 27.8\ mM^{-1} \cdot cm^{-1}$ as a measure of the amount of cytochrome present, until a better value is available. On this basis, the cytochrome concentration of the enzyme solution used to obtain the spectra of Fig. 1 was 1.06 μM. The solution contained 12.2 units of enzyme per ml after activation, and 1.9 mg protein per ml, by the method of Lowry, giving a specific activity of 6.4 units/mg. The turnover number with respect to cytochrome was $12.2/1.06 \cdot 10^{-3} = 11500$ moles nitrate reduced per mole of cytochrome per min. The solution contained $1.7 \cdot 10^6$ μg protein per μmole cytochrome. The best enzyme preparation obtained to date with the procedure previously described and here employed, had a specific activity of 9.9 units per mg, and $1.2 \cdot 10^6$ μg protein per μmole cytochrome.

To see if the enzyme activity is proportional to the cytochrome, we have examined preparations at the stage of the protamine sulfate extract and the subsequent $(NH_4)_2SO_4$ fractions, and calculated turnover numbers per mole of cytochrome in the manner described. The specific activities of the fractions examined ranged from 0.066 to 9.9 (*i.e.* a variation of 150-fold). Fractions from six different cell extracts were examined. The calculated turnover numbers ranged from 9200 to 13500, with an average value of 11200. The agreement is good, in view of the uncertainty in the measurement of the amount of nitrate reductase present. Although a constant level of activity is reached in the activation medium, there is still some question about the extent to which the enzyme may have been denatured during the long activation process.

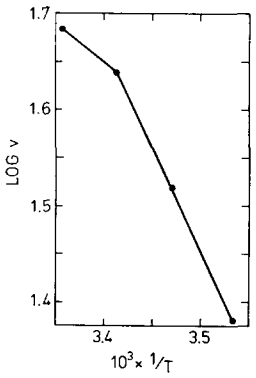
Temperature dependence

An Arrhenius plot showing the dependence of the nitrate reductase reaction rate on temperature between 10 and 25 °C is shown in Fig. 2. These measurements were made with an enzyme preparation which had been extensively activated. Between 10 and 20 °C, there was a linear relationship between log v and $1/T$, but the slope decreased between 20 and 25 °C. From the linear part of the curve, an activation energy, $E = 9800$ cal was calculated. Q_{10} (10–20 °C) = 1.81. Measurements made with other enzyme preparations gave values for E ranging from 9200 to 11000 cal, with Q_{10} (10–20 °C) ranging from 1.75 to 1.95.

Inhibitors

Except for the variations noted, all kinetic measurements were made under the conditions of the enzyme assay as described under Experimental. The initial reaction velocity was determined within the first min after addition of enzyme to the reaction mixture previously equilibrated to 20 °C. Solutions of inhibitors were freshly prepared, and neutralized, if necessary, immediately before use.

The reaction velocity of the nitrate reductase varies with nitrate concentration



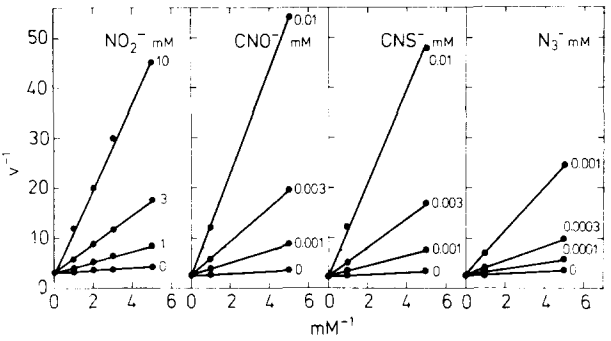


Fig. 2. Dependence of nitrate reductase reaction rate on temperature. Measurements were made with suitable aliquots of a solution containing 4.4 mg protein, of spec. act. 9.9, per ml. The reaction rate, v, is given in μmoles/ml per min.

Fig. 3. Double reciprocal plots of reaction velocity *versus* nitrate concentration in the presence of nitrite, cyanate, thiocyanate and azide. The ordinate shows the reciprocal of the initial reaction velocity, expressed as μmoles NADH oxidized per min per ml of enzyme preparation. The abscissa shows the reciprocal of the mM nitrate concentration. Each of the lowest curves represents the reaction in the absence of inhibitor. The numbers adjacent to the curves represent the mM concentration of inhibitor, as indicated. The enzyme preparations used for measurements with each of the different inhibitors were similar but not identical. Thus, V was 0.327, 0.386, 0.380, and 0.386 unit/ml for the measurements with nitrite, cyanate, thiocyanate and azide, respectively. From the data shown, the following constants were calculated: K_m (nitrate) $= 8.4 \cdot 10^{-5}$ M; K_i (nitrite) $= 2.8 \cdot 10^{-4}$ M; K_i (cyanate) $= 2.15 \cdot 10^{-7}$ M; K_i (thiocyanate) $= 2.55 \cdot 10^{-7}$ M; K_i (azide) $= 5.2 \cdot 10^{-8}$ M.

in the manner expected for a substrate showing typical Michaelis–Menten kinetics. Azide, cyanate, thiocyanate and nitrite all inhibit the enzyme reaction reversibly, and all are competitive with nitrate. The calculated K_m for nitrate, and the calculated K_i values for these inhibitors are given in the legend for Fig. 3, which shows some of the data used for making the calculations.

Cyanide and hydroxylamine are good inhibitors of nitrate reductase, but the manner of their action is different from that of the inhibitors for which data are given in Fig. 3. Both cyanide and hydroxylamine behave similarly, except that cyanide is somewhat more effective at a given concentration. With these reagents, time is required for the interaction of inhibitor and enzyme, and the presence of nitrate delays the development of the inhibition: the higher the nitrate concentration, the greater the delay. Maximum inhibition is achieved most rapidly when the inhibitor is incubated with the enzyme in the presence of NADH and absence of nitrate, prior to commencement of the reaction by nitrate addition.

The behavior of HCN is illustrated in Fig. 4, which shows a plot of the change in absorbance with time during the course of the oxidation of NADH by nitrate in the presence of HCN under a variety of conditions. The uninhibited reaction is shown, for comparison, in Curve 4. For the measurements of Fig. 4A, enzyme and HCN were added simultaneously to start the reaction. Fig. 4B shows the results obtained when HCN was incubated with the enzyme in the presence of NADH for 3.5 min before the reaction was started by addition of nitrate. The curves of Fig. 4A are concave, showing that inhibition increased with time. In contrast, the curves of Fig. 4B tend to be

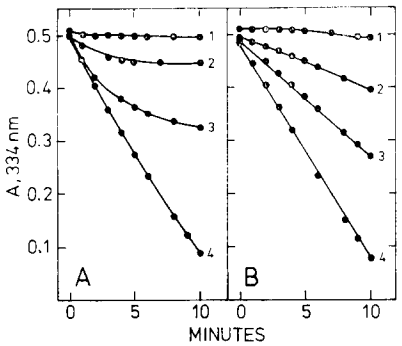


Fig. 4. Inhibition of nitrate reductase by HCN. Conditions were those of the standard assay system, except as noted. A. A freshly neutralized solution of KCN was introduced into the bottom of a cuvette already containing buffer, NADH and water. An aliquot of enzyme solution was added to the top of the solution, the reaction mixture was stirred briefly, and readings were begun. Curve 1, $3.3 \cdot 10^{-4}$ M KNO_3, $1 \cdot 10^{-5}$ M HCN; Curve 2, $3.3 \cdot 10^{-3}$ M KNO_3, $1 \cdot 10^{-5}$ M HCN; Curve 3, $1.7 \cdot 10^{-2}$ M KNO_3, $1 \cdot 10^{-5}$ M HCN; Curve 4, $3.3 \cdot 10^{-3}$ M KNO_3, no HCN. B. Freshly neutralized KCN was mixed with enzyme in a reaction mixture which was complete except for nitrate. After incubation for 3.5 min, nitrate was mixed in and readings were begun. Curve 1, $3.3 \cdot 10^{-3}$ M KNO_3, $1 \cdot 10^{-6}$ M HCN; Curve 2, $3.3 \cdot 10^{-3}$ M KNO_3, $2 \cdot 10^{-7}$ M HCN; Curve 3, $3.3 \cdot 10^{-3}$ M KNO_3, $1 \cdot 10^{-7}$ M HCN; Curve 4, $3.3 \cdot 10^{-3}$ M KNO_3, no HCN.

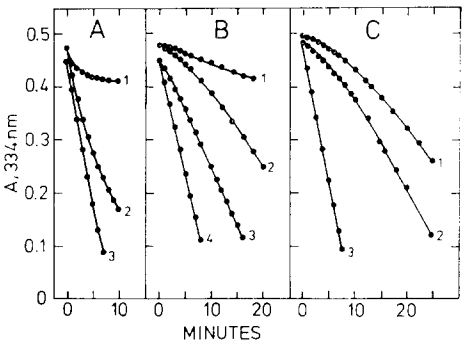


Fig. 5. Inhibition of nitrate reductase by hydroxylamine. Conditions were those of the standard assay system, except as noted. A. Reaction was started by adding enzyme to an otherwise complete reaction mixture containing hydroxylamine in the indicated amounts. Curve 1, $3.3 \cdot 10^{-4}$ M KNO_3, $3.3 \cdot 10^{-4}$ M NH_2OH or $3.3 \cdot 10^{-3}$ M KNO_3, $3.3 \cdot 10^{-3}$ M NH_2OH; Curve 2, $3.3 \cdot 10^{-3}$ M KNO_3, $3.3 \cdot 10^{-4}$ M NH_2OH; Curve 3, $3.3 \cdot 10^{-3}$ M KNO_3, no NH_2OH or $3.3 \cdot 10^{-3}$ M KNO_3, $3.3 \cdot 10^{-5}$ M NH_2OH. B. Hydroxylamine and enzyme were mixed into a reaction mixture which was complete except for nitrate. After incubation for 2 min, NO_3^- was mixed in and readings were begun. Curve 1, $3.3 \cdot 10^{-4}$ M KNO_3, $1 \cdot 10^{-4}$ M NH_2OH; Curve 2, $6.7 \cdot 10^{-3}$ M KNO_3, $1 \cdot 10^{-4}$ M NH_2OH; Curve 3, $3.3 \cdot 10^{-3}$ M KNO_3, $3.3 \cdot 10^{-5}$ M NH_2OH; Curve 4, $3.3 \cdot 10^{-3}$ M KNO_3, no NH_2OH. C. Procedure same as in B, but with 7 min prior incubation of enzyme and inhibitor. Curve 1, $3.3 \cdot 10^{-3}$ M KNO_3, $1 \cdot 10^{-4}$ M NH_2OH; Curve 2, $3.3 \cdot 10^{-3}$ M KNO_3, $3.3 \cdot 10^{-5}$ M NH_2OH; Curve 3, $3.3 \cdot 10^{-3}$ M KNO_3, no NH_2OH.

convex, showing a decline of inhibition with time. This latter effect, which suggests a slow reactivation of the inhibited enzyme, can be seen more clearly in the experiments of Fig. 5, which shows selected measurements of the inhibition of nitrate reductase by hydroxylamine.

Fig. 5A shows how the inhibition by hydroxylamine increases with time when the reaction is started by adding enzyme to the otherwise complete reaction mixture. The course of the development of the inhibition depends on the nitrate concentration as well as on the concentration of hydroxylamine. In the experiments shown in Figs 5B and 5C, the inhibition was first established by prior incubation of enzyme with inhibitor and NADH, and the reaction was started by addition of nitrate. The experiment of Fig. 5B was carried out with partially purified enzyme which was incubated with inhibitor and NADH for 2 min prior to initiation of the reaction. During this prior incubation, the enzyme lost 23 % of its activity in the absence of inhibitor. The experiment of Fig. 5C was carried out with the enzyme at the stage of the first $(NH_4)_2SO_4$ fractionation. With this more stable enzyme, prior incubation of enzyme with inhibitor and NADH was continued for 7 min. The inhibition then established by a given amount of hydroxylamine was larger than after 2 min; and the reactivation

of the inhibited enzyme was more striking (*cf.* Curve 2 of Fig. 5C with Curve 3 of Fig. 5B).

Some results of preliminary tests with a variety of other possible inhibitors have been assembled in Table I. Some of these substances had no effect; others gave a clear inhibition, but have not been examined in sufficient detail to permit a description of the type of inhibition obtained (except for pyridoxal phosphate, see Discussion).

TABLE I

MISCELLANEOUS TESTS OF POSSIBLE INHIBITORS OF NITRATE REDUCTASE

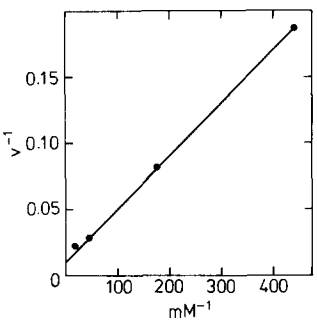
Unless otherwise noted, the inhibitor was mixed with the reagents of the standard assay system, and the reaction was started by adding enzyme.

Substance tested	*Inhibition* (%)	*Comments*
Argon, 1 atm	None	Reactions run in Warburg vessels, with measurements of NO_2^- formed.
CO, 1 atm	None	Control in air
Antimycin, 6.7 μg/ml*	None	
2-*n*-Heptyl-4-hydroxyquinoline-*N*-oxide, 3.3 μg/ml	None	
Orthophenanthroline, 10^{-3} M	9	
Hydrazine, 10^{-2} M	39	$1.6 \cdot 10^{-4}$ M KNO_3
	15	$3.3 \cdot 10^{-3}$ M KNO_3
Semicarbazide, 10^{-2} M	35	$1.6 \cdot 10^{-4}$ M KNO_3
	10	$3.3 \cdot 10^{-3}$ M KNO_3
NH_4Cl, $5 \cdot 10^{-2}$ M	None	
Urea, $3 \cdot 10^{-2}$ M	None	
$3 \cdot 10^{-1}$ M	14	
Formamide, 10^{-3} M	None	
10^{-2} M	10	
L-Histidine, 10^{-2} M	10	
L-Asparagine, 10^{-2} M	None	
N-Carbamyl-DL-aspartate, 10^{-3} M	6	
Glycine hydroxamate, $2 \cdot 10^{-3}$ M	22	$3.3 \cdot 10^{-4}$ M KNO_3
	9	$6.7 \cdot 10^{-3}$ M KNO_3
Hypoxanthine, $5 \cdot 10^{-3}$ M	31	Reaction started
$1.7 \cdot 10^{-3}$ M	4	with nitrate
Potassium xanthogenate ($C_2H_5O \cdot CS \cdot SK$), 10^{-3} M	14	$3.3 \cdot 10^{-4}$ M KNO_3
	7	$3.3 \cdot 10^{-3}$ M KNO_3
Pyridoxal phosphate, $3.3 \cdot 10^{-4}$ M**	62	

* Prior incubation of enzyme and inhibitor for 5 min.
** Prior incubation of enzyme and inhibitor for 10 min.

The cytochrome c reductase (diaphorase) activity

Like the nitrate reductase of *Chlorella fusca*, moulds and higher plants (see Discussion), the nitrate reductase of the Berlin strain is associated with a cytochrome *c* reductase (= diaphorase) activity, *i.e.* all preparations catalyze the reduction of mammalian cytochrome *c*, 2,6-dichlorophenolindophenol, and ferricyanide by NADH. In the present studies, attention was focussed on cytochrome *c* as a substrate for this reaction. The reduction of cytochrome *c* by NADH showed Michaelis–Menten kinetics with respect to cytochrome *c*. From the data plotted in Fig. 6, $K_m = 4.0 \cdot 10^{-5}$ M was calculated.



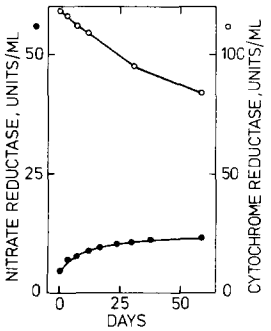


Fig. 6. Double reciprocal plot of reaction velocity *vs* cytochrome *c* concentration. The ordinate shows the reciprocal of the initial reaction velocity, expressed as μmoles of cytochrome *c* reduced per min per ml of enzyme preparation. The abscissa shows the reciprocal of the mM cytochrome *c* concentration. Measurements were made as described under Methods. Conditions were those of the standard assay, except as noted.

Fig. 7. Changes with time of cytochrome reductase and nitrate reductase activities. Upper curve, cytochrome reductase, lower curve nitrate reductase. A sample of the best enzyme fraction, shortly after preparation, was supplemented with nitrate, phosphate, dithioerythreitol and EDTA, as described under Methods, and incubated at 4 °C, to obtain maximum activation of the nitrate reductase. At the times indicated, aliquots were taken for measurement of nitrate reductase and cytochrome reductase activity as described under Methods. The ordinates show the units/ml, calculated for the original enzyme solution.

Because of the gradual activation of nitrate reductase, the ratio of cytochrome reductase activity to nitrate reductase activity varied enormously, from about 500 to 1 in crude extracts, prior to activation of nitrate reductase, down to about 9:1 in partially purified fractions with activated nitrate reductase. During the activation of nitrate reductase in the activation medium described under Methods, the cytochrome *c* reductase activity gradually declined. Fig. 7 shows the course of the activation of the nitrate reductase activity of the final best enzyme fraction in the activation medium, and the associated decline in the cytochrome *c* reductase activity. Because the latter activity does not reach a constant value during the activation of the nitrate reductase, we used the ratio of the initial cytochrome *c* reductase activity, to the final maximum activity of the nitrate reductase after activation, as a measure of the relation of the two activities. Assays of all fractions obtained throughout the course of the enzyme fractionation showed that the cytochrome *c* reductase was following the nitrate reductase activity, and was a better (faster) measure of the amount of nitrate reductase activity present than the nitrate reductase assay itself. A ratio of about 9:1 for initial cytochrome *c* reductase activity to activated nitrate reductase activity was observed in all fractions obtained after the first $(NH_4)_2SO_4$ fractionation.

The NADH–cytochrome *c* reductase activity was not inhibited by cyanide, hydroxylamine, azide, cyanate or thiocyanate. The tests were carried out under conditions which gave at least 80 % inhibition of nitrate reductase. Antimycin and 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide, tested as described in Table I, likewise had no inhibitory effect on cytochrome *c* reductase.

Tests with other substrates have not been extensive. The NADH–dichlorophenolindophenol reductase activity and the NADH–ferricyanide reductase activity of the final fractions were high. Dichlorophenolindophenol showed Michaelis–Menten kine-

tics, with $K_m = 1.3 \cdot 10^{-4}$ M. The maximum velocity was 5 times as fast as the nitrate reductase activity in the few fractions tested. HCN and azide did not inhibit this reaction. With $K_3Fe(CN)_6$, a concentration of $7.5 \cdot 10^{-5}$ M gave the maximum reaction rate. At this concentration, 1 unit of nitrate reductase catalyzed the reduction of 11 μmoles of ferricyanide by NADH per min, under the standard assay conditions without nitrate. Higher concentrations of ferricyanide inhibited the reaction. Neither cyanide nor azide inhibited the reduction of ferricyanide by NADH.

Inhibition of nitrate reductase by cytochrome c

Added nitrate did not cause detectable inhibition of the cytochrome *c* reductase component of the enzyme. It was possible, however, to demonstrate an inhibition of nitrate reduction by oxidized cytochrome *c*.

The conditions were those of the standard assay system in the presence and absence of 10^{-4} M cytochrome *c*. After equilibration at 20 °C for 10 min, the reactions were started by the addition of 0.003 units of an enzyme preparation containing 6 units of nitrate reductase and 47 units of cytochrome reductase per mg protein. After 10 min, the reaction was stopped by the addition of 0.3 ml of 25 % (w/v) $ZnSO_4$ and the amount of nitrite formed was determined as previously described[1]. Under these conditions, 0.030 μmole of NADH was oxidized and 0.030 μmole of NO_2^- was formed in the reaction without added cytochrome *c*, while in the reaction with added cytochrome *c*, 0.14 μmole of cytochrome *c* was reduced and 0.012 μmole of NO_2^- was formed; *i.e.* nitrate reduction was inhibited 60 % by cytochrome *c* under these conditions. Separate experiments have shown that no significant inhibition of nitrate reduction occurs in the presence of reduced cytochrome *c*. Since the reduction of cytochrome *c* is more rapid than the reduction of nitrate, and since it is impractical to use a high concentration of cytochrome *c*, the inhibition of nitrate reductase by cytochrome *c* could best be measured if the reduced cytochrome *c* were reoxidized as rapidly as possible, but such measurements have not been attempted. The inhibition of nitrate reductase by oxidized cytochrome *c* provides further evidence that the cytochrome reductase and nitrate reductase activities are associated in the same enzyme complex.

DISCUSSION

We have previously called attention to the differences between the nitrate reductase of the Berlin strain of *Chlorella*, and the nitrate reductase of *Chlorella fusca*[2]: namely (1) that the former enzyme has no requirement for added FAD, whereas the latter enzyme requires added FAD, and (2) that the former enzyme is present in crude extracts in the form of an almost inactive precursor which can readily be activated, which is not the case for the enzyme from *Chlorella fusca*, tested under identical conditions. With respect to both of these properties, the nitrate reductase of *Chlorella fusca* is more similar to the nitrate reductases of higher plants and fungae, generally[9,18,19], whereas the nitrate reductase of the Berlin strain is different. In spite of such differences, the present study mainly corroborates that the assimilatory nitrate reductases of algae, higher plants[18], and fungae[9, 19] constitute a family of enzyme "complexes", with closely related though not identical properties.

Separate lines of evidence support the view that assimilatory nitrate reductases

generally contain two catalytic centers, closely associated in the same molecule, but subject to independent modification *in vivo* and *in vitro*. The cytochrome reductase represents one of these components, which activates the pyridine nucleotide (NAD or NADP, depending on the enzyme). The nitrate activating moiety, containing molybdenum, constitutes the other component. The facts are consistent with an electron transport scheme in which cytochrome *c* accepts electrons at a site prior to the site at which nitrate reduction occurs.

Kinsky and McElroy[20] noted the close association, during purification, of the NADPH–cytochrome *c* reductase and nitrate reductase of *Neurospora*, and showed that cytochrome *c* inhibited the reduction of nitrate. Similar results have here been obtained with the nitrate reductase of the Berlin strain of *Chlorella*. Kinsky[21] showed further that the NADPH–cytochrome *c* reductase of *Neurospora* was inducible by nitrate. Sorger[22, 23] amplified these investigations with *Neurospora*, and studied the phenomenon genetically, with mutants. Similar genetic studies have been made with the nitrate reductase of Aspergillus[24, 25] and include the isolation of mutants which lack nitrate reductase but possess a nitrate-inducible cytochrome *c* reductase. More recently, Wray and Filner[26] demonstrated that the normal induction of nitrate reductase by nitrate in barley shoots is accompanied by induction of cytochrome *c* reductase, and that administration of tungstate to the plants results in formation of an enzyme complex which is functional only with respect to cytochrome *c* reductase. Notton and Hewitt[27, 28] in a study with spinach leaves, showed further that inhibition by tungstate of the induction of nitrate reductase involves formation of a tungstoprotein analogue of the normal molybdoprotein enzyme. The tungstoprotein analogue is fully active as a diaphorase, though it lacks nitrate reductase activity. Studies with *Chlorella fusca*[6] confirmed that the physiological competition between tungsten and molybdenum is due to the fact that both metals can be incorporated by the cells into the nitrate reductase complex. The enzyme complexes of both metals are active as diaphorases; whereas only the molybdo complex is active as a nitrate reductase.

The fresh extracts of the Berlin strain of *Chlorella* call to mind the above phenomena, since the diaphorase activity is high, and the nitrate reductase activity very low. It is not impossible that this low activity of the nitrate reductase moiety is due, perhaps in part, to a substitution of tungsten for molybdenum, since the microelements added to the growth medium include 0.06 μM tungsten, as Na_2WO_4, as well as 0.09 μM molybdenum, as $(NH_4)_6Mo_7O_{24}$. The activation of the nitrate reductase occurs best in the presence of added nitrate and phosphate; and the latter may contain a trace of molybdenum[29]. No clear cut stimulation of the activation was observed, however, when molybdate was substituted for phosphate or added to the phosphate (*cf.* ref. 30).

Another hypothesis visualizes the activation as a process associated with the release of a firmly bound inhibitor. The main evidence for this possibility is the resemblance between the activation of the natural enzyme precursor and the reactivation of the enzyme after inhibition by hydroxylamine and by HCN (*cf.* Curve 1 of Fig. 5C with Curve A of Fig. 1, Vennesland and Jetschmann[1]). Furthermore, the addition of $NiSO_4$ to fresh extracts of the Berlin strain of *Chlorella* results in a 3–6-fold stimulation of the nitrate reductase activity. This effect is very similar to the effect of nickel salts on the nitrate reductase activity of sorghum extracts, described by Maranville[31], who concluded that, in sorghum, the nickel salt acts by binding the

HCN released by hydrolysis of the cyanogenic glycoside, dhurrin. We have no evidence, however, for the presence of a similar cyanide precursor in *Chlorella*. Furthermore, the stimulation by $NiSO_4$ is small in comparison with the 50-fold activation of the enzyme in the presence of phosphate and nitrate.

We have also used pyridoxal and pyridoxal phosphate to see whether these aldehydes, which would be expected to combine with both cyanide and hydroxylamine, might not have a stimulatory effect on the activation of the nitrate reductase precursor. No trace of stimulation was observed. Instead, it was found that pyridoxal phosphate (but not pyridoxal) was an inhibitor of nitrate reductase. At $3.3 \cdot 10^{-4}$ M, this substance caused an inhibition of 62%. The inhibition developed slowly, requiring about 10 min to reach its maximum value in the standard assay system. The development of inhibition was the same whether the enzyme was kept oxidized by nitrate addition, or reduced by NADH addition, during prior incubation with inhibitor, before commencement of assay. The cytochrome reductase was inhibited to about the same extent as the nitrate reductase. The inhibition by pyridoxal phosphate may be related to the type of inhibition which has been described for ADP[32, 33] which mainly appears to affect the NADH activating moiety of the enzyme. The high concentration of pyridoxal phosphate required for inhibition does not favor the view that this inhibition is of physiological importance.

Our results showing that inhibitors such as cyanide, cyanate and azide (*cf.* ref. 34), which compete with nitrate, have no inhibitory effect on the cytochrome *c* reductase component of the nitrate reductase of the Berlin strain of *Chlorella*, are in line with previous studies of the *Neurospora* enzyme[8] and of the enzyme from higher plants[35, 36]. Our demonstration that nitrite behaves as a reversible competitive inhibitor of nitrate is at variance, however, with the conclusions of Garrett and Nason[8] regarding the *Neurospora* enzyme, and with those of Schrader and Hageman[37], who state that nitrite does not inhibit the nitrate reductase of corn seedlings. It should be noted, though, that K_i for nitrite is larger than K_m for nitrate (Fig. 3); so that the inhibition by nitrite can not readily be measured with an assay system which depends on measurement of the amount of nitrite formed. In a preliminary report on the nitrate reductase of tomato leaf, Eaglesham and Hewitt[38] have reported that nitrite inhibits noncompetitively with nitrate.

The *b*-type cytochrome associated with the nitrate reductase of the Berlin strain of *Chlorella* follows the enzyme activity closely during the course of the purification, as shown by the fact that the turnover number (after activation) with respect to cytochrome was a reasonably constant value, for preparations which differed by 150-fold in specific activity. The participation of the cytochrome in the oxidation–reduction reactions catalyzed by the enzyme can readily be demonstrated. The reduction of the cytochrome by added NADH is too rapid to measure by ordinary methods, as already mentioned[1]. The reoxidation by nitrate is also very rapid, but the inhibitors which compete with nitrate also inhibit the reoxidation of the cytochrome by nitrate, so that the rate of the reoxidation can be reduced to almost any desired level. Preliminary measurements of this type show that the cytochrome remains completely reduced in the presence of nitrate and inhibitor, as long as a small amount of NADH is present.

The similarity of the nitrate reductase of the Berlin strain of *Chlorella* to the nitrate reductase of *Neurospora*[8] has been emphasized repeatedly. It seems improbable

that the nitrate reductases from two such different sources should happen fortuitously to be associated with a cytochrome which is unrelated functionally. Relatively few nitrate reductases of algae, higher plants and fungae have been purified to a sufficient extent to permit identification of a cytochrome prosthetic group, so the question of the presence or absence of a cytochrome in most of these enzymes remains open. An exception is the nitrate reductase of *Aspergillus*, which has been extensively purified[19] and found to contain no cytochrome. The dissimilatory or "respiratory" nitrate reductase of *Escherichia coli* likewise contains no cytochrome after purification[39], but in these cells the induction of nitrate reductase has been shown to be accompanied by the appearance of a *b*-type cytochrome[39,40] which is present in a particulate preparation containing the nitrate reductase moiety also. The assimilatory nitrate reductase of the Berlin strain of *Chlorella* apparently represents a variation on a pattern, and confirms the conclusion already reached by Garrett and Nason that the distinction between assimilatory and respiratory nitrate reductase can not be made on the basis of the presence or absence of a cytochrome.

ACKNOWLEDGEMENTS

We are indebted to Rainer Stevens and Cornelia Hesselberger for technical assistance.

NOTE ADDED IN PROOF (Received May 8th, 1972)

The Berlin strain of *Chlorella* has recently been reclassified as *Chlorella vulgaris* Beijerinck (see ref. 2).

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