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THE NITRATE REDUCTASE OF *CHLORELLA PYRENOIDOSA*

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SUMMARY

1. A nitrate reductase (NADH:nitrate oxidoreductase, EC 1.6.6.1), which catalyzes the reduction of nitrate to nitrite by NADH, has been found to be present in fresh extracts of nitrate-grown *Chlorella pyrenoidosa* in the form of a precursor of low activity. The enzyme precursor can be activated *in vitro* over 100-fold in a slow process which is accelerated by added nitrate and phosphate buffer of low pH.

2. A partial purification of nitrate reductase is described. The enzyme is activated during the purification process itself. The partially purified enzyme contains a cytochrome, which is similar to the cytochrome associated with the assimilatory nitrate reductase of Neurospora, studied by A. NASON and his associates.

INTRODUCTION

The first step in the assimilation of nitrogen from nitrate is generally regarded as the reduction of nitrate to nitrite[1]. A Neurospora enzyme, which catalyzes the reduction of nitrate to nitrite by NADPH, has been extensively purified by GARRETT AND NASON[2,3] and has been shown to contain a cytochrome. Leaves and algae have been found to contain an enzyme which catalyzes the reaction shown in Eq. 1[1,4-7],

$$NO_3^- + NADH + H^+ \rightarrow NO_2^- + NAD^+ + H_2O \quad (1)$$

and a preliminary note has outlined a purification of a nitrate reductase (NADH:nitrate oxidoreductase, EC 1.6.6.1) from Chlorella[6] without details.

The present studies describe a partial purification and a study of some of the properties of the nitrate reductase of *Chlorella pyrenoidosa*[6-8]. This enzyme was present in our crude algal extracts mainly in the form of an inactive pro-enzyme, which could readily be activated. The activation process will be described, and preliminary evidence will be presented for the presence in the partially purified enzyme preparation of a cytochrome similar to the cytochrome of the Neurospora enzyme.

MATERIALS AND METHODS

Pyridine nucleotides were obtained from Boehringer, protamine sulphate from

Nordisk Insulin Laboratorium. Nitrite was measured with sulfanilamide and *N*-(naphthyl) ethylene diamine hydrochloride[9]. If necessary, protein was removed prior to nitrite determination by adding 0.1 vol. 25% $ZnSO_4$ and 0.1 vol. 1.3 M NaOH. The sample was diluted 2- or 3-fold with water. After 10 min, the precipitate was removed by centrifugation, and an aliquot of the supernatant was used for the analysis. Phosphate buffers were prepared from KH_2PO_4 and Na_2HPO_4.

Preparation of Chlorella extracts

The strain of *Chl. pyrenoidosa* employed was originally obtained from Professor Harder, Göttingen[10]. Stock cultures have been grown autotrophically with nitrate as a nitrogen source for many years. The Chlorella cells were grown in continuous light in mass culture[11] or under the conditions described for 1-day north cells[12] (see also ref. 13) in a stream of 5% CO_2 in air on K medium *plus* microelements[14]. K medium has the composition: 5 g $MgSO_4 \cdot 7H_2O$, 2.5 g KH_2PO_4, 2 g NaCl, 2 g KNO_3, and 0.5 g $Ca(NO_3)_2 \cdot 4H_2O$ per l. The initial pH is 4.3; the concentration of nitrate is 0.022 M.

The cells were harvested by centrifugation, washed once with water, suspended in water, and disrupted by ultrasonication[15]. The dark green supernatant obtained after removal of debris by centrifugation (10 min at 5000 × *g*) had a chlorophyll content equivalent to that of 200 μl cells per ml. It was frozen and stored at −20° until needed.

Enzyme assay

The enzyme was assayed by measuring the rate of oxidation of NADH by nitrate. Absorbance of NADH was measured at 334 nm with an Eppendorf colorimeter with an attached thermostat to maintain the temperature of the cuvette contents at 20°. The standard assay system contained 0.5 ml 0.5 M phosphate buffer (pH 7.6 or 7.7), 0.2 ml 0.1 M KNO_3, 0.2–0.4 mg NADH and water to a final volume of 3.0 ml. After temperature equilibration, the reaction was started by adding enzyme, and a series of measurements at 0.5 or 1-min intervals was made for a total of 2 or 3 min or longer if desired. Depending on the preparation assayed, the rate of oxidation of NADH in the assay might decrease with time, increase with time or stay constant with time. The initial rate was taken as a measure of the enzyme present. The assay conditions provided an optimal pH with phosphate buffer and saturating concentrations of nitrate and NADH. The reaction rate was not limited by the NADH concentration until the absorbance had decreased to about 0.12. At 334 nm, 1 μmole NADH in 3 ml, with optical path, $d = 1$ cm, gives an absorbance of 2.0. The change in absorbance per min divided by 2 therefore gives the μmoles NADH oxidized per min by the added enzyme.

Frequent checks were made to compare the amount of NADH oxidized in the assay with the measured amount of nitrite formed. This was done during enzyme purification and enzyme activation. Agreement was usually within less than 5% of the total quantities measured.

A unit of enzyme is defined as that amount which catalyzes the oxidation of 1 μmole NADH per min in the standard assay system. The assay gave values linearly proportional to the amount of enzyme tested over a broad range (0.001–0.05 unit or more per assay; with larger amounts of enzyme, larger amounts of NADH were added). After the protamine sulfate fractionation, the absorbance at 280 nm was taken as a

measure of the amount of protein, with the approximation that a solution containing 1 mg protein per ml has an absorbance of 1.0 in a cuvette with an optical path of 1 cm[16,17].

RESULTS

The activation of nitrate reductase in crude extracts

The standard assay procedure could not be applied directly to the Chlorella extracts because of the suspended green grana. To remove the grana from fresh extracts, high speed centrifugation (Spinco centrifuge, 1 h at 100 000 × *g*) was employed. If the extracts were frozen and thawed, the grana formed a coagulum more readily, and centrifugation for 30 min at 30 000 × *g* (Sorvall) gave a clear, straw-colored or pale-green supernatant. The nitrate reductase activity of fresh extracts was usually in the range of 0.01–0.025 unit/ml at the beginning of the assay. If the assay reaction was

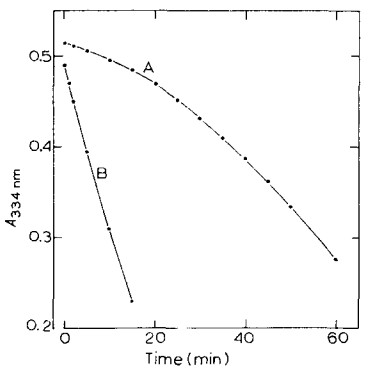

Fig. 1. Activation of nitrate reductase. Fresh sonicated Chlorella extract was clarified by centrifugation at 100 000 × *g*. Sample A contained 2.3 ml extract *plus* 0.7 ml water. Sample B contained 2.3 ml extract *plus* 0.5 ml 0.5 M phosphate, pH 7.7, and 0.2 ml 0.1 M KNO_3. After incubation at 4° for 16 h, 0.1 ml of each sample was assayed in the standard assay system. The figure shows the change in absorbance with time during the assay.

continued over a period of several hours, however, it became apparent that the rate of the oxidation of NADH was steadily increasing with time, during the assay, though the temperature was kept constant. After storage at 4° for 16–24 h, this activation of the enzyme during the assay became noticeably faster. When phosphate buffer and nitrate were added to the extract, a steady increase in activity occurred during storage.

These facts are illustrated in the experiment of Fig. 1, which shows the change in absorbance during the assay of 0.1 ml of each of two incubation mixtures which had been stored at 4° for 16 h. Both incubation mixtures contained identical amounts of the same Chlorella extract. For Curve A, the extract had no supplement except water; for Curve B, the extract had been made 0.083 M in phosphate (pH 7.7) and 0.0067 M in KNO_3.

The activation of nitrate reductase was stimulated by addition of phosphate alone or of KNO_3 alone, but was faster when both were added together, as shown in

TABLE I

ACTIVATION OF NITRATE REDUCTASE IN CHLORELLA EXTRACTS

Fresh, sonicated Chlorella extract was frozen, thawed the next day, cleared of insoluble material by centrifugation, and dialyzed for 4 h against 0.001 M Tris–HCl buffer, pH 8.0. Four reaction mixtures were prepared, Each contained 1 ml dialyzed extract *plus* water and additions as indicated to a final volume of 1.3 ml. The samples were incubated at 22°, and 0.1 ml was withdrawn for assay in the standard assay system at the times indicated. Units are calculated for the total reaction mixture of 1.3 ml.

Additions	*Nitrate reductase (units)*		
	0 h	*3.5 h*	*19 h*
None	0.043	0.12	0.25
0.0385 M phosphate, pH 6.2		0.18	0.58
0.0154 M KNO_3		0.17	0.79
0.0385 M phosphate, pH 6.2, and 0.0154 M KNO_3		0.17	1.07

Table I. The activation was much greater in the experiment of Table I than in the experiment of Fig. 1 because the former was carried out at higher temperature and lower pH. Additions of KCl to a concentration of 0.033 M had no activating effect.

The effect of pH on the activation is shown in Fig. 2. The results of assays, calculated as units/ml of sonicated extract, are plotted against time of incubation of the enzyme with nitrate and buffers of different pH. Tris buffer was used at pH 8.0. All other pH's were buffered with phosphate. The curves of Fig. 2A were obtained with freshly prepared extract. The curves of Fig. 2B were obtained on the next day with the same extract stored in the meantime at 4°. The initial rate of activation was greatest at the lowest pH values (5.1 and 5.5), but the maximum activity reached in

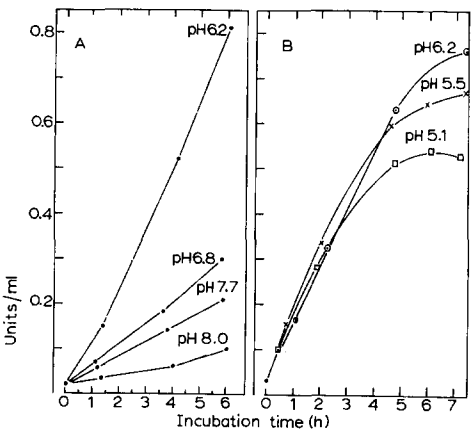

Fig. 2. Dependence of activation on pH. Grana were removed from frozen and thawed sonicated extract of Chlorella by strong centrifugation. Activation incubation mixtures were prepared by mixing 1 ml extract with 0.2 ml 0.10 M KNO_3 and 0.1 ml 0.5 M phosphate of the indicated pH, or 0.1 ml 1.0 M Tris (pH 8.0). Incubation was at 22°. Aliquots were removed for enzyme assay at the times indicated. The units are calculated for the total volume of the reaction mixture, equivalent to 1.0 ml of the original Chlorella extract. A. Activation incubation mixtures prepared immediately with fresh Chlorella extract. B. Activation incubation mixtures prepared 24 h later with same Chlorella extract stored at 4°.

7 h was greatest at pH 6.2. Comparison of A and B shows that the extract which had been aged for a day had about the same initial enzyme content as the fresh extract, but the initial rate of the activation was somewhat greater for the older extract. Later, however, the activation rate also declined faster with time for the older extract. The lower the pH, the more rapid was this decline and the subsequent inactivation of the activated enzyme.

We are clearly dealing with a complex process. The amount of enzyme activity at any given time reflects the net result of an activation process (which probably occurs in at least two steps) and of the inactivation of the active enzyme. A low pH gives the most rapid initial activation, but the maximum number of units obtained is greater at higher pH values where the active enzyme is more stable.

Extracts stored at 4° with phosphate (pH 7.6) and nitrate showed a slow but steady increase in activity which lasted for several weeks. The highest activity observed was 1.5 units/ml.

The rate of activation appeared to be independent of protein concentration except at high dilution where the activated enzyme was more unstable. Addition of NADH in the absence of nitrate led to a loss of enzyme activity. This inactivation was greatest with highly activated enzyme. Addition of cysteine or dithiothreitol had no substantial effect on the activation except for a small stabilizing effect on highly activated preparations. Dialysis as such likewise had little effect other than that associated with salts added to the dialyzing fluid.

Phosphate and nitrate were not specifically required for the activation of nitrate reductase. The enzyme was also activated during $(NH_4)_2SO_4$ fractionation as described in the following section.

Partial purification of nitrate reductase

A good partial purification of nitrate reductase from sonicated Chlorella extract was achieved by a three-step procedure consisting of: (1) an $(NH_4)_2SO_4$ fractionation; (2) a protamine fractionation; and (3) a second $(NH_4)_2SO_4$ fractionation.

The $(NH_4)_2SO_4$ fractionations were carried out by addition of the calculated volumes of a solution of reagent grade $(NH_4)_2SO_4$, saturated at 4° and containing 0.01 M NH_4OH, to bring the pH to 7. Solutions were cooled in an icebath; dialysis and centrifugation were carried out at 4°.

(1) First $(NH_4)_2SO_4$ fractionation. About 400 ml of sonicated Chlorella extract were conveniently employed without prior removal of grana by centrifugation and without prior activation of the enzyme. The protein which precipitated between 0.25 and 0.45 saturation with $(NH_4)_2SO_4$ was collected by centrifugation and dissolved in water to one-third the volume of the original extract. The solution was dialyzed against three or four changes of 0.001 M NH_4OH for at least 4 h or overnight when desired.

(2) Fractionation with protamine sulfate. A 2% solution of neutral protamine sulfate was added dropwise with stirring to the dialyzed enzyme solution. A voluminous precipitate was formed at first, leaving the enzyme in solution. The nitrate reductase was present in the last protein fraction precipitated by the protamine. The brown color of this protein fraction was a convenient guide to the fractionation. The amount of protamine sulfate required to precipitate inert protein without precipitating enzyme was dependent on the amount of salt remaining in the protein solution

and could be determined beforehand on a small scale. After 4 h of dialysis, about 0.1 vol. 2% protamine sulfate could be added to a given volume of protein solution before the activity began to disappear from the supernatant. After overnight dialysis, only about 0.01 vol. of the protamine sulfate was necessary. These figures are only approximations. Since the brown protein fraction desired was the last to be precipitated, the final fraction was brought down with an excess of about 0.05 vol. 2% protamine sulfate to ensure complete precipitation.

The brown precipitate containing the enzyme was collected by centrifugation and washed with 0.02 M Tris–HCl buffer (pH 8.0). The sticky mass was triturated in the buffer with a glass rod, then transferred to a single centrifuge tube, and the washings were poured off after centrifugation. The enzyme was conveniently extracted from the precipitate with about 20 ml of 0.1 M phosphate (pH 6.8). The precipitate was triturated with a glass rod in the extraction medium at intervals, over a period of 2 or 3 h, to ensure complete extraction. The bulk of the brown pigment went into solution, leaving a grayish-colored residue. A second extraction with a smaller volume of phosphate could be made. Alternatively, 0.1 M Tris–HCl (pH 8.0) was used for the extraction. In this case, more time (up to 24 h) was required before the extraction was complete. The extract of the protamine sulfate precipitate contained little nucleic acid ($A_{280\ nm}/A_{260\ nm} = 1.3$–$1.5$).

(3) Second $(NH_4)_2SO_4$ fractionation. The yellow-brown enzyme solution, clarified by centrifugation, was diluted with water to give an absorbance at 280 nm of 2.5 in a 1-cm cuvette. The second $(NH_4)_2SO_4$ fractionation was carried out like the first except that fractions were collected at 0–0.28, 0.28–0.37 and 0.37–0.5 saturation with $(NH_4)_2SO_4$. The bulk of the nitrate reductase activity was in the second fraction. The first fraction contained much catalase, and the third fraction contained much yellow-brown protein, rich in iron but with relatively little enzyme activity.

The second fraction, dissolved in 5 ml water, could be stored in the frozen state for months with no loss of activity. The enzyme lost activity rapidly when stored as a precipitate under concentrated ammonium sulfate.

Table II summarizes the results of two fractionations. In the first, the fractionation procedure was carried through as rapidly as possible with no intentional activation of the enzyme before the final stage. Assays were performed as soon as the fractions were prepared, and units were calculated from the initial rates measured in the assay system. The measured number of enzyme units increased at every stage of the fractionation, and the protein fraction obtained after the second $(NH_4)_2SO_4$ fractionation could still be further activated. In the second fractionation, the enzyme was activated with phosphate and nitrate after the first $(NH_4)_2SO_4$ fractionation. After this stage, activity was lost in the subsequent fractionation steps, and the final protein fraction was resistant to further activation.

It is noteworthy that final yields and specific activities were about the same in the two fractionations. It appears that the proenzyme and the enzyme are not separated during the fractionation, though this conclusion has not been rigorously tested. Tabulation of enzyme units measured, as in Table II, gives no indication of the proenzyme content of a given preparation, but the behavior of the enzyme in the assay system often gives an approximate indication of the extent to which the activity can be increased. Highly activated enzyme is relatively unstable. The rate of oxidation of NADH by nitrate decreases with time in the assay. In contrast, the activity of the

TABLE II

PARTIAL PURIFICATION OF NITRATE REDUCTASE

Fractionation procedure is described in text.

Prep.	*Fraction*	*Vol. (ml)*	*Total enzyme units measured (μmoles/min)*	*Specific activity (units/mg)*
I	Chlorella extract	420	8.4	
	Step I			
	Undialyzed	140	28	
	Dialyzed	154	33	
	Step II	35.5	59	0.05
	Step III			
	Before activation*	5.0	76	1.53
	After activation*		135	2.80
	Final yield: 0.33 unit/ml Chlorella extract			
II	Chlorella extract	390	9.5	
	Step I			
	Undialyzed	130	22	
	Dialyzed	150	36	
	After activation**	120	250	
	After activation and dialysis***	126	170	
	Step II	22.5	112	0.095
	Step III	5.0	108	3.54
	Final yield: 0.275 unit/ml Chlorella extract			

* For activation, 1 part enzyme solution was added to 9 parts 0.5 M phosphate (pH 6.8), and the solution was incubated at 4°. The units/ml diluted enzyme reached a maximum and began decline after 45 h as shown:

Time (h)	*Units/ml diluted enzyme*
0	1.51
3	1.77
4.5	1.88
5.5	2.06
21	2.45
27	2.54
45	2.78
69	2.70

** To 150-ml solution were added 75 ml 0.5 M phosphate (pH 6.8) and 15 ml 0.1 M KNO_3. The solution was stored at room temperature 3.5 h, then at 4° for 16 h, and again at room temperature for 6 h. At this point, the activity had increased to a total of 192 units. Then $(NH_4)_2SO_4$ was added to 0.5 saturation, and the protein collected by centrifugation was redissolved in 120 ml water. The total activity increased to 250 units. The solution was dialyzed overnight against 0.001 M NH_4OH.

first ammonium sulfate fraction increases steadily with time, both before and after dialysis and during storage at 4°. With enzyme samples assayed at this stage, there is often a marked increase with time in the rate of oxidation of NADH by nitrate in the assay.

Absorption spectrum

After the second $(NH_4)_2SO_4$ fractionation, spectrophotometric examination showed that the fractions with the highest enzyme content contained a cytochrome (oxidized form) which was rapidly reduced on addition of NADH and rapidly reoxidized on addition of nitrate. The absorption spectra before and after reduction with dithionite are shown in Fig. 3. The α band had a maximum at 556 nm; the β band, at 525 nm. The reduction by dithionite gave an absorption spectrum similar to that obtained by addition of NADH. Addition of nitrate resulted in reappearance of the oxidized form. The NADH and nitrate were added at the concentrations employed in the standard assay system. No attempt has been made to measure the rate of reaction

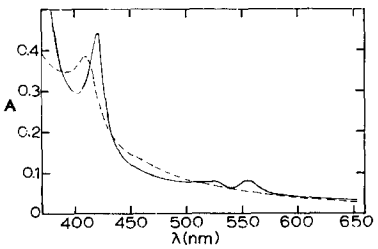

Fig. 3. Cytochrome absorption spectrum associated with nitrate reductase. The solution contained 4.2 mg protein (specific activity 1.3) per ml 0.036 M phosphate (pH 7.6). The enzyme protein was prepared as described in the text, except that the last $(NH_4)_2SO_4$ fraction was collected between 0.3 and 0.35 saturation. The protamine precipitate was extracted with 0.1 M Tris buffer (pH 8.0), and no activation step was used during the preparation. Optical path, 1 cm. ———, after reduction with 1 mg $Na_2S_2O_4$; — — —, prior to reduction.

of the cytochrome with NADH and nitrate. It was complete within the time required to mix the sample and make the spectrophotometric readings.

Effect of temperature

Between 10 and 20° a Q_{10} of 2.77 was observed, giving an activation energy, $E =$ 17 000 cal (calculated from the Arrhenius equation). The reaction rate at 30° was only a little faster than that at 20°. These measurements were made with enzyme at the stage of the first $(NH_4)_2SO_4$ fractionation, and the figures are provisional.

The oxidation of NADH in the absence of added nitrate

If the nitrate was omitted from the nitrate reductase assay, there was always a measurable oxidation of NADH in the presence of added enzyme. This "blank" reaction was most striking in the case of unfractionated extracts. It could be reduced in amount but not eliminated by dialysis of the extracts. It occurred with the most highly purified fractions as well as with the crude extracts, amounting to approximately 20% of the total activity measured in crude extracts and about 5% of the total activity measured with the most purified preparations.

This blank reaction did not represent NADH oxidase activity but was due to the oxidation of NADH by nitrate present in the enzyme preparations and perhaps also in the reagents. The blank was not subtracted from the assay value.

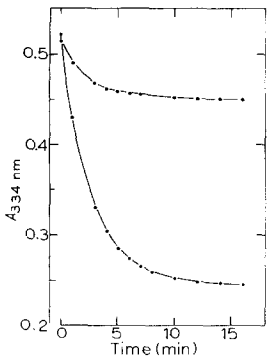

Fig. 4. Determination of nitrate with nitrate reductase. Condition as in assay system, with 0.11 unit enzyme, specific activity, 1.75. Upper curve, no added nitrate; lower curve, 0.100 μmole KNO_3 added.

Determination of nitrate with nitrate reductase

The reaction catalyzed by nitrate reductase is not measurably reversible and goes to completion. The partially purified nitrate reductase could therefore be used as a reagent for the specific determination of small amounts of nitrate. An excess of enzyme was required. Conditions used were those of the standard assay system, with the unknown sample to be analyzed instead of with added nitrate. A blank determination with all reagents except the unknown was necessary. With 0.1 unit of enzyme and 0.1 μmole of nitrate, the reaction reached completion in 10–15 min, as shown in Fig. 4. When the final change in absorbance of the blank was subtracted from the final change in absorbance observed with the added nitrate, the calculated amount of NADH oxidized was equal to the amount of nitrate added. The theoretical amount of nitrite was formed.

DISCUSSION

The cytochrome associated with the Chlorella nitrate reductase had absorption maxima in the reduced form at 556, 525 and 420–423 nm. The position of the Soret band in the oxidized form was at 409–411 nm. This compares with 412–413 nm for the maximum of the Soret band in the oxidized form, and 557, 528 and 423–424 nm for the absorption maxima of the reduced form of the cytochrome associated with the nitrate reductase of Neurospora[2]. The specific activity of 3.54 μmoles/min per mg at 20° of the Chlorella enzyme (Table II) may be compared with the value of 20 μmoles/min per mg at 28° reported for the purified Neurospora enzyme[3]. The activated Chlorella enzyme could not be assayed at 28° because of its instability at that temperature, but an extrapolation from data at lower temperatures gave 2.15 for the ratio of the rate at 28° to the rate at 20°. Thus, as a gross approximation, the most highly purified preparation of the Chlorella enzyme had a specific activity 0.4 as great ($3.54 \times 2.15/20 = 0.38$) as the best Neurospora enzyme preparation of GARRETT AND NASON[2,3].

A rough comparison can be made of the ratio of cytochrome content to enzyme activity for the two enzyme preparations on the basis of the absorbance at the maxi-

mum of the Soret band. The Chlorella enzyme (first preparation of Table II) had an absorbance at 411 nm of 0.043 for a solution containing 1 unit enzyme per ml, light path, $d = 1$ cm. With the correction for temperature made as previously described, the absorbance was $0.043/2.15 = 0.020$ for a solution containing 1 unit corrected to 28° per ml. From the data reported by GARRETT AND NASON[2], we calculated an absorbance at 412 nm of $0.054/3.2 = 0.017$ for a solution containing 1 unit/ml. These values agree as well as could be expected, especially since the Chlorella enzyme preparations contained variable amounts of material giving absorption not due to cytochrome in the region of the Soret band. Such figures do not prove that cytochrome is necessary for the enzyme reaction, and much remains to be done to characterize the prosthetic group of the Chlorella enzyme and other nitrate reductases.

The role of flavin in nitrate reductase action is not clear. The Neurospora enzyme is reported to have a requirement for added flavin, which must be included in the assay system[2,3]. ZUMFT *et al.*[6] have reported a strong stimulatory effect of FAD on Chlorella nitrate reductase. Our experience has been that added FMN or FAD causes no clear stimulation of the reduction of nitrate by NADH in the assay of the partially purified Chlorella enzyme. Occasional small stimulatory effects (10–30%) observed when FAD or FMN was added to cruder enzyme preparations were difficult to reproduce, and consequently difficult to evaluate. Attempts to resolve the enzyme into active apoenzyme and free flavin with acid $(NH_4)_2SO_4$ were unsuccessful. Further purification of the enzyme is required, however, before the presence of bound flavin can be excluded. The recent experiments of ZUMFT *et al.*[6], showing that added FAD protects nitrate reductase against thermal inactivation, may lead to eventual clarification of the nature of the flavin involvement.

The nature and significance of the *in vitro* activation of nitrate reductase is not yet clear. LOSADA *et al.*[7] have reported that 1–2 h prior exposure of Chlorella cells to ammonia inactivated the nitrate reductase but that the enzyme was subsequently reactivated in cell-free extracts during storage for several hours at 0°. In our experiments, the algae were grown autotrophically on nitrate as the sole source of nitrogen and harvested during active growth; no ammonia was added to inactivate or repress the enzyme.

The stimulatory effect of nitrate on the activation calls to mind the frequently described[1,4,5] adaptive response of the nitrate reductase of intact cells to nitrate addition, but the latter response is reported to be associated with *de novo* synthesis of protein, whereas the activation of the nitrate reductase activity of Chlorella extracts occurs under conditions where protein synthesis can be excluded. The response of the activation process in extracts to nitrate suggests either that the enzyme is activated in the oxidized form and/or that it is stabilized in the oxidized form. The latter interpretation is supported by the observed instability of the activated enzyme in the presence of added NADH without added nitrate. It is not impossible that the relatively inactive enzyme and/or enzyme precursor present in the fresh Chlorella extracts represents the native enzyme combined with a firmly bound inhibitor which serves as a regulator of the *in vivo* process of nitrate reduction in the manner suggested by SYRETT AND MORRIS[18].

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