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Structural and Functional Relationships of Enzyme Activities Induced by Nitrate in Barley

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1. Nitrate induces the development of NADH–nitrate reductase (EC 1.6.6.1), $FMNH_2$–nitrate reductase and NADH–cytochrome *c* reductase activities in barley shoots. 2. Sucrose-density-gradient analysis shows one band of NADH–nitrate reductase (8S), one band of $FMNH_2$–nitrate reductase activity (8S) and three bands of NADH–cytochrome *c* reductase activity (bottom layer, 8S and 3.7S). Both 8S and 3.7S NADH–cytochrome *c* reductase activities are inducible by nitrate, but the induction of the 8S band is much more marked. 3. The 8S NADH–cytochrome *c* reductase band co-sediments with both NADH–nitrate reductase activity and $FMNH_2$–nitrate reductase activity. Nitrite reductase activity (4.6S) did not coincide with the activity of either the 8S or the 3.7S NADH–cytochrome *c* reductase. 4. $FMNH_2$–nitrate reductase activity is more stable ($t_{\frac{1}{2}}$ 12.5min) than either NADH–nitrate reductase activity ($t_{\frac{1}{2}}$ 0.5min) or total NADH–cytochrome *c* reductase activity ($t_{\frac{1}{2}}$ 1.5min) at 45°C. 5. NADH–cytochrome *c* reductase and NADH–nitrate reductase activities are more sensitive to *p*-chloromercuribenzoate than is $FMNH_2$–nitrate reductase activity. 6. Tungstate prevents the formation of NADH–nitrate reductase and $FMNH_2$–nitrate reductase activities, but it causes superinduction of NADH–cytochrome *c* reductase activity. Molybdate overcomes the effects of tungstate. 7. The same three bands (bottom layer, 8S and 3.7S) of NADH–cytochrome *c* reductase activity are observed irrespective of whether induction is carried out in the presence or absence of tungstate, but only the activities in the 8S and 3.7S bands are increased. 8. The results support the idea that NADH–nitrate reductase, $FMNH_2$–nitrate reductase and NADH–cytochrome *c* reductase are activities of the same enzyme complex, and that in the presence of tungstate the 8S enzyme complex is formed but is functional only with respect to NADH–cytochrome *c* reductase activity.

The induction of nitrate reductase activity by nitrate in higher plants is well documented (Beevers & Hageman, 1969). A survey by Beevers, Flesher & Hageman (1964) showed that, with the exception of soya bean, NADH was the preferred electron donor for nitrate reductase in the 16 plant species studied. However, $FMNH_2$–nitrate reductase activity can also be detected in tissue extracts of spinach (Paneque, Del Campo, Ramirez & Losada, 1965) and other higher plants. Schrader, Ritenour, Eilrich & Hageman (1968) have shown that the $FMNH_2$- and NADH-linked activities are induced in an approximately 1:1 ratio in corn, but have different stabilities. Since these two activities also bore a 1:1 relationship to each other during partial purification, they concluded that the two activities were probably located on the same protein moiety.

The objectives of the present paper are threefold: first, to report the presence of a nitrate-inducible NADH–cytochrome *c* reductase activity in a higher plant, barley, comparable with the nitrate-inducible NADPH–cytochrome *c* reductase of *Neurospora* (Kinsky, 1961); secondly, to describe the structural and functional relationships between this newly described NADH–cytochrome *c* reductase, and the other nitrate-inducible enzyme activities of higher plants, namely NADH–nitrate reductase, $FMNH_2$–nitrate reductase and methyl viologen–nitrite reductase; thirdly, to describe the basis for the inhibition of the formation of nitrate reductase activity by tungstate (Heimer, Wray & Filner,

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1969). The results suggest that NADH–cytochrome *c* reductase, NADH–nitrate reductase and $FMNH_2$–nitrate reductase activities are partial activities of one enzyme complex, that nitrate reductase and nitrite reductase activities are properties of different protein moieties, and that in the presence of tungstate the nitrate reductase apoenzyme is formed but is functional only with respect to NADH–cytochrome *c* reductase activity. A preliminary account of some of this work has been presented (Wray, Filner & Ries, 1969).

MATERIALS AND METHODS

Chemicals. All common chemicals were of the highest quality available and were obtained from either the Fisher Scientific Co., Fairlawn, N.J., U.S.A., the Mallinckrodt Chemical Works, St Louis, Mo., U.S.A., or the J. T. Baker Chemical Co., Phillipsburgh, N.J., U.S.A. Catalase (ox liver; EC 1.11.1.6) was obtained from the Worthington Biochemical Corp., Freehold, N.J., U.S.A. FMN (sodium salt), FAD (disodium salt) and NADH (disodium salt) were from the Sigma Chemical Co., St Louis, Mo., U.S.A.; cytochrome *c* (horse heart; A grade) was from Calbiochem, Los Angeles, Calif., U.S.A.; sucrose (ribonuclease-free) was from Mann Research Laboratories, New York, N.Y., U.S.A.; methyl viologen was from K and K Laboratories Inc., Plainview, N.Y., U.S.A.; Sephadex G-25 was from A.B. Pharmacia, Uppsala, Sweden.

Plant material and growth conditions. Seeds of barley, *Hordeum vulgare* L. cultivar Himalaya, were obtained from Professor Nilan of Washington State University, Pullman, Wash., U.S.A., through Professor J. E. Varner of this laboratory. Seedlings were grown at 22°C in pots containing vermiculite in a constant-temperature cabinet illuminated with 2000 ft-candles of continuous fluorescent light supplemented with tungsten light. The pots were 11.4cm wide and 8.3cm deep, and 20 seeds were sown per pot. Seeds were initially treated with 250ml of half-strength Hoagland's nutrient solution (Hoagland & Arnon, 1938) deficient in nitrate, followed by 100ml of this solution when the seedlings were 3 days old. When the seedlings were 5 days old they were treated with 100ml of half-strength Hoagland's nutrient solution containing 3mM-KNO_3. In the experiments involving the effect of tungstate, the seeds were treated at planting with 250ml of half-strength Hoagland's nutrient solution lacking added nitrate and molybdate and containing 100μM-sodium tungstate. The plants were treated with 100ml of the same solution at 3 days, and when the seedlings were 5 days old they were treated with 100ml of the same solution containing 3mM-KNO_3. Separate experiments showed that omission of molybdate from the half-strength Hoagland's solution had little effect on the induction kinetics of nitrate reductase, presumably owing to contamination of the other mineral salts with molybdate.

Tissue extraction. Shoots were removed from the plants at the time-intervals indicated in the figures and tables after treatment of the plants with nitrate. The shoots were weighed, and then ground in a porcelain mortar with 0.1M-tris–HCl–1mM-cysteine buffer, pH 7.5. Extractions were carried out at 4°C in the cold-room. The brei was centrifuged at 12000*g* for 20min and the supernatant was used for enzyme assays.

Enzyme assay procedures. All enzymes were assayed under conditions such that the rate of reaction was proportional to time and amount of enzyme at 25°C and pH 7.5.

NADH–nitrate reductase. This activity was assayed essentially by the procedure of Sanderson & Cocking (1964). The reaction mixture contained 0.5ml of 0.1M-potassium phosphate buffer, pH 7.5, 0.1ml of 0.1M-KNO_3, 0.1ml of 1mM-NADH, 0.2ml of enzyme and 0.1ml of deionized distilled water, giving a final volume of 1.0ml. At the end of the 15min incubation period, nitrite formation was measured by adding 1.0ml of 1% (w/v) sulphanilamide in 3M-HCl and 1.0ml of 0.02% (w/v) *N*-(1-naphthyl)ethylenediamine dihydrochloride. The tube contents were mixed and the precipitated protein was sedimented in a bench centrifuge for 5min at top speed (3000*g*). After 15min the extinction was measured at 540nm in a Gilford spectrophotometer. Extinction values were then converted into amounts of nitrite by means of a previously established standard curve. Enzyme was added to the control tube after addition of sulphanilamide.

$FMNH_2$–nitrate reductase. The assay was essentially that described by Paneque *et al.* (1965). The assay was performed in the following manner: 0.4ml of 0.1M-potassium phosphate buffer, pH 7.5, 0.3ml of 2mM-FMN, 0.1ml of 0.1M-KNO_3, 0.1ml of enzyme and 0.05ml of deionized distilled water were pipetted into the incubation tubes. The tubes were gently flushed with N_2 and 0.05ml of $Na_2S_2O_4$ (10mg/ml) in 95mM-$NaHCO_3$ was added. The tubes were shaken until the yellow FMN colour was bleached (i.e. the FMN reduced to $FMNH_2$) and then incubated for 15min in unstoppered tubes. At the end of the incubation period, the tubes were vigorously mixed on a Vortex mixer to reoxidize the $FMNH_2$ to FMN (i.e. mixed until the yellow FMN colour reappeared). Nitrite formation was then measured as in the assay of NADH–nitrate reductase. The control tube was incubated as above but without addition of $Na_2S_2O_4$.

NADH–cytochrome c *reductase.* This enzyme was assayed spectrophotometrically. The reaction mixture contained 0.2ml of 0.1M-potassium phosphate buffer, pH 7.5, 0.02ml of aq. 2% (w/v) cytochrome *c* (horse heart), 0.08ml of 1mM-NADH, enzyme and deionized distilled water to a final volume of 0.4ml. The reduction of cytochrome *c* was measured by following the rate of increase in extinction at 550nm with a Gilford spectrophotometer equipped with a linear chart recorder. NADH was omitted from the control cuvette.

Nitrite reductase. This enzyme was assayed by a modification of the method of Chroboczek-Kelker (1969). The assay was performed in the following manner: 0.3ml of 0.1M-potassium phosphate buffer, pH 7.5, 0.1ml of 20mM-KNO_2, 0.1ml of 10mM-methyl viologen, enzyme and water to a final volume of 0.8ml were pipetted into the assay tubes. The tubes were flushed with N_2 and the reaction was started by the addition of 0.2ml of $Na_2S_2O_4$ (10mg/ml) in 0.29M-$NaHCO_3$. The tubes were then stoppered with rubber serum-bottle caps, incubated for the desired time-period, unstoppered and the reaction was stopped by mixing the contents of the tubes vigorously on a Vortex mixer until the methyl viologen was oxidized.

A 0.1ml portion of the reaction mixture was taken and pipetted into 2.9ml of deionized distilled water. The nitrite was determined as described for the assay of NADH–nitrate reductase. The disappearance of nitrite from the reaction mixture was taken as a measure of nitrite reductase activity.

Nitrate assay. The method of Lowe & Hamilton (1967) was used with slight modification. The assay utilizes soya-bean-nodule bacteroids for reduction of nitrate to nitrite and subsequent quantitative determination of the latter. Soya-bean nodules were obtained from 6–8-week-old soya-bean plants. The plants were grown in a mixture of vermiculite and gravel under artificial-daylight conditions. Before being planted, the seeds were inoculated with a commercial nitrogen-fixing inoculant (gift from the Nitragin Co., Milwaukee, Wis., U.S.A.). The nodules were collected and extracted as described by Lowe & Hamilton (1967). The assay of nitrate was performed in the following manner: 0.5ml of 0.1M-potassium succinate, pH6.8, tissue extract and deionized distilled water to a final volume of 0.8ml were pipetted into the assay tube. The assay was started by the addition of 0.2ml of the undiluted stock bacterial suspension. After incubation for 30min at 45°C the reaction was stopped by the addition of the nitrite-determination reagents and the reaction mixture cleared by centrifugation at 27000*g* for 10min. The extinction at 540nm was measured and nitrate was determined from a standard curve. Under the conditions described above, reduction of nitrate to nitrite by the bacteria was linear between 0 and 100nmol of nitrate/tube.

Sephadex G-25 treatment of tissue extracts. The method used was essentially that described by Sanderson & Cocking (1964). Treatment with Sephadex was carried out by passing 2.0ml of 12000*g* supernatant through a column (1cm × 30cm) containing 6.0g of Sephadex G-25. Elution was carried out at 4°C with 0.1M-tris–HCl buffer, pH7.5, containing 1mM-cysteine. The fractions containing enzyme were used for the experiments involving the effect of heat and thiol-blocking reagents on the nitrate-induced enzyme activities.

Sucrose-density-gradient centrifugation. The procedure used was essentially that of Martin & Ames (1961). After sedimentation for 6h at 65000rev./min (Spinco model L2-65B ultracentrifuge with SW65 rotor) in 0.1M-tris–HCl buffer, pH7.5, recovery of $FMNH_2$–nitrate reductase activity and of NADH–cytochrome *c* reductase activity was about 10%, and recovery of NADH–nitrate reductase activity was even poorer, being between 0 and 5%. Essentially complete recovery could be obtained in sucrose solutions prepared in 0.1M-potassium phosphate buffer, pH7.5, containing FAD (10μM), cysteine (1mM) and EDTA (1mM). However, this concentration of cysteine interfered in the NADH–cytochrome *c* reductase assay by reducing the substrate, cytochrome *c*. To assay NADH–cytochrome *c* reductase after gradient fractionation cysteine was therefore omitted from the sucrose solutions. For the nitrate reductase assays, cysteine (final concentration 1mM) was added to the reaction mixtures. This procedure gave 80–100% recovery of all three enzyme activities.

Tissue extract from plants treated with nitrate for 20h was layered on top of the gradient, which consisted of 4.4ml of a linear 5–20% (w/v) sucrose gradient layered over 0.8ml of 50% (w/v) sucrose. The sucrose solutions were buffered in 0.1M-potassium phosphate buffer, pH7.5, containing 10μM-FAD and 1mM-EDTA. The gradients were centrifuged at 65000rev./min at 0°C for 6h in a Spinco L2-65B ultracentrifuge with SW65 rotor. After centrifugation the gradients were fractionated from the bottom of the tube by using a no. 20 hypodermic needle. Either 30 fractions of 10 drops each or 20 fractions of 15 drops each were collected. The sedimentation coefficient of enzymes was determined by comparison with ox liver catalase, as described by Martin & Ames (1961).

RESULTS

Detection of nitrate reductase and cytochrome c reductase activities, and their electron-donor specificities. The soluble protein fraction of barley plants that have been exposed to nitrate will catalyse the reduction of nitrate to nitrite if the electron donor offered is $FMNH_2$ or NADH (Fig. 1), but not if it is NADPH. The reduction of cytochrome *c* is also catalysed by this fraction, with either NADH or NADPH as electron donor, but the reaction rate with NADH (69nmol of cytochrome *c* reduced/min per ml of extract) is about 8 times faster than with NADPH. Both nitrate reductase activities are

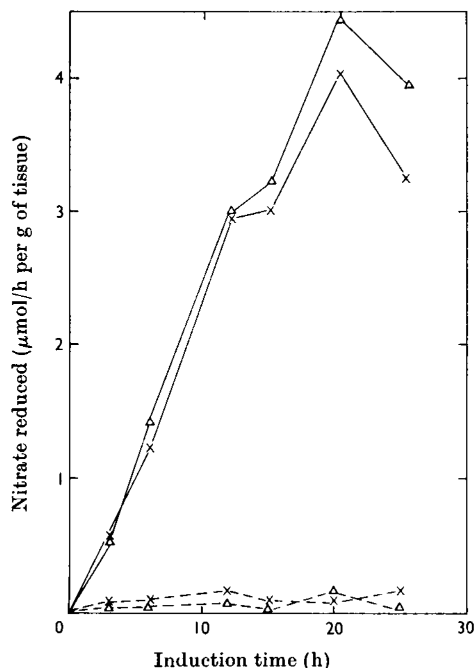


Fig. 1. Induction of NADH–nitrate reductase activity (×) and $FMNH_2$–nitrate reductase activity (△) by nitrate in the absence and presence of 100μM-sodium tungstate. Plants were treated as described in the Materials and Methods section. ——, Without tungstate; ----, with tungstate.

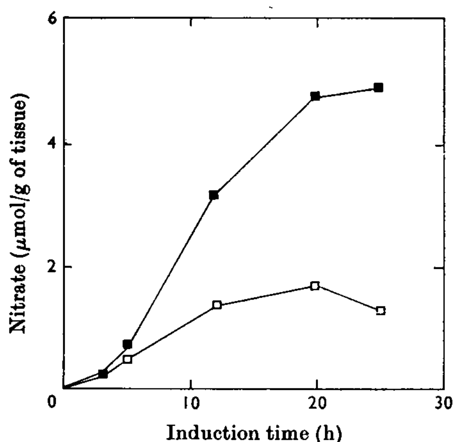


Fig. 2. Nitrate content of shoots from plants treated (■) or not treated (□) with 100μM-sodium tungstate.

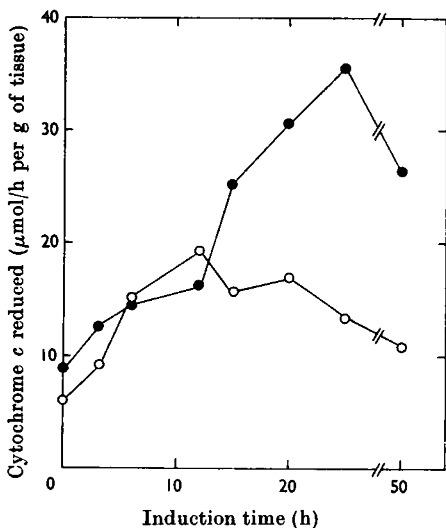


Fig. 3. Induction of NADH–cytochrome c reductase activity by nitrate in the absence (○) and presence (●) of 100μM-sodium tungstate. Plants were treated as described in the Materials and Methods section.

usually absent from the soluble protein fraction of plants not exposed to nitrate, but occasionally low concentrations of both nitrate reductase activities (0–75nmol of nitrate reduced/h per g of tissue) are detected, probably a consequence of the fact that low concentrations of nitrate (approx. 80 nmol of nitrate/g of tissue) are sometimes detected in shoot extracts from plants not treated with nitrate. In contrast, NADH–cytochrome c reductase activity is readily detected in the soluble protein fraction from tissue not treated with nitrate, but it (approx.

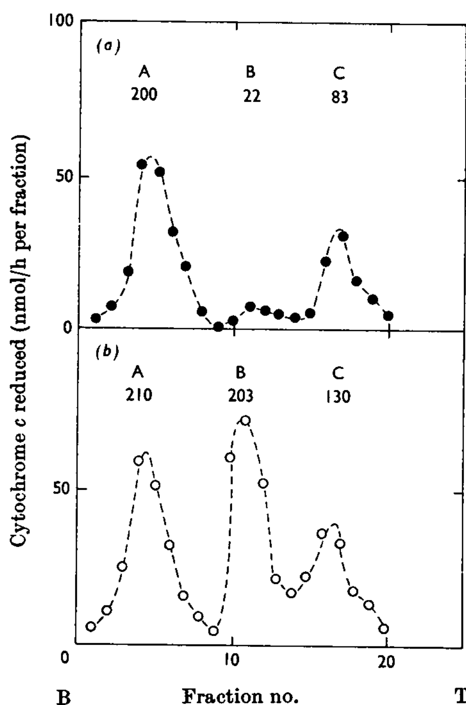


Fig. 4. Distribution of NADH–cytochrome c reductase activity detected after sucrose-density-gradient centrifugation of 12000g shoot supernatant from plants (a) not treated and (b) treated with nitrate. Sedimentation was under standard conditions. Tissue supernatant (0.2ml) was layered on top of the gradient. B is the bottom and T is the top of the gradient. The bands of activity are lettered A, B, and C and the numbers refer to the NADH–cytochrome c reductase activity in each band.

6nmol of cytochrome c reduced/h per g of tissue) is only 20–30% of that found in extracts from plants treated with nitrate.

Kinetics of induction of NADH–nitrate reductase, $FMNH_2$–nitrate reductase and NADH–cytochrome c reductase. Induction was brought about by the addition of 100ml of half-strength Hoagland's nutrient solution containing 3mM-potassium nitrate to pots containing 5 day old barley plants. Zero time represents the time at which nitrate was added to the plants.

After addition of nitrate, NADH–nitrate reductase activity and $FMNH_2$–nitrate reductase activity begin to become detectable in the soluble protein fraction (Fig. 1). There is no apparent lag before the increases begin, and the increases are approximately linear for 24h. The ratio of NADH–nitrate reductase to $FMNH_2$–nitrate reductase is near one. Nitrate also increases at an approximately linear rate over the first 24h (Fig. 2). The

activity of NADH–cytochrome *c* reductase also begins to increase on addition of nitrate to the plants (Fig. 3).

Sucrose-density-gradient centrifugation. Three bands of NADH–cytochrome *c* reductase activity were detected after sedimentation in the sucrose gradient of 12000*g* shoot supernatant from plants not treated with nitrate (Fig. 4*a*); a major band A, which sedimented to the 50% sucrose layer at the bottom of the gradient, and two lighter bands, B and C, which possessed sedimentation coefficients of 8S and 3.7S respectively. The NADH–cytochrome *c* reductase activity associated with band B increases greatly in extracts prepared from plants treated with nitrate (Fig. 4*b*). A small increase in the activity associated with band C is also observed (Fig. 4*b*). This small increase in the activity of band C was routinely observed, and an additional increase in activity is seen in plants treated with nitrate in the presence of tungstate (see below).

Only one band of NADH–nitrate reductase activity was detected in the 12000*g* supernatant of extracts from plants treated with nitrate and this co-sedimented with band B of NADH–cytochrome *c* reductase activity at 8S (Fig. 5). Only one band of $FMNH_2$–nitrate reductase activity was detected and this also co-sedimented at 8S with the inducible NADH–cytochrome *c* reductase activity (Fig. 6). The fact that the three enzyme activities induced by nitrate co-sediment suggests that the three activities are possessed by the same protein moiety.

Heat-stability studies. Samples of tissue supernatant were kept at 45°C and samples were removed at time-intervals and assayed for enzyme activity. The three activities exhibited different stabilities (Fig. 7). $FMNH_2$–nitrate reductase activity was the most stable, the half-time of inactivation being 12.5min. NADH–nitrate reductase activity and NADH–cytochrome *c* reductase activity were inactivated with half-times of 0.5 and 1.5min respectively. The addition of nitrate had no effect on the rates of inactivation.

Thiol requirement. Of the three activities examined, NADH–nitrate reductase activity was the most sensitive to *p*-chloromercuribenzoate (Table 1). The inhibition of both nitrate reductase activities

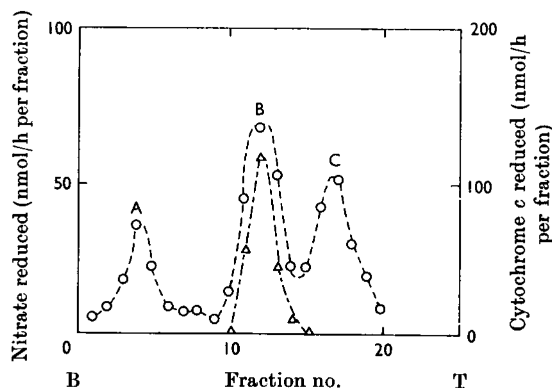


Fig. 6. Distribution of NADH–cytochrome *c* reductase activity (○) and $FMNH_2$–nitrate reductase activity (△) detected after sucrose-density-gradient centrifugation of 12000*g* shoot supernatant from plants treated with nitrate. Sedimentation was under standard conditions. Tissue supernatant (0.2ml) was layered on top of the gradient. B is the bottom and T is the top of the gradient.

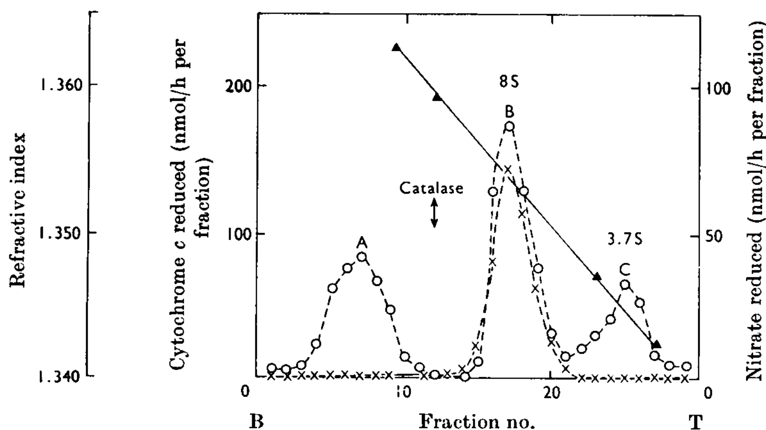


Fig. 5. Distribution of NADH–cytochrome *c* reductase activity (○) and NADH–nitrate reductase activity (×) after sucrose-density-gradient centrifugation of 12000*g* shoot supernatant from plants treated with nitrate. Sedimentation was under standard conditions. Tissue supernatant (0.2ml) was layered on top of the gradient. B is the bottom and T is the top of the gradient. ▲, Refractive index of the gradient.

was reversed by cysteine but this reversal was much more marked in the case of the $FMNH_2$-linked activity. NADH–cytochrome *c* reductase activity was also inhibited by *p*-chloromercuribenzoate, but reversal by cysteine could not be tested since cysteine interferes with the assay.

Effect of tungstate in vitro *on NADH–nitrate reductase and $FMNH_2$–nitrate reductase activities.* When extract from plants treated with nitrate in the absence of tungstate was incubated for 5min with concentrations of sodium tungstate up to 1mM before assay, no effect of tungstate on either NADH–nitrate reductase or $FMNH_2$–nitrate reductase activity could be detected.

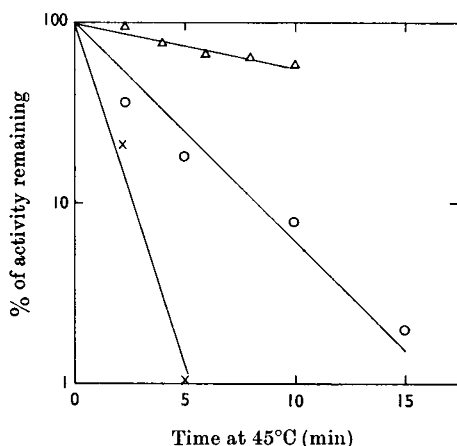


Fig. 7. Thermal stability of $FMNH_2$–nitrate reductase activity (△), NADH–nitrate reductase activity (×) and NADH–cytochrome *c* reductase activity (○) at 45°C. The 12000*g* shoot supernatant from plants treated with nitrate was used after passage through Sephadex G-25.

Induction of nitrate reductase activity in the presence of tungstate. Tungstate at 100μM in half-strength Hoagland's nutrient solution inhibits the development of nitrate reductase activity otherwise observed in 5-day-old barley shoots in response to nitrate, with minimal effect on plant growth. Higher concentrations of tungstate inhibit root and shoot development as well as nitrate uptake (Heimer *et al.* 1969). The inhibition of the formation of nitrate reductase activity is observed regardless of whether NADH or $FMNH_2$ is used as electron donor in the enzyme assay (Fig. 1).

Induction of NADH–cytochrome c *reductase activity in the presence of tungstate.* NADH–cytochrome *c* reductase activity is superinduced two- to three-fold in the presence of tungstate over that induced by nitrate in the absence of tungstate (Fig. 3). Increases up to fivefold have been observed in some experiments. The nitrate content of the shoots also increases in the presence of tungstate (Fig. 2).

Sucrose-density-gradient analysis of the NADH–cytochrome c *reductase activity induced in the presence of tungstate.* The activity associated with band A appears to be independent of the presence of tungstate during induction. However, the activity associated with both bands B and C is increased in plants treated with nitrate in the presence of tungstate (Fig. 8). Sedimentation of a mixture of extracts from plants treated with nitrate in the presence and absence of tungstate shows the same three bands and there was no apparent band broadening due to tungstate. We conclude that the

Table 1. *Effect of* p*-chloromercuribenzoate and cysteine on the activities of NADH–nitrate reductase, $FMNH_2$–nitrate reductase and NADH–cytochrome* c *reductase in the* 12000*g* *shoot supernatant from barley plants treated with nitrate for* 20h.

Final concn. (μM)		% of inhibition		
p-Chloromercuribenzoate	Cysteine	NADH–nitrate reductase	FMNH–nitrate reductase	NADH–cytochrome *c* reductase
0	0†	0	0	0
0	1000‡	0	0	*
10	0	100	86	100
10	1000	28	0	—
50	0	100	82	—
50	1000	50	16	—
100	0	100	81	100
100	1000	65	14	—
200	0	100	84	—
200	1000	79	19	—

* NADH–cytochrome *c* reductase cannot be assayed in the presence of this concentration of cysteine.
† 100% value for assays without cysteine.
‡ 100% value for assays with cysteine.

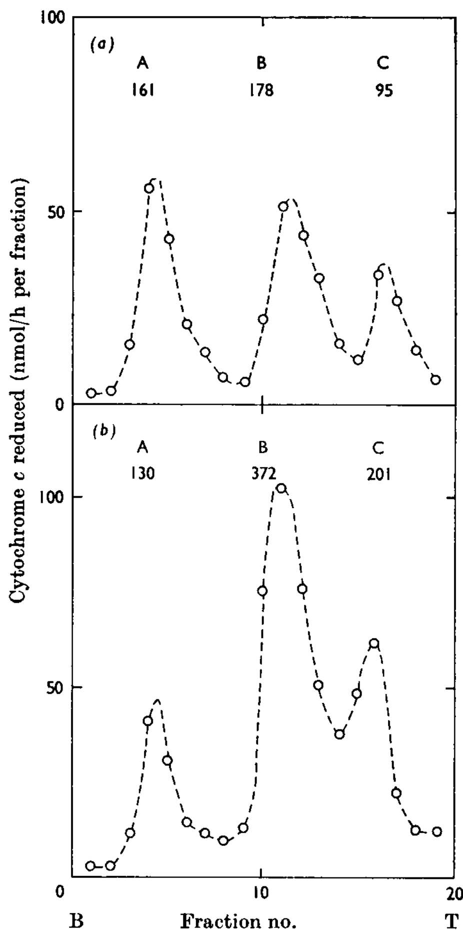


Fig. 8. Distribution of NADH–cytochrome *c* reductase activity detected after sucrose-density-gradient centrifugation of 12000*g* shoot supernatant from plants treated with nitrate in (*a*) the absence or (*b*) the presence of 100μM-sodium tungstate. Sedimentation was under standard conditions. Tissue supernatant (0.2ml) was layered on top of the gradient. B is the bottom and T is the top of the gradient. The bands of activity are lettered A, B and C and the numbers refer to the NADH–cytochrome *c* reductase activity in each band.

8S NADH–cytochrome *c* reductase activity associated with nitrate reductase is formed in the presence of tungstate. This implies that in the presence of tungstate the entire nitrate reductase enzyme complex is formed but lacks nitrate reductase activity.

Reversal of the tungstate effects by molybdate. If extracts from plants treated with nitrate and tungstate are preincubated for 5 min with concentrations of molybdate up to 1mM before assay, no reactivation of either nitrate reductase occurred. Similarly, sedimentation of tissue extracts from plants treated with nitrate and tungstate through a sucrose density gradient containing 0.5mM-molybdate for 6h failed to reactivate either nitrate reductase activity. The effects of tungstate are, however, prevented *in vivo* when molybdate is given to the plants at the same time as tungstate. The superinduction of NADH–cytochrome *c* reductase activity is reversed by molybdate, increasing concentrations of molybdate bringing the activity down to the control value or below (Fig. 9*a*). A similar effect is obtained for the nitrate content of the shoot (Fig. 9*b*). Less than 0.2μM-molybdate is effective in obtaining some reversal of the effect of 100μM-tungstate in inhibiting the formation of both nitrate reductase activities (Figs. 9*c* and 9*d*).

Sucrose-density-gradient analysis of methyl viologen–nitrite reductase. Only one band of methyl viologen–nitrite reductase, sedimenting at 4.6S, was detected in the sucrose gradient after centrifugation of 12000*g* shoot supernatant from plants treated with nitrate (Fig. 10).

DISCUSSION

Although the induction of nitrate reductase activity in higher plants in response to nitrate has been demonstrated by many other workers (see reviews by Beevers & Hageman, 1969; Filner, Wray & Varner, 1969) no clear demonstration of an increase in NADH–cytochrome *c* reductase activity in response to nitrate (Fig. 3) has previously been reported. Spencer (1959) has observed that a sevenfold purified nitrate reductase preparation from wheat also possessed NADH–cytochrome *c* reductase activity but in view of the degree of purity of the preparation the significance of this observation is obscure. However, evidence for induction of NADPH–cytochrome *c* reductase activity by nitrate has been established in *Neurospora* by Kinsky (1961) and in *Aspergillus* by Cove & Coddington (1965).

Sorger (1966) was the first to demonstrate that *Neurospora* nitrate reductase co-sedimented with NADPH–cytochrome *c* reductase and suggested that both activities were located on the same protein. Garrett & Nason (1969) confirmed this and further showed that a 500-fold purified preparation of *Neurospora* nitrate reductase possessed NADPH–nitrate reductase, $FADH_2$–nitrate reductase, NADPH–cytochrome *c* reductase and methyl viologen–nitrate reductase activities and that all the activities co-sedimented at 8S.

We have shown in this paper that nitrate induces the development of NADH–nitrate reductase, $FMNH_2$–nitrate reductase and NADH–cytochrome *c* reductase activities in barley and that the three induced activities co-sediment at 8S. Since

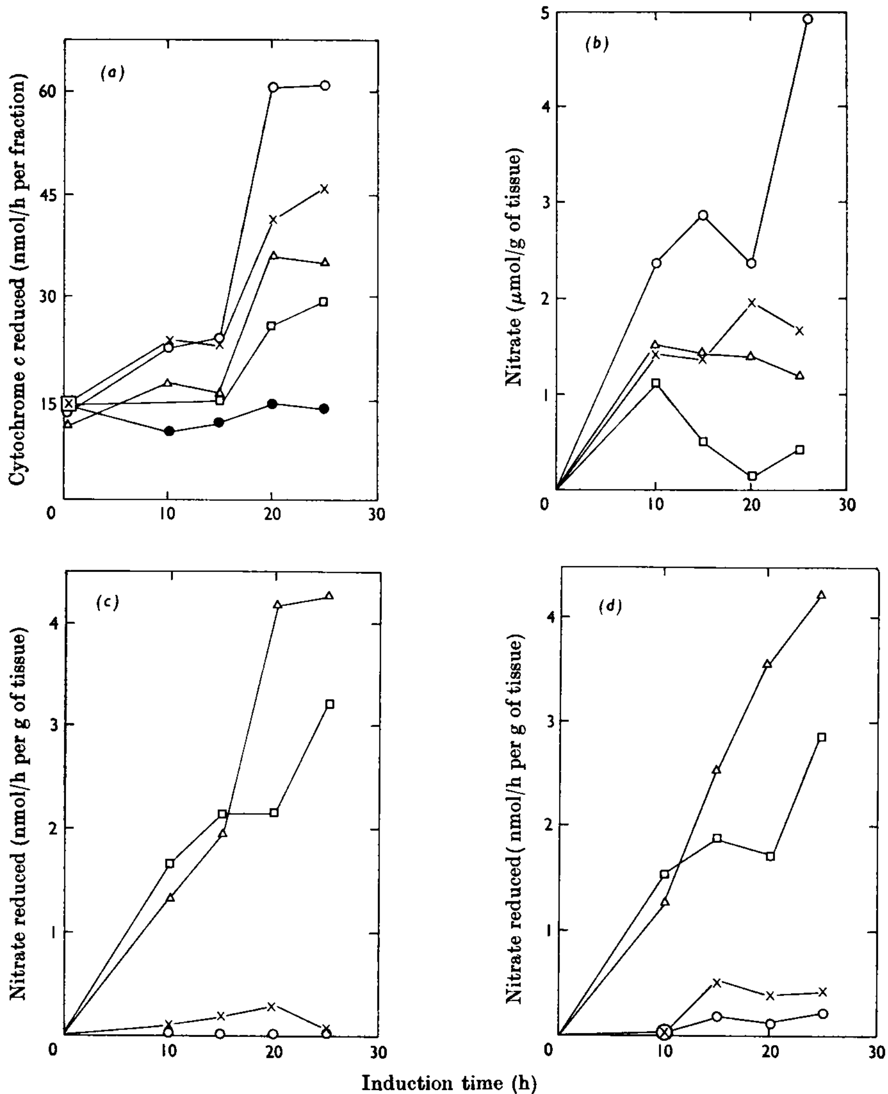


Fig. 9. Antagonism by molybdate of the effects of tungstate on (*a*) NADH–cytochrome *c* reductase, (*b*) nitrate, (*c*) NADH–nitrate reductase and (*d*) $FMNH_2$–nitrate reductase contents in barley shoots. Plants were treated for 5 days as described in the Materials and Methods section, with nitrate-less nutrient solutions of the following composition; half-strength Hoagland's nutrient solution lacking added molybdate (△), the same solution plus 100μM-sodium tungstate (○), the same solution plus 100μM-sodium tungstate plus 100μM-sodium molybdate (□) or half-strength Hoagland's solution (0.187μM-molybdate) plus 100μM-sodium tungstate (×). At 5 days the plants were treated with the same solutions containing 3mM-potassium nitrate. ●, Nitrate-less nutrient only.

both nitrate reductase activities co-sediment at 8S it appears that one protein is capable of using either NADH or $FMNH_2$ as electron donor in the enzymic reduction of nitrate to nitrite. It seems unlikely that three different enzymes possessing the same sedimentation coefficient would be induced by nitrate. The major difference between the barley enzyme and the fungal enzyme is that NADPH appears to be the preferred electron donor for the *Neurospora* enzyme.

The three nitrate-induced activities observed here respond differently to various inhibitors and this

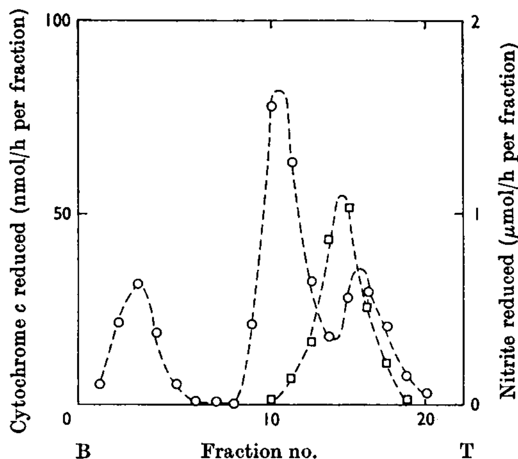
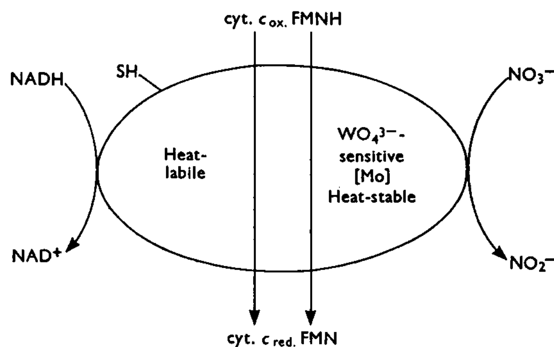


Fig. 10. Distribution of NADH–cytochrome *c* reductase activity (○) and methyl viologen–nitrite reductase activity (□) detected after sucrose-density-gradient centrifugation of 12000*g* shoot supernatant from plants treated with nitrate. Sedimentation was under standard conditions. Tissue supernatant (0.2ml) was layered on top of the gradient. B is the bottom and T is the top of the gradient. The recovery of methyl viologen–nitrite reductase activity was 55%.



Scheme 1. Speculative model of the barley nitrate reductase enzyme complex (cf. model in Schrader *et al.* 1968).

differential inhibition has led to some conclusions with respect to the interrelationships of these activities in the enzyme complex (Scheme 1). The marked effect of the thiol-blocking reagent *p*-chloromercuribenzoate on both NADH–nitrate reductase activity and NADH–cytochrome *c* reductase activity suggests that one or more thiol groups are involved between NADH and the site at which cytochrome *c* reductase feeds into the electron-transport sequence of the enzyme. The less marked effect of *p*-chloromercuribenzoate on $FMNH_2$–nitrate reductase activity suggests the presence of a less accessible thiol group between nitrate and the site at which $FMNH_2$ feeds into the electron-transport sequence. Alternatively the tertiary structure of that part of the enzyme responsible for $FMNH_2$–nitrate reductase activity may depend on the functional state of the exposed thiol group between NADH and the cytochrome *c* site. The heat-denaturation experiments (Fig. 7) suggest that the enzyme consists of at least two components of different stability. That part of the molecule responsible for NADH–cytochrome *c* reductase activity is more heat-labile than the part responsible for transferring electrons from $FMNH_2$ to nitrate. Although only $FMNH_2$–nitrate reductase activity can be detected in tissue extracts after 15min at 45°C, no apparent dissociation of the protein occurs, since subsequent sucrose-density-gradient analysis does not show any detectable change in the sedimentation of this more stable activity. However, this activity becomes less stable to sedimentation after heat treatment. Recoveries after 6h of sedimentation are 20–30% rather than the usual 80–100% for unheated enzyme.

The above results have led us to the view that the barley nitrate reductase is an enzyme complex consisting of at least two components of different stability measurable as NADH–cytochrome *c* reductase and $FMNH_2$–nitrate reductase, and that the overall reaction of the enzyme complex is the reduction of nitrate to nitrite with NADH as electron donor. Our results are not consistent with the suggestion of Paneque & Losada (1966) that the enzyme cannot use NADH directly, but support the conclusion reached by Schrader *et al.* (1968) based on purification studies.

We previously reported that the formation of $FMNH_2$–nitrate reductase activity is prevented in the presence of the molybdate analogue tungstate (Heimer *et al.* 1969). The present paper shows that tungstate also prevents the formation of NADH–nitrate reductase activity (Fig. 1). However, the 8S NADH–cytochrome *c* reductase induced by nitrate is still produced (Fig. 3). The formation of the 8S NADH–cytochrome *c* reductase activity in the presence of tungstate suggests that the nitrate reductase protein is formed but is not functional with respect to nitrate reductase activity. Tungstate appears to affect that part of the complex lying between nitrate and the site at which $FMNH_2$ feeds into the electron-transport sequence. Supporting evidence that the enzyme is not functional *in vivo* is perhaps provided by the fact that nitrate accumulates to a higher concentration in tungstate-treated plants than in untreated plants (Fig. 2). Nitrate may be considered to be a gratuitous inducer of nitrate reductase in the presence of tungstate.

How does tungstate cause the enzyme to be non-functional? Although the expression of nitrate reductase activity is prevented if induction is carried out in the presence of tungstate, the metal has no effect on nitrate reductase activity *in vitro*. This suggests that the inhibition *in vivo* is not due to simple heavy-metal inactivation of the enzyme. Since molybdate overcomes the tungstate inhibition *in vivo* (Figs. 9*c* and 9*d*) but not *in vitro* there appear to be two possible reasons for the non-functional state of the enzyme. One is that tungstate prevents the insertion of molybdenum into the complex and the other is that tungsten is incorporated into the complex in place of molybdenum. Either event would be expected to adversely affect the electron-transport sequence of the complex. Attempts to demonstrate labelling of the 8S NADH–cytochrome *c* reductase band with [^{189}W]tungstate in a sucrose density gradient were not successful. However, this negative result must be treated with caution since difficulty was experienced in removing free [^{189}W]tungstate from the tissue before sedimentation and this led to a high background of radioactivity in the gradient. Both repeated ammonium sulphate precipitation and passage through Sephadex G-25 were tried in attempts to decrease the background radioactivity without complete success. Another problem may be that the commercially available [^{189}W]tungstate is not of sufficiently high specific radioactivity to allow detection of labelling without further purification of the enzyme.

The requirement for molybdenum as well as for nitrate in the induction of nitrate reductase activity has been shown by Afridi & Hewitt (1964), but the nature of the role played by molybdenum in the formation of functional enzyme is unknown. Molybdenum may act together with nitrate as a co-inducer or it may function solely as a prosthetic group in the enzyme. On the other hand it may do both. The experiments reported here suggest that molybdenum is not required for the formation of nitrate reductase apoenzyme. However, it must be recognized that low concentrations of molybdenum sufficient to act as an inducer may be present in plants treated with tungstate. Alternatively tungstate itself may be an analogue of molybdate that can act as a co-inducer.

The situation in barley where tungstate causes nitrate reductase to be non-functional although 8S NADH–cytochrome *c* reductase activity is over-produced is somewhat analogous to the situation obtaining in nitrate reductase-negative mutants of *Aspergillus*. Such mutants possess constitutive contents of cytochrome *c* reductase. Some of the mutants also lack xanthine dehydrogenase activity, perhaps due to the inability to produce a common factor related to the molybdenum prosthetic group of these enzymes (Pateman, Cove, Rever & Roberts, 1964). Apparently the lack of functional nitrate reductase results in the constitutive formation of cytochrome *c* reductase, and suggests that nitrate reductase is involved in the regulation of its own formation (Cove & Pateman, 1969). Mutants with similar properties have been found in *Neurospora* (Sorger & Giles, 1965) and *Escherichia coli* K12 (Venables, Wimpenny & Cole, 1968). The super-induction of the 8S NADH–cytochrome *c* reductase activity observed in barley in the presence of tungstate may also be a manifestation of a role for functional nitrate reductase in the regulation of its own formation. On the other hand, nitrate accumulates to elevated concentrations in tungstate-treated plants (Fig. 2), and the increased activity of NADH–cytochrome *c* reductase may simply be due to the higher concentration of inducer accumulating in the organism.

A further unexplained observation is the relationship between the 8S enzyme complex and the 3.7S NADH–cytochrome *c* reductase, which is induced to some extent in response to nitrate. The 3.7S enzyme may represent a precursor protein of nitrate reductase or a dissociation product. It is noteworthy that *nit*-1 nitrate reductase-negative mutants of *Neurospora crassa* produce only the NADH–cytochrome *c* reductase component of the nitrate reductase complex and that this component sediments at 3.7S instead of the usual 6.8S for the wild-type enzyme (Sorger, 1966).

The 3.7S enzyme is not a partial activity of nitrite reductase (4.6S) (Fig. 10), the other well known nitrate-induced enzyme (Ingle, Joy & Hageman, 1966). The observation that nitrate reductase and nitrite reductase are physically separable by density-gradient centrifugation does not of course preclude the possibility that they may be associated with each other *in vivo*. This is however, unlikely, since Ritenour, Joy, Bunning & Hageman (1967) have reported that nitrite reductase is associated with the chloroplast, whereas nitrate reductase is localized in the cytoplasm. There thus appears to be no evidence at the moment for the presence of a nitrate reductase–nitrite reductase complex in higher plants analogous to that reported to occur (and dubbed 'nitrosome') in yeast (Sims, Folkes & Bussey, 1968).

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