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NITRITE, HYDROXYLAMINE AND SULPHITE REDUCTASES IN WHEAT LEAVES

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Key Word Index—*Triticum vulgare*; Gramineae; wheat leaf; nitrite, hydroxylamine and sulphite reductases.

Abstract—The inclusion of cysteine and Na-EDTA in the extracting buffer lowered the activity of sulphite reductase extracted from wheat leaves while nitrite and hydroxylamine reductases were not so affected. Maximum activity for the three enzymes was achieved with reduced methyl viologen as the electron donor. The three enzyme activities were found in the chloroplasts. Nitrite reductase was detected in the leaves of the seedlings only when grown with nitrate and exposed to light. Sulphite and hydroxylamine reductases were not, however, influenced by either of these treatments. These results suggest that nitrite reductase is a distinct enzyme and is not associated with sulphite reductase and hydroxylamine reductase in wheat leaves.

INTRODUCTION

Nitrite and sulphite reductases from higher plants have several common features. In the leaves of higher plants both the enzymes are located in chloroplasts [1–3], require reduced ferredoxin for their activity [4–7] and catalyse reductions involving the transfer of six electrons [7, 8]. Murphy *et al.* [9] have shown that nitrite reductase from spinach leaves and sulphite reductase from *E. coli* contain sirohaem as a prosthetic group. This haem-like chromophore absorbs at 582–589 nm [9]. Sulphite reductases from higher plants also exhibit this spectral property [10, 11]. In several organisms purified preparations of sulphite reductase reduce nitrite [12–15] as well as hydroxylamine [16–19]. It has been suggested that the reduction of sulphite, nitrite and hydroxylamine might be catalysed by a single enzyme [12–14, 19–23]. In this paper, the relationship between these three activities is examined and it is concluded that nitrite reductase in wheat leaves is a distinct enzyme from hydroxylamine reductase and sulphite reductase.

RESULTS

Extraction conditions. Activities of nitrite and hydroxylamine reductases were not affected by including either cysteine (10^{-3} M) or cysteine and Na-EDTA (10^{-4} M) in the extracting buffer. However, in the presence of cysteine a much lower sulphite reductase activity was recorded which declined further on supplementing the phosphate buffer with Na-EDTA. In control experiments it was found that cysteine (10^{-3} M) did not interfere with the chemical test for sulphide.

Electron donors. The reduction of sulphite, nitrite and hydroxylamine was mediated by reduced viologen dyes and in each case reduced methyl viologen (MVH) was more effective than reduced benzyl viologen (BVH). Reduced flavin nucleotides ($FMNH_2$ and $FADH_2$) were also utilized to a similar extent by the three reductases, although the activities observed were much lower than for those with MVH. Hydroxylamine reductase, however, utilized BVH more effectively than did the other two enzymes (Table 1). On passing the leaf homogenate (S_{10}) through Sephadex G-10

Table 1. Relative efficiency of reduced viologen dyes and reduced flavin nucleotides for the activities of nitrite, hydroxylamine and sulphite reductases from leaves of wheat plants

Electron donor	Enzyme activities* Nitrite reductase A†	Nitrite reductase B‡	Hydroxylamine reductase A	Hydroxylamine reductase B	Sulphite reductase A	Sulphite reductase B
Methyl viologen (reduced)	100	96	100	100	100	95
Benzyl viologen (reduced)	30	27	60	34	28	30
$FADH_2$	14	13	28	23	20	22
$FMNH_2$	14	13	28	26	15	10

Crude leaf homogenate was passed through a Sephadex G-10 column. The enzyme activity in the crude leaf homogenate and Sephadex G-10 treated extract was determined using different electron donors as described in the Experimental. The activities in the leaf homogenate with reduced methyl viologen as an electron donor were: nitrite reductase and hydroxylamine reductase 0·86 μmol NO_2^- and 0·68 μmol NH_2OH reduced respectively per mg protein in 30 min, and sulphite reductase activity was 18 nmol of S^{2-} produced per mg protein in 60 min.

* Expressed as percentage of the activity in the crude extracts with reduced methyl viologen as electron donor.

† Activity in the crude leaf homogenate.

‡ Activity after passing the extract through Sephadex G-10.

column, the BVH-dependent hydroxylamine reductase activity was considerably diminished. On eluting the column with water and adding the concentrated effluent to the reaction mixture, BVH-hydroxylamine reductase was partially restored. Both MVH- and BVH-dependent hydroxylamine reductase activities had the same pH optimum of 7·8 in 0·1 M phosphate buffer but exhibited differences in their stability on storage. Thus at 0°, after 24 hr the extract retained 65% of the original MVH-hydroxylamine reductase but only 40% when BVH was the reductant.

Thermal stability. Nitrite and sulphite reductases were fairly stable enzymes and lost about 5% of their activities on heating the extract for 20 min at 45°. Under these conditions, however, MVH- and BVH-dependent hydroxylamine reductase activities decreased by 25 and 38% respectively.

Intracellular localization. When chloroplasts were isolated in aqueous media, about 20% of the total hydroxylamine reductase and sulphite reductase activities were found in these preparations and nitrite reductase activity was not detected. The three enzyme activities were, however, found in the soluble fraction (Table 2). When non-aqueous solvents were used to isolate the chloroplasts the bulk of the three enzymes were located in these organelles (Table 3). The ratios of nitrite, hydroxylamine and sulphite reductases to chlorophyll content in the various fractions of the non-aqueous leaf preparation were more or less constant. This suggests that the enzyme activities in the non-chloroplast fractions resulted

Table 2. Intracellular localization of the enzymes in leaves of wheat fractionated by an aqueous technique

Fraction	Nitrite reductase* A	Nitrite reductase* B	Hydroxylamine reductase* A	Hydroxylamine reductase* B	Sulphite reductase† A	Sulphite reductase† B
Crude leaf extract	5·10 (100)‡	0·30	4·20 (100)	0·28	141 (100)	8·3
Chloroplasts	—	—	0·77 (18)	0·20	32·6 (23)	8·4
Mitochondria	—	—	0·27 (6·0)	0·15	10·1 (7)	5·7
Cytoplasm	4·80 (94)	0·52	3·46 (82)	0·36	103·2 (73)	10·9

A, activity per g fr. weight; B, activity per mg protein.

* μmol of substrate reduced in 30 min.

† nmol of sulphide formed in 60 min.

‡ Values in parentheses represent enzyme activity in each fraction as a percentage of that in the crude leaf homogenate. These values were calculated by the formula $x/v \times V/W$, where x is amount of substrate reduced in reaction mixture, v is volume of extract used for assay, V is volume in which each fraction was suspended and W is fr. wt. of tissue used for preparing the fractions.

Table 3. Intracellular localization of the enzymes in wheat leaves fractionated by non-aqueous techniques

Fraction	Nitrite reductase* A	Nitrite reductase* B	Hydroxylamine reductase* A	Hydroxylamine reductase* B	Sulphite reductase† A	Sulphite reductase† B	Cytochrome *f* reductase‡ A	Cytochrome *f* reductase‡ B
Crude leaf homogenate	0·67	14·21	0·22	4·63	12·00	79	2·14	44·7
$d = <1{\cdot}32$	0·72	10·41	0·27	4·88	18·00	82	2·74	47·0
$d = 1{\cdot}32$–$1{\cdot}36$	0·18	12·00	0·06	4·20	5·27	84	0·78	50·0
$d = >1{\cdot}36$	0·18	12·00	0·06	4·20	5·27	84	0·78	50·0

A, activity per mg protein; B, activity per mg chlorophyll. Freeze-dried leaves of 12-day-old plants were homogenized in a mixture of CCl_4 and C_6H_6 (density = 1·32) and then fractionated in mixtures of CCl_4 and C_6H_6 of increasing densities as described in Experimental.

* μmol of substrate reduced in 30 min.

† nmol of S^{2-} formed in 60 min.

‡ μmol ferricyanide reduced per min.

from leaching of these enzymes from the chloroplasts. This was confirmed by a similar distribution for another chloroplast enzyme, cytochrome *f* reductase, which was used as a marker.

Effect of nutritional conditions. Nitrite reductase was found in the leaf extracts only when the seedlings were supplied with nitrate (Table 4). The activities of hydroxylamine reductase and sulphite reductase were not, however, influenced by the inclusion of either nitrate or sulphate in the nutrient solution. Thus nitrite reductase was formed only when nitrate was present in the medium while the other two enzymes were apparently constitutive.

Effect of light. Exposure of the plants to light had a significant effect on the activity of nitrite reductase in the leaves (Table 5). The seedlings grown in light had a 26-fold higher enzyme activity than that of etiolated plants. The seedlings grown in light and transferred to the dark immediately after supplying nutrient solution containing nitrate also exhibited very low nitrite reductase activity. Thus a continued exposure of the seedlings to light was essential for the synthesis of nitrite reductase in the leaves. In contrast, light had no effect on hydroxylamine and sulphite reductases.

DISCUSSION

In several organisms the capacity to reduce nitrite, hydroxylamine and sulphite has been found to be associated with the same protein [12–14, 19–23]. In the present studies possible relationship between these three activities in wheat leaves has been investigated. In crude leaf homogenates the BVH-linked hydroxylamine reductase had a much higher activity than that of either BVH-sulphite or nitrite reductase. However, on passing the extract through Sephadex G-10, the BVH-linked hydroxylamine reductase activity was markedly reduced whereas those for nitrite and sulphite reductase were unchanged by the column treatment.

The results reported here provide information about the subcellular location of these enzymes.

Table 4. Effect of nutritional conditions on the activities of nitrite, hydroxylamine and sulphite reductases in the leaves of wheat seedlings

Solution supplied to the seedlings	Nitrite reductase (μmol NO_2^- reduced per 30 min) A	Nitrite reductase (μmol NO_2^- reduced per 30 min) B	Hydroxylamine reductase (μmol NH_2OH reduced per 30 min) A	Hydroxylamine reductase (μmol NH_2OH reduced per 30 min) B	Sulphite reductase (nmol S^{2-} formed per hr) A	Sulphite reductase (nmol S^{2-} formed per hr) B
Distilled water	0	0	6·40	0·45	181	13·10
KNO_3 (10 mM)	10·40	0·71	6·40	0·44	210	14·10
$MgSO_4$ (5 mM)	0	0	6·24	0·37	210	13·40
$KNO_3 + MgSO_4$	12·60	0·74	6·24	0·38	210	13·40

Nine-day-old wheat plants were supplied with nutrient solutions as specified in the table. After 72 hr the leaves were harvested and the activities of the enzymes determined as described in Experimental.
A, per g fr. wt; B, per mg protein.

Table 5. Effect of light on the activities of nitrite, hydroxylamine and sulphite reductases in the leaves of wheat seedlings

Light treatment	Nitrite reductase (μmol NO_2^- reduced per 30 min)		Hydroxylamine reductase (μmol NH_2OH reduced per 30 min)		Sulphite reductase (nmol S^{2-} formed per hr)	
	A	B	A	B	A	B
Continuous light	14·3	0·86	7·51	0·47	166	9·43
Light → dark	2·20	0·15	6·71	0·47	149	10·40
Etiolated plants	0·55	0·04	5·42	0·44	112	9·32

Two sets of wheat seeds were germinated in light. A third set of seeds was kept in complete darkness. After 9 days, the plants were supplied with the nutrient solution and one set of plants, which had been growing in light, was immediately transferred into the dark (denoted as light → dark). After 72 hr the activities of nitrite, hydroxylamine and sulphite reductases were determined in leaf extracts.

A, activity per g fr. wt; B, activity per mg protein.

Fractionation by aqueous solvents showed that nitrite reductase leaked from chloroplasts to a greater extent than did sulphite and hydroxylamine reductases. This result suggests that nitrite reductase is a distinct enzyme. This suggestion is strengthened further by data on the effect of nutritional conditions and light on the activities of these three enzymes. The leaf extracts of the seedlings grown either without nitrate or without light reduced only sulphite and hydroxylamine (Tables 4 and 5). If the reduction of the three substrates is mediated by the same enzyme, these extracts would be expected to reduce nitrite as well. The results also indicate that nitrite reductase activity can vary independently of those of sulphite and hydroxylamine reductases. These observations confirm that in wheat leaves nitrite reductase is a distinct enzyme as is the case with other plants [6, 10, 11].

The present investigations show that hydroxylamine reductase was not affected by different nutritional conditions and light treatments. This contrasts with the effect on nitrate [7, 24] and nitrite [24] reductases in higher plants. The work of other investigators [7, 25, 26] indicates that hydroxylamine reductase activity in higher plants is not essential for the assimilation of nitrate. In *Neurospora crassa*, a constitutive hydroxylamine reductase was found to function as a sulphite reductase and this enzyme was distinct from a hydroxylamine reductase induced by nitrate [18]. In most of the experiments described herein hydroxylamine and sulphite reductases responded in a similar way. Both the activities were not influenced by supplying either nitrate or sulphate to the plants or by light treatments and they leached out of the chloroplasts to the same extent in aqueous media. The purified preparations of sulphite reductase from higher plants have been shown to invariably reduce hydroxylamine as well [5, 10, 11, 20, 21]. These observations taken along with the constitutive nature of hydroxylamine reductase in wheat leaves suggest that, as in *Neurospora crassa*, the activities of hydroxylamine reductase and sulphite reductase might be associated with the same enzyme. The observed differences in their thermal stability and the effect of including cysteine and EDTA in the extraction buffer on these two activities could be due to differences in the catalytic properties of the active site for these two substrates or two separate active sites might be involved. Such a possibility has been suggested by Asada *et al.* [11] as well.

EXPERIMENTAL

Plant material. Wheat plants (Var. *Insignia*) were grown in vermiculite in an illuminated growth cabinet maintained at 25°. The plants were exposed to a light of 50 g cal/cm²/day for 14 hr daily. Etiolated seedlings were grown in complete darkness. The 9-day-old plants were supplied daily with a nutrient solution containing 10 mM KNO_3 and 2·5 mM $MgSO_4 . 7 H_2O$ and 72 hr later the leaves were harvested for extracting the enzymes.

Preparation of leaf homogenate. Two g of leaves were macerated in 5 ml of 0·1 M Pi buffer (pH 7·5) in a chilled pestle and mortar. The slurry was squeezed through 4 layers of muslin and the homogenate centrifuged at 10000 *g* for 20 min. The supernatant fraction (S_{10}) was the source of the crude enzymes.

Isolation of chloroplasts. (a) *Aqueous technique.* Eight g of leaf material were gently ground in a chilled pestle and mortar with 20 ml of 0·1 M Pi buffer (pH 7·5) containing 0·15 M NaCl and 0·5 M sucrose. Slurry was squeezed through 4 layers of

muslin. Various sub-cellular fractions were obtained by differential centrifugation, according to the procedure described by Pierpoint [27].

(b) *Non-aqueous technique.* The method of Stocking [28] was followed except that the densities of CCl_4–C_6H_6 mixtures used were different. The freeze-dried leaves were extracted in CCl_4–C_6H_6 mixture having a density of 1·32. The second fraction was obtained between the densities 1·32–1·36. The supernatant from the second fraction contained cellular components having density greater than 1·36. These fractions were dried under vacuum and suspended in 0·01 M Pi buffer (pH 7·5) and were kept at 0° overnight. An aliquot was withdrawn from each fraction for determining chlorophyll and protein content and the remainder was centrifuged at 10000 *g* for 10 min. The supernatant fraction thus obtained was used for determining the enzyme activities.

Assay of enzyme activities. Activities of nitrite reductase and hydroxylamine reductase were determined from the rate of utilization of their respective substrates. Sulphite reductase was assayed by following the formation of sulphide. The assays were conducted anaerobically in small tubes (7·5 × 30 mm) fitted with Suba-seals. Except for the different substrates, the reaction mixture for determining the activities of the three enzymes was the same. The reaction mixture in a final volume of 2 ml contained, in μmol: phosphate buffer (pH 7·5), 100; methyl viologen, 0·6; enzyme preparation 0·3–0·7 ml. In addition the substrates for nitrite reductase, hydroxylamine reductase and sulphite reductase were 1·5 μmol $NaNO_2$, 2·5 μmol NH_2OH and 1·0 μmol Na_2SO_3 respectively. The reaction was started by adding 7·5 μmol of freshly prepared $Na_2S_2O_4$ in 1% w/v $NaHCO_3$. After 30 min incubation at 28°, the reaction was terminated by shaking the tubes vigorously until MVH was completely oxidized. Tubes containing boiled extracts and also without MV served as controls. Nitrite and hydroxylamine were determined by the colorimetric method of Griess–Ilosvay and Csaky as described by Hewitt and Nicholas [29]. Sulphide was estimated by Gilboa–Garber's procedure [30]. In some of the experiments BV (0·4 μmol), FAD (0·3 μmol) and FMN (0·3 μmol) were also used as electron carriers. Cytochrome *f* reductase was assayed by the method of Forti [31]. Chlorophyll was determined by the procedure of Arnon [32], and proteins were estimated as described by Lowry *et al.* [33].

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