Some Properties of Two Forms of Nitrite Reductase from Corn (Zea mays L.) Scutellum

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Summary. Nitrite reductase from corn scutellum—a non-chlorophyllous tissue—can use methyl viologen, benzyl viologen or ferredoxin as electron donor. Little or no reduction occurs with nicotinamide or flavin nucleotides. Activity is inhibited by p-chloromercuribenzoate and by cyanide. Organic chelates, with the exception of bathocuproine disulphonate and bathophenanthroline disulphonate, are not inhibitory. Ammonia is the reaction product. Ion exchange chromatography resolves the nitrite reductase activity into two peaks which apparently represent two forms of the enzyme. Both have a molecular weight of 61–63000 as determined by molecular exclusion chromatography, and a pH optimum of 6.7–6.8. Although their properties are generally similar, they show a marked difference in thermal stability, ionic charge and behaviour during isoelectric focusing. Nitrite reductase is found largely in the soluble fraction although some particulate activity is also obtained. Both forms of the enzyme are present in the soluble and particulate fractions.

Introduction

Present evidence concerning the intracellular location and electron donor requirements of the leaf nitrite reductase suggests that the reduction of nitrite is most probably coupled to photosynthetic electron transport at the level of ferredoxin. This subject has been reviewed by Hewitt, Hucklesby and Betts (1968) and by Beevers and Hageman (1969). Recent experiments by Swader and Stocking (1971) seem to support the chloroplastic location of nitrite reduction. This simple view is challenged however by the studies of Grant, Atkins and Canvin (1970) and Grant and Canvin (1970).

Nitrite reduction in non-green tissues must be expected to depend upon a different energy source and to use an electron donor other than ferredoxin, since ferredoxin seems to be present only in chlorophyllous tissue. The nature of the process in non-chlorophyllous tissues is not well understood. The involvement of ATP in some types of nitrite reduction is supported by the studies of Bourne and Miflin (1970). Nitrite reductase activity in extracts of tomato roots (Sanderson and Cocking, 1964) and barley roots (Miflin, 1967) was demonstrated using synthetic compounds—the reduced viologen dyes—as electron donors.
Nitrite Reductase from Corn

Although the physiological electron donor has not yet been described, it is interesting to notice that ferredoxin from a leaf source can substitute for the viologen dyes in the case of enzymes from corn scutellum (Hucklesby and Elsner, 1969) and barley roots (Miflin, 1970a).

The intracellular location of the barley root nitrite reductase has been studied by Miflin (1970b). Activity was associated with both soluble and particulate fractions. It is not clear at present whether this indicates the presence of two enzymes differently localized or whether the soluble enzyme is derived from particles broken during the extraction procedure. Enzyme preparations made from leaves by total extraction in hypotonic solutions and subsequently fractionated by the standard techniques for soluble enzymes appear to contain only one nitrite reductase (Ramirez et al., 1966; Hucklesby and Hewitt, 1970).

Materials and Methods

Plant Material. Corn seed (Ohio 43 × B14 from Crowe's Hybrid Seed Co., Milford, Illinois, U.S.A.) was germinated on paper towels with a nutrient solution containing 30 mM KNO₃, 0.4 mM KH₂PO₄, 0.8 mM CaCl₂, 1.6 mM MgSO₄, 0.2 mM K₂SO₄, 1.14 mg/l H₃BO₃, 3.62 mg/l MnCl₂, 4 H₂O, 1.4 mg/l ZnSO₄·7 H₂O, 0.16 mg/l CuSO₄·5 H₂O, 0.08 mg/l H₃MoO₄·4 H₂O, 0.42 mg/l FeEDTA. The seedlings were harvested after 4 days' growth at 30°C and the scutella were removed by hand.

Chemicals. Special chemicals were obtained from the following sources: N,N'-dibenzyl-4,4'-dipyridylium dichloride (benzyl viologen), sodium dithionite from British Drug Houses, Poole, England; N,N'-dimethyl-4,4'-dipyridylidium dichloride (methyl viologen) from Sigma Chemical Co., St. Louis, Mo., U.S.A.; 4,7-diphenyl-1,10-orthophenanthroline disulphonate (bathophenanthroline disulphonate); 2,9-dimethyl-4,7-diphenyl-1,10-orthophenanthroline disulphonate from Eastman Kodak, Rochester 3, N.Y., U.S.A.; palladized asbestos from Engelhard Inc., Newark, 14, N.J., U.S.A.; DEAE-cellulose (Cellex D, exchange capacity 0.76 meq/g) from Bio-Rad, Richmond, California.

Ferredoxin. This was prepared from leaves of Datura stramonium L. essentially as described by Joy and Hageman (1966). The principal modifications were the use of 50–75% concentration limits during the acetone precipitation, and the insertion of a final chromatographic step on a DEAE-cellulose column. Ferredoxin was eluted from this column with a linear gradient of chloride concentration from 0.2 to 0.8 M.

Preparation of Enzyme Extracts. Excised corn scutella were homogenized for 90 s in a homogenizer (Vertis Research Equipment, Gardiner, New York) at 1/2 line voltage with 4 volumes (ml/g) of extracting medium containing 0.025 M potassium phosphate, 5 mM EDTA and 1 mM cysteine hydrochloride, adjusted to a final pH of 8.8. After homogenisation the pH was approximately 7.5. The homogenate was filtered through 4 layers of muslin and centrifuged at 27000 g for 30 min. The supernatant was dialysed for 2–4 h against a solution of 0.02 M potassium phosphate, pH 7.7, containing 0.1 mM dithiothreitol, and then applied to a column of DEAE-cellulose (15 × 1.7 cm) equilibrated with the same buffer. The column was eluted at a constant pH of 7.7 by a linear gradient of sodium chloride passing from zero to 0.2 M in a volume of 150 ml. The flow rate through the column was 20 ml/h. Potassium phosphate and dithiothreitol concentrations were maintained
at 0.02 M and 0.1 mM respectively during the elution. Fractions were collected and assayed for nitrite reductase.

**Assay of Nitrite Reductase.** Benzyl or methyl viologen reduced with dithionite were used as electron donors for the enzyme. The disappearance of nitrite or formation of ammonia was followed.

Method 1. This was based on the method of Miflin (1967) and was found to be more sensitive than Method 2. Enzyme preparation (0.05—0.2 ml) was added to a solution containing 0.3 mg methyl viologen, 0.5 μmol sodium nitrite and 0.3 mmol potassium phosphate, at a final pH of 6.8. The reaction was started by the addition of 0.5 ml sodium dithionite (72 mg in 10 ml of 0.05 M potassium phosphate, pH 6.9), giving a final volume of 3.0 ml. After 10—20 min incubation at 30°C, the reaction was stopped by agitation of the reaction mixture with a Vortex mixer (Scientific Industries, Springfield, Mass., U.S.A.). Two aliquots (0.25 ml) of the reaction mixture were assayed for nitrite by the addition of 4 ml of a solution containing 0.5% sulphanilamide, 0.01 M N-(1-naphthyl ethylene diamine dihydrochloride) and 1.5 N HCl.

Method 2. This resembled Method 1, except that benzyl viologen (0.3 mg) was substituted for methyl viologen. The pH of the reaction mixture was 7.5 before addition of the sodium dithionite (0.5 ml of solution containing 7.2 mg/ml of dithionite in 0.05 M potassium phosphate, pH 6.5).

Method 3. For the assay of nitrite reductase in column eluates a quick and sensitive small scale assay was used. Enzyme preparation (0.05—0.2 ml) was added to a solution containing 10 μmol potassium phosphate, 0.1 μmol sodium nitrite and 0.14 μmol benzyl viologen at a final pH of 7.5. The reaction was started by the addition of 0.1 ml sodium dithionite (72 mg in 10 ml of 0.05 M potassium phosphate, pH 6.5). After 10—20 min incubation at 30°C 7.5 ml water was added and the solution agitated for 10 s with a Vortex mixer. Nitrite was estimated as described above.

Ammonia was determined by the microdiffusion method of Cedrangingo, Salvatore, Cimino and Zappia (1965) in conjunction with the colourimetric method described by Conway (1962). Protein was precipitated by the addition of an equal volume of 10% trichloroacetic acid. The precipitate was redissolved in 0.1 M sodium hydroxide and protein determined by the method of Lowry, Rosebrough, Farr and Randall (1951).

Nitrite reductase was induced in scutella from ammonia-grown plants by immersion for 5½ h in aerated solutions of 0.05 M potassium phosphate, pH 5.5, containing 1 mM potassium nitrite.

The various solutions used for isotonic extraction of nitrite reductase were as follows:—A. 0.5 M sucrose, 0.05 M potassium phosphate, pH 7.5, 1 mM cysteine hydrochloride. B. Tris-HCl, 0.05 M, pH 7.5, 0.4 M sucrose, 1 mM dithiothreitol, 0.01 M KCl, 0.1 mM MgCl₂, 1% bovine serum albumin (adapted from Breidenbach and Beevers, 1967). C. 0.2 M sucrose, 0.3 M mannitol, 0.05 M potassium phosphate, pH 7.8, 0.005 M EDTA, 1% polyvinyl pyrrolidone, 0.005 M MgCl₂, 1 mM GSH (Miflin, 1970). Tissue was ground gently with chilled mortar and pestle using four volumes (v/w) of isotonic solution at 0°C. The extract was strained through four layers of muslin. Cell debris etc. was removed by centrifugation at 270 or 500 g for 10 min and then the supernatant fraction recentrifuged at 20000 g. The pellet from this centrifugation was resuspended in isotonic medium and the particulate fraction again separated by centrifugation at 20000 g. The final pellet, resuspended in hypotonic medium (0.05 M potassium phosphate, pH 7.5) and the supernatant from the first 20000 g centrifugation were assayed for nitrite reductase by Method 2.

**Iso-Electric Focusing.** This was carried out in a 110 ml LKB column, at 4°C, with sucrose as non-ionic solute. The anode was at the bottom of the column.
Results

Electron Donors. The scutellum nitrite reductase accepts electrons from benzyl viologen or methyl viologen reduced with dithionite (Table 1). Reduced nicotinamide nucleotides, flavin nucleotides and several other redox compounds are ineffective. Ferredoxin prepared from a leaf source substitutes readily for the viologens. Some reaction occurs between dithionite-reduced ferredoxin and nitrite in the absence of enzyme, representing 5-15% of the activity of the complete system. Ferredoxin reduced enzymatically by glucose-6-phosphate and NADP can also donate electrons for nitrite reduction (Table 1); the necessary intermediary enzymes for this electron transfer—that is glucose-6-phosphate dehydrogenase and an NADP specific enzyme which transfers electrons to ferredoxin—are present in the scutellum extracts. Although this system consists entirely of physiological components, it cannot be regarded as the in vivo system since the ferredoxin is supplied from an

Table 1. Trial of electron donors with nitrite reductase of corn scutellum extracts. The reaction mixture contained 15 μmol potassium phosphate pH 7.5, 500 nmol sodium nitrite in all cases, in a final volume of 3.0 ml. Other quantities were 1.83 mmol benzyl viologen, 3.6 mg sodium dithionite, 7.5 μmol NADPH, 20 μmol glucose 6-phosphate, 20 μmol glyceraldehyde-3-phosphate, 1.5 μmol FMN, 1.5 μmol ascorbate, 0.23 mg ferredoxin partially purified from leaves of Datura stramonium L. Menadione dissolved only partially, and its concentration is not known. The enzyme preparation was an unpurified extract prepared by homogenization and centrifugation only. 0.5 ml of this was added to the reaction mixture. The table shows data extracted from several experiments.
Fig. 1. Separation of two peaks of nitrite reductase activity by gradient elution from DEAE-cellulose. Corn scutellum extracts were prepared and fractionated on DEAE-cellulose as described in "Methods". 0.1 ml aliquots of the eluate fractions were assayed for nitrite reductase using Method 3. Incubation was for 15 min. 5.5 ml fractions were collected from the column.

extra-scutellar source and is not thought to be a constituent of non-green tissues.

Separation of Nitrite Reductase Enzymes. Corn scutellum tissue was homogenized in 0.025 M potassium phosphate, pH 8.8, and chromatographed on a column of DEAE-cellulose as described in "Methods". Two separate peaks of nitrite reductase activity (Fig. 1) were eluted at chloride concentrations of 0.18 and 0.23 M respectively, in a highly reproducible and predictable manner. The enzymes responsible for the peaks of activity will be referred to as NiR₁ and NiR₂, indicating the order of their elution from the column. For corn scutellum tissues of the hybrid used in these experiments (Ohio 43 × B14), the ratio of peak heights (NiR₁/NiR₂) was usually 1.0–1.25, i.e. there was some tendency for NiR₁ to be slightly more active (fresh weight basis) than NiR₂. Extracts of corn leaves chromatographed in the same manner showed only one peak of activity.

In order to check whether the resolution of nitrite reductase into components is an artefact, the gradient elution from DEAE-cellulose was repeated on enzyme preparations which had been prepared by a range of extraction methods. Patterns of elution indistinguishable from those described in the preceding paragraph were obtained after the following treatments:

1. Homogenization of the tissue in higher concentrations of potassium phosphate, (0.1 M, pH 7.5) + 1 mM cysteine-HCl + 5 mM EDTA.
2. Homogenization in 0.025 M tris-acetate, pH 8.5 + 10 mM cysteine-HCl + 5 mM EDTA.

3. Homogenization in potassium phosphate (0.025 M, pH 8.8) + 1 mM cysteine-HCl + 5 mM EDTA, followed by acetone precipitation (40–60% v/v). The acetone-precipitated protein was redisolved in 20 ml of potassium phosphate 0.02 M, pH 7.7 + 0.1 mM dithiothreitol for introduction to the column.

4. As (3), but substituting ammonium sulphate (0–70%) for acetone in the precipitation step. After resuspension of the ppt. the preparation was dialyzed against 0.02 M potassium phosphate, pH 7.7 + 0.1 mM dithiothreitol before introduction to the column.

5. Homogenization at −12°C in 10 vol (v/w) of cold acetone to make an acetone powder. After drying, this powder was extracted with 20 vol (v/w) of 0.02 M potassium phosphate buffer, pH 7.7 + 0.1 mM dithiothreitol for introduction to the column.

6. Extraction in isotonic sucrose solutions. This procedure is discussed in the section on subcellular localization.

7. Rechromatography of both enzymes. Preparations of NiR₁ and NiR₂ were made by gradient elution from a DEAE-cellulose column, as described in “Methods”. The two enzymes were then dialysed, adsorbed on separate columns of DEAE-cellulose, and eluted again in the same manner. One peak of activity was obtained from each column. Elution volumes were unaltered by the rechromatography.

**Induction.** The induction of nitrite reductase has been observed previously in leaves (Ingle, Joy, and Hageman, 1966) and in non-green tissues (Hucklesby and Eisner, 1969; Ferrari and Varner, 1970).

Corn scutella from four-day old seedlings germinated with 5 mM ammonium chloride as the nitrogen source display no major peaks of nitrite reductase activity when eluted from DEAE-cellulose. A similar fractionation after 5½–7 h immersion in an aerated solution of nitrite, which gives maximal induction of activity, revealed both peaks of nitrite reductase activity (0.18 M and 0.23 M) in the column eluates, indicating that both enzymes 1 and 2 are nitrite-inducible. Total nitrite reductase activity of scutella following induction under these conditions was 60–70% of that obtained from scutella of nitrate-grown seedlings.

Although the peaks of induced nitrite reductase activity were eluted at the same chloride concentrations as those obtained from nitrate-grown material, the ratio of activities (NiR₁/NiR₂) was quite different. The ratios in three experiments were 0.64, 0.50 and 0.51 (mean = 0.55); by contrast, material grown on nitrate exhibited ratio values of 1.0 to 1.25. The cause of this difference is unknown.

**Molecular Weight.** The two enzymes were separated by gradient elution from DEAE-cellulose and then chromatographed consecutively.
Fig. 2. Effect of cyanide concentration on activity of corn scutellum nitrite reductase enzymes. The enzymes NiR₁ and NiR₂ were separated by chromatography on DEAE-cellulose. The reaction mixture was 1.4 mg sodium dithionite, 200 μmol sodium nitrite, 17 μmol potassium phosphate, pH 7.5 and 0.29 μmol of benzyl viologen in a final volume of 1.0 ml. Cyanide was added immediately before assay. 0.1 ml aliquots of enzyme were assayed. Incubation was for 20 min.

Reaction Product. Both enzymes catalyse the reduction of nitrite to ammonia. In three experiments, ammonia was obtained from nitrite with 98, 108 and 101 per cent recovery (mean ±102) for NiR₁ and 96, 115 and 110 per cent recovery (mean ±107) for NiR₂. Purification of either NiR₁ or NiR₂, approximately 100-fold, does not alter the stoichiometry of conversion of nitrite to ammonia.

Inhibitors. The effect of a range of inhibitors is shown in Table 2. Cyanide was inhibitory to both enzymes (50% inhibition at 8 × 10⁻⁵ M, 100% at 5 × 10⁻⁴ M). Curves relating cyanide concentration to activity (Fig. 2) corresponded very closely for the two enzymes. Inhibition by cyanide was immediate, which, together with the severity of inhibition, suggests the involvement of a metal at the active centre. Carbon monoxide was moderately inhibitory. Metal-chelating reagents with the exception of bathophenanthroline disulphonate and bathocuproine disul-
Table 2. Effect of inhibitors on nitrite reductases from corn scutellum. The two nitrite reductases were separated by DEAE-cellulose chromatography. Nitrite reductase was assayed by Method 2. Inhibitors were added to the reaction mixture immediately before assay. Incubation was for 20 min. The full chemical names of bathophenanthroline and bathocuproine are given in "Materials". Carbon monoxide was bubbled through a solution of the dithionite-reduced enzyme and activity was compared with a control through which nitrogen was passed.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration of inhibitor (mM)</th>
<th>Activity-% control NiR₁</th>
<th>Activity-% control NiR₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA</td>
<td>1</td>
<td>108</td>
<td>92</td>
</tr>
<tr>
<td>Diethylidithiocarbamate (Na)</td>
<td>1</td>
<td>115</td>
<td>106</td>
</tr>
<tr>
<td>8-Hydroxyquinoline</td>
<td>1</td>
<td>97</td>
<td>91</td>
</tr>
<tr>
<td>α,α'-Dipyridyl</td>
<td>1</td>
<td>106</td>
<td>104</td>
</tr>
<tr>
<td>o-Phenanthline</td>
<td>1</td>
<td>107</td>
<td>96</td>
</tr>
<tr>
<td>Bathophenanthroline disulphonate</td>
<td>1</td>
<td>34</td>
<td>48</td>
</tr>
<tr>
<td>Bathocuproine disulphonate</td>
<td>1</td>
<td>47</td>
<td>33</td>
</tr>
<tr>
<td>Potassium cyanide</td>
<td>0.1</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Carbon monoxide</td>
<td></td>
<td>74</td>
<td>50</td>
</tr>
<tr>
<td>Hydrazine sulphate</td>
<td>1</td>
<td>106</td>
<td>94</td>
</tr>
<tr>
<td>Isonicotinic acid hydrazide</td>
<td>1</td>
<td>84</td>
<td>94</td>
</tr>
<tr>
<td>p-Chloromercuribenzoate</td>
<td>0.5</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>p-Chloromercuribenzoate + GSH (2.5 mM)</td>
<td>0.5</td>
<td>85</td>
<td>83</td>
</tr>
</tbody>
</table>

Cyanate were not inhibitory. Reagents which react with carbonyl groups did not affect enzyme activity or were only slightly inhibitory. Little or no inhibition was observed in the presence of 1 mM arsenite, 1 mM arsenate, 1 mM fluoride, antimycin A, rotenone or n-heptyl-4-hydroxyquinoline N-oxide.

Ammonium persulphate—a gelling agent frequently used in acrylamide gel electrophoresis—was completely inhibitory to both enzymes at 10⁻⁵ M.

Effect of Time, Enzyme Concentration and Mixing of Enzymes. The amount of nitrite reduced was linearly related to time of reaction and to enzyme concentration. Activities of the two enzymes were additive over a range of enzyme concentrations. No evidence of interaction was obtained.

Nitrite Concentration. Values of Km for NiR₁ were 0.084, 0.040 and 0.035 mM (mean = 0.053 mM) and for NiR₂ were 0.060, 0.052 and 0.018 mM (mean = 0.045 mM) for three separate preparations from corn scutella. These values fall within the range of Km values for leaf nitrite reductase (10⁻⁴ to 10⁻⁶ M) reported by other workers. At concentrations of nitrite above 0.1 mM, the kinetics of both forms of enzyme are of non-Michaelis-Menten type, and require further investi-
Table 3. Thermal inactivation of nitrite reductase enzymes. The two nitrite reductase enzymes were separated by DEAE-cellulose chromatography. Enzyme preparation was maintained at the stated temp. for 10 min and then rapidly cooled to 0°C. Activity was measured by method 2, using 0.3 ml aliquots of enzyme preparation. Incubation was for 15 min at 30°C. Nitrite reductase activity is given as percentage of the activity of enzyme preparation which had been maintained at 0°C throughout the experiment.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Activity, % control at 0°C</th>
</tr>
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<tbody>
<tr>
<td>Nitrite reductase 1</td>
<td>Nitrite reductase 2</td>
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<tr>
<td>-----------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>30.5</td>
<td>93</td>
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<tr>
<td>36.5</td>
<td>88</td>
</tr>
<tr>
<td>47.0</td>
<td>23</td>
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<td>3</td>
</tr>
<tr>
<td>57.0</td>
<td>0</td>
</tr>
<tr>
<td>62.0</td>
<td>4</td>
</tr>
</tbody>
</table>

In 1968, Hewitt, Hucklesby and Betts noted the wide variation in reported Km values for leaf nitrite reductase, but were unable to relate discrepancies to particular experimental conditions.

Effect of pH. Both nitrite reductase enzymes have maximal activity at pH 6.7–6.8 (Fig. 3). Below pH 6.5 a chemical reaction, recalling that described by Hucklesby and Hewitt (1970) for benzyl viologen, occurs between reduced methyl viologen and nitrite. Appropriate blanks were used to correct for loss of nitrite by chemical reaction in the pH range 6.0—6.5.

Effect of Heat. Enzyme NiR₁ was inactivated more readily than NiR₂ by moderate rises in temperature (Table 3). The difference was especially marked in the range 47–57°C.

Intracellular Localization. Scutellum tissue was gently ground with isotonic solution at 0°C. Various types of extracting solution were used; their composition and the extraction technique is given in Methods.

The supernatant from the first centrifugation at 20000 g and the final particulate fraction were assayed for nitrite reductase. Most of the activity was found in the supernatant fractions. The amount of activity associated with the particulate fraction was variable and usually small, although not insignificant. The mean per cent total activity found in the particulate fraction was 17.0 in eleven experiments, with a maximum of 37.4 and a minimum of 1.5 per cent.

At least a portion of the nitrite reductase activity therefore appears to be particulate in nature. Miflin (1970b) has discussed the significance
Fig. 3. Activity-pH curve for corn scutellum nitrite reductases. The two enzymes were separated by DEAE-cellulose chromatography. Aliquots of the enzymes were assayed by Method 1. Reaction mixtures were buffered with potassium phosphate throughout. Incubation was for 15 min. The observed pH values of the reaction mixtures, measured with a Beckman "Expandomatic TM" pH-meter after completion of the assay, are plotted against activity. NiR₁ ——, NiR₂ ———

of the presence of soluble and particulate nitrite reductase activity in barley root extracts. The supernatant activity observed with both corn scutellum and barley root extracts may represent enzyme which has solubilized during preparation. Another possibility is the location of nitrite reductase in both cytoplasm and particles. If the latter were true of scutellum nitrite reductase, NiR₁ and NiR₂ could represent differently localized enzymes. In order to test this, particulate and soluble fractions were prepared as described above using Miflin's (1970b) isotonic solutions for initial extraction and for washing and resuspension of the pellet from the 20000 g centrifugation. Although the particulate activity obtained in this experiment was only 13% of the total, enough scutellum material (15 g) was used to yield sufficient activity for subsequent chromatography. After dialysis with hypotonic solution (0.02 M potassium phosphate, pH 7.7, + 1 mM glutathione) the supernatant and particulate fractions were chromatographed concurrently on columns
Fig. 4. Distribution of nitrite reductase after 21 h of iso-electric focusing at 500 V with pH 3–6 Ampholytes. 2 ml fractions were collected, and 0.2 ml aliquots assayed for nitrite reductase —○— (Method 3). —●— pH value

of DEAE-cellulose having similar dimensions. These columns were eluted in parallel with linearly increasing concentration of chloride dispersed from a common gradient maker. Running rates were synchronized by use of a Buchler "Polystaltic" pump. The particulate and supernatant fraction when chromatographed gave identical elution patterns of NiR₁ and NiR₂. The activity ratios NiR₁/NiR₂ were 1.15 and 1.13 for the soluble and particulate fractions respectively.

Nitrite Reductase Content of Related Genetic Materials. Seeds of Zea mays L. vars Ohio 43 and B14 were obtained from Crowe's Hybrid Seed Co., Milford, Illinois, U.S.A. These varieties represent the inbred parental lines of the hybrid seed Ohio 43 × B14 used in the experiments described above. Enzyme extracts were prepared from scutella of four-day old seedlings and nitrite reductase activity was fractionated on a DEAE-cellulose column as described in "Methods". The two enzymes NiR₁ and NiR₂ were obtained from extracts of both varieties, eluting at 0.16 and 0.21 M chloride concentrations respectively. The ratios of activities \( \frac{\text{NiR}_1}{\text{NiR}_2} \) differed between the varieties. Values of 2.20 and 1.07 were obtained from B14 and Ohio 43 respectively.

Iso-Electric Focusing. Nitrite reductase (NiR₂) is obtained in a single symmetrical peak of activity after iso-electric focusing (Fig. 4). The iso-electric point is approximately 4.5. By contrast, NiR₁ did not survive iso-electric focusing.

Discussion

Scutellum extracts contain two nitrite reductase enzymes, both of which are induced by the substrate. That these are genuine constituents
of the scutellum, rather than extraction artefacts, is suggested by the high degree of reproducibility observed in their preparation by ion exchange chromatography, with respect both to ratio of activities of the two enzymes and their position in the elution pattern. Further evidence is provided by the maintenance of their chromatographic characteristics following extraction by a range of methods. Two similar forms of nitrite reductase have been obtained from all non-chlorophyllous tissues of corn (*Zea mays* L.) so far examined, while green tissues from the same species contain only one (this laboratory, unpublished).

No difference could be detected in molecular weight as measured by molecular exclusion chromatography. The two enzymes therefore do not represent components of different molecular weight in an oligomeric system: neither is one enzyme formed from the other by cleavage into sub-units during extraction. The possibility that both are sub-units of a larger functional enzyme is improbable, since no activity was found in the earlier eluting fractions from the column of Sephadex G 100.

The physiological electron donor for the scutellum nitrite reductase is unknown. The two enzymes can accept electrons from benzyl or methyl viologen or from leaf ferredoxin. Menadione and FMNH$_2$ have a slight tendency to donate electrons to the system. A slow reaction in the presence of high concentrations of FMNH$_2$ has been noted with the nitrite reductase from leaves of marrow and spinach (Hewitt *et al.*, 1968; Hucklesby and Hewitt, 1970). Nitrite reductases 1 and 2 do not seem to have different electron donor requirements. This is in marked contrast to the two nitrate reductases of the scutellum which differ in specificity towards NADH and NADPH (Hucklesby and Elsner, 1969). Hydroxylamine reductase enzymes from the same tissue also can be distinguished by their electron donor requirements (unpublished).

Both enzymes responded in a similar manner to a range of inhibitors, especially cyanide (Table 2, Fig. 2). The sensitivity of both nitrite reductase enzymes to inhibition by cyanide, in conjunction with the failure of several organic chelating reagents to impair the reaction, suggests the presence of a metal which is tightly co-ordinated into the enzyme structure. Inhibition was obtained with bathophenanthroline disulphonate and bathocuproine disulphonate, which have particularly high affinities for iron (Fe$^{2+}$) and copper (Cu$^{+}$) respectively. Bathophenanthroline disulphonate is an inhibitor of nitrite reductase from marrow leaves (Hucklesby, Hewitt, and James, 1970). The nature of this inhibition is being investigated. Compounds reacting with carbonyl groups have little or no effect on either enzyme. On the other hand, p-chloromercuribenzoate inhibits both NiR$_1$ and NiR$_2$. Glutathione affords protection against inhibition.
The enzymes NiR₁ and NiR₂ do not seem to represent soluble and particulate nitrite reductases. Similarities in electron donor requirement, inhibition properties, pH optimum and Km value for nitrite suggest the absence of fundamental differences in the nature of their active centres. The differences so far noted—in thermal stability, ionic charge and in behaviour during iso-electric focusing—are compatible with a difference in structure of the amino acid chain. Unfortunately, the nature of the scutellum limits the availability of tissues and at present precludes purification on a scale large enough to subject the enzymes to amino acid analysis. It is possible that the two forms of nitrite reductase are differently regulated. In this connection it should be noticed that both forms of the enzyme give non-hyperbolic relationships between nitrite concentration and activity.

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References


