The Purification and Properties of Nitrite Reductase from Higher Plants, and its Dependence on Ferredoxin

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1. NADPH-dependent nitrite reductase from the leaves of higher plants was purified at least 70-fold and separated into two enzyme fractions. The first enzyme, a diaphorase with ferredoxin-NADP-reductase activity, is required only to transfer electrons from NADPH to a suitable electron acceptor, which then donates electrons to nitrite reductase proper. 2. Purified nitrite reductase accepted electrons from ferredoxin (the natural donor) or from reduced dyes. Ferredoxin was reduced by illuminated chloroplasts or dithionite, or by NADPH when diaphorase was present. The purified enzyme did not accept electrons directly from NADPH. 3. Ferredoxins purified from maize, spinach or *Clostridium* were interchangeable in the nitrite-reductase system. 4. Nitrite reductase had K_m 0.15 mm for nitrite. The pH optimum varied with plant and method of assay. The preparation had low sulphite-reductase activity. Ammonia was the product of nitrite reduction. 5. For some plants, the assay of crude preparations with NADPH was limited by diaphorase and the addition of diaphorase gave a better estimate of nitrite-reductase activity. A simple method of assay is described that uses dithionite with benzyl viologen as electron donor.

The enzyme nitrite reductase is classified as being NADPH-dependent (EC 1.6.6.4), and enzyme systems involving nitrite and reduced nicotinamide nucleotide have been described (Nason, Abraham & Averbach, 1954; Vaidyanathan & Street, 1959; Roussos & Nason, 1960). In many cases no correspondence was found between nitrite loss and ammonia production, and Sanderson & Cocking (1964) have outlined various criticisms of the earlier work. The observations of Hageman, Cresswell & Hewitt (1962) and Cresswell, Hageman, Hewitt & Hucklesby (1965) indicated that extracts of *Cucurbita* contained a system that utilized substrate amounts of the reduced dye BV† as electron donor for the reduction of nitrite to ammonia. The system was also operative with NADPH plus catalytic amounts of BV and an endogenous diaphorase, and it was suggested (Hageman *et al.* 1962) that the BV was substituting for an unidentified natural cofactor.

A connexion between light and nitrite reduction has been reported (C. F. Cresswell, unpublished work cited by Hageman *et al.* 1962). With the

observations that naturally occurring electron-transporting proteins, the ferredoxins, were important not only in bacteria (Mortenson, Valentine & Carnahan, 1963) but also in photosynthetic mechanisms of higher plants (Davenport, 1960; Tagawa & Arnon, 1962; Hill & San Pietro, 1963), it became apparent that ferredoxin might also be involved in nitrite reduction. A number of workers have reported reduction of nitrite by illuminated chloroplasts in systems that presumably contained ferredoxins, or to which ferredoxins had been added (Husisige & Satoh, 1961; Hewitt & Betts, 1963; Losada, Paneque, Ramirez & Del Campo, 1963; Sanderson & Cocking, 1964). In addition, Losada *et al.* (1963) and Paneque, Ramirez, Del Campo & Losada (1964) found that an enzyme with 'TPN-reductase' activity brought about reduction of nitrite when added ferredoxin was reduced by *Clostridium* hydrogenase, and they considered the possibility that NADPH-nitrite reductase might involve reversal of the 'ferredoxin-TPN reductase' (EC 1.6.99.4) described by Tagawa & Arnon (1962) and Shin, Tagawa & Arnon (1963). Further complication in the nature of nitrite reductase came with the finding that a bacterial nitrite reductase (Lazzarini & Atkinson, 1961) was in reality a sulphite reductase (EC 1.8.1.2) (Kemp, Atkinson, Ehret & Lazzarini, 1963).

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† Abbreviations: BV, benzyl viologen (*NN'*-dibenzyl-4,4-bipyridylum dichloride); GPD, glucose 6-phosphate dehydrogenase.

In the present paper a new assay for nitrite reductase is described that uses a chemical electron donor. Evidence is presented that nitrite reductase proper, from maize and spinach, is distinct from sulphite reductase and NADPH-ferredoxin reductase, although the latter enzyme is required when NADPH is the electron donor. An attempt was made to clarify the role of ferredoxin and other electron-transferring factors in nitrite reduction, and the properties of the purified nitrite reductase are outlined.

MATERIALS AND METHODS

Plant material. Fresh spinach (*Spinacea oleracea*) was obtained from the grocery store or field; prominent midribs and petioles were removed. Maize (*Zea mays*) was grown in soil in a greenhouse for about 4 weeks (young tissue) or was obtained from field-grown plants (old tissue).

Preparation of nitrite reductase and ferredoxin. The method is based on a procedure for ferredoxin preparation used in E. J. Hewitt's Laboratory (personal communication) that is similar to that of San Pietro & Lang (1958) and Tagawa & Arnon (1962). All operations were carried out as near to 0° as possible. Fresh leaf tissue was frozen (−25°) and crushed in a precooled mortar. Cold tris-HCl buffer, pH 7.8, was added at the rate of 1 ml. of 0.7M-buffer/100g. of fresh spinach or 100 ml. of 0.05M-buffer/100g. of fresh young maize; older maize tissue was homogenized in a blender for 2 min. with twice the amount (w/v) of the dilute (0.05M) tris-HCl buffer, pH 7.8. The mixture was squeezed through four layers of cheesecloth. Acetone at −25° was added to give 33% (v/v) concentration, and the resulting precipitate was removed by centrifugation and discarded. More acetone was added to the supernatant to bring the concentration to 75% (v/v). The precipitate, collected by centrifugation, was dissolved in 0.07M-tris-HCl buffer, pH 7.3, usually in a volume equivalent to about 20 ml./100g. of original tissue. This solution was dialysed overnight against three changes of at least 40 vol. of the same buffer. After dialysis the solution was made 0.2M with respect to NaCl and run on to a column of DEAE-cellulose previously equilibrated with the 0.07M-buffer. The effluent that ran through the column was a crude preparation of nitrite reductase with several other enzymes, but was free from ferredoxin, which remained on the column. This crude enzyme preparation was used in many of the experiments described below. By washing the column with tris-HCl buffer, pH 7.3, of increasing molarity, a preparation of ferredoxin was eluted at about 0.9M-buffer. Enzymes and ferredoxin were stored in small batches at −25°. Further purification of nitrite reductase is described below.

For all but the preliminary work, a single batch of spinach ferredoxin was prepared and purified by rechromatography on DEAE-cellulose.

Crude diaphorase. Diaphorase for use with the NADPH-GPD assay was prepared by heating small batches of the crude enzyme preparation described above at 60° for 5 min. Precipitated protein was removed by centrifugation.

Assay of nitrite reductase. (1) NADPH-GPD-linked nitrite-reductase system. NADPH was generated by means of GPD. Both BV and ferredoxin were included as electron

carriers. The assay mixture (3 ml.) contained (μmoles): potassium phosphate buffer, pH 7.5, 120; KNO₂, 0.9; MgCl₂, 10; BV, 1.0; NADP⁺, 0.3. Also included were: 0.3–0.6 unit of GPD; 0.05 ml. of spinach ferredoxin (containing 2.5 mg. of protein/ml.); 0.2 ml. of spinach diaphorase. The reaction mixture was placed in Thunberg tubes standing in ice. After addition of the enzyme the reaction was started by adding 4 μmoles of glucose 6-phosphate (sodium salt), and the tubes were evacuated and incubated at 30° for 30 min. or less. The reaction was terminated by mixing 1 ml. portions with 0.2 ml. of M-zinc acetate, 5 ml. of 95% (v/v) ethanol then was added and the resulting precipitate, containing NADPH and some protein, was removed by centrifugation (Hewitt & Nicholas, 1964). Samples (1 ml.) of the supernatant were assayed for nitrite by mixing with 1 ml. of 1% (w/v) sulphanilamide in 1.5N-HCl and then adding 1 ml. of 0.02% N-(1-naphthyl)ethylenediamine dihydrochloride (Hageman & Flesher, 1960). After 20 min. *E*₅₄₀ was determined. Boiled-enzyme controls were always included.

(2) Use of chemically reduced BV as electron donor (dithionite assay). This method is simple, inexpensive and independent of the diaphorase that was found to be necessary for full activity in the preceding assay. The assay mixture contained (μmoles): potassium phosphate buffer, pH 7.5, 120; KNO₂, 0.9; BV, 1.0. The reaction mixture was placed in 150 mm. × 10 mm. tubes that were kept in ice until initiation of the reaction after enzyme had been added. The reaction was started in timed sequence by adding, with minimum turbulence, about 7.5 μmoles of sodium dithionite in 0.6 ml. of water, bringing the final volume to 3 ml. The tubes were immediately plugged with rubber serum stoppers, briefly evacuated through a hypodermic needle, shaken gently to mix the contents and incubated at 30° for 30 min. The reaction was stopped by opening the tubes and shaking them vigorously on a Vortex mixer (Scientific Industries, Springfield, Mass., U.S.A.) until at least 5 sec. after the blue colour of the reduced BV had disappeared. Samples (0.2 ml.) were pipetted into colour reagent [1 ml. of 1% sulphanilamide in 1.5N-HCl, 1 ml. of 0.02% N-(1-naphthyl)ethylenediamine dihydrochloride and 0.8 ml. of water]. *E*₅₄₀ was read after 20 min. Reagent-blank and boiled-enzyme controls were included. The incubation could be carried out in open tubes but reproducibility was then poorer, possibly owing to some oxidation during gentle shaking in the water bath.

Assay of diaphorase. The method was essentially similar to that of Avron & Jagendorf (1956). A final volume of 3 ml. contained (μmoles): potassium phosphate buffer, pH 7.5, 120; 2,6-dichlorophenol-indophenol, 0.15. The reaction was started by adding 0.5 μmole of NADPH, and *E*₆₂₀ was followed for 4 min. on a Beckman model DB recording spectrophotometer.

Enzyme units. One unit of nitrite reductase is defined as the amount of enzyme causing the reduction of 1 μmole of nitrite/min. One 'unit' of diaphorase activity is defined as the amount of enzyme that causes a change in the *E*₆₂₀ value of 1/min.

Illuminated chloroplasts. Washed spinach chloroplasts were prepared by the method of Allen, Whatley & Arnon (1958). Preparations were illuminated in a water bath at 30° with 850–950 ft.-candles of tungsten light.

Protein. Protein was precipitated in 5% (w/v) trichloro-

acetic acid, removed by centrifugation, dissolved in 0.1 N-NaOH and assayed by the method of Lowry, Rosebrough, Farr & Randall (1951).

Ammonia. Ammonia was determined by the ninhydrin method of Yemm & Cocking (1955).

Sulphide. Sulphide was measured by the method of Ellis (1964).

Materials. Sources were as follows: BV was from British Drug Houses Ltd., Poole, Dorset; GPD was from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.; NADPH was from Biochemicals Inc., Milwaukee, Wis., U.S.A.; NADP⁺ was from Sigma Chemical Co., St Louis, Mo., U.S.A.; dithiothreitol (Cleland's reagent) was from Calbiochem, Los Angeles, Calif., U.S.A.; *Clostridium ferredoxin* was from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Methyl viologen was a gift from California Chemical Co., Richmond, Calif., U.S.A.

RESULTS

Dithionite assay

Although the assay involving NADPH and GPD as well as the reduced-BV procedure of Hageman *et al.* (1962) are satisfactory for the assay of nitrite reductase, both are too laborious for routine operation. The nitrate-reductase assay described by Sadana & McElroy (1957) suggested that dithionite coupled with BV might serve as electron donor in a nitrite-reducing system. It was found that dithionite would reduce BV, ferredoxin and methyl viologen and could in this way supply electrons for nitrite reduction (Table 1). However, ferredoxin and methyl viologen sometimes gave non-enzymic reduction with boiled-enzyme controls and were therefore unsuitable for routine use. Although FAD was reduced, as indicated by colour change, little reduction of nitrite occurred. As shown in Table 1, 7.5 μ moles of dithionite gave sufficient reserve of reducing power in the standard assay, and no benefit was obtained by increasing the concentration. It should be emphasized that

it is essential that all residual dithionite is oxidized by vigorous shaking in air before samples are taken for colour development, otherwise colour yield is seriously affected.

The rate of loss of nitrite was higher in the dithionite assay than in the NADPH-nitrite-reductase method. Although the ratio of the rates does not appear to change for a particular species with increasing purification of the enzyme, there was some variation from species to species (see Table 3). In the dithionite assay, nitrite reduction was linear with time, after a slight lag (Fig. 1), and was linear with enzyme concentration up to a rate of loss of nitrite of 1 μ mole/hr.

Crude enzyme preparations may contain nitrate reductase, and under exceptional circumstances

Fig. 1. Effect of time on the dithionite assay of crude spinach nitrite reductase.

Table 1. *Dithionite as electron donor for spinach nitrite reductase: effects of various concentrations and cofactors*

The assay method was as described in the Materials and Methods section, with appropriate substitutions; 0.1 ml. of crude spinach enzyme was used. For comparison with other assays with similar amounts of enzyme, see Table 4.

Cofactor	Dithionite (μ moles)	Nitrite-reductase activity (m μ moles of NO ₂ ⁻ reduced/min.)	
		Enzyme	Boiled enzyme
BV (1.0 μ mole)	3.75	3.33	—
BV (1.0 μ mole)	7.5	17.0	—
BV (1.0 μ mole)	15	16.9	—
BV (1.0 μ mole)	7.5	16.9	0
Purified ferredoxin (0.05 ml.)	7.5	21.1	1.13
Methyl viologen (1.0 μ mole)	7.5	21.0	7.03
FAD (0.2 μ mole)	7.5	0.53	—

may also contain substrate amounts of nitrate, which might be reduced to nitrite and so decrease the accuracy of the assay. However, as shown by Cresswell *et al.* (1965), BV will not act as electron donor for nitrate reduction when more than 25% reduced. When excess of dithionite is present, BV is at least 50% reduced and there is no danger of interference from nitrate reduction.

Diaphorase in the NADPH-nitrite-reductase system

Paneque *et al.* (1964) showed that 'TPN-reductase' or diaphorase might be important in nitrite reduction. Since nitrite reductase is much more heat-labile than diaphorase, it is possible by controlled heating to obtain a crude preparation of diaphorase free from nitrite reductase (Table 2). Such a preparation from spinach was added to the NADPH-GPD assay of nitrite reductase from several species. Table 3 shows that only the crude spinach preparation had sufficient diaphorase to support maximum nitrite-reducing ability. Maize, radish (*Raphanus sativus*) and especially pigweed

(*Amaranthus retroflexus*) showed considerable increases in activity with added diaphorase. It was also apparent that with increasing purification of spinach nitrite reductase there was an increasing dependence on added diaphorase, but the dithionite assay, being independent of diaphorase, was unaffected by increasing purification. It was therefore obvious that NADPH-dependent nitrite reductase is a two-enzyme system, and that nitrite reductase proper needed not only cofactors but also a second enzyme to show activity with NADPH. The section on further purification (below) also provides evidence that the diaphorase and nitrite reductase are independent enzymes. It is evident that, for many plant extracts, a more accurate estimate of nitrite-reductase activity with NADPH as the electron donor will be obtained only with the addition of a diaphorase preparation.

The crude diaphorase also mediated reduction of BV and 2,6-dichlorophenol-indophenol by NADPH. It had ferredoxin-NADP-reductase activity when assayed with a washed chloroplast preparation (San Pietro, 1963). The diaphorase

Table 2. *Effect of heating (at 60° for 5 min.) on nitrite-reductase and diaphorase activities*

The assay method was as described in the Materials and Methods section; 0.1 ml. of crude enzyme was used.

		Nitrite-reductase activity (NADPH-GPD assay) (μ moles of NO_2^- reduced/min.)	Diaphorase activity ($\Delta E_{620}/\text{min.}$)
Maize enzyme	Before heating	15.2	0.43
	After heating	< 0.90	0.36
Spinach enzyme	Before heating	21.2	0.80
	After heating	< 0.73	0.76

Table 3. *Importance of diaphorase in NADPH-dependent nitrite reduction*

The NADPH-GPD assay was carried out on various enzyme preparations, with and without the addition of spinach diaphorase, prepared as described in the Materials and Methods section. Activities estimated by the dithionite assay (without diaphorase) are also shown.

Enzyme	Nitrite-reductase activity (μ moles of NO_2^- reduced/mg. of protein/min.)			Percentage increase of activity with diaphorase in NADPH-GPD assay
	Dithionite assay	NADPH-GPD assay		
		No addition	Diaphorase added	
Maize (crude)	130.4	32.4	58.6	81
Spinach (crude)	29.3	27.1	27.3	0.7
Spinach (partial purification)	—	27.3	104.1	281
Spinach (purified*)	213.3	21.0	186.6	789
Pigweed (crude)	52.6	7.70	30.0	290
Radish (acetone-dried powder)	—	1.50	2.76	84

* 0.45 III fraction (Table 8).

obtained during enzyme purification, e.g. 'Column 2: Run through' in Table 8, had properties similar to those of the crude diaphorase and was equally effective in restoring NADPH activity to the purified nitrite reductase.

Effect of various electron donors and cofactors on nitrite-reductase activity

In Table 4 are compared the effectiveness of a number of reducing systems as electron donors for nitrite reduction. For uniformity and for comparison, this and some other Tables contain experimental results obtained with a single 'crude' spinach extract (see the Materials and Methods section). Similar experiments were carried out with different purified preparations from spinach and maize and, except where noted, similar results and phenomena were observed as long as diaphorase was added in any experiments involving NAD(P)H.

Table 4 shows that reduced BV served as a direct electron donor for nitrite reduction, and the addition of FAD or ferredoxin had no beneficial effect. With illuminated washed chloroplasts as electron source ferredoxin was essential, as already reported by Losada *et al.* (1963), and the addition of ADP also enhanced the reduction of nitrite, suggesting that the process was coupled to photophosphorylation. BV added with ferredoxin almost completely prevented nitrite reduction with illuminated chloroplasts, possibly by an 'uncoupling'

of electron transport through a cyclic reoxidation of the extremely autoxidizable dye by oxygen produced in the cleavage of water. FAD with ferredoxin had little effect *in vacuo*, but in air nitrite reduction was markedly decreased, presumably by a similar autoxidation with atmospheric oxygen. With some crude spinach preparations a small but repeatable reduction of nitrite by NADH was found. This reaction required either BV or ferredoxin as cofactor, but exhibited no additional reduction of nitrite when both cofactors were added together.

With the NADPH-GPD system, BV alone was more effective than ferredoxin as cofactor in nitrite reduction (Table 4), although at higher concentrations ferredoxin increased in effectiveness. A trace of activity was found with FAD and 2,6-dichlorophenol-indophenol. It was surprising to find that, when ferredoxin was added with BV, FAD or 2,6-dichlorophenol-indophenol, there was a marked increase in nitrite-reductase activity and this was considerably greater than the sum of reductions with either of the reagents alone. For this synergistic effect, ferredoxin was a necessary component. The synergism with BV was always found, in spinach and in maize, with both crude and purified enzymes. Greater synergism with FAD was frequently found with crude spinach preparations, but tended to disappear with purification. No FAD synergism was detected in a limited number of experiments with maize. Table 5

Table 4. *Reduction of nitrite by equivalent amounts of crude spinach enzyme with various electron donors and cofactors*

All assays contained, in addition to the components listed, phosphate buffer, pH 7.5 (120 μ moles), and KNO₂ (0.9 μ mole), and were carried out *in vacuo*. The assay systems used were: (a) to phosphate, nitrite (a total of 2.25 μ moles) and enzyme in 1.5 ml., 6.0 ml. of 0.04 M-phosphate buffer containing 22 μ moles of BV, about 50% reduced with H₂ and catalyst (Hageman *et al.* 1962) was added *in vacuo*; (b) 0.2 ml. of washed chloroplast suspension was added to phosphate, nitrite and enzyme (total volume 3.0 ml.); (c) 6 μ moles of NADH were added to phosphate, nitrite and enzyme (total volume 3.0 ml.); (d) BV and ferredoxin were omitted from the standard mix, but were replaced as indicated. After 30 min. at 30°, samples were taken, added to zinc acetate to stop the reaction and processed as described in the Materials and Methods section.

Addition	Nitrite-reductase activity (m μ moles of NO ₂ ⁻ reduced/min.)			
	(a) Electron donor Reduced BV	(b) Illuminated chloroplasts	(c) NADH	(d) NADPH-GPD
None	22.6	0.73	0	0
Ferredoxin (0.05 ml.)	17.5	16.5	2.33	3.67
BV (0.5 μ mole)	—	0	2.90	14.6
FAD (0.3 μ mole)	20.1	1.37	< 0.17	2.47
Ferredoxin (0.05 ml.) + BV (0.5 μ mole)	—	0.90	3.13	> 28.3
Ferredoxin (0.05 ml.) + FAD (0.3 μ mole)	—	17.1	—	20.7
Ferredoxin (0.05 ml.) + ADP (1 μ mole)	—	21.2	—	—
ADP (1 μ mole)	—	0.97	—	—
2,6-Dichlorophenol-indophenol (0.5 μ mole)	—	—	—	1.0
Ferredoxin (0.05 ml.) + 2,6-dichlorophenol-indophenol (0.5 μ mole)	—	—	—	16.8

shows the synergistic effect of various concentrations of BV with a constant amount of ferredoxin.

The lighter colour of the assay solutions with BV in the absence of ferredoxin suggested that BV was more reduced in the presence of ferredoxin than without it. This was confirmed by following the rate of reduction of BV (E_{560}), with and without ferredoxin, by the NADPH-GPD system *in vacuo*. The rate of reduction was enhanced in three ways: the time before reduction began was slightly shorter, the rate of reduction was faster (four- to five-fold) and the final degree of reduction was greater (two- to three-fold). The possibility that the increased reduction of BV may explain the synergistic effect is discussed below.

The effectiveness, in nitrite reduction, of the spinach ferredoxin was compared with that of ferredoxin prepared from maize and of a commercial preparation from *Clostridium* (Table 6). The three preparations gave rise to nitrite reduction with illuminated chloroplasts, NADPH-GPD and dithionite as electron donors. On a protein basis the spinach ferredoxin was the most efficient in each system. The relative efficiencies with different

donors varied with the ferredoxins, which were apparently of different structure (see Fig. 2).

Further purification and extraction of nitrite reductase

The results of attempts to purify the enzyme on DEAE-cellulose columns are summarized in Tables 7 and 8. The specific activity of both maize and spinach enzyme could be increased at least 70-fold. The enzyme, especially from maize, was labile and activity was constantly lost during purification, but the diaphorase was relatively stable. Even so, the nitrite reductase could be substantially freed of the diaphorase involved in the NADPH system (see also Table 3). The purified enzyme would accept electrons from ferredoxin reduced with chloroplasts or dithionite, or from BV reduced by dithionite or hydrogen and palladium catalyst. The diaphorase was therefore essential only for the transfer of electrons from NADPH to ferredoxin or BV.

Experience with nitrate reductase (Beever, Fletcher & Hageman, 1964) suggested that protec-

Table 5. *Effect of BV concentration on synergism in the NADPH-GPD assay of crude spinach nitrite reductase*

Nitrite-reductase activity ($\mu\text{moles of NO}_2^-$ reduced/min.)			
BV (μmoles)	BV alone	BV + ferredoxin (0.05 ml.) added	Synergism (ferredoxin + BV) – (ferredoxin alone + BV alone)
0	—	6.53	—
0.1	1.80	16.2	7.87
0.25	4.20	20.0	9.27
0.5	10.2	21.7	4.97
1.0	12.7	25.8	6.57
2.0	19.8	30.0	3.67

Table 6. *Effect of different sources of ferredoxin on nitrite reduction by crude spinach enzyme with different electron donors*

The assay systems used were: (a) as in Table 4; (b) as in the Materials and Methods section, with BV included; (c) as in the Materials and Methods section, with BV omitted.

Nitrite-reductase activity						
(mmoles of NO_2^- reduced/min./0.05 ml. of ferredoxin)			(mmoles of NO_2^- reduced/min./mg. of ferredoxin protein)			
Electron donor ...	(a)	(b)	(c)	(a)	(b)	(c)
Ferredoxin source	Illuminated chloroplasts	NADPH-GPD	Dithionite	Illuminated chloroplasts	NADPH-GPD	Dithionite
Spinach	16.3	7.27	13.9	129.0	57.5	110.0
Maize	7.48	6.0	—	59.9	48.0	—
<i>Clostridium</i>	20.2	6.97	14.6	53.8	20.2	42.2

Fig. 2. Absorption curves of maize (A), spinach (B) and *Clostridium* (C) ferredoxins (see the text for details). The curves are displaced vertically for clarity.

tion of thiol groups might also aid extraction and stability of nitrite reductase. Addition to the extraction medium of cysteine, and also, at lower concentrations, of dithiothreitol (Cleland, 1964), increased the activity of nitrite reductase from maize (Table 9) but had little effect on extraction from spinach. It was noted that extraction of ferredoxin from maize, as judged by density and size of the band adsorbed on DEAE-cellulose, was markedly decreased by the presence of cysteine, and none could be detected in preparations extracted with 10mM-cysteine. Cysteine increased the extraction of flavoprotein compounds, as indicated by the brilliant yellow colour of the final 'crude' preparations. For maize, cysteine was more beneficial in extraction from older field-grown tissue than in extraction from young tissue from the greenhouse.

Properties of nitrite reductase

K_m value. The *K_m* for nitrite, of nitrite reductase, purified from both maize and spinach, was 0.1–0.2mM, with both the dithionite and NADPH-GPD assays (Fig. 3).

Optimum pH. The pH-activity curves with both assays, and also with substrate concentrations

Table 7. Purification of nitrite reductase from maize

A 33–75%-(v/v)-acetone precipitate, redissolved and dialysed, was prepared from the homogenate as described in the Materials and Methods section, and added to the first DEAE-cellulose column (2.2 cm. × 10 cm.). The 'Run through' and 'Wash I (0.35M-tris)' (tris buffer, pH7.3) fractions were combined, diluted to 4 vol. and added to a second DEAE-cellulose column (1.7 cm. × 10 cm.). The enzymes were then eluted with tris buffer, pH7.3, with stepwise increases in molarity. Nitrite reductase was assayed by the NADPH-GPD method. Units are as defined in the Materials and Methods section.

Fraction	Vol. (ml.)	Protein (mg./ml.)	Nitrite reductase			Total diaphorase 'units'	Ratio: Units of nitrite reductase × 10 Units of diaphorase
			(milliunits/ ml.)	(total units)	(milliunits/ mg. of protein)		
Original homogenate	340	8	47.35	16.1	5.91	146.2	1.1
Column 1:							
Run through	100	4	91.3	9.13	22.8	46	1.98
Wash I (0.35M-tris)	50		92.1	4.6		13.5	3.4
Wash II (0.35M-tris)	40		11.85	0.47		0.4	—
Wash III (0.35M-tris)	40		5.45	0.22		0.4	—
(run through + wash I transferred to column 2)							
Column 2:							
Run through	600		0.51	0.31		< 2	—
Wash I (0.10M-tris)	100		0.20	0.02		1.0	0.2
Wash II (0.15M-tris)	150		1.02	0.15		39	0.04
Wash III (0.20M-tris)	50		0.54	0.03		7	0.07
Wash IV (0.25M-tris)	60	0.18	63.8	3.82	355.0	6.6	5.79
Wash V (0.25M-tris)	60	0.07	11.75	0.70	167.0	1.5	4.67
Wash VI (0.25M-tris)	60		2.7	0.16		0.6	2.67
Wash VII (0.40M-tris)	100		0.83	0.08		1	—

Table 8. *Purification of nitrite reductase from spinach*

A 33–75%-(v/v)-acetone precipitate, redissolved and dialysed, was prepared from a homogenate as described in the Materials and Methods section, and added to the first DEAE-cellulose column (2.2 cm. × 12 cm.). The 'Run through' was diluted (to 3 vol.) and added to the second DEAE-cellulose column (1.8 cm. × 10 cm.). Fractions eluted with 0.15 M- and 0.2 M-tris buffer, pH 7.3, were combined and diluted to 3 vol. and added to a third column of DEAE-cellulose (1.7 cm. × 10 cm.). Fractions were eluted with tris buffer, pH 7.3, as indicated. Nitrite reductase was assayed by the NADPH-GPD method. Units are as defined in the Materials and Methods section.

Fraction	Vol. (ml.)	Protein (mg./ml.)	Nitrite reductase			Total diaphorase 'units'	Ratio: Units of nitrite reductase × 10 Units of diaphorase
			(milliunits/ ml.)	(total units)	(milliunits/ mg. of protein)		
Original homogenate	880	11.6	32.7	28.8	2.82	—	—
Column 1:							
Run through and wash with 0.07 M-tris	150		135.5	20.3		—	—
(run through + wash transferred to column 2)							
Column 2:							
Run through	550		12.2	6.71		176	0.38
Wash I (0.15 M-tris)	90		44.7	4.02		(~20)	(~2)
Wash II (0.20 M-tris)	100	0.54	58.7	5.87	109	2.5	23.4
Wash III (0.40 M-tris)	60		20.9	1.25		0.9	13.9
(wash I + wash II transferred to column 3)							
Column 3:							
Run through	600		32.4	1.94		10.2	1.9
Wash I (0.15 M-tris)	30	0.56	48.4	1.45	86.4	3.0	4.8
Wash II (0.15 M-tris)	50		20.7	1.03		5.5	1.9
Wash III (0.45 M-tris)	25	0.52	97.3	2.43	187.1	1.0	24.3
Wash IV (0.45 M-tris)	30		9.7	0.29		< 0.3	—

Table 9. *Effect of cysteine concentration on the extraction of nitrite reductase from maize*

Leaves of 2-week-old plants were ground in solid CO₂ and thawed with an equal amount (v/w) of 0.07 M-tris buffer, pH 7.3, containing cysteine at the concentrations shown. A 33–75%-(v/v)-acetone precipitate was prepared and dissolved and dialysed before assay.

Concn. of cysteine (mM)	Nitrite-reductase activity (mμmoles of NO ₂ ⁻ reduced/min.)
0	13.7
0.1	16.3
1	19.1
10	19.5

of NADPH, were determined for different enzyme preparations. The curves in Fig. 4 show no sharp optimum with any system. For spinach the optimum varies with the assay method, being about pH 6.6 with NADPH, pH 7.0 with NADPH-GPD and pH 7.8 with dithionite. The grana

Fig. 3. Plot of substrate concentration divided by velocity of reaction, against substrate concentration (nitrite), of various nitrite-reductase preparations: ■, spinach (crude), NADPH-GPD assay; ○, maize (purified), dithionite assay; ●, spinach (crude), dithionite assay.

Fig. 4. Effect of pH on the activity of various crude nitrite-reductase preparations with different assay methods: Δ , maize, dithionite assay; $\circ$, maize, NADPH assay (substrate amounts); $\blacktriangle$, spinach, NADPH-GPD assay; $\blacksquare$, spinach, NADPH assay (substrate amounts); $\bullet$, spinach, dithionite assay. The curves are displaced vertically for clarity.

Table 10. *Sulphite-reductase activity of nitrite-reductase preparations*

The usual NADPH assay was carried out, but nitrite was replaced by $10\mu\text{moles}$ of Na_2SO_3 .

Enzyme	Sulphide produced ($\mu\text{moles/min.}$)	Nitrite reduction by equivalent amount of enzyme ($\mu\text{moles/min.}$)
Maize (crude)	1.5	25.5
Spinach (crude)	2.5	65.3
Spinach (partially purified)	1.8	72.7

system used by Husisige & Satoh (1961) had optimum pH 7. Although not optimum for all systems, the normal assay pH of 7.5 gave good activity with all systems and preserved continuity with early experiments of this series and of other workers.

Yield of ammonia. The natural reaction product appears to be ammonia (Lazzarini & Atkinson, 1961; Sanderson & Cocking, 1964). It was necessary to check that the assay methods used in the present work, which measured substrate loss, assayed

reduction of nitrite to ammonia, and that the nitrite loss was not caused by any other chemical or enzymic reaction. Experiments with spinach and maize enzymes showed that in both NADPH-GPD and dithionite assays the values for ammonia production and nitrite loss were of the same order. In a series of experiments the yield of ammonia averaged 85% (range 75–108%).

Sulphite-reductase activity. Table 10 shows that the sulphite-reductase activity, which was at the limits of detection by the method used, was negligible compared with the nitrite-reductase activity of the preparations.

DISCUSSION

Early work on nitrite reductase has been extensively documented by Hewitt & Nicholas (1964). The present work has confirmed the importance of ferredoxin in the nitrite-reducing system, as already described by Hewitt & Betts (1963), Losada *et al.* (1963) and Paneque *et al.* (1964). We have shown that ferredoxin from different higher plants and bacteria is interchangeable in both photoreduction and NADPH-mediated reduction of nitrite, and is the only natural cofactor that has been found to be active in the nitrite-reducing system. Ferredoxin is a single-electron donor (Tagawa & Arnon, 1962).

The reduction of nitrite with NADPH requires two enzymes: (i) a diaphorase, and (ii) nitrite reductase, plus the necessary participation of ferredoxin or an artificial dye as cofactor. The ferredoxin-NADP-reductase activity of the preparation obtained by Paneque *et al.* (1964) can be assumed to be due to an enzyme equivalent to the diaphorase found in our preparations. The evidence suggests that the diaphorase fraction, obtained during purification of nitrite reductase, contains the ferredoxin-NADP reductase described by Shin *et al.* (1963). Shin *et al.* (1963) found that ferredoxin-NADP reductase would also catalyse the oxidation of NADPH by NAD^+ (i.e. transhydrogenase activity), ferricyanide, FAD and indophenol dyes (diaphorase activity). As discussed by Shin *et al.* (1963), it is probable that the diaphorase activities described by Avron & Jagendorf (1956) and Keister, San Pietro & Stolzenbach (1960) are due to the same enzyme, although other diaphorases of different identity have been described (e.g. Lazzarini & San Pietro, 1964). It is likely that the diaphorase described in the present work is also a manifestation of the same enzyme of Shin *et al.* (1963), and this agrees with its role in the coupling of a two-electron donor (NADPH) to single-electron donors (ferredoxin and BV). The transhydrogenase activity found by Shin *et al.* (1963) may account for the small nitrite reduction that

Scheme 1.

we have observed with NADH. These results indicate that the electron-transport paths involved in nitrite reduction can be summarized as shown in Scheme 1.

Steps 1 and 2 are carried out by the diaphorase; steps 5 and 6 involve direct transfer to nitrite reductase, and are dependent on the concentration of the reduced electron donor (see below). Under natural conditions it is likely that photoreduction of nitrite (steps 3 and 5) is the normal system in leaves. It has been shown that nitrite infiltrated into leaves is rapidly reduced only in light (C. F. Cresswell, unpublished work, cited by Hageman *et al.* 1962). Unpublished work in this Laboratory (by G. Ritenour) shows that in leaf tissue nitrite reductase is located in the chloroplasts. The work of several authors shows that light has a role in nitrate assimilation, but appears to be only indirectly involved in the reduction of nitrate to nitrite (Mendel & Vissar, 1951; review by Kessler, 1964; Beevers, Schrader, Flesher & Hageman, 1965). In contrast, light may have a direct role in the reduction of nitrite, and this is supported by the facts that the cofactor ferredoxin is also involved in the processes of photoreduction by chloroplasts (Tagawa & Arnon, 1962) and that nitrite reduction is readily coupled to an illuminated chloroplast system. However, some nitrite reductase has been extracted from non-photosynthetic tissue (Sanderson & Cocking, 1964). Observations that nitrogenous material in ascending sap of apple trees is organic in Nature (Bollard, 1956) and that excised roots can grow with nitrate or nitrite as the sole nitrogen source (Hannay, Fletcher & Street, 1959) suggest that reduction of nitrate to ammonia must occur in roots and that non-photosynthetic reduction of nitrite must be involved. Such a system could be driven by NADPH from the pentose phosphate cycle, but would depend on the presence of diaphorase and ferredoxin or a similar cofactor. However, the

characteristics of nitrite reductase and ferredoxin (if any), and their intracellular location, in root tissue are not known.

The synergism of ferredoxin and a second factor in nitrite reduction has not been reported previously and needs some explanation. With the NADPH-GPD system (redox potential $E'_0 - 280\text{mv}$; Glaser & Brown, 1955) it can be calculated that only about 1.5% of the ferredoxin ($E'_0 - 420\text{mv}$) will be reduced, and this could account for the relatively low nitrite reduction with ferredoxin alone. It is possible that the reduced ferredoxin can transfer electrons efficiently to BV (E'_0 approx. -350mv), and it has been observed that BV is reduced faster and to a greater extent with ferredoxin present. BV can act as direct electron donor to nitrite reductase, as shown by the fact that BV reduced with dithionite or hydrogen will drive the reaction. These facts suggest that synergism is due to the more efficient transfer of electrons from ferredoxin-reduced BV, involving steps 1, 4, 6 in Scheme 1. Cresswell *et al.* (1965) have shown that for BV reduced with hydrogen the rate of nitrite reduction is directly dependent on the degree of reduction of the dye. This may also apply for ferredoxin, and the efficient reduction of nitrite with ferredoxin and illuminated chloroplasts could result from the greater degree of reduction of ferredoxin by chloroplasts than by NADPH.

The less frequently observed FAD synergism probably cannot be explained in the same way as the BV synergism. FAD reduced by dithionite, NADPH or hydrogen and a catalyst has not given more than a trace of activity with nitrite reductase. Atebrine (0.5mm), an inhibitor of FAD-mediated electron transport, had little effect on the dithionite-BV assay of crude preparations of nitrite reductase but caused a small (20%) inhibition with an NADPH (substrate amounts)-ferredoxin-BV system. This suggests that any involvement of FAD is not with nitrite reductase proper but at

the earlier step mediated by diaphorase, itself a flavoprotein (Shin *et al.* 1963). The fact that purification caused disappearance of FAD synergism, rather than increasing dependence, may indicate that a third enzyme is involved.

The K_m values obtained in the present work (0.2 mM-NO₂⁻) are similar to those found by Husisige & Satoh (1961) for spinach and by Lazzarini & Atkinson (1961) for bacterial extracts. Both Sanderson & Cocking (1964) and Cresswell *et al.* (1965) have obtained values of approx. 1 μ M when short (6 min. or less) incubation times were used. It is not surprising that different K_m values are found, since values for apparent K_m will reflect differences in enzyme preparation, duration of assay, electron donor and its reduction state, and a number of other experimental factors. Short times of assay would emphasize any lag before the reaction rate becomes linear and would underestimate the value of K_m . Our experiments were run for longer times and errors due to change of substrate concentration during the assay may have caused overestimation of the K_m value. However, only for the lowest concentrations was there a marked decrease in substrate concentration during the assay.

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