Nitrite and Hydroxylamine Reduction in Higher Plants

FRACTIONATION, ELECTRON DONOR AND SUBSTRATE SPECIFICITY OF LEAF ENZYMES, PRINCIPALLY FROM VEGETABLE MARROW (*CUCURBITA PEPO* L.)

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Nitrite reductase was purified between 760- and 1300-fold from vegetable marrow (*Cucurbita pepo* L.) and residual hydroxylamine reductase activity was low or negligible by comparison. With ferredoxin as electron donor, nitrite loss and ammonia formation at pH 7.5 were stoichiometrically equivalent. Crude nitrite reductase preparations showed negligible activity with NADPH as electron donor maintained in the reduced state by glucose 6-phosphate, whereas by comparison, activity was high when either ferredoxin or benzyl viologen were also present and reduced by the NADPH-glucose 6-phosphate system, whereas FMNH₂ produced variable and relatively low activity under the same conditions. At pH values below 7, non-enzymic reactions occurred between reduced benzyl viologen and nitrite, and intermediate reduction products were inferred to be produced instead of ammonia. Activity with ferredoxin (0.1 mM), reduced by chloroplast grana in the light, was 25 times that produced with ferredoxin (40 μ M) reduced with NADPH and glucose 6-phosphate. For an approximate molecular weight 61 000–63 000 derived by chromatography on Sephadex G-100 and G-200, and a specific activity of 46 μ mol of nitrite reduced/min per mg of protein with light and chloroplast grana, a minimum turnover number of 3×10^3 mol of nitrite reduced/min per mol of enzyme was found. Two hydroxylamine reductases were separated on Sephadex gels. One (HR1) was initially associated with nitrite reductase during gel filtration but disappeared during later fractionation. This HR1 fraction showed nearly comparable activity with reduced benzyl viologen, ferredoxin or FMNH₂. The other (HR2), of molecular weight approx. 35 000, reacted with reduced benzyl viologen but showed negligible activity with ferredoxin or NADPH. Activity with FMNH₂ was associated with an irregular trailing boundary during gel filtration, with much diminished activity in the HR2 region. Activity with NADPH was about 30% of that with FMNH₂, reduced benzyl viologen or ferredoxin and was considered to reside in fraction HR1. Hydroxylamine yielded ammonia under all assay conditions. No activity with hyponitrite or sulphite was observed with reduced benzyl viologen as electron donor in either the nitrite reductase or the hydroxylamine reductase systems, but pyruvic oxime produced about 4% of the activity of hydroxylamine.

The quantitative reduction of nitrite and hydroxylamine to ammonia by plant leaf enzymes with BV⁺† as electron donor was reported by Hageman, Cresswell & Hewitt (1962) and Cresswell, Hageman & Hewitt (1962) and described in detail by Cresswell, Hageman, Hewitt & Hucklesby (1965). As the result of a suggestion by Nason (1962), ferredoxin was identified as the physio-

logical electron donor for these enzymes (Hewitt & Betts, 1963; Huzisige, Satoh, Tanaka & Hayasida, 1963; Losada, Paneque, Ramirez & Del Campo, 1963; Paneque, Del Campo & Losada, 1963). Partial purification and properties of nitrite reductase were described from several plants (Paneque, Ramirez, Del Campo & Losada, 1964; Losada, Paneque, Ramirez & Del Campo, 1965; Betts & Hewitt, 1966; Joy & Hageman, 1966; Ramirez, Del Campo, Paneque & Losada, 1966; Shin & Oda, 1966). Betts & Hewitt (1966) showed that nitrite reductase and hydroxylamine reductase in spinach

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† Abbreviations: BV and BV⁺, benzyl viologen and its reduced (free-radical) form respectively.

were located in chloroplasts and reported a tentative separation of these enzymes by chromatography on Sephadex G-200. This observation was of interest in relation to the status of hydroxylamine as an intermediate in nitrite reduction under conditions where ammonia was produced more rapidly from nitrite than from hydroxylamine at comparable substrate concentrations (Cresswell *et al.* 1965; Betts & Hewitt, 1966).

Further attempts to separate nitrite reductase and hydroxylamine reductase in spinach and marrow leaf preparations revealed the existence of multiple hydroxylamine reductase fractions, one of which is associated in variable amounts with nitrite reductase. The present paper describes work carried out with vegetable marrow (*Cucurbita pepo*) and a few experiments with spinach (*Spinacia oleracea*), with special reference to protein fractionation, and electron donor and substrate specificity. Preliminary accounts of this work have appeared (Hewitt & Hucklesby, 1966; Hewitt, Hucklesby & Betts, 1966). A related investigation with spinach enzymes was reported in a recent symposium (Hewitt, Hucklesby & Betts, 1968).

EXPERIMENTAL

Plant material. Plants used for enzyme preparations were grown for 3–5 weeks in sand culture with a nitrate-type Long Ashton nutrient solution supplied three times daily by automatic irrigation (Hewitt, 1966), in a controlled environment providing 16h light as 16000 lx from a mixture of 80W daylight and de-luxe warm-white fluorescent tubes and 25W tungsten lights at 24°C, or grown for 4–6 weeks by similar methods during May–August in a greenhouse. The partially expanded leaves were used. Older leaves were used for preparing ferredoxin, plastocyanin and ferredoxin–NADP oxidoreductase (EC 1.6.99.4), referred to here as diaphorase.

Materials. These were obtained from the following sources: FMN, NADP⁺ (sodium salt), D-glucose 6-phosphate (sodium salt), reduced thioctic (α -lipoic) acid, GSH, bovine serum albumin (Sigma Chemical Co., St Louis, Mo., U.S.A., and London S.W.6, U.K.); hydrogen, argon (British Oxygen Co. local depot, Brislington, Bristol, U.K.); *NN'*-dibenzyl-4,4'-bipyridylum dichloride (benzyl viologen), palladized asbestos (5% Pd), guanidinium chloride, chymotrypsinogen, salmine (protamine) sulphate, glucose 6-phosphate dehydrogenase (EC 1.1.1.49) [100 μ mol of NADP⁺ reduced/min per ml as suspension in 3.5M-(NH₄)₂SO₄] (BDH Chemicals Ltd., Poole, Dorset, U.K.); human γ -globulin (AB Kabi, Stockholm, Sweden); hydroxyapatite, menadione, horse cytochrome *c* (Calbiochem, London W.1, U.K.); ovalbumin, myoglobin (Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K.); L-ascorbic acid (Roche Products Ltd., London W.1, U.K.); glucagon (Eli Lilly and Co., Indianapolis, Ind., U.S.A.); Whatman DE50 DEAE-cellulose (W. and R. Balston Ltd., Maidstone, Kent, U.K.); Sephadex gels [Pharmacia (G.B.) Ltd., London W.5, U.K.]; Norit charcoal (Verkoop Centrale,

Amsterdam C, The Netherlands). Sodium hyponitrite was prepared by Mr B. A. Notton by the method of Addison, Gamlen & Thompson (1952) and metals were removed from (NH₄)₂SO₄ (Hewitt & Nicholas, 1964).

BV (3.5mm in 50mm-potassium phosphate buffer, pH7.5) was partially reduced to the free radical BV[•] with H₂ and palladized asbestos (Hageman *et al.* 1962) in the apparatus illustrated by Hewitt & Nicholas (1964). Concentration and percentage reduction were measured either by titration into KMnO₄ (Hageman *et al.* 1962) or with H₂O₂ as follows. Evacuated Thunberg cuvettes containing 0.5–3 μ mol of H₂O₂ in 0.05ml in the side arm were charged with 3.0ml of partially reduced 3.5mm-BV. The light-absorption spectra were recorded in a Unicam SP.800 spectrophotometer before and after tipping. Values of ϵ_{mM} were calculated to be 5.7 at 560nm, 1.0 at 700nm and 0.6 at 780nm from the linear relationships between decrease in extinction and amount of H₂O₂ added.

Plastocyanin was prepared from marrow and ferredoxin was obtained from marrow or spinach (Hewitt *et al.* 1968) for use with enzymes from these respective sources. Ferredoxin was relatively pure by spectrophotometric criteria (Tagawa & Arnon, 1962; Fry & San Pietro, 1963; San Pietro, 1963). The diaphorase obtained from these preparations was free of nitrite reductase activity and hydroxylamine reductase activity. Average specific activity was about 1 μ mol of BV or FMN reduced/min per mg of protein under argon in the presence of 8mm-glucose 6-phosphate, 0.2mm-NADP⁺ (10min pre-reduced) and excess of glucose 6-phosphate dehydrogenase in 50mm-potassium phosphate buffer, pH7.5. It was used in three- to five-fold excess relative to the electron-transport rate anticipated for the nitrite reductase preparation to be assayed.

Chloroplast grana were prepared from greenhouse-grown spinach rather than from marrow because of their greatly superior stability at 0°C (Vernon & Zaugg, 1960). Nitrite reductase and hydroxylamine reductase activities were eliminated by repeated washing in 3mm-tris-HCl buffer, pH7.8, and heating for 5min at 55°C (Hewitt *et al.* 1968).

Assays of enzyme activity. Several assays of activity recorded as stated below were used according to circumstances.

(1) BV (21 μ mol reduced 35–50%) was dispensed in 6ml of 50mm-potassium phosphate buffer, pH7.5, into enzyme in evacuated Thunberg tubes each holding 2 μ mol of substrate in the side arm and incubated for 25min at 27°C.

(2) BV (1.8 μ mol) was reduced to equilibrium by 10min incubation with glucose 6-phosphate (20 μ mol), NADP⁺ (0.5 μ mol), excess of glucose 6-phosphate dehydrogenase and diaphorase. The latter was added to the Thunberg tubes immediately before evacuation and purging with argon to minimize autooxidation. Substrate (2 μ mol) was tipped and incubated as above in a total volume of 2.5ml of 50mm-potassium phosphate buffer, pH7.5.

(3) BV (2ml of 3.5mm solution reduced to 40–50%) was dispensed as in (1) into potassium phosphate buffer, pH7.5, in evacuated matched Thunberg tubes (15mm diam.) or 1cm Thunberg cuvettes, to a final volume of 4ml with the enzyme. The initial extinction at 700nm was measured in an EEL Spectra or in a Unicam SP.800

spectrophotometer. Substrate ($2\mu\text{mol}$) was tipped and the change in extinction during reoxidation of the $\text{BV}^{\bullet}$ was followed.

(4) As (2), but BV was replaced by FMN ($1\mu\text{mol}$).

(5) As (2), but BV was replaced by ferredoxin ($0.1\mu\text{mol}$).

(6) Ferredoxin ($0.3\mu\text{mol}$) was continuously photo-reduced under anaerobic conditions (Losada, Whatley & Arnon, 1961). The medium contained heat-treated chloroplast grana ($750\mu\text{g}$ of chlorophyll), ascorbic acid ($30\mu\text{mol}$), 2,6-dichlorophenol-indophenol ($0.2\mu\text{mol}$) and plastocyanin ($0.01\mu\text{mol}$) in 50mm-tris-HCl buffer, pH 7.5, in a total volume of 3ml. Substrate ($2\mu\text{mol}$ in 0.2ml) was placed in the side arm of the 15mm Thunberg tube and the contents were evacuated and purged with argon. The substrate was tipped and the tube was illuminated in a glass water bath usually for 3min at 20°C with a 250W tungsten projector lamp providing 80 000 lx inside the tube as measured by a photovoltaic diode (Mullard BPY10) calibrated with an EIL Lightmaster photometer. The tube was covered in aluminium foil between illumination and sampling. Controls in darkness or with enzyme omitted showed negligible loss of nitrite. Loss of hydroxylamine without formation of ammonia occurred during illumination in the absence of ferredoxin unless strict anaerobic conditions were maintained (Hewitt *et al.* 1968), but the reaction between nitrite and reduced ferredoxin without added enzyme (Joy & Hageman, 1966) was not consistently detected in our system.

(7) As (4) or (5), but with BV, FMN or ferredoxin omitted, i.e. with NADPH alone as the electron donor.

Activity was linear with respect to time and protein concentration in the assay systems described here. Where several comparative assays were planned, portions of preparations were pipetted into Thunberg tubes and frozen at -18°C until just before use to ensure uniformity and minimal denaturation.

Activity is recorded in terms of loss of substrate used in a specified time or rate of reoxidation of electron donor as stated. Specific-activity units are defined as loss of substrate ($\mu\text{mol}/\text{min}$ per mg of protein) at 27°C for the dark systems (1, 2, 4, 5 and 7) and at 20°C for the photochemical system (6). On an equivalent specific activity basis defined in terms of loss of substrate the stoichiometric reduction of nitrite to ammonia implies a threefold higher rate of electron transport than for the reduction of hydroxylamine.

Nitrite was determined after diazotization by the Griess reaction and measurement of extinction at 540nm; hydroxylamine was determined as nitrite after oxidation by iodine in alkaline solution and ammonia was determined by the phenol-hypochlorite method, all as described previously (Hewitt & Nicholas, 1964; Cresswell *et al.* 1965). Hyponitrite was determined as hydroxylamine by difference between boiled and unboiled samples (Yamafuji & Osajima, 1961; Hewitt & Nicholas, 1964). Interference by NADPH in the determination of nitrite or hydroxylamine (Hewitt & Nicholas, 1964) was negligible. Substrate loss was expressed as the difference between determinations at zero time and after the assay period, with calibration curves prepared in the absence of enzyme.

Protein was precipitated in 10% (w/v) trichloroacetic acid, redissolved in 0.1M-NaOH and determined by the method of Lowry, Rosebrough, Farr & Randall (1951),

and, when necessary in dilute purified preparations, spectrophotometrically (Warburg & Christian, 1941; Patterson, 1964) with serum albumin and casein as standards. Chlorophyll was determined by the method of Arnon (1949).

Chromatography and electrophoresis. Sephadex-gel columns were prepared and calibrated (Andrews, 1964, 1965) with two mixtures: (i) serum albumin, chymotrypsinogen and cytochrome *c*; (ii) γ -globulin, ovalbumin, myoglobin and glucagon. Preparations were applied to columns as described in the figures or tables. For comparative assays with different electron donors each 3ml fraction of eluate was subdivided and frozen at 18°C and then thawed immediately before use.

DEAE-cellulose (Whatman DE50) was washed in 1M-NaOH, 0.2M-HCl and water on Buchner funnels, resuspended in 0.2M-tris-HCl buffer, pH 7.5, and diluted to 10mm-tris-HCl buffer at 0°C until required. ECTEOLA- or aminoethyl-cellulose offered no advantages. Hydroxyapatite (Tiselius, Hjertén & Levin, 1956) had very low flow rates and commercial hydroxyapatite was preferable. It was supported in 2cm-diam. columns on Sephadex G-25.

Electrophoresis was performed in 7% cross-linked polyacrylamide gel at pH 8.3 in a Shandon disc-electrophoresis apparatus and polymerized with ammonium persulphate (Ornstein & Davis, 1964; Reisfeld, Lewis & Williams, 1962). Protein was stained with Naphthalene Black 12B in 7% (v/v) acetic acid and the gel was destained electrophoretically.

RESULTS

Enzyme purification. Leaves (700g) were macerated for 90s in 2 vol./wt. proportions of 20mm-potassium phosphate buffer, pH 7.5, containing 5mm-cysteine hydrochloride and 0.5mm-EDTA. The extract was strained through two layers of organdie, charcoal was added (5g/80ml), stirred and centrifuged immediately at $5000g_{\text{av.}}$. Protein precipitated between 48 and 70% saturation with ammonium sulphate was redissolved and centrifuged in 75ml of 10mm-potassium phosphate buffer, pH 7.5, containing 50mm-sodium chloride to minimize possible adsorption effects (Andrews, 1965) and applied to a column of Sephadex G-100. Fractions eluted by the same solution were monitored by assay method (3) to isolate the nitrite reductase and separate hydroxylamine reductase fractions described in detail below. The fraction containing both nitrite reductase and that part of the hydroxylamine reductase activity referred to below as HR1 was applied directly to a column of hydroxyapatite equilibrated with 10mm-potassium phosphate buffer, pH 7.5, and washed with 6 column vol. of 20mm-potassium phosphate buffer, pH 7.5. The eluate obtained with 40mm-potassium phosphate buffer, pH 7.5, was applied directly to a column of DEAE-cellulose equilibrated with 50mm-potassium phosphate buffer, pH 7.5, and was washed with 34mm-sodium citrate in 10mm-tris-hydrochloric

Table 1. *Purification of nitrite reductase and associated hydroxylamine reductase present in leaf extracts of vegetable marrow*

The enzymes were assayed by method (5) with ferredoxin (40 μ M) reduced by NADPH (0.2 mM) and glucose 6-phosphate as the electron donor, as described in the Experimental section. Incubation was for 25 min at 27°C. Total activity represents loss of substrate (μ mol in 25 min) possible for the whole fraction and specific activity μ mol reduced/min per mg of protein. Abbreviations: NR, nitrite reductase; HR, hydroxylamine reductase.

Fraction	Total volume (ml)	Total activity		Specific activity		Yield		Activity ratio NR/HR
		NR	HR	NR	HR	NR	HR	
(1) Initial extract	1630	19.7	15.5	0.0025	0.002	100	100	1.25
(2) (NH ₄) ₂ SO ₄ ppt. 48–70% satn.	50	25.0	15.6	0.024	0.015	127	100	1.6
(3) Sephadex G-100, 1 litre column	160	11.5	2.5	0.046	0.010	58	16	4.6
(4) Hydroxyapatite, 15 ml column	80	3.6	1.25	0.28	0.095	18	8.1	3.3
(5) DEAE-cellulose, 5 ml column	20	1.9	0.64	1.18	0.40	9.6	4.1	2.9
(6) Sephadex G-100, 280 ml column (best 10 ml)	10	0.49	0.074	3.29	0.49	2.5	0.5	6.7
Purification ratio				1310	245			

acid buffer, pH 7.5, until no further protein was eluted. The enzyme fraction was eluted with 0.2 M-sodium citrate in 10 mM-tris-hydrochloric acid buffer, pH 7.5. This was applied to a column of Sephadex G-100 equilibrated in 10 mM-potassium phosphate buffer, pH 7.5, containing 50 mM-potassium chloride and collected in 10 ml fractions at 20 ml/h. These five steps achieved 700–1300-fold increases in specific activity (see Table 1).

Electrophoresis of nitrite reductase fraction (5) from DEAE-cellulose revealed the presence of a strong band (mobility relative to Bromophenol Blue 0.49) and much weaker bands (M 0.32, 0.41 and 0.61). Fraction 6 was too dilute to examine. Electrophoresis of a similar preparation from spinach (Hewitt *et al.* 1968), corresponding to fraction 6 but omitting the use of hydroxyapatite (870-fold increase in specific activity; 34 μ g/ml) revealed pronounced 'twin' bands (M 0.65) and one much fainter band (M approx. 0.4).

Addition of charcoal after extraction and passage through Sephadex G-25 after precipitation by ammonium sulphate often enhanced activity and removed substances with light-absorption at about 350 nm. Dialysis, addition of ascorbate, citrate or solid polyvinylpyrrolidone or protamine precipitation were less effective. In the experiments reported in Table 1 Sephadex G-100 was used instead of Sephadex G-25 to separate the HR1 fraction from the other major hydroxylamine reductase fraction designated HR2 below. Precipitation by ammonium sulphate apparently removed a factor that inhibited nitrite reductase (Table 1). Acetone

fractionation, although satisfactory with spinach (Ramirez *et al.* 1966; Hewitt *et al.* 1968), was unsatisfactory with marrow, and even after dialysis acetone-treated preparations interfered with hydroxylamine determinations.

Table 1 shows the effects of fractionation on hydroxylamine reductase activity. The assay method with ferredoxin differentiates, as shown below, between HR1 activity associated with the nitrite reductase fraction and another hydroxylamine reductase fraction designated HR2 for which BV[•] and dithionite are the only reductants yet found. As the HR2 fraction was largely eliminated at the first Sephadex G-100 stage values at and after this stage relate to fraction HR1 only.

Comparison of activity observed with two assay methods, i.e. (5) with ferredoxin (40 μ M total) reduced about 15% by NADPH and glucose 6-phosphate at 27°C, or (6) with ferredoxin (0.1 mM total) reduced more completely by washed and heated chloroplast grana, light and ascorbic acid at 20°C, showed large differences (Table 2). Method (6) gave 14–28 times as much activity for nitrite reductase and 3–12 times as much for fraction HR1 in spite of being run at a lower temperature.

Chromatography on Sephadex gels and electron-donor specificity. The results of chromatography on Sephadex G-100 are shown in Figs. 1 and 2. When assayed with BV[•] as electron donor (method 1) nitrite reductase activity appeared in a single peak with molecular weight approx. 63 000, whereas hydroxylamine reductase was eluted in at least two major peaks with molecular weights approx. 60 000

Table 2. Comparison of specific activities and purification of nitrite reductase and hydroxylamine reductase HR1 activities by using two assay methods

The assay methods used were: method (5) with ferredoxin ($40\mu\text{M}$) reduced about 15% by NADPH and glucose 6-phosphate, and method (6) with ferredoxin (0.1mM) reduced with chloroplast grana, light and ascorbic acid as described in the Experimental section. Specific activities are expressed as μmol of substrate reduced/min per mg of protein. The fraction numbers correspond to those shown in Table 1. Abbreviations: NR, nitrite reductase; HR, hydroxylamine reductase.

Fraction	Assay method (5), ferredoxin with NADPH at 27°C for 25 min					Assay method (6), ferredoxin with chloroplast grana at 20°C for 3 min				
	Specific activity		Purification ratio		Activity ratio NR/HR	Specific activity		Purification ratio		Activity ratio NR/HR
	NR	HR	NR	HR		NR	HR	NR	HR	
(1) Initial extract	0.0025	0.002	—	—	1.25	0.069	0.017	—	—	4.2
(5) DEAE-cellulose eluates (5ml)	(a)	0.92	0.32	366	160	2.9	26.8	2.38	389	24.5
	(b)	2.00	1.07	800	558	1.9	47.6	2.24	691	13.6
	(c)	1.28	0.21	512	105	6.1	34.8	6.49	505	39.4
(6) Sephadex G-100 eluates (10ml)	(a)	3.29	0.49	1310	245	6.7	46.3	1.20	668	17.0
	(b)	—	—	—	—	35.0	0	506	<1	>100

Fig. 1. Chromatography of nitrite reductase (NR) and hydroxylamine reductases (HR1 and HR2) from vegetable marrow [50–65%-saturated- $(\text{NH}_4)_2\text{SO}_4$ fraction] on Sephadex G-100 equilibrated in 0.03M-potassium phosphate buffer, pH 7.5, containing 0.1M-KCl. The column dimensions were 61 cm $\times$ 2.45 cm (290ml). The flow rate was 15ml/h and the sample volume was 6ml; 3ml fractions were subdivided for comparative assays with BV (36% reduced) by method (1). Activity is shown as the change in extinction at 540nm after 25min, which is a measure of substrate loss. ■, Nitrite reductase activity; ●, hydroxylamine reductase activity.

and 35000 (Fig. 1). Additional hydroxylamine reductase peaks with molecular weights approx. 120000 and 23000 were also observed. The principal hydroxylamine reductase peak with mole-

cular weight 60000 has been designated HR1. The activity with molecular weight 35000 has been designated HR2 (Hewitt & Hucklesby, 1966; Hewitt *et al.* 1968).

Fig. 2. Chromatography of hydroxylamine reductase activity from vegetable marrow on Sephadex G-100 carried out as described for Fig. 1 but with a column of 254ml and a sample volume of 9ml. Activity was assayed in subdivided 3ml fractions by two methods: FMNH₂ (0.25mM) (method 4) or ferredoxin (40μM) (method 5) as electron donors reduced by NADPH and glucose 6-phosphate and shown as for Fig. 1. The position of the HR1 peak and the expected position of the HR2 peak are indicated by the arrows. O, FMNH₂ assay; ●, ferredoxin assay.

Fig. 3. Chromatography of nitrite reductase (NR) and hydroxylamine reductase (HR1 region) from vegetable marrow on Sephadex G-200 carried out as described for Fig. 1. The column dimensions were 88cm×3.1cm (660ml), the flow rate was 30ml/h and the sample volume was 18ml; 3ml fractions were subdivided for assay of both systems with ferredoxin (55μM) by method (5). Activity is expressed as in Fig. 1. For clarity the symbols for separate points have been omitted and the elution profiles are drawn through each adjacent point for the complete set of 3ml fractions. —, Nitrite reductase; ---, hydroxylamine reductase.

When the assays for hydroxylamine reductase were repeated with ferredoxin (method 5) or with FMNH₂ (method 4), the elution pattern shown in Fig. 2 was different from that obtained with BV*. The peaks and leading boundaries for fraction HR1 still coincided in location and relative activity for both assays with estimated molecular weights 60000–61000. The second clear HR2 peak found with BV* was, however, absent. In the ferredoxin assay the HR1 peak only was observed and it was eluted with a symmetrical boundary profile. In the FMNH₂ assay the rear boundary was trailing and irregular, with indications of minor peaks including some activity in the HR2 region and possibly at about molecular weight 44000, where slight activity was also observed with ferredoxin (Figs. 2 and 3). The correspondence between the principal peaks and boundaries for nitrite reductase and fraction HR1 assayed with ferredoxin (method 5) is shown in Fig. 3 for one of two preparations chromatographed on Sephadex G-200. The peaks showed molecular weights about 61000. There appeared, however, to be differences in the trailing boundaries in the region of molecular weight 45000 approx. The coincidence of peaks for marrow nitrite reductase assayed with BV* and fraction HR1 assayed with ferredoxin after passage through

Sephadex G-200 was shown previously (Hewitt & Hucklesby, 1966; Hewitt *et al.* 1968). Similar relative patterns were observed with BV* and ferredoxin assays for nitrite reductase and hydroxylamine reductase activities in spinach preparations (Hewitt *et al.* 1968). In one of these experiments with spinach, for which the coincidence of hydroxylamine reductase and nitrite reductase activities with ferredoxin had been shown to occur as a major peak at molecular weight 63 000–65 000 approx., the activity of hydroxylamine reductase in another portion of the same preparation chromatographed separately and assayed with FMNH₂ was distributed in a series of decreasing peaks extending to molecular weight 20 000 approx. Activity in the region of fraction HR1 was only 45% of that with ferredoxin, whereas activity in the region of fraction HR2 was 75% of that of HR1 with FMNH₂.

There is therefore a pronounced difference in electron-donor specificity between the three systems, namely fraction HR1, which reacts nearly equally with BV*, ferredoxin and FMNH₂, fraction HR2, which reacts principally or perhaps only with BV* and dithionite, and, as shown previously (Hewitt & Hucklesby, 1966; Hewitt *et al.* 1968), nitrite reductase, which reacts nearly equally well with BV* and ferredoxin and to a much smaller extent with FMNH₂ (Table 3), with which last substance the activity is variable, ranging from almost 0% up to 35% of that with BV* or ferredoxin. The activity with FMNH₂ of hydroxylamine reductase, shown from Fig. 2 to be principally fraction HR1, was 4–10 times that of nitrite

reductase for preparations that showed comparable nitrite reductase and hydroxylamine reductase activities with BV* as electron donor. The reaction between hydroxylamine and FMNH₂ (0.5 mM) reduced 87% by the diaphorase system was essentially enzymic. With boiled or no enzyme under argon about 10% of the normal loss of hydroxylamine was found.

The effectiveness of NADPH alone as electron donor is shown in Table 3. The activity of nitrite reductase with NADPH alone (method 7) was only 3% of that with BV* when BV was reduced by NADPH and was therefore present at the same redox level, whereas the activity of hydroxylamine reductase with NADPH alone was about 30% of that with either BV*, FMNH₂ or ferredoxin when present at similar redox values. Similar results were obtained with a spinach preparation (Table 3).

The inability of fraction HR2 to function with ferredoxin as electron donor and the fact that it had low activity with FMNH₂ prompted a search for some other physiological type of electron donor. Tests with ascorbate alone or together with mammalian cytochrome *c*, or plastocyanin, or with α -lipoic acid, or menadione (or its bisulphite) reduced by NADPH and the diaphorase, or chlorogenic acid or caffeic acid gave negative results. Dithionite served as an electron donor for fraction HR2 but did not reduce hydroxylamine non-enzymically. Potassium borohydride was not effective. Dithionite, borohydride and BV* produced changes in the light-absorption spectrum of HR2 preparations (Hucklesby, Hewitt & James,

Table 3. *Comparative activities of nitrite reductase and hydroxylamine reductase (HR1 + HR2) corresponding to preparations of fraction (2) (Table 1) when assayed with different electron-donor systems*

The electron-donor systems were: (2) BV* (0.7 mM) reduced by NADPH (0.2 mM) and glucose 6-phosphate; (4) FMNH₂ (0.4 mM) and (5) ferredoxin (40 μ M) reduced by the same system as (2); (7) NADPH (0.2 mM) and glucose 6-phosphate only. Expts. 1 and 2 were done with marrow and Expt. 3 with spinach preparations. Comparative activities are expressed as μ mol of substrate loss in 25 min. The last two lines show means, standard errors (as % of mean) and numbers of assays for 23 experiments with several marrow preparations. The non-orthogonal analysis of transformed data accommodated differences in actual and specific activities of preparations obtained or used on different occasions and the incompleteness of sets resulting from comparisons made with only two or three combinations of the four possible electron-donor systems. Significant differences are shown at the following confidence levels: *** 0.1%; ** 1%; * 5%; N.S., not significant. Significant differences for nitrite reductase: BV* $\leq$ ferredoxin *** FMNH₂ *** NADPH; significant differences for hydroxylamine reductase: BV* $\leq$ ferredoxin $\leq$ NADPH; BV*, FMNH₂ *** NADPH. Abbreviations: NR, nitrite reductase; HR, hydroxylamine reductase.

Assay method ...	(2) BV*		(4) FMNH ₂		(5) Ferredoxin		(7) NADPH	
Expt. no.	NR	HR	NR	HR	NR	HR	NR	HR
1 (Marrow)	1077	1200	101	1021	—	—	21	345
2 (Marrow)	913	825	71	578	325	638	—	—
3 (Spinach)	526	553	122	293	515	432	0	113
Means for 23 expts. with marrow	622 *	946	77 ***	743	368 N.S.	474	20 ***	260
S.E.M. as % of mean (no. of assays)	13.8 (11)	14.5 (10)	11.8 (15)	12.7 (13)	17.3 (7)	20.5 (5)	20.5 (4)	13.2 (12)

Table 4. *Yield of ammonia from nitrite or hydroxylamine obtained with preparations of different purification ratios with respect to nitrite reductase and different specific activities produced under different assay conditions for respective substrates*

The assay conditions were: (1) with 3.6mM-BV (35% reduced), (2) with BV* (0.7mM), (4) with FMNH₂ (0.4mM), and (5) with ferredoxin (40μM) all reduced by NADPH (0.2mM) and the glucose 6-phosphate system; (6) ferredoxin (0.1mM) reduced with chloroplast grana, light and ascorbic acid; (7) NADPH (0.2mM) alone, reduced with glucose 6-phosphate. Preparations 1–4 were not treated with Sephadex G-100 to separate the multiple hydroxylamine reductases (HR1 and HR2), which show different specificity for BV*, FMNH₂ or ferredoxin as electron donors; preparations 5–7 contained only HR1 as described in the text. Preparation 3 was from spinach, the others were from marrow. Data for preparation 1 were obtained by E. J. Hewitt & G. F. Betts (unpublished work).

Prep. no.	Purification ratio for nitrite reductase	Assay method	Substrate	Specific activity for substrate and assay method	Substrate loss (nmol)	Ammonia gain (nmol)
1	~ 10	(2)	Nitrite	0.009	913	701
			Hydroxylamine	0.008	825	815
		(4)	Nitrite	0.0007	71	100
			Hydroxylamine	0.006	578	510
		(5)	Nitrite	0.003	325	250
			Hydroxylamine	0.006	628	486
2	~ 10	(4)	Hydroxylamine	0.007	702	761
		(7)	Hydroxylamine	0.002	221	260
3	~ 10	(6)	Nitrite	0.082	1670	1640
			Hydroxylamine	0.032	669	635
4	74	(1)	Nitrite	0.66	795	877
5	720	(6)	Nitrite	46.3	416	450
			Hydroxylamine	1.2	11	33
6	760	(6)	Nitrite	35.1	295	312
7	1310	(5)	Nitrite	3.3	1232	1171
			Hydroxylamine	0.49	212	380

1970). The hydroxylamine reductase activity obtained in the presence of NADPH alone (Table 3) can be ascribed to the fraction HR1 having relatively wide electron-donor specificity because in several experiments where fraction HR2 was separated from HR1, its activity with ferredoxin or with menadione, menadione bisulphite or α -lipoic acid, each reduced in the presence of the NADPH–glucose 6-phosphate system, was negligible as compared with activity with BV* reduced in the same system.

Yield of ammonia. Yields of ammonia from nitrite (Table 4) in the enzymic system at pH 7.5 were practically quantitative regardless of purification factor, residual HR1 activity, method of assay, or rate of reduction. A similar conclusion was drawn for the product of fraction HR1 or combined fraction HR1 and fraction HR2 activities for several electron-donor systems with wider limits of error. These results are in agreement with those reported for spinach (Hewitt *et al.* 1968).

Turnover number. A minimum turnover number for nitrite reductase of 3×10^3 mol of nitrite reduced/min per mol of enzyme at 20°C corresponding to an electron-transport rate of 1.8×10^4 electrons/min per mol of enzyme was calculated on the basis of the maximum specific activity of 47.6 and the estimated molecular weight 63000.

Stability relative to pH, temperature and storage. The activity of nitrite reductase was more sensitive to denaturation at low pH than that of hydroxylamine reductase (Fig. 4). In two thermal-denaturation tests (Fig. 5) nitrite reductase activity was over 90% denatured by 10min at 55°C whereas hydroxylamine reductase activity was inactivated about 25–40% at 55°C and only 50–85% at 60°C. Heats of denaturation calculated from Fig. 5 were 91 and 106kcal/mol for nitrite reductase and 19 and 37kcal/mol for the unresolved HR1 and HR2 system. Preparations stored after fractionation to the stage of DEAE-cellulose were often stable for several weeks at –18°C but lost activity unpredictably.

Effects of pH on activity. Assay mixtures were prepared by using BV* as electron donor in 1mM-potassium phosphate buffer, pH 7.5, which was adjusted to various pH values by the presence of appropriate citrate, phosphate or borate buffers (0.1M) in the Thunberg tube. Nitrite does not react non-enzymically with BV* at an appreciable rate at pH 7.5 when reduced less than 60% (Cresswell *et al.* 1965). At pH values below 7 nitrite and BV* react non-enzymically with increasing rapidity as the pH decreases. Enzymic and non-enzymic activities were measured together by method (1)

Fig. 4. Effect of pH on the denaturation of nitrite reductase and mixed hydroxylamine reductases (HR1 and HR2) from vegetable marrow [50–65% saturated- $(\text{NH}_4)_2\text{SO}_4$ and Sephadex G-25 fraction]. Preparations were held at the pH values shown for 15 min before restoration to pH 7.5 for assay with reduced benzyl viologen by method (1). Activity is shown as substrate loss (μmol) in 25 min. ■, Nitrite reductase activity; ●, hydroxylamine reductase (HR1 plus HR2) activity.

Fig. 5. Effect of temperature on the denaturation of nitrite reductase and mixed hydroxylamine reductases (HR1 and HR2) from vegetable marrow obtained as described for Fig. 4. Preparations were held at the temperatures shown for 10 min, cooled rapidly, centrifuged and assayed by method (1) as for Fig. 4. Activity is shown as substrate loss (μmol) in 25 min. □ and ■, Nitrite reductase activity; ○ and ●, hydroxylamine reductase (HR1 plus HR2) activity; two separate preparations were used.

and values for the non-enzymic reaction alone were subtracted as shown in Fig. 6. A broad optimum between pH 6.0 and 7.5 was found for the enzymic system. As non-enzymic reaction was just detectable at pH 7.0 the value of pH 7.5 adopted by Cresswell *et al.* (1965) was adhered to for the remainder of the work.

Whereas at pH 7.5 nitrite is quantitatively reduced enzymically to ammonia, other products must be produced in the non-enzymic reaction at lower pH values. Thus at pH 4.5 in the absence of enzyme 2.86 μmol of $\text{BV}^\bullet$ was reoxidized for a loss of 1.75 μmol of nitrite instead of an expected loss of only 0.48 μmol if ammonia were the product. The stoichiometry of $<2 \text{ e}^-/\text{NO}_2^-$ ion indicates that some nitric oxide was produced. In three further tests where all nitrite was lost in the presence of excess of $\text{BV}^\bullet$ (35% reduced BV) 20–23% was recovered as hydroxylamine or hypo-

nitrite or both and 5% or less was identified as hydroxylamine after boiling. The stoichiometry of these experiments predicts over 70% production of nitric oxide and less than 10% of ammonia. It is concluded that non-enzymic reactions with $\text{BV}^\bullet$ occur with nitrous acid (HONO) and enzymic ones with nitrite (NO_2^-) ions.

No non-enzymic reaction between $\text{BV}^\bullet$ and hydroxylamine was observed at pH values between 4.0 and 9.0. However, in three experiments with the unresolved HR1 and HR2 systems no clear or consistent pH optima were observed.

Effects of guanidinium chloride or urea. Preparations containing nitrite reductase/fraction HR1 and fraction HR2 were incubated for 10 min with different concentrations of guanidinium chloride in 0.1 M-tris-hydrochloric acid buffer at pH 7.5, then dialysed for 3 h against 0.1 M-tris-hydrochloric acid. The activity of nitrite reductase assayed by re-oxidation of $\text{BV}^\bullet$ (method 3) was inhibited 80 and 90% by 2 M- and 3 M-guanidinium chloride respectively, whereas hydroxylamine reductase activity, which comprised both fraction HR1 and fraction HR2, was inhibited only 25 and 30%. When ferredoxin was used as electron donor (method 5) the HR1 activity was inhibited 38 and 50% by the 2 M- and 3 M-guanidinium treatments. In 1.5 M-guanidinium chloride, nitrite reductase and total

Fig. 6. Effect of pH on the activity of nitrite reductase from vegetable marrow obtained as described for Fig. 4. Buffer systems were: 0.1M-citrate, pH 4.5–6.0, 0.1M-phosphate, pH 6.0–8.0, 0.1M-borate-KCl, pH above 8.0. A 1ml volume of buffer was mixed with 5ml of BV[•] (35% reduced) in 1mM-phosphate buffer, pH 7.5, and 0.5ml of enzyme solution. The pH was remeasured after assay and actual pH ranges were as indicated. The curve for enzyme activity (●) is the arithmetical difference between values for non-enzymic (Δ) and combined non-enzymic and enzymic rates (□).

hydroxylamine reductase assayed by BV[•], and fraction HR1 assayed by ferredoxin were all inhibited to similar extents of 21–23%. Similar results were obtained with nitrite reductase when dialysis was omitted but a slow non-enzymic reaction between nitrite, BV[•] and guanidinium chloride was also observed. Treatment with urea at 6M concentration for 10min in 50mM-potassium phosphate buffer, pH 7.5, caused less than 30% inhibition of nitrite reductase when tested without dialysis by reoxidation of BV[•] added subsequently so that the urea was diluted to 1.5M. In a similar experiment with 6.4M-guanidinium chloride, inhibition was 100%. The possibility that the electron-transfer sequence in nitrite reductase might have been interrupted by guanidine treatment was tested by measuring the ratio of BV[•] reoxidized to nitrite lost. A mean value of 6.6 from three experiments compared with a theoretical value of 6.0 indicated that reduction to ammonia was complete in spite of inhibition. Ammonia formation was not

determined because guanidinium chloride controls produced too much ammonia for reliable estimates of enzymic activity, but interference with nitrite estimation was negligible.

Substrate specificity. (i) Sulphite. The respective nitrite reductase/fraction HR1 and fraction HR2 systems (stage 3 of Table 1) were tested separately for ability to reduce sulphite with BV[•] as electron donor. A sulphite reductase in marrow utilizes BV[•] and is precipitated by 48%-saturated ammonium sulphate together with a cofactor, which is separated by retardation on Sephadex G-25 (E. J. Hewitt, D. P. Hucklesby & D. M. James, unpublished work). The cofactor was added to nitrite reductase/fraction HR1 or to fraction HR2 preparations together with their normal substrates (0.5mM) or sulphite (1.5mM) or both. Activity was assayed by reoxidation of BV[•] (method 3). No sulphite reductase activity was detected in either enzyme system with or without the added cofactor, although the activity with the normal substrates was high. Sulphite was not inhibitory to nitrite reductase, but fractions HR1 and HR2 were both inhibited 40% in systems where rates were linear for 30min (E. J. Hewitt & D. M. James, unpublished work).

(ii) Hyponitrite. Hyponitrite is unstable in aqueous solutions at pH 7.5, producing nitrous oxide (Anderson, 1963), so that hyponitrite reductase activity cannot be measured by hyponitrite disappearance, and reoxidation of BV[•] (method 3) was used in a Thunberg tube with a double side arm. Sodium hyponitrite (2mM as tested), free of nitrite and hydroxylamine (Hewitt & Nicholas, 1964), was tipped into an unresolved nitrite reductase/fraction HR1 plus fraction HR2 preparation (Table 1, stage 2) with BV[•]. There was no reaction, as shown by non-reoxidation of BV[•] for 15min, although subsequent addition of nitrite (0.5mM) from the second side arm showed that the nitrite reductase activity was high. In two further experiments, where hyponitrite was incubated with BV[•] and an active nitrite reductase preparation for 25min, no reoxidation of the BV[•] was observed, but hyponitrite still remained in the reaction mixture at the end of the experiments. Hyponitrite was therefore still present in the previous test when nitrite was tipped and it was not significantly inhibitory to nitrite reductase activity.

(iii) Oximes. An unresolved nitrite reductase/fraction HR1 plus fraction HR2 preparation of high activity was assayed by reoxidation of BV in the presence of oximes of pyruvic acid or α -oxoglutaric acid or acetone. Pyruvic oxime (1mM), characterized by melting point and i.r. spectrum, produced about 4% of the activity observed with hydroxylamine at 0.7mM, but the other two oximes were inactive.

DISCUSSION

The maximum specific activities for highly purified nitrite reductase from vegetable marrow (46.3–47.6 μmol reduced/min per mg of protein) and comparable values from spinach (Hewitt *et al.* 1968) are more than ten times those previously reported for higher plants. The higher specific activities obtained in these experiments probably result from the electron-donating system used, namely ferredoxin in the presence of the ascorbate–indophenol couple together with plastocyanin and chloroplast grana and illuminated by light of high intensity under anaerobic conditions. This system has the advantage of achieving maximal reduction of ferredoxin without generating inhibitory by-products.

The nitrite reductase activity obtained with ferredoxin in the light–grana system was about 25 times that in the dark–glucose 6-phosphate system (Table 2), whereas the ratio of concentrations of reduced ferredoxin in the two systems is calculated to have been probably about 15:1. In a normal kinetic system a 100-fold change in concentration from one-tenth to ten times the K_m value would be expected to produce an approximately tenfold change in activity. The difference between six-tenths and nine times the K_m concentration of about 10 μM (Hewitt *et al.* 1968; A. R. Crofts & E. J. Hewitt, unpublished work) calculated as reduced ferredoxin would be expected to produce only a threefold difference in activity. The electron-donating capacity of the dark system as measured by rate of reduction of FMN or by use of $\text{BV}^\bullet$ as electron donor was in substantial excess of the requirement of the nitrite reductase activity and would not be expected to have been a limiting factor. The suggestion that highly reduced ferredoxin may react non-enzymically with nitrite (Joy & Hageman, 1966) was not important here when washed and heated grana were used (Hewitt *et al.* 1968). The spinach nitrite reductase preparation obtained by Ramirez *et al.* (1966) was intermediate in specific activity between values obtained in the light–grana and dark systems for preparations of comparable purity tested by Hewitt *et al.* (1968). Studies with *Chromatium* ferredoxin (Arnon, 1968) indicate that ferredoxin reduced by dithionite as used by Ramirez *et al.* (1966) might be intermediate in reduction between that obtained in the light and the dark reduction systems (Hewitt *et al.* 1968, and this work). The activity of nitrite reductase with ferredoxin may therefore be subject to a similar relationship to redox values to that inferred to occur with $\text{BV}^\bullet$ (Cresswell *et al.* 1965). The lower sensitivity of the HR1 system to the method of ferredoxin reduction corresponds with the less-marked effect of degree of reduction to $\text{BV}^\bullet$ on

the unresolved hydroxylamine reductase systems (Cresswell *et al.* 1965) and with the lower K_m value (1 μM) for ferredoxin in the HR1 system in spinach (Betts & Hewitt, 1966).

The variable but low activity of nitrite reductase in the presence of FMNH_2 observed in several preparations, in spite of previous reports to the contrary (Cresswell *et al.* 1965; Joy & Hageman, 1966; Ramirez *et al.* 1966), suggests that an additional dissociable factor may be needed to link FMNH_2 with the nitrite reductase system. Presumably it is not needed by the fraction HR1 system, which shows consistently high activity with FMNH_2 (Table 3). Contamination by ferredoxin in the diaphorase could account for the appreciable activity of FMNH_2 with the nitrite reductase system, but the procedures used make this unlikely.

The hydroxylamine reductase activity of these preparations was provided by two or possibly more fractions differing in molecular weight and in electron-donor specificity. The fraction designated HR2, of lower molecular weight than nitrite reductase, behaved as a distinct protein and no efficient physiological electron donor for it was found. Its activity with $\text{BV}^\bullet$ but not with ferredoxin resembled behaviour of nitrogenase from soya-bean-nodule bacteroids (Klucas & Evans, 1968) and spinach nitrate reductase (Del Campo, Paneque, Ramirez & Losada, 1963). The fraction designated HR1 has a molecular weight close to that of nitrite reductase, from which it has not been separated in marrow or spinach preparations (Hewitt *et al.* 1968). The small separation of peak elution values for nitrite reductase and fraction HR1 during elution from Sephadex G-200 and G-100 was obtained with the use of $\text{BV}^\bullet$ for the assays (Hewitt & Hucklesby, 1966). It was considered later (Hewitt *et al.* 1966) and appears from the use of ferredoxin in the present work (Fig. 3) that this separation could have resulted from mutual supplementation of overlapping boundaries of HR1 and HR2 fractions so that their peaks are 'pulled' together, whereas the single nitrite reductase peak is correctly indicated in the $\text{BV}^\bullet$ system and the fraction HR1 and nitrite reductase peaks are both correctly indicated in the ferredoxin system in which fraction HR2 is unreactive. In the marrow preparations ferredoxin produced little hydroxylamine reductase activity in the region beyond the HR1 peak, but in spinach preparations apparently consistent and noteworthy hydroxylamine reductase peaks were found at molecular weight about 40 000 and 22 000 (Hewitt & Hucklesby, 1966; Hewitt *et al.* 1968).

There was a progressive loss of fraction HR1 during the purification of nitrite reductase. The final HR1 activity was 15% of the hydroxylamine reductase activity in the ferredoxin assay system

with glucose 6-phosphate and NADP⁺ and only 1–2.5% in the ferredoxin system with chloroplast grana and light (Table 2). Similar decreases in hydroxylamine reductase activity relative to that of nitrite reductase were obtained with spinach preparations (Ramirez *et al.* 1966; Hewitt *et al.* 1968). It is uncertain at present whether this loss represents a separation of fraction HR1 and nitrite reductase or a differential inactivation of the hydroxylamine-reducing component of a complex enzyme system. Differential sensitivity to denaturation by heat or low pH does not necessarily distinguish between these alternatives.

Certainly the loss of HR1 activity from purified nitrite reductase does not interfere with the stoichiometric conversion of nitrite into ammonia by both the marrow and the spinach enzymes. As shown in Table 4 and elsewhere (Cresswell *et al.* 1965; Betts & Hewitt, 1966; Hewitt *et al.* 1968; Joy & Hageman, 1966; Ramirez *et al.* 1966) this stoichiometry holds regardless of rate of reaction, electron donor, specific activity or degree of protein purification up to 1300-fold. The evidence indicates that one protein mediates the transfer of 6 electrons and that free hydroxylamine is not involved as an intermediate in the reaction. Failure to reduce hyponitrite excludes this as a potential intermediate, a result in accordance with conclusions of Frear & Burrell (1958). Hyponitrite did not inhibit marrow nitrite reductase, in contrast with the *Neurospora* enzyme (Nicholas, 1959). Generalizations on the participation of hyponitrite and hydroxylamine in a physiological sequence for nitrite reduction by plants, based on studies of fungal enzymes (Nicholas, 1959), are not substantiated by our work.

Multiple hydroxylamine reductase proteins occur in *Neurospora crassa* (Leinweber, Siegel & Monty, 1965) and multiple nitrite reductase systems occur in *Escherichia coli* (Kemp & Atkinson, 1966). Multiple functions for apparently a single protein were shown for nitrite and sulphite reduction in *E. coli* (Lazzarini & Atkinson, 1961; Kemp, Atkinson, Ehret & Lazzarini, 1963), for hydroxylamine and sulphite reduction in *E. coli*, yeast, *N. crassa* and higher plants (Mager, 1960; Tamura, 1964; Siegel, Leinweber & Monty, 1965; Asada, Tamura & Bandurski, 1968), for nitrite and hydroxylamine reduction in *E. coli* (Kemp & Atkinson, 1966) and for all three substrates in yeast (Yoshimoto & Sato, 1968). Although there is considerable 'overlapping' of specificity in this class of enzymes, neither the nitrite reductase/fraction HR1 nor the fraction HR2 preparations obtained from marrow would reduce sulphite with BV⁺ although the cofactor necessary for sulphite reductase was added. Sulphite was, however, inhibitory to both hydroxylamine reductase frac-

tions but not to nitrite reductase activity. Sulphite reductase of marrow and spinach is separated from nitrite reductase/fraction HR1 and fraction HR2 by precipitation with ammonium sulphate (E. J. Hewitt, D. P. Hucklesby & D. M. James, unpublished work). The hydroxylamine reductase activity remaining in purified spinach sulphite reductase (Asada *et al.* 1968) may therefore be distinct from the HR1 and HR2 fractions described here. Alternatively the enzymes may show different specificity for methyl viologen and benzyl viologen used respectively by Asada *et al.* (1968) and ourselves; specificity of this nature occurs with nitrate reductase (Del Campo *et al.* 1963).

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