The Reduction of Nitrate, Nitrite and Hydroxylamine to Ammonia by Enzymes from *Cucurbita pepo* L. in the Presence of Reduced Benzyl Viologen as Electron Donor

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(Received 10 March 1964)

1. Enzyme systems from *Cucurbita pepo* have been shown to catalyse the reduction of nitrite and hydroxylamine to ammonia in yields about 90–100%. 2. Reduced benzyl viologen serves as an efficient electron donor for both systems. Activity of the nitrite-reductase system is directly related to degree of dye reduction when expressed in terms of the function for oxidation–reduction potentials, but appears to decrease to negligible activity below about 9% dye reduction. 3. NADH and NADPH alone produce negligible nitrite loss, but NADPH can be linked to an endogenous diaphorase system to reduce nitrite to ammonia in the presence of catalytic amounts of benzyl viologen. 4. The NADH- or NADPH-nitrate-reductase system that is also present can accept electrons from reduced benzyl viologen, but shows relationships opposite to that for the nitrite-reductase system with regard to effect of degree of dye reduction on activity. The product of nitrate reduction may be nitrite alone, or nitrite and ammonia, or ammonia alone, according only to the degree of dye reduction. 5. The relative activities of nitrite-reductase and hydroxylamine-reductase systems show different relationships with degree of dye reduction and may become reversed in magnitude when effects of degree of dye reduction are tested over a suitable range. 6. Nitrite severely inhibits the rate of reduction of hydroxylamine without affecting the yield of ammonia as a percentage of total substrate loss, but hydroxylamine has a negligible effect on the activity of the nitrite-reductase system. 7. The apparent K_m for nitrite (1 μM) is substantially less than that for hydroxylamine, for which variable values between 0.05 and 0.9 mM (mean 0.51 mM) have been observed. 8. The apparent K_m values for reduced benzyl viologen differ for the nitrite-reductase and hydroxylamine-reductase systems: 60 and 7.5 μM respectively. 9. It is concluded that free hydroxylamine may not be an intermediate in the reduction of nitrite to ammonia by plants, and a possible mechanism for reduction of both compounds by the same enzyme system is discussed in the light of current ideas relating to other organisms.

The biological reduction of nitrate to ammonia has been assumed to proceed in stages each involving two electrons. Nitrite is often observed as an end product of nitrate reduction by plant enzymes *in vitro* although ammonia is ultimately formed *in vivo*. Hydroxylamine has been regarded as a likely intermediate in the reduction of nitrite to ammonia where this process has been demonstrated. This subject has been extensively reviewed, e.g. by Nason & Takahashi (1958), Nicholas (1957, 1958, 1959), Webster (1959), McKee (1962) and Nason (1962).

In an investigation of nitrate reduction by en-

zymes in leaf extracts of vegetable marrow (*Cucurbita pepo* L.) we sought to use reduced benzyl viologen as a substitute for NADH as the electron donor. The results at first were difficult to interpret because of inconsistency in the behaviour of the same, or identically treated, preparations, used on the same or on successive days. Thus separate preparations having identical nitrate-reductase (NADH-nitrate oxidoreductase, EC 1.6.6.1) activities as determined by measurements of production of nitrite as the sole end product with NADH would produce nil to large amounts of nitrite from nitrate when assayed with reduced benzyl viologen, and sometimes small amounts of ammonia. Further, these same preparations might, when used on different occasions, either fail to reduce added nitrite or reduce it to ammonia with great rapidity. These

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results indicated the presence of an active nitrite-reductase system that had hitherto remained undetected in plants (Hageman, Cresswell & Hewitt, 1962). As is shown below, the different types of behaviour noted above were found to be all related to the probable degree of benzyl viologen reduction, when expressed in terms used to calculate the oxidation-reduction potential of the system. In view of the recent descriptions (Tagawa & Arnon, 1962; Valentine, Brill, Wolfe & San Pietro, 1962; Valentine, Jackson & Wolfe, 1962; Whitely & Woolfolk, 1962; Losada, Paneque, Ramirez & Del Campo, 1963; Mortenson, Valentine & Carnahan, 1962; Paneque, Del Campo & Losada, 1963) of systems in which benzyl viologen and ferredoxins, the iron-containing proteins obtained from several sources, have been shown to exhibit similar electron-transferring functions, we think our results are of interest and are relevant to studies in progress in several Laboratories (Hewitt & Betts, 1963; Losada *et al.* 1963; Paneque *et al.* 1963) on systems that utilize ferredoxins in nitrite and hydroxylamine reduction by plants. Experience obtained with reduced benzyl viologen may be useful in helping to elucidate the mechanism of action of the natural single-electron carrier in nitrite and hydroxylamine metabolism.

Our preliminary results (Hageman *et al.* 1962) and further observations reported briefly (Cresswell, Hageman & Hewitt, 1962) suggested a reappraisal of the status of hydroxylamine as an intermediate in nitrite reduction to ammonia. This problem is considered in relation to some kinetic aspects of nitrite and hydroxylamine reduction to ammonia.

MATERIALS AND METHODS

Plants. Seedlings of *Cucurbita pepo* var. Bushy Type (Suttons Seeds Ltd., Reading) were grown in sand culture in a controlled environment chamber with a day length of 18 hr. and a light-intensity of 1100 lumens/ft.² at plant level at 26.6° in the light and at 20° in the dark. The nutrient solutions, including those deficient in micronutrients, were prepared as described by Hewitt (1952); nitrogen was supplied solely in the nitrate form.

Reagents. The following chemicals and enzyme preparations were obtained from sources indicated: FMN, FAD, GSH, the sodium salts of NAD⁺ and NADP⁺, enzymically reduced NADH and NADPH, D-glucose 6-phosphate, the enzyme glucose 6-phosphate dehydrogenase (yeast *Zwischenferment*) (EC 1.1.1.49), NADP⁺ and NADPH were given by Sigma Chemical Co.; L-cysteine hydrochloride, EDTA (disodium salt), diphenylthiocarbazone, NN'-dibenzyl-4,4'-bipyridylium dichloride (benzyl viologen), sulphanilamide, N-1-naphthylethylenediamine dihydrochloride, tris and all other inorganic chemical reagents used were from British Drug Houses Ltd.; palladized asbestos (5% Pd) was from Hopkin and Williams Ltd. Ammonium sulphate was heavily contaminated by heavy metals, and saturated solutions were purified by extraction at room

temperature with dithizone at pH 7.5, and 8-hydroxyquinoline at pH 5 as described by Hewitt & Nicholas (1964). The pH was then raised by adding ammonia solution until free ammonia could be detected.

Reduction of NN'-dibenzyl-4,4'-bipyridylium dichloride. The dye (3.65 mM) dissolved in 0.05 M-potassium phosphate buffer, pH 7.5, was reduced by hydrogen and palladized asbestos in the apparatus described by Hageman *et al.* (1962) and Hewitt & Nicholas (1964), and the extent of reduction was determined by titration into potassium permanganate in an evacuated Thunberg tube as previously described. The extent of dye reduction is expressed either as % of BVH* or as the logarithm of the ratio of reduced to oxidized dye or as the concentration of BVH which is independent of the expressions above.

Enzyme preparation. Rapidly expanding leaves from plants between 3 and 5 weeks old were used in preparing the enzymes. The leaves were harvested, washed with deionized water, dried on clean filter paper and chilled in a cold room at 2° for 15–30 min. One part (by wt.) of tissue and 3 vol. of 0.1 M-tris buffer, pH 7.8, containing disodium EDTA (0.4 mM) and L-cysteine hydrochloride (5 mM) were homogenized in an MSE Ato-Mix blender for 90 sec. at full speed. All operations were carried out so that the enzyme preparations remained cold (0–2°). The homogenate was squeezed through two layers of muslin, and centrifuged at 15000 g_{av.} for 15 min. Cold saturated (NH₄)₂SO₄ solution containing 1 mM-L-cysteine hydrochloride and 1 mM-KOH was added to the supernatant solution to give 66% saturation and left for 30 min. before centrifuging at 3000 g for 30 min. The supernatant solution was discarded and the precipitate was resuspended in cold 0.1 M-potassium phosphate buffer, pH 7.5, containing L-cysteine hydrochloride (1 mM), in a volume usually equivalent to the original weight of fresh tissue.

The enzyme solution was placed in Visking dialysing tubing that had previously been soaked in 10 mM-GSH for several hours, and dialysed against 1 mM-cysteine in 0.03 M-phosphate buffer, pH 7.5, with three changes each of 3 l. for a total period of at least 15 hr. The enzyme solution was then centrifuged at 15000 g_{av.} for 30 min. and decanted into several chilled small polythene bottles. Some preparations were separated from (NH₄)₂SO₄ by passage through Sephadex G-50 (Pharmacia, Uppsala, Sweden) in a column or by adding solid ground Sephadex and then collecting the enzyme solution by centrifugation. If the enzyme preparation was required immediately it was placed in ice; otherwise it was stored at –15°. Nitrate-reductase, nitrite-reductase and hydroxylamine-reductase activities were stable for several weeks at –15°, but activity deteriorated significantly in a few hours at 0°. During long experiments, frozen preparations were crushed in the bottles and small amounts were thawed at intervals.

Assay of enzyme activity. Enzyme assays were done at 27° in Thunberg tubes under anaerobic conditions for periods between 5 and 25 min. according to circumstances, loss of substrate being a function of time. Assays were made at pH 7.5 in 0.05 M-potassium phosphate buffer. Between 0.1 and 0.5 ml. of enzyme solution containing 4–15 mg. of protein/ml. was used according to activity and the objects of the experiment; activity was proportional to enzyme protein. Assays were made in duplicate and showed satisfactory agreement. In all cases the inclusion of boiled

* Abbreviations: BVH, reduced benzyl viologen; BV, benzyl viologen.

enzyme preparations showed that the reactions considered here were enzymic under the conditions specified.

Nitrate-reductase activity was measured by both nitrite and ammonia formation. Nitrite-reductase and hydroxylamine-reductase activities were measured both as substrate loss and ammonia formation until satisfactory evidence of their stoichiometry was obtained.

Nitrate, when used, was present at a concentration of 6 mM. The usual concentrations of nitrite or hydroxylamine were 0.28 mM, except where otherwise stated. Nitrite reductase was saturated by substrate under these conditions but hydroxylamine reductase was not.

When NADPH (or NADH) was used as electron donor at concentrations of 1 mM or more, the nucleotides were removed with zinc acetate and ethanol as described by Hewitt & Nicholas (1964) before estimating substrate losses. Preparations contained an active glucose 6-phosphate-NADPH dehydrogenase and an NADPH-specific diaphorase (Avron & Jagendorf, 1956) that Hageman *et al.* (1962) showed readily reduced BV. These enzymes, supplemented on some occasions by *Zwischenferment* from yeast, formed an efficient system linking glucose 6-phosphate to nitrite reduction or hydroxylamine reduction in the presence of catalytic quantities of NADP and BV as described in the Results section. When glucose 6-phosphate (20 μ moles) was added and NADPH concentration was decreased to 0.1 mM, usually in the presence of small amounts of BVH, as shown in the Results section, nitrite and hydroxylamine were determined without prior removal of NADPH. These assays were carried out in a total volume of 2.5–3.0 ml. When partially reduced benzyl viologen was used as the sole electron donor in substrate amounts, 6.0 ml. of dye was usually added in the manner described (Hageman *et al.* 1962). The assays were terminated by opening the Thunberg tubes and shaking for a few seconds to oxidize all the BVH. The actual volumes of reaction mixture were determined to ± 0.05 ml. after withdrawing portions for various determinations. No reaction occurred between the substrates and hydrogen under the conditions used with precautions to exclude any pallidized asbestos from the assay mixtures.

Nitrite was determined by a slightly modified method of Evans & Nason (1953) as described by Candela, Fisher & Hewitt (1957). Ammonia was determined in portions (1.0 ml.) by the microdiffusion method of Conway (1957) and colorimetric measurement by the alkaline phenate-hypochlorite procedure of Russell (1945). Light-extinction was measured at 625 $m\mu$. The estimation of hydroxylamine was at first carried out by the method of Csáky (1949), but serious interference occurred when BV was present, apparently because of pyridinium iodide formation. This reaction was decreased to negligible importance by using iodine at pH 8.5 $\pm$ 0.2, whereas hydroxylamine was oxidized nearly stoichiometrically to nitrite provided that the concentration of BV did not exceed 0.25 mM. The method used was based on that of Yamafuji & Akita (1952) and has been described in a modified form suited to the present work (Hewitt & Nicholas, 1964). The extinction coefficient at 540 $m\mu$ for nitrite was the same in either the above method or the usual Griess-Ilosvay method. The same extinction coefficient was obtained for nitrite with the Griess-Ilosvay method in the presence or absence of hydroxylamine. The amounts of nitrite and hydroxylamine present in a mixture was therefore determined by first estimating nitrite by the Griess-Ilosvay method and subtracting this extinction

value from that produced by the two together in the alkaline iodine method. The appropriate factor for hydroxylamine derived from calibration data obtained in the presence of BV and nitrite (Hewitt & Nicholas, 1964) was then used to calculate changes in hydroxylamine concentrations. When the effects of pyruvic oxime formation on enzyme activity were investigated a further modification of the method was found necessary. This consisted in adding 0.3 ml. of a solution of iodine [1.25% (w/v) dissolved in the presence of 9.5% (w/v) KI] to the test solution made up to 6.7 ml. with 0.16 M-potassium phosphate, pH 8.5, and then adding 0.05 ml. of N-KOH, which partially bleached the iodine colour. The mixture was heated in a water bath to 80° and left to cool at room temperature before addition of the other reagents (Hewitt & Nicholas, 1964). By this method hydroxylamine alone yielded 90% of the extinction at 540 $m\mu$ of an equivalent amount of nitrite; in the presence of pyruvate the yield was 100% and diacetyl monoxime also yielded 100% of the extinction of nitrite. Yamafuji & Akita (1952) observed that oximes yielded more nitrite than free hydroxylamine by their method. The method of Frear & Burrell (1955), which would have provided an independent check on hydroxylamine alone, could not be used in the presence of BV.

Protein estimation. Protein was determined by Folin-Ciocalteu method as described by Lowry, Rosebrough, Farr & Randall (1951) and was calibrated with standard casein. Protein was precipitated in 5% (w/v) trichloroacetic acid and stored until convenient, when it was redissolved in 0.1 N-NaOH before estimation.

RESULTS

Nature and products of reaction

Over the range of dye reduction corresponding to the points in Figs. 1 and 3 the reactions resulting in substrate disappearance were strictly enzymic. When about 75% of the dye was reduced, i.e. when $\log([BVH]/[BV]) = 0.5$, a chemical reduction of nitrite occurred but yields of ammonia were low.

Within the range of dye reduction in Figs. 1 and 3, the conversion of nitrite or hydroxylamine into ammonia was nearly complete (Tables 1 and 2). The recovery of ammonia from nitrite alone was $90 \pm 2.9\%$ for 12 experiments, that from hydroxylamine was $85 \pm 4.0\%$ for 17 experiments, and the total recovery of ammonia when nitrite and hydroxylamine were both present was $91 \pm 2.4\%$ for 20 experiments that involved values of $\log([BVH]/[BV])$ between 1.15 and 0.08.

Ammonia was also produced from nitrite in quantitative yield when NADPH and catalytic amounts of BVH were used with glucose 6-phosphate in place of substrate amounts of BVH, as shown in Table 3.

Effect of BVH concentration and percentage dye reduction on enzyme activity

The effect on enzyme activity of different concentrations of BVH in a partially reduced dye may

Table 1. *Loss of nitrite or hydroxylamine and production of ammonia when substrates were present either separately or together in different concentrations in the presence of benzyl viologen*

Assays were carried out in duplicate under anaerobic conditions in a volume of 7.2 ml. at 27° for 25 min. with 7.8 mg. of protein as described in the Materials and Methods section, the same enzyme preparation being used in both experiments. Values are given as mμmoles.

Initial substrate given		log ([BVH]/[BV]) 1̄.81			log ([BVH]/[BV]) 1̄.64		
		Substrate loss		Ammonia gained	Substrate loss		Ammonia gained
Nitrite	Hydroxylamine	Nitrite	Hydroxylamine		Nitrite	Hydroxylamine	
2000	0	1050	—	1005	606	—	486
2000	400	998	63	903	568	75	532
2000	2000	960	105	1153	627	119	771
400	2000	391	268	633	395	166	553
0	2000	—	514	527	—	478	375
400	0	395	—	303	400	—	391
0	400	—	154	107	—	158	110

Table 2. *Loss of nitrite or hydroxylamine and production of ammonia when substrates were present either alone or together with different values for benzyl viologen reduction such that the relative activities of nitrite reductase and hydroxylamine reductase are reversed.*

Assays were carried out under anaerobic conditions with 13.3 mg. of protein in 7.2 ml. at 27° for 25 min., as described in the Materials and Methods section.

log ([BVH]/[BV])	Substrate given (mμmoles)		Substrate loss (mμmoles)		Ammonia gained (mμmoles) (% recovery)		Percentage inhibition of hydroxylamine reductase
	Nitrite	Hydroxylamine	Nitrite	Hydroxylamine	(mμmoles)	(% recovery)	
1̄.31	2000	0	245	—	244	100	—
	2000	2000	225	20	248	101	93
	400	2000	212	48	231	89	84
	0	2000	—	296	288	97	—
0.08	2000	0	1173	—	1078	92	—
	2000	2000	1105	101	1187	98	76
	400	2000	391	204	568	95	51
	0	2000	—	415	407	98	—

Table 3. *Production of ammonia from nitrite with NADPH as electron donor and catalytic amounts of reduced benzyl viologen, in comparison with the system with reduced benzyl viologen alone or NADH*

The NADPH was placed in the side arm of the Thunberg tube and mixed with the contents of the tube (buffer, substrate and enzyme) after evacuation. The benzyl viologen oxidized (BV) or partially reduced (BVH) dye was admitted as described in the Materials and Methods section. No glucose 6-phosphate was added in these experiments.

Expt. no.	Electron donors (μmoles)	Nitrite loss (mμmoles)	Ammonia gained	
			(mμmoles)	(yield %)
1	BVH only, 9.5	1180	957	81
	NADPH, 6.8, + BV, 0.6	510	600	117
	NADPH, 13.6, + BV, 0.3	540	506	94
	NADPH only, 6.8	0	0	—
2	BVH only, 12.6	794	757	95
	BVH only, 1.3	0	0	—
	NADPH, 3.0, + BV, 1.3	313	282	90
	NADPH 3.0, + BVH, 1.3	439	464	105
	NADH, 3.0, + BV, 1.3	0	0	—

reflect the combined effects of three possible factors, namely the redox potential of the BV-BVH system, the actual concentration of reduced dye in relation to the saturation of the enzyme, and possible competition between BV and BVH for the enzyme site.

Effect of BVH concentration on nitrite reductase.

The effect of concentration of BVH on nitrite reduction was studied in two ways. In Fig. 1 constant volumes of BV + BVH present at a total concentration of 3.65 mM but of varying degrees of reduction (% of BVH) were introduced into the Thunberg tube containing nitrite, buffer and enzyme as described in the Materials and Methods section. The results of several experiments with preparations of comparable activity showed a linear relationship between extent of nitrite loss in a given time and degree of dye reduction when expressed as

Fig. 1. Relationship between activity of nitrite reductase and proportion of benzyl viologen reduced expressed as $\log([BVH]/[BV])$. Results are shown for a random collection of four preparations (represented by four symbols) having comparable activity, and containing between 6 and 13 mg. of protein/ml. Each was assayed at several different values of $\log([BVH]/[BV])$ calculated from titration against permanganate of a 3.65 mM solution of the dye reduced catalytically to various extents and used as electron donor in substrate amounts as described in the Materials and Methods section.

$\log([BVH]/[BV])$. Since this expression is related to that for the standard electrode potential in the following manner: $E = E'_0 - 0.06 \log([BVH]/[BV])$ ($e^- = 1$), the relationship indicates that nitrite-reductase activity as measured by nitrite loss and ammonia formation was directly proportional to the redox potential of the BV-BVH system over the range tested. The results suggest also that, when dye reduction was less than about 10%, i.e. $\log([BVH]/[BV]) < 1$, nitrite-reductase activity was negligible (Figs. 1, 2 and 3). Further evidence to

Fig. 2. Relationship between activity of nitrite reductase and ratio of constant concentration of BVH to varying concentrations of BV. Dye was reduced catalytically to about 50% and a constant amount (8 μmoles of BVH) was added as described in the Materials and Methods section to Thunberg tubes containing different amounts of oxidized BV to produce different values of $\log([BVH]/[BV])$. Δ and $\circ$ represent results of separate experiments.

Fig. 3. Differential effects of degree of reduction of benzyl viologen on nitrite or hydroxylamine reduction. Results are shown for two different preparations represented by $\blacksquare$, $\square$ and $\blacktriangle$, $\triangle$ respectively when assayed for nitrite-reductase ($\square$, $\triangle$) and hydroxylamine-reductase ($\blacksquare$, $\blacktriangle$) activities at the same time for different values of $\log ([BVH]/[BV])$ with substrate quantities of 3.65 mM-benzyl viologen solution as described in the Materials and Methods section.

support the conclusion drawn from the results of the experiments of Fig. 1 was obtained in another manner, namely by observing the effects on nitrite reduction of a constant concentration of BVH when supplied in the presence of different concentrations of oxidized BV. For this purpose 9.65 μ equiv. of BVH, present in 6 ml. of partially reduced dye, i.e. with $\log ([BVH]/[BV]) = \bar{1}.91$, as described in the Materials and Methods section, was introduced into Thunberg tubes to which enzyme, substrate, buffer and varying amounts, or none at all, of oxidized BV had already been added. In this manner a range of values of $\log ([BVH]/[BV])$ from $\bar{1}.13$ to $\bar{1}.91$ was obtained similar to those shown in Fig. 1, but with a constant BVH concentration comparable with the highest values present in the previous experiments. The maximum total concentration of dye

was about 4 mg./ml., which was not of itself inhibitory to the enzyme. Results of two experiments presented in Fig. 2 show that there was a linear relationship between quantity of nitrite reduced in a given period of time and the initial value of $\log ([BVH]/[BV])$. Extrapolation to zero nitrite reduction indicated as before that activity tended to a negligible value when dye reduction was less than about 10%, in spite of the relatively high concentration of BVH present.

The assumption that initial activity is linearly related to $\log ([BVH]/[BV])$ in a system where such activity causes a progressive decrease with time in the ratio $([BVH]/[BV])$ is in accordance with the observation that the mean activity measured over an interval of time tends to be linearly related to the initial value of the ratio. We are indebted to Mr G. M. Clarke of the Statistics Section at Long Ashton for confirming the validity of this conclusion. It does, however, fail to hold when initial activity is so great that most of the available BVH is utilized during the reaction, as occurred for the two highest points in the upper line of Fig. 2.

The value of $\log ([BVH]/[BV])$ as distinct from the actual concentration of BVH seems to be the principal rate-limiting factor at low values of nitrite-reductase activity (Fig. 1). This was confirmed in another manner. As described in the Materials and Methods section, our preparations contained both a highly active diaphorase (Avron & Jagendorf, 1956) and a glucose 6-phosphate dehydrogenase. These systems, in the presence of added glucose 6-phosphate and NADP⁺, maintained catalytic amounts of dye in the reduced state, the concentration of which could be calculated by the method explained in the section describing the measurement of apparent K_m for BVH. The results in Table 4 show that when the concentration of reduced dye in this system (B) was maintained as low as 0.35 mM, i.e. that corresponding to $\log ([BVH]/[BV]) = \bar{1}.15$ for the conditions used in Fig. 1, the reduction of nitrite was as rapid as that which occurred in system A when the concentration of BVH was between 1.2 and 1.7 mM and the total dye was approximately 50% reduced, i.e. $\log ([BVH]/[BV]) = \bar{1}.95$ to 0.02. When less-reduced dye was used in system A, i.e. $\log ([BVH]/[BV]) = \bar{1}.31$ to $\bar{1}.51$, the rates were much less than in system B in spite of the higher concentrations and quantities of BVH still present in system A.

As the dye in system B was not reduced to the maximum extent possible until a few minutes after starting the assays by tipping NADP⁺ or glucose 6-phosphate from the side arm, the average rates shown in Table 4 were in fact somewhat less than the maximum possible under steady-state conditions. In system A also the average rates shown for the nominal values of $\log ([BVH]/[BV])$ were less than

Table 4. *Effects on the activity of nitrite-reductase and hydroxylamine-reductase systems with partially reduced benzyl viologen either alone in substrate quantities (A) or in catalytic amounts in the presence of a reducing system (B)*

The amounts of BVH used are shown as $\log ([\text{BVH}]/[\text{BV}])$, as concentration and as total quantity. The assays were carried out under anaerobic conditions at 27° as described in the Materials and Methods section. In the reducing system (B) glucose 6-phosphate (5 mM), and NADP (0.3 mM), glucose 6-phosphate dehydrogenase and NADPH diaphorase were also present.

Preparation	System	Benzyl viologen*			Enzyme activity (as substrate loss) (mμmoles/min./mg. of protein)	
		$\log ([\text{BVH}]/[\text{BV}])$	Concn. of BVH		Nitrite reductase	Hydroxylamine reductase
			(mM)	Total BVH (μmoles)		
(i) Fe- and Mn-deficient plants	A	1.31	0.62	3.45	1.8	9.9
	A	0.02	1.66	11.9	7.7	12.5
	B	0.07	0.34	0.95	8.1	13.1
(ii) Mn-deficient plants	A	1.98	1.60	11.5	14.8	8.7
	B	0.07	0.36	0.95	12.1	7.3
(iii) Fe-deficient plants	A	1.95	1.56	11.2	6.9	6.0
	B	0.07	0.60	1.90	6.8	6.2
(iv) Full nutrient plants	A	1.90	1.23	8.85	20.8	8.5
	A	1.51	0.65	4.68	2.4	7.5
	B	0.07	0.35	0.95	17.4	7.3

* Values of $\log ([\text{BVH}]/[\text{BV}])$ and corresponding values for concentration of BVH and total BVH are calculated either from titration with permanganate for system A or from data obtained for equilibrium conditions in the NADP-glucose 6-phosphate system B as described in the text in connexion with the determination of K_m for BVH.

the initial rates which would have been expected under steady-state conditions for the reasons explained above in connexion with the results in Fig. 2.

Effect of BVH concentration on hydroxylamine reductase. The data of Fig. 3 show the effect of concentration of BVH on hydroxylamine-reductase activity as compared with that on nitrite reductase assayed simultaneously on the same preparations. Hydroxylamine reductase was much less sensitive to changes in amount of BVH than nitrite reductase. It was observed frequently, as shown in Fig. 3 and Table 2, that whereas nitrite was reduced at a slower rate than hydroxylamine when the concentration of BVH gave $\log ([\text{BVH}]/[\text{BV}])$ values below 1.4, the reverse relationship existed when $\log ([\text{BVH}]/[\text{BV}])$ was between about 1.7 and 0.1. At intermediate values around 1.5 the two substrates were reduced at similar rates. As stated above, yields of ammonia from either substrate were around 90%, and it therefore follows that nitrite could be reduced to ammonia either more or less rapidly than hydroxylamine, depending only on the degree of dye reduction.

Effects of BVH concentration on nitrate reductase. Although the present paper is principally concerned with the characteristics of nitrite-reductase and hydroxylamine-reductase systems, it is relevant to draw attention to the effects of concentration of BVH on nitrate reductase, present in the same preparations as those used above, in view of the confusing results mentioned in the introduction.

The results of three experiments similar to those described for the effects of concentration of BVH on the other two enzymes are shown in Fig. 4. There was a striking and entirely opposite type of effect of concentration of BVH, expressed as $\log ([\text{BVH}]/[\text{BV}])$, on nitrate-reductase activity which was obviously independent of any possible effects on enzyme saturation by BVH. When the degree of dye reduction exceeded a value of 1.5 for $\log ([\text{BVH}]/[\text{BV}])$ nitrate-reductase activity was negligible. At values between 1.4 and 1.1 nitrite accumulated and ammonia was also formed. At values below 1.1 no ammonia appeared but nitrite production was maximal for the range of dye-reduction values used. Errors in ammonia estimations where amounts produced were small probably decreased their precision more than those of nitrite, and thus the range of dye reduction over which actual ammonia production was detected varied in the three experiments. The general conclusion, however, that nitrate reductase and nitrite reductase present in the same preparation may produce only nitrite, or nitrite and ammonia together, or ammonia only, or may fail entirely to produce either, according only to the degree of dye reduction seems justified and explains completely the confusing situation encountered in earlier stages of the work. Inspection of Figs. 3 and 4 and Tables 1 and 2 together suggests that there is a relatively narrow range of dye-reduction values $\log ([\text{BVH}]/[\text{BV}]) = 1.2$ to 1.4 within which nitrate will form nitrite, nitrite will be reduced

Fig. 4. Relationship between nitrate reduction to nitrite or ammonia and degree of benzyl viologen reduction expressed as $\log ([BVH]/[BV])$. A 3.65 mM solution of dye was reduced catalytically to different extents as shown by titration against permanganate and used as electron donor in substrate amounts as described in the Materials and Methods section. The left-hand part of the Figure shows production of nitrite ($\circ$, $\square$, $\triangle$) and ammonia ($\bullet$, $\blacksquare$, $\blacktriangle$) measured simultaneously in three separate experiments represented by the different symbols. The right-hand part of the Figure shows the calculated nitrate loss as sum of nitrite and ammonia production.

and form ammonia, and hydroxylamine will be reduced and form ammonia at comparable rates.

Apparent Michaelis constants for nitrite and hydroxylamine

The apparent Michaelis constants were determined as shown in Figs. 5 and 6 by the graphical methods of Lineweaver & Burk (1934) as described by Dixon & Webb (1958). A value about $1 \mu\text{M}$ was consistently obtained for nitrite. The nitrite-reductase system was practically saturated at a concentration of 0.05 mM , and concentrations above 0.5 mM produced substrate inhibition. Values obtained for hydroxylamine in Fig. 6 were less consistent than for nitrite, and for ten experiments giving linear reciprocal plots they ranged from 0.05 to 0.9 mM (mean $0.51 \pm 0.24 \text{ mM}$). The apparent K_m for hydroxylamine nevertheless appeared to be much greater than that for nitrite.

Fig. 5. Effect of hydroxylamine on nitrite-reductase activity. Hydroxylamine, when present, was at an initial concentration of 0.3 mM . Substrate quantities of BVH were used as electron donor as described in the Materials and Methods section. The results are plotted according to one of the methods described by Dixon & Webb (1958). V was calculated as μmoles of nitrite lost/min./mg. of protein in 20 min. and S as concentration of NO_2^- (μM). $\bullet$, Hydroxylamine present; $\circ$, hydroxylamine absent.

Fig. 6. Lineweaver-Burk plots for hydroxylamine-reductase preparations obtained on different occasions or from plants grown under different nutritional conditions: $\square$, $\circ$, separate full nutrient plants; $\triangle$, Cu-deficient plants; $\bullet$, Mn-deficient plants. Substrate quantities of BVH were used as described in the Materials and Methods section. V was calculated as μmoles of hydroxylamine lost/min./mg. of protein in 20 min.

Effects of nitrite and hydroxylamine present together on hydroxylamine-reductase and nitrite-reductase activities

As our preliminary work (Hageman *et al.* 1962) had raised some doubt about the status of hydroxylamine as an intermediate in nitrite reduction to ammonia, we investigated in more detail the interaction between the two compounds with respect to ammonia production and kinetic behaviour. Relevant data are presented in Tables 1, 2 and 5 and in Fig. 5. Nitrite reduction to ammonia occurred at rates that were practically independent of the presence or absence of hydroxylamine; in 13 experiments covering a range of hydroxylamine/nitrite ratios between 10:1 and 0.2:1, under conditions where nitrite concentrations were approaching saturation, the observed mean inhibition of nitrite reductase by hydroxylamine was only $3 \pm 2.9\%$, i.e. it did not differ significantly from zero. As shown in Fig. 5 hydroxylamine had an almost negligible effect on the apparent K_m for nitrite, raising it from 1.0 to 1.4 μM . By contrast, however, the presence of nitrite sharply decreased the rate of ammonia production from the mixture of substrates compared with the expected rate for the sum of the separate nitrite-reductase and hydroxylamine-reductase activities. Simultaneous estimations of substrate loss and ammonia formation showed that hydroxylamine reductase was severely inhibited by the presence of nitrite; in 14 experiments the mean inhibition was $81 \pm 7.1\%$. The degree of inhibition by nitrite appeared to be independent of either the initial nitrite concentration or of the rate of nitrite reduction, whether expressed on a protein basis or as controlled by percentage dye reduction, provided that (i) some nitrite (about 20 μmoles in 7.2 ml.) still remained by the end of the assay period and (ii) nitrite-reductase activity was appreciable. However, as shown in Table 5, when percentage dye

reduction was decreased to values such that nitrite-reductase activity was negligible, hydroxylamine-reductase activity, which was still considerable, was inhibited by nitrite much less than at concentrations of BVH which also permitted rapid nitrite reduction. This was clear not only in terms of percentage inhibition but also of actual hydroxylamine loss. One other point arising from Table 1 is that the percentage inhibition of hydroxylamine reduction in the presence of nitrite decreased as the concentration of hydroxylamine was decreased from 2000 to 400 μmoles in 7.2 ml. Although three experiments (not presented here) were performed to investigate this relationship in more detail, the inconsistent kinetic behaviour of the hydroxylamine-reductase system noted above precluded the determination with any confidence of the effect of nitrite on apparent values of K_m or V_{max} for the hydroxylamine-reductase system.

Apparent Michaelis constants for BVH

The relationship between $\log([BVH]/[BV])$ and enzyme activities made impracticable the determination of K_m merely by increasing the concentration of BVH without corresponding increase in that of BV. The preparation of several solutions providing different concentrations of BVH with the ratio $[BVH]/[BV]$ practically constant was achieved as an approximation by using the endogenous NADPH-diaphorase and glucose 6-phosphate-dehydrogenase systems (supplemented by adding *Zwischenferment*) in the presence of NADP^+ and glucose 6-phosphate to reduce the dye to a constant equilibrium value. This was reached within 15 min. under anaerobic conditions as shown by measurements at 780 μ of light-extinction of the solution in a 1 cm. cuvette attached to the Thunberg tube; the maximum extinction when no

Table 5. *Inhibition of hydroxylamine reductase by nitrite in relation to the presence or absence of nitrite-reductase activity*

Assays were carried out under anaerobic conditions for 25 min. using substrate quantities of benzyl viologen as described in the Materials and Methods section. The degree of dye reduction was adjusted in accordance with the relationship shown in Fig. 1 to produce either negligible or considerable nitrite-reductase activity in two preparations showing either intrinsically fairly high (A) or relatively low (B) nitrite-reductase activity and fairly high hydroxylamine-reductase activities on a protein basis. Each system contained 2000 μmoles of hydroxylamine and the same quantity of nitrite when added.

Preparation	$\log([BVH]/[BV])$	Substrate loss ($\mu\text{moles}/\text{mg. of protein}$)			Percentage inhibition by nitrite
		Nitrite	Hydroxylamine	Hydroxylamine nitrite	
A	1.71	195	264	61	77
	1.03	0	132	80	40
B	1.84	23	177	51	72
	1.11	0	97	71	26

further change occurred was proportional to the concentration of BV provided initially. It was therefore possible to obtain a range of values for BVH concentration each with practically the same value of $\log ([BVH]/[BV])$, which was determined as follows. On the basis of the increase in light-extinction at 320 $m\mu$ for the cyanide-addition reaction at pH 10.0 (Colowick, Kaplan & Ciotti, 1951), and freedom from NAD^+ as detected chromatographically (Sigma Chemical Co. assay sheet), it was found from change in extinction at 340 $m\mu$ in the absence of BV that the $NADP^+$ was 90% reduced when equilibrium was attained at pH 7.5. Taking E'_0 for $NADPH$ at pH 7.5 (27°) as -0.332 v (Mansfield-Clark, 1960) and as -0.363 v for 90% reduction, and E'_0 of benzyl viologen as -0.359 v (pH-independent) (Michaelis & Hill, 1933a,b), we calculate that the BV should have been 54% reduced when equilibrium was reached in the $NADPH$ system; this value was used to calculate the concentration of BVH produced for each amount of BV originally taken.

When it was considered that equilibrium had been established for dye reduction in a series of initially differing amounts of BV, the substrate (nitrite or hydroxylamine) was tipped in from the side arm of the Thunberg tube and the assay was continued for 20 min. It was assumed on the basis of observations of dye colour and light-extinction that BVH oxidized by nitrite or hydroxylamine was reduced again fast enough to prevent the occurrence of appreciable changes in the initial concen-

tration of BVH at any given value. The apparent K_m for BV in the $NADPH$ -diaphorase system was determined in separate experiments and found to be 0.44 mM; saturation occurred at about 4 mM-BV. However, the rate of BV reduction in this system was still rapid enough in selected preparations to ensure that this reaction was not a rate-limiting factor in the reduction of nitrite or hydroxylamine to ammonia. The results with the procedure just described are shown for the nitrite-reductase and hydroxylamine-reductase systems in Fig. 7. The values for the apparent K_m were 60 μM for nitrite and 7.5 μM for hydroxylamine. The values of BVH in Figs. 1, 2 and 3 for which nitrite-reductase activity tended to zero were therefore still six times that of the apparent K_m .

Effects of some inhibitors

Results of inhibitor tests are given in Table 6. Cyanide at pH 7.5 in concentrations between 1 and 5 mM was severely inhibitory to both systems. Effects of lower concentrations suggested that the nitrite-reductase system was the more sensitive of the two. Inhibition by azide at pH 7.5, when corrections were introduced for chemical reaction between azide and nitrite (Hewitt & Hallas, 1959), was negligible at concentrations up to 0.2 mM for nitrite-reductase and 0.7 mM for hydroxylamine-reductase systems. Higher concentrations cannot be used reliably when nitrite or hydroxylamine are estimated colorimetrically (Villanueva, 1959; Hewitt & Hallas, 1959); the manometric procedure described by Hewitt & Hallas (1959) was not used in view of the negative results obtained with lower concentrations. Several other inhibitors of metal-enzyme systems, i.e. 1,1'-bipyridyl, 8-hydroxyquinoline, sodium diethyldithiocarbamate, o-phenanthroline and EDTA (disodium salt), were practically inert and no evidence for the presence of metal components was obtained in these studies. The effects of cyanide are not conclusive evidence in this respect, and it is relevant to note that Senez & Pichinoty (1958c) made the remarkable and emphatic statement that 1 mM-potassium cyanide severely inhibited the non-enzymic reoxidation of BVH by nitrite. It is possible that a cyanide addition compound analogous to that formed with $NADP^+$ is produced (Colowick *et al.* 1951). Senez & Pichinoty (1958a) found that hydrazine at 10 and 1.0 mM inhibited hydroxylamine reduction 65 and 23% respectively in cells of *Desulfovibrio desulfuricans*. Walker & Nicholas (1961) reported that hydrazine acted as a competitive inhibitor of hydroxylamine reductase of *Pseudomonas aeruginosa*; concentrations of 1.7 and 0.33 mM inhibited 82 and 37% respectively. Several tests were made with nitrite-reductase and hydroxylamine-reductase

Fig. 7 Relation between nitrite-reductase (Δ) or hydroxylamine-reductase ($\circ$) activities and concentration of BVH for constant degree of dye reduction shown as reciprocal plots by one of the methods described by Dixon & Webb (1958). V was calculated as $m\mu$ moles of nitrite or hydroxylamine lost/min./mg. of protein in 20 min.; actual rates were low enough to be rate-limiting in the overall system in which glucose 6-phosphate, $NADPH$, glucose 6-phosphate dehydrogenase and the diaphorase were used to maintain catalytic amounts of BVH in the required concentrations as described in the Materials and Methods section. S is shown as concentration of BVH (μM).

Table 6. *Effects of some inhibitors on nitrite-reductase and hydroxylamine-reductase activities*

Assays were carried out at pH 7.5 under anaerobic conditions at 27° for 25 min. with reduced benzyl viologen as the electron donor in substrate quantities as described in the Materials and Methods section. Further details of the use of azide, thiol reagents and pyruvate are given in the text. Values indicate percentage inhibition compared with control systems, except where stimulation occurred as shown, for inhibitors given at the concentration (mM) shown for each system.

Inhibitor	Nitrite-reductase system		Hydroxylamine-reductase system	
	(mM)	(% inhibition)	(mM)	(% inhibition)
Potassium cyanide	5	100	5	100
	1	100	2.2	78
	0.1	85	0.51	68
	0.015	10	0.22	55
			0.07	17
Sodium azide	0.2	0	0.7	0
<i>p</i> -Chloromercuribenzoate	5	78	0.5	47
	0.2	14	0.5 (+ 2.5 mM-GSH)	24
	0.2 (+ 1 mM GSH)	0		
Phenylmercuric acetate	0.2	90	0.5	73
	0.2 (+ 1 mM-GSH)	0	0.5 (+ 2.5 mM-GSH)	22
Hydrazine sulphate	4	0	4	10-15
	1.5	0	1.5	0
Sodium pyruvate	3.3	0	3.5	20
	1.25	0	1.25	7
<i>o</i> -Phenanthroline	1	10	1	0
1,1'-Bipyridyl	1	9	1	10% stimulation
8-Hydroxyquinoline	1	0	1	5% stimulation
Sodium diethyldithiocarbamate	1	18% stimulation	1	9
EDTA (disodium salt)	1	0	1	12

Table 7. *Effects of some micronutrient deficiencies on activity of nitrite-reductase and hydroxylamine-reductase preparations*

Severity of the deficiency condition of the plants is indicated as slight, moderate or severe in terms of visible symptoms. Necrotic leaves were omitted from the preparations. Assays were carried out under anaerobic conditions at 27° for 25 min., as described in the Materials and Methods section with substrate quantities of partially reduced benzyl viologen as shown.

Origin of preparation and severity of deficiency	Protein (mg./g. fresh wt.)	log ([BVH]/[BV])	Nitrite reductase (mμmoles/min./ mg. of protein)	Hydroxylamine reductase (mμmoles/min./ mg. of protein)
(i) Plants 5 weeks old; preparation stored 10 days at -15°				
Full nutrient plants	16.1	1.66	2.9	1.3
Severely Fe-deficient plants	5.6	1.66	0.4	36.5
Severely Mn-deficient plants	6.0	1.66	2.7	26.3
Moderately Zn-deficient plants	6.1	1.66	3.9	12.7
Slightly Cu-deficient plants	8.4	1.66	3.5	2.0
(ii) Plants 7 weeks old; preparation stored 20 days at -15°				
Full nutrient plants	9.0	1.88	5.7	—
		1.64	—	3.9
Moderately Cu-deficient plants	7.3	1.88	7.0	—
		1.64	—	38.7
(iii) Plants 4 weeks old; preparation stored 24 hr. at -15°				
Full nutrient plants	10.4	1.88	21.0	8.5
Moderately Fe-deficient plants	9.0	1.88	14.7	15.2
Slightly Mn-deficient plants	11.7	1.88	14.8	8.7
Moderately Fe- and Mn-deficient plants	10.4	1.88	13.8	18.4

systems in the present work, but no evidence of marked inhibition by hydrazine present at 4 mM was obtained with either. Sodium pyruvate at concentrations up to 3.3 mM made no difference to the activity of nitrite reduction, and hydroxylamine reduction was inhibited to a maximum of 20%.

The inhibition by phenylmercuric acetate and *p*-chloromercuribenzoate suggested the presence of functional SH groups. Phenylmercuric acetate was the more effective of the two. Inhibition of nitrite reductase was fully reversed by 1 mM-GSH, but that of hydroxylamine reductase was less completely reversed by 2.5 mM-GSH even though inhibition was less severe with phenylmercuric acetate than for nitrite reductase.

Effects of micronutrient elements on enzyme activity

The addition of iron, manganese, copper, zinc or molybdenum at concentrations between 0.01 and 0.1 mM to extensively dialysed preparations obtained after precipitation by ammonium sulphate had no effect on the activity of either nitrite or hydroxylamine reduction when BVH was used. The requirement for any dissociable metal cofactor in either system therefore seems unlikely.

The results of separate experiments obtained when plants were grown with full nutrient or metal-deficient treatments under comparable conditions are shown for activities on a protein basis in Table 7.

Iron deficiency, when severe, caused a marked decrease in nitrite-reductase activity. Less acute deficiency of iron or of iron and manganese together also caused appreciable decreases in specific activity. None of the other deficiencies, manganese, copper or zinc, alone caused marked decreases in specific activity of the nitrite-reductase system.

The effects of metal deficiencies on the hydroxylamine-reductase system were entirely different. With few exceptions, specific activities of preparations from metal-deficient tissues were substantially increased compared with those from plants receiving full nutrient.

DISCUSSION

The relationships between the extent of reduction of BV and nitrite-reductase activity which was associated with practically stoichiometric production of ammonia may be most simply explained by assuming that the overall rate of the reaction:

is directly proportional to the redox potential of the benzyl viologen system over a certain range. The standard electrode potential for the BV/BVH couple, which involves a single-electron reaction, has been given as $E'_0 = -0.359$ v (Michaelis & Hill,

1933a,b; Homer, Mees & Tomlinson, 1959; Homer & Tomlinson, 1959) and is independent of pH. Less negative values between -0.31 and -0.32 v quoted by the manufacturers are regarded as incorrect and the result of failure to exclude oxygen entirely during measurement. When dye reduction was less than about 9%, for which the expression $E = E'_0 - 0.06 \log ([\text{BVH}]/[\text{BV}])$ has a value of -0.30 v, nitrite-reductase activity was negligible, whereas for values exceeding about -0.395 v (78% reduction) chemical reduction of nitrite was inferred and ammonia production was no longer stoichiometrically related to nitrite loss (Hageman *et al.* 1962). Senez & Pichinoty (1958c) reported that nitrite reacted non-enzymically with BVH that had been produced by extensive reduction by a hydrogenase system, and was therefore probably reduced to a more negative potential than that for which only an enzymic reaction has been observed by us. Hydroxylamine also, according to Senez & Pichinoty (1958b), was reduced chemically to ammonia by BVH that had been produced by dithionite or a hydrogenase system.

The conditions for the enzymic reduction of nitrate to nitrite contrasted remarkably with those for nitrite and hydroxylamine in that activity decreased as $\log ([\text{BVH}]/[\text{BV}])$ increased and was negligible for values greater than 1.5 for which the electrode potential would be about -0.33 v. Whereas nitrate-reductase systems that contain molybdenum accept electrons freely either from two-electron donors, e.g. the NADPH-FAD sequence, or from a single-electron donor (BVH), the enzymic reduction of nitrite in *C. pepo* requires the mediation in an obligatory manner of a single-electron donor. This can be provided by BVH or alternatively, as stated in the introduction, by ferredoxin, which is also a single-electron donor (Tagawa & Arnon, 1962). If nitric oxide (Fewson & Nicholas, 1961) and/or a nitroxyl radical are intermediates in nitrite reduction to ammonia at least two single-electron steps would then be involved and a single-electron mediator might well be necessary in accordance with the Michaelis principle (cf. Mansfield-Clark, 1960, pp. 340-342).

The enzymic production of ammonia from nitrite in nearly stoichiometric quantities regardless of a fourfold difference in the rate of the reaction (Table 2) suggests either that the initial reduction of nitrite is the rate-limiting step in the overall reaction or that an intermediate product is severely inhibitory to nitrite reduction, and that the further reduction of this intermediate is the rate-limiting step. The results of experiments in which nitrite and hydroxylamine were present together exclude hydroxylamine as such an inhibitory intermediate. Hydrazine may similarly be excluded from this category with respect to either nitrite-reductase or

hydroxylamine-reductase systems in *Cucurbita*. Hyponitrite may, however, require consideration in the present context. Nicholas (1959) reported that this compound, but not hydroxylamine, severely inhibited the nitrite-reductase system of *Neurospora*. We have obtained evidence for the formation in some experiments of a compound, which yields nitrite after treatment with the alkaline iodine reagent as reported for hyponitrite by Yamafuji & Osajima (1961), that occurs in amounts between 5 and 10% of the nitrite reduced (E. J. Hewitt & D. P. Hucklesby, unpublished work).

The status of hydroxylamine as a possible intermediate in the reduction of nitrite to ammonia requires consideration. If free hydroxylamine is involved its rate of reduction when present in concentrations that could be produced from nitrite should not be less than the rate of the overall reaction in which ammonia yields attained 90% of nitrite loss. Our results show that this requirement is not necessarily fulfilled when BVH is the electron donor in an enzymic system since nitrite loss and ammonia production can occur more rapidly than the corresponding changes for hydroxylamine present at equivalent concentrations when the same enzyme preparation is used on the same occasion. The apparent K_m for hydroxylamine is substantially greater than that for nitrite, so the nitrite-reductase system is saturated at a concentration that, if converted instantaneously into hydroxylamine, would be barely half-saturating for this substrate. The presence of nitrite severely inhibits hydroxylamine reduction, probably in a reversible manner, since hydroxylamine loss was much greater when practically all the nitrite present initially had disappeared during the assay period (Tables 1 and 2). Senéz & Pichinoty (1958a) observed a similar reversible inhibition of hydroxylamine reduction by *D. desulfuricans*.

Lazzarini & Atkinson (1961) could not detect the production of hydroxylamine by an isotope-dilution technique when unlabelled hydroxylamine was added to, and later recovered from, the system in *Escherichia coli* during the reduction of ^{15}N -labelled nitrite. Further, Mager (1960), Lazzarini & Atkinson (1961) and Kemp, Atkinson, Ehret & Lazzarini (1963) have collectively concluded on good evidence that in *E. coli* the reduction of nitrite or hydroxylamine to ammonia and of sulphite to hydrogen sulphide are manifestations of a single enzyme system in which transfer of up to six electrons occurs without the production of any free intermediates. In *Cucurbita* the independent effects of micronutrient deficiencies, the differences in apparent K_m values for BVH and the differential effects of percentage reduction of BV suggest, but (as indicated below for the second and third points) may not in fact require, that separate enzymes are concerned

in the reduction of nitrite and hydroxylamine to ammonia in plants.

In the mechanism proposed by Kemp *et al.* (1963) nitrite combines via the ionized oxygen atom with a hydroxyl group in the enzyme, an OH^- ion being released. The intermediate complexes produced at each stage of reduction remain bound via an oxygen atom until ammonia is released. Hydroxylamine forms hydroxamic acids with certain configurations containing hydroxyl groups, e.g. hemiacetal, and we suggest that it might combine thus with the same prosthetic group as nitrite but initially it would be combined via the nitrogen instead of an oxygen atom. A rearrangement would then be required to produce the same structure as an intermediate formed from nitrite reduction, before reduction to ammonia. This scheme would account for direct inhibition of hydroxylamine reduction by nitrite and for increased inhibition when nitrite is undergoing reduction (Table 5), because still less free enzyme would be available. Hydroxamate formation and rearrangement might account for an apparently large K_m for hydroxylamine and the irregular kinetic behaviour. The scheme also accommodates the differential or independent effects of percentage dye reduction in nitrite-reductase and hydroxylamine-reductase activities if it is assumed that the first step in nitrite reduction is rate-limiting for this activity, whereas the rate of rearrangement of the hydroxamic acid is the rate-limiting step in the hydroxylamine reduction. It is conceivable that different configurations in the successive intermediate complexes corresponding to successive stages of reduction would be associated with different affinities of the enzyme for BVH, and in this way different apparent K_m values for BVH might occur for the two activities. The observed relative magnitude of the apparent K_m values would be compatible with that for nitrite being associated with the first step and that for hydroxylamine with the last. The different effects of micronutrient deficiencies on the two activities and different ratios of these activities for comparable values of $\log([\text{BVH}]/[\text{BV}])$ (Table 4) are not so simply explained by the scheme presented above.

Mager (1960) suggested that a carbonyl group was present at the active site. A tautomerism between an enolic and carbonyl configuration would be compatible with most of our observations and would additionally accommodate the effects of cyanide, which could compete with hydroxylamine if oxime formation were involved and could also shift the equilibrium from the enolic form reactive with nitrite to the inactive cyanhydrin with which the nitrite could not compete. The relative sensitivities of the two systems to cyanide would be compatible with this suggestion.

Regardless of the mechanism which can be pro-

posed, our results appear contrary to the classical idea that free hydroxylamine occurs as an intermediate in nitrite reduction by higher plants. This view is supported by the results of physiological experiments with algae described by Syrett (1954) and Kessler (1957*a,b*). The requirement for a single-electron mediator and the doubtful evidence for the participation of metals suggest that the mechanism differs in important respects from that inferred by Nicholas (1957, 1958, 1959) to occur in fungi, where a number of metal-dependent NAD(P)H-flavine enzymes have been described.

The tenure by R. H. H. of a year's sabbatical leave from the University of Illinois and the award of the Boots Scholarship to C. F. C. that enabled them to participate in this work are gratefully acknowledged. We are also grateful to Mr E. F. Watson and Mr B. A. White for their help in growing the plants used in this work, to Dr A. Couper of the Department of Biological Chemistry for the helpful discussions in connexion with the redox systems involved and to the Sigma Chemical Co. for generous gifts of NADP⁺ and NADPH.

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