PARTIAL PURIFICATION AND CHARACTERIZATION OF NITRATE REDUCTASE INACTIVATORS FROM MAIZE ROOTS

by

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DECLARATION

I hereby declare that the following thesis is based on work carried out by myself, that the thesis is of my own composition, and that no part of it has been presented previously for a higher degree.

The research was carried out in the Department of Biochemistry and Microbiology of the University of St. Andrews under the direction of Dr. J.L.Wray.

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SUMMARY

The maize nitrate reductase complex was found to have a sedimentation coefficient of 7.7S, and a smaller species of 6.8S was also observed, which had NADH-nitrate reductase, NADH-cytochrome c reductase and MVH-nitrate reductase activities. 3-4S NADH-cytochrome c reductase species were also present in maize nitrate reductase preparations, extending previous observations to maize.

Two nitrate reductase inactivators were partially purified from 5 day old maize roots and were also observed in 10 day old maize leaves.

Nitrate reductase inactivator I from roots was inhibited by PMSF and casein, but not by BSA, EDTA, 1,10-phenanthroline or leupeptin and was therefore considered to be a serine proteinase. Polyacrylamide disc-gel electrophoresis of inactivator I preparations suggested that nitrate reductase inactivation and azocasein degradation were properties of a single protein band. The pH optimum for azocasein degradation was at pH 9.5. Nitrate reductase inactivator I had a Stokes radius of 2.9nm, a sedimentation coefficient of 4.7S and a calculated molecular weight of 56,200. It inactivated all activities of the nitrate reductase complex although MVH-nitrate reductase was inactivated more slowly than NADH-nitrate reductase and NADH-cytochrome c reductase activities. An approximately 6.8S species possessing MVH-nitrate reductase, NADH-nitrate reductase and NADH-cytochrome c reductase activities appeared during nitrate reductase inactivation.

Nitrate reductase inactivator II from roots was unable to degrade azocasein but this did not rule out the possibility that it could be a proteinase. It was inhibited by EDTA and 1,10-phenanthroline but not by BSA, casein or leupeptin, while PMSF was only slightly inhibitory. Inactivator II, which had a sedimentation coefficient of 4.8 S, inactivated NADH-nitrate reductase and NADH-cytochrome c reductase but did not affect MVH-nitrate reductase activity.

A nitrate reductase inactivator was also present in the roots and shoots of barley seedlings. It was inhibited by EDTA, 1,10-phenanthroline and, to a

lesser extent, by leupeptin, but not by PMSF, BSA or casein.

The mechanism of inactivation of nitrate reductase by these inactivators was discussed.

ABBREVIATIONS

BSA	bovine serum albumin
cyt c	cytochrome c
DCPIP	dichlorophenolindophenol
EDTA	ethylenediamine tetraacetic acid
FAD	flavin adenine dinucleotide - oxidised form
$FADH_2$	flavin adenine dinucleotide - reduced form
FMN	flavin mononucleotide - oxidised form
$FMNH_2$	flavin mononucleotide - reduced form
Methyl viologen	1,1'-dimethyl-4,4'-bipyridilium dichloride
MVH-NR	reduced methyl viologen nitrate reductase
NAD	nicotinamide adenine dinucleotide - oxidised form
NADH	nicotinamide adenine dinucleotide - reduced form
NADH-NR	NADH-nitrate reductase
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NR	nitrate reductase
pCMB	p-chloromercuribenzoate
PMSF	phenylmethylsulphonyl fluoride
TEMED	N,N,N',N'-tetramethylethylene diamine
Tris	tris-hydroxymethylmethylamine

FIGURES AND TABLES

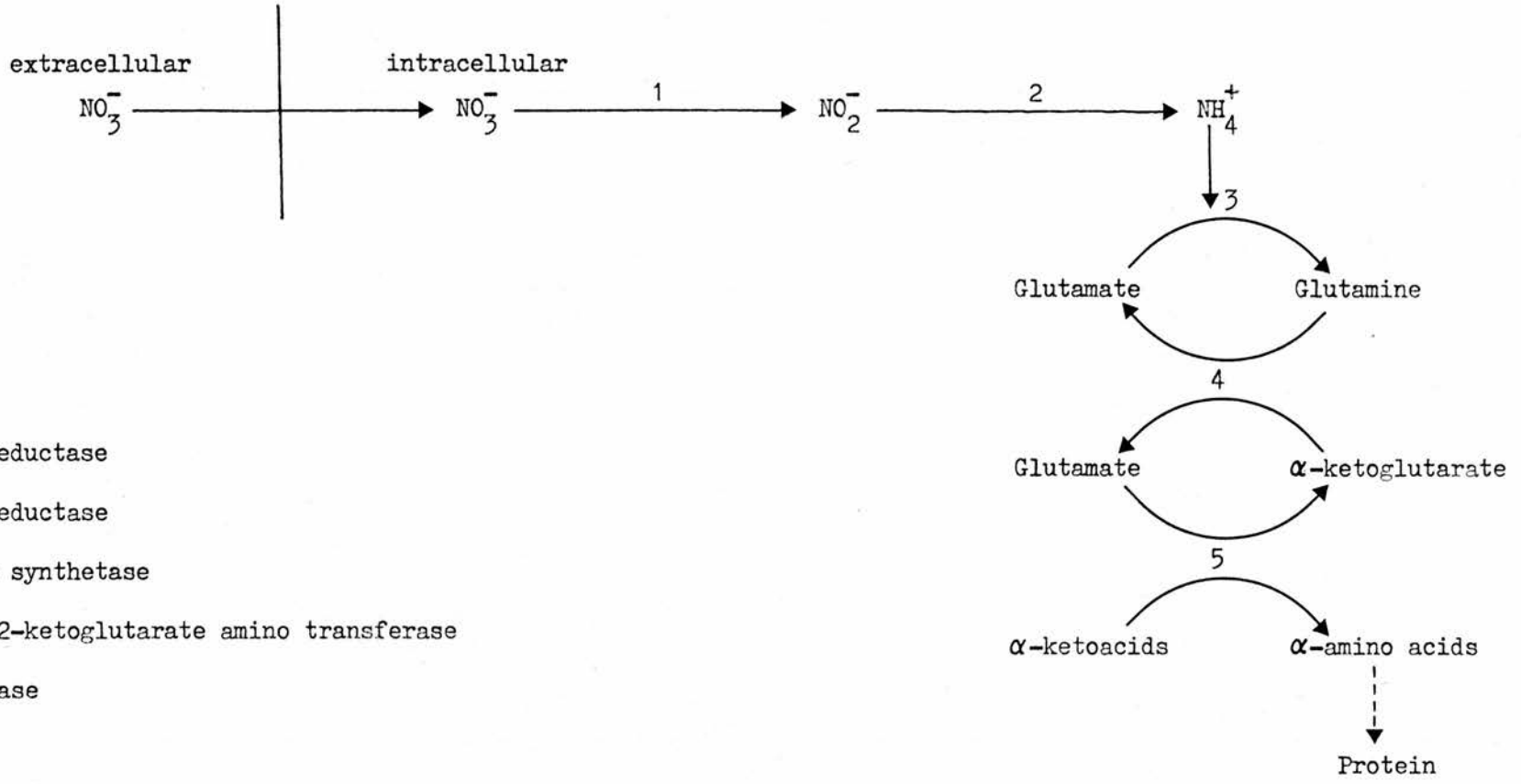
CHAPTER 2 Characterization of nitrate reductase inactivator I

CHAPTER 3 Inactivation of nitrate reductase by nitrate reductase inactivator I

CONTENTS

INTRODUCTION

Figure 1. Pathway of Nitrate Assimilation



Key:

1 Nitrate reductase

2 Nitrite reductase

3 Glutamine synthetase

4 Glutamic:2-ketoglutarate amino transferase

5 Transaminase

et al., 1977). The presence of nitrate reductase in roots demonstrated that light was not an absolute requirement for induction of the enzyme (Beevers and Hageman, 1969) but both light and nitrate were required for maximal nitrate reductase activity (Harel et al., 1977). In some species, cytokinins and gibberellic acid substituted for light in nitrate reductase induction (Rijven and Parkash, 1971) and could replace or enhance the effect of nitrate as the inducer (Kende et al., 1971; Parkash, 1972). This may be important in the development of nitrate reductase activity in non-green tissue.

NADH is the physiological electron donor in the majority of higher plants (Beevers et al., 1964; Schrader et al., 1968) while nitrate reductases from algae, bacteria, fungi and yeasts utilise NADPH. In most higher plants NADPH could act as an electron donor only in the presence of an NADP:ferredoxin reductase and FMN (Paneque and Losada, 1966) but several species have been reported to utilise NADPH directly, particularly Lemna minor, maize and soybean (Hageman and Hucklesby, 1971). It was thought that nitrate reductase from young soybean leaves could utilise both NADH and NADPH as electron donors (Evans and Nason, 1953) but this was later found to be due to the presence of two separate nitrate reductases (Jolly et al., 1975). Campbell (1976) also demonstrated the presence in maize scutellum of two nitrate reductases which had different affinities for nitrate, one exhibiting maximal activity with NADH, the other with NADPH.

Nitrate reductase can also reduce nitrate using $FMNH_2$, $FADH_2$ or reduced viologen dyes as electron donors (Cresswell et al., 1965; Schrader et al., 1968; Wray and Filner, 1970) and in addition has NADH dehydrogenase activity whereby it can reduce cytochrome c (Wray and Filner,1970), DCPIP (Schrader et al., 1968) ferricyanide, methylene blue, benzoquinone and menadione (Smarelli and Campbell, 1979). Initially it was thought that NADH-nitrate reductase and $FMNH_2$-nitrate reductase represented the activities of two separate enzymes but it proved impossible to separate them (Schrader et al., 1968) and it was deduced that NADH-nitrate reductase, $FMNH_2$-nitrate reductase and NADH-cytochrome c reductase

were activities of a single protein moiety (Wray and Filner, 1970). This was supported by the observation that all three activities were induced in parallel in the presence of nitrate.

Nitrate reductase from higher plants is thought to be an asymmetrical, cigar-shaped molecule with an axial ratio (in barley) of 11:1 (Small and Wray, 1980) and a wide variety of molecular weights have been reported in the range 160,000-600,000 (Hewitt, 1975). The nitrate reductase used for the work described in this thesis was obtained from maize and barley. Molecular weights of 160,000 for maize nitrate reductase (Hageman and Hucklesby, 1971) and 221,000 (Kuo et al., 1980a) or 203,000 (Small and Wray, 1980) for barley nitrate reductase have been estimated. Barley nitrate reductase is thought to consist of two subunits of molecular weight 100,000 each (Kuo et al., 1980a; Small and Wray, 1980) although spinach nitrate reductase was proposed to consist of four smaller subunits with molecular weights of about 40,000 each (Notton and Hewitt, 1979).

The nitrate reductase complex contains FAD (Hewitt and Notton, 1980) and cytochrome b_{557} (Guerrero et al., 1977; Notton et al., 1977; Somers et al., 1982), recent work with fungal nitrate reductase suggesting the presence of two moles of FAD and two moles of cytochrome b_{557} per mole of enzyme (Pan and Nason, 1978). Fungal nitrate reductase would appear to be essentially similar to that in higher plants (Pan and Nason, 1976, 1978; Garrett and Nason, 1967, 1969). Molybdenum is also an essential requirement for nitrate reductase activity (Nicholas and Nason, 1954; Notton and Hewitt, 1971) and, in fungi, was shown to be associated with a small polypeptide of molecular weight 1,000 (Lee et al., 1974). The molybdenum-containing component is bound noncovalently to the enzyme complex and is common to nitrate reductase, xanthine oxidase and sulphite oxidase (Johnson, 1980). Substitution of molybdenum by tungsten produced an inactive enzyme with respect to NADH-nitrate reductase and $FMNH_2$-nitrate reductase activities, but in barley or maize leaves NADH-cytochrome c reductase activity was superinduced (Wray and Filner, 1970; Aslam and Oaks, 1976) and this was

thought to be due to an accumulation of nitrate which acted as a gratuitous inducer of the enzyme complex. The effect of tungsten could be overcome by molybdenum *in vivo* but not *in vitro* (Wray and Filner, 1970).

In contrast to the situation in higher plants and fungi, nitrate reductase from *Chlorella vulgaris* is thought to consist of three subunits of molecular weight 90,000 each, resulting in an enzyme complex of molecular weight 280,000. There is some evidence to suggest that the enzyme may be dimeric and is under the control of a concentration-dependent association-dissociation reaction which favours the monomeric form at low nitrate reductase concentrations. The enzyme contains 2-3 FAD and haem units per molecule (probably one of each per subunit) and the complex has an overall globular shape (Giri and Ramadoss, 1979).

The activities of the nitrate reductase complex do not exhibit identical stabilities. $FMNH_2$-nitrate reductase was more heat-stable than NADH-nitrate reductase (Schrader *et al.*, 1968) suggesting that the component involved in binding NADH was less stable than that binding $FMNH_2$. Wray and Filner (1970) also found that NADH-cytochrome c reductase was slightly more heat-stable than NADH-nitrate reductase, but much less stable than $FMNH_2$-nitrate reductase. (In contrast, however, Sherrard and Dalling (1979) considered that the site of acceptance of electrons from NADH was the most stable part of the nitrate reductase complex.)

NADH-nitrate reductase activity could be partially stabilized by exogenous cysteine implying the involvement of a sulphydryl group in NADH binding (Schrader *et al.*, 1968; Wray and Filner, 1970) and further evidence was provided by the observation that inhibition of NADH-nitrate reductase and NADH-cytochrome c reductase activities by low concentrations of pCMB was reversed by cysteine (Wray and Filner, 1970). $FMNH_2$-nitrate reductase and MVH-nitrate reductase were much less sensitive to pCMB. Nitrate reductase was inhibited by azide, cyanide and organic chelating agents (Hageman and Hucklesby, 1971; Smarelli and Campbell, 1979) resulting in the abolition of all activities except NADH

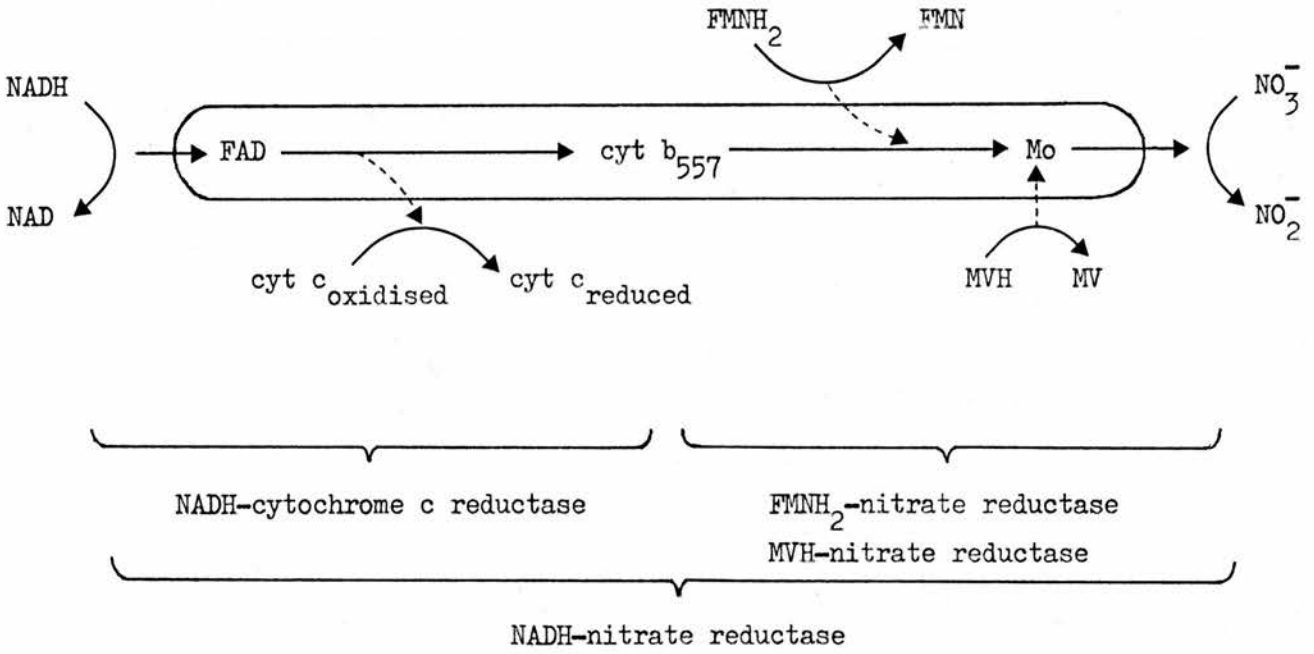
dehydrogenase.

The differential effects of the various inhibitors, together with susceptibility to heat treatment, would seem to divide the nitrate reductase complex into two functionally distinct parts: an NADH-oxidising region responsible for NADH-cytochrome c reductase activity and a nitrate-reducing region responsible for $FMNH_2$-nitrate reductase and MVH-nitrate reductase activities. However the fact that MVH-nitrate reductase was more heat-stable than $FMNH_2$-nitrate reductase suggests that MVH and $FMNH_2$ do not donate electrons at the same site (Wray et al., 1979). It has been suggested that MVH may donate electrons directly to the molybdenum-containing component (Hewitt et al., 1976). The pathway of electron flow during nitrate reduction and the relationship of the activities of the nitrate reductase complex are shown in Figure 2.

Nitrate reductase activity is highly unstable both in vivo (Schrader et al., 1968; Travis et al., 1969; Oaks et al., 1972) and in vitro (Wray and Filner, 1970; Wallace, 1973a, 1975a; Brown et al., 1981), and this instability could be due to inhibition, degradation, dissociation or denaturation of the nitrate reductase complex, or a combination of these processes. Cotton nitrate reductase activity in vitro was most stable in the presence of both NADH and nitrate therefore it was suggested that substrate availability may have a role in determining the in vivo stability of nitrate reductase (Tischler et al., 1978).

Many authors have reported the use of exogenous protein for in vitro stabilization of nitrate reductase activity (Schrader et al., 1974; Schrader and Vogelzang, 1974; Wallace, 1975a; Purvis et al., 1976; Sherrard and Dalling, 1978). Extraction of tissue in buffers containing 3% BSA or casein stabilized nitrate reductase from several species (Schrader and Vogelzang, 1974; Sherrard and Dalling, 1978) and resulted in an overall increase in nitrate reductase activity especially from older tissue, but delayed addition of BSA to extracts was equally effective (Schrader and Vogelzang,1974; Purvis et al., 1976). It

Figure 2. Pathway of Electron Flow in Nitrate Reductase



The exact sites of electron donation and acceptance are not certain.

was suggested that the effect of exogenous protein was either to prevent the binding of inhibitors to the nitrate reductase complex or to protect nitrate reductase from proteolytic enzymes and was not due to an increase in the amount of nitrate reductase extracted (Schrader et al., 1974; Schrader and Vogelzang, 1974; Purvis et al., 1976; Sherrard and Dalling, 1978). Schrader et al. (1974) found that retarded decay of nitrate reductase activity in the presence of exogenous protein was not due to an osmotic effect during the extraction procedure. In the presence of BSA or Dowex 1-Cl, which is known to remove phenolic enzyme inhibitors from solution (Lam and Shaw, 1970), Purvis et al. (1976) observed an increased nitrate reductase activity which was not caused by either an increase in the amount of enzyme extracted or increased enzyme stability, but which they considered was due to the removal of phenolic compounds by formation of inert hydrogen-bonded complexes. The factor responsible for in vitro loss of nitrate reductase activity was not removed or inhibited by either Dowex 1-Cl or BSA. Casein was able to increase the stability of nitrate reductase but less enzyme could be extracted from tissue in its presence (Sherrard and Dalling, 1978).

It has been suggested that protein concentration may be important in maintaining nitrate reductase stability since dilution of extracts from cotton cotyledons resulted in a more labile enzyme (Tischler et al., 1978) while the high protein concentrations in the presence of exogenous protein might prevent dissociation of the nitrate reductase complex (Schrader et al., 1974; Sherrard and Dalling, 1978). However, Brown et al. (1981) found that although BSA stabilized barley nitrate reductase in extracts from older seedlings by preventing breakdown of the nitrate reductase complex, the effect was unlikely to be due to the increased protein concentration since stability was not decreased by dilution.

Nitrate reductase activity from the mature region of the maize root was much less stable than that from the root tip (Oaks et al., 1972; Wallace, 1973a, 1975a) and the in vitro activity was much more unstable than that measured

in vivo (Wallace, 1975a). Schrader *et al.* (1974) proposed that proteolytic degradation might be responsible for the exponential decay of nitrate reductase activity *in vitro* but Purvis *et al.* (1976) were unable to relate increased instability of nitrate reductase from cotton cotyledons to an increase in proteolytic activity. Barley nitrate reductase was least stable during darkness *in vivo* and may have been subject to proteolysis as it was inactivated by a process requiring protein synthesis since inactivation was inhibited by cycloheximide (Travis *et al.*, 1969).

Although non-specific degradation by proteinases from other cell compartments was not ruled out, it was proposed that loss of nitrate reductase activity *in vitro* was caused by exposure to specific inactivating systems in the crude extract (Pan and Marsh, 1972; Oaks *et al.*, 1972; Wallace, 1973b; Schrader *et al.*, 1974). Two broad types of nitrate reductase inactivators have been reported in the literature, the term nitrate reductase inactivator being used to describe any protein capable of inactivating nitrate reductase. One type inactivates nitrate reductase by proteolysis while the other associates with the nitrate reductase complex in such a way as to cause a loss of activity.

Pan and Marsh (1972) detected a protein-like macromolecular inhibitor of nitrate reductase in maize roots which was thought to be relatively specific for nitrate reductase, while Wallace (1973b) observed a nitrate reductase inactivator in mature maize roots (whether or not they had been grown in the presence of nitrate) which was able to promote inactivation of the relatively stable nitrate reductase in root tip extracts. This inactivator was also present in root tips, (although its activity in crude extracts was masked by an inhibitor (Wallace, 1975b, 1978)), scutella and leaves of maize and in the roots and shoots of pea seedlings (Wallace, 1975a). The inactivator was heat-labile, although less so than nitrate reductase, and was considered to be a serine proteinase since it was inhibited by PMSF (Wallace, 1974). It was also inhibited by casein (Wallace, 1975a) but not by pCMB or cysteine (Wallace, 1974, 1975b) while EDTA and 1,10-phenanthroline had only a minor inhibitory effect (Wallace, 1974; Shannon and Wallace, 1979). The molecular weight of the

inactivator has been reported as 44,000 (Wallace, 1974) and 54,000 (Shannon and Wallace, 1979).

The maize nitrate reductase inactivator was able to inactivate nitrate reductase from pea leaves and Neurospora crassa in addition to that from maize but had no effect on nitrate reductase from Pseudomonas denitrificans or Nitrobacter agilis suggesting a major structural difference between the nitrate reductases of prokaryotic and eukaryotic cells (Wallace, 1975b). The inactivator, which was located in the cytosol (Wallace, 1974), was originally thought to inactivate NADH-cytochrome c reductase activity much more slowly than NADH-nitrate reductase (Wallace, 1973b) but results had not taken into account the high level of constitutive NADH-cytochrome c reductase activity present in the nitrate reductase sample (Wallace and Johnson, 1978). The inactivator was found to inactivate all activities of the nitrate reductase complex (Wallace, 1973b, 1975b) but the NADH-cytochrome c reductase component appeared to be the main site of action, whereas in Neurospora crassa nitrate reductase only the NADPH-cytochrome c reductase activity was inactivated suggesting that the Neurospora $FMNH_2$-nitrate reductase moiety differed from that in maize (Wallace, 1975b). The inactivator was initially considered to be specific for nitrate reductase since it had no influence on several other enzymes (Wallace, 1973b) but it was later found to inactivate yeast tryptophan synthase (Wallace, 1978) and to have proteolytic activity towards azocasein (Wallace, 1978; Shannon and Wallace, 1979) and haemoglobin (Shannon and Wallace, 1979) although no peptidase activity was detected (Wallace, 1974).

Despite causing complete inhibition of the inactivator, PMSF only partially prevented loss of nitrate reductase activity in extracts from older maize seedlings but casein was more effective in stabilizing nitrate reductase (Wallace, 1975a). A similar phenomenon was observed in barley extracts (Brown et al., 1981) where PMSF retarded, and BSA prevented, breakdown of the nitrate reductase complex which suggests that more than one nitrate reductase inactivating system may operate in vitro. It is however possible that both the

maize and barley extracts contained high concentrations of phenolic compounds which would be removed by hydrogen-bonding to exogenous protein (Loomis and Battaile, 1966).

Walls et al. (1978) proposed the existence of two mechanisms for nitrate reductase inactivation in Neurospora crassa, the operation of which depended on the nitrogen-nutritional status of the mycelia, although they were not considered specific to nitrate reductase. Two heat-labile nitrate reductase inactivators were observed in Neurospora extracts (Sorger et al., 1978) both of which were proteolytic and caused the loss of NADPH-nitrate reductase activity while activating MVH-nitrate reductase activity. Inactivator I hydrolysed casein but not Azocoll, was inhibited by EDTA and was present under all conditions tested, while inactivator II was inhibited by PMSF, hydrolysed Azocoll and was present only in nitrogen-starved mycelia.

Yeast proteinases A and B which inactivate tryptophan synthase (Katsunuma et al., 1972; Saheki and Holzer, 1974) have also been shown to inactivate maize nitrate reductase (Wallace, 1978). The properties of yeast proteinase B were similar to those of the maize nitrate reductase inactivator except that it was inhibited by pCMB, hydrolysed Azocoll, and had a molecular weight of 32,000 (Wallace, 1978). Proteinase A was an acid proteinase.

A proteolytic nitrate reductase inactivating factor was detected in 8 day old wheat seedlings only after partial purification since two nitrate reductase stabilizing factors were also present (Sherrard et al., 1979a, 1979b). The inactivating factor, of molecular weight 37,500, was inhibited by 1,10-phenanthroline, iodoacetamide, trypsin inhibitors, casein and BSA but not by PMSF or EDTA. It was more active towards NADH-nitrate reductase than towards $FMNH_2$-nitrate reductase or MVH-nitrate reductase, and NADH-ferricyanide reductase was least affected by it (Sherrard et al., 1979b).

A nitrate reductase inactivating factor in rice (Kadam et al., 1974; Yamaya and Ohira, 1976, 1977, 1978a, 1978b) had no proteolytic activity towards casein, BSA or nitrate reductase (Yamaya and Ohira, 1977) and was therefore

considered to be a nitrate reductase-binding protein rather than a proteinase. The heat-labile inactivating factor was inhibited by 1,10-phenanthroline, EDTA and pCMB (Yamaya and Ohira, 1977) but not by BSA, casein, cysteine, nitrate or PMSF (Kadam et al., 1974; Yamaya and Ohira, 1977). It had a molecular weight of 200,000 and was thought to be fairly specific for nitrate reductase although it was also able to inactivate xanthine oxidase, a molybdoflavoprotein (Yamaya and Ohira, 1977).

The rice inactivating factor inhibited NADH-nitrate reductase, NADH-cytochrome c reductase and $FMNH_2$-nitrate reductase activities, but had no effect on MVH-nitrate reductase activity (Kadam et al., 1974; Yamaya and Ohira, 1976, 1977, 1978a). It was ineffective in the presence of NADH and therefore appeared to bind only to the oxidised form of nitrate reductase, particularly since NADH did not protect nitrate reductase in the presence of either nitrate or cytochrome c. NADH was also able to reverse the effect of the inactivating factor (Yamaya and Ohira, 1978b).

A heat-labile nitrate reductase inhibitor (of molecular weight 31,000) was isolated from young soybean leaves (Jolly and Tolbert, 1978) and did not seem to be a proteinase although its mode of action was uncertain. It differed from other inactivators in that it was subject to photoinactivation, which was thought to occur via a reversible conformational change in the inhibitor. The soybean nitrate reductase inhibitor was unaffected by pCMB, PMSF, soybean and ovomucoid trypsin inhibitors, EDTA or 1,10-phenanthroline. It inhibited all activities of nitrate reductase except NADH-cytochrome c reductase, and was therefore considered to act on the nitrate-reducing region of the nitrate reductase complex. It did not inhibit NADPH-nitrate reductase and was thought to be specific for NADH-nitrate reductase.

In addition to the NADH-cytochrome c reductase activity possessed by the nitrate reductase complex, smaller nitrate-inducible NADH-cytochrome c reductase species having sedimentation coefficients of 3-4S have been reported (Wray and Filner, 1970; Wallace and Johnson, 1978; Small and Wray, 1979, 1980). These

species, which in barley had molecular weights of 40,000 (3.1S) and 61,000 (3.8S), were originally thought to be subunits of nitrate reductase (Small and Wray, 1979; Wray et al., 1979) but were recently proposed to represent functionally intact domains of the nitrate reductase complex released by proteolytic cleavage at exposed hinge regions (Small and Wray, 1980; Brown et al., 1981). An additional species of molecular weight 160,000 (6.8S) which had both NADH-nitrate reductase and NADH-cytochrome c reductase activities was also observed in extracts of the primary leaves from 5 and 6 day old barley seedlings (Brown et al., 1981). Similar NADH-cytochrome c reductase species were produced by tryptic cleavage of nitrate reductase (Brown et al., 1981) and it was therefore thought that breakdown of the nitrate reductase complex in crude extracts to yield smaller NADH-cytochrome c reductase species was the result of proteolytic attack by an endogenous proteinase.

Exposure of maize nitrate reductase to the proteolytic maize nitrate reductase inactivator resulted in a differential loss of the activities of the nitrate reductase complex (Wallace, 1975b) although very little was known of the mechanism of inactivation. The maize inactivator might have attacked nitrate reductase by limited proteolysis to release smaller enzymically-active fragments like those observed in barley extracts (Small and Wray, 1980; Brown et al., 1981), therefore the aim of the work described in this thesis was to:

a) purify nitrate reductase inactivating activity from maize,

b) determine its properties,

c) further characterize the mechanism of inactivation of nitrate reductase, using sucrose density gradient centrifugation to study the products of inactivation and look for evidence of the formation of smaller species, in an attempt to determine the sites of attack by the inactivator and thus to extend Wallace's work in the light of recent observations in barley.

Two nitrate reductase inactivators were isolated from maize roots and leaves, and another was prepared from barley leaves.

MATERIALS AND METHODS

MATERIALS

Maize (Zea mays L. cv. LG11) seeds were obtained from Bassaguard Botanic Gardens, University of St. Andrews, and barley (Hordeum vulgare cv. Golden Promise) seeds from William Watt & Sons, Cupar, Fife.

The following were obtained from the Sigma Chemical Company: alcohol dehydrogenase (yeast); azocasein; bovine serum albumin (fraction V); casein; catalase (bovine liver); Coomassie brilliant blue G; cysteine (free base); cytochrome c (horse heart, type III); dithiothreitol; flavin adenine dinucleotide (disodium salt, grade III); leupeptin (hemisulphate); methylene bis-acrylamide; methyl viologen; myoglobin (whale skeletal muscle, type II); NAD (yeast, grade III); NADH (yeast, grade III); phenylmethylsulphonyl fluoride; 1,10-phenanthroline (monohydrate).

Sephadex G200, CNBr-activated Sepharose 4B and blue Dextran 2000 were obtained from Pharmacia.

Biogel A1.5m was obtained from Biorad Laboratories Ltd.

CM-cellulose was obtained from Whatman Biochemicals Ltd.

All other chemicals were of the highest grade available.

GROWTH OF PLANT MATERIAL

Maize (Zea mays L. cv. LG11) was mainly used in this work. Barley (Hordeum vulgare cv. Golden Promise) was used in some experiments.

Maize roots used in the preparation of nitrate reductase inactivators were grown under aseptic conditions. Plastic bowls (internal dimensions 26.5 x 32 x 14cm) were sterilized by rinsing briefly with 70% ethanol and irradiating with ultraviolet light for 16 hours, prior to pouring 1 litre of autoclaved 1% agar (made up in half Hoagland nutrient solution (Table 1) containing 5mM KNO_3) into each bowl. Seeds were surface sterilized in 70% ethanol for 3 minutes, stirred in a 40% Domestos solution for 10 minutes and rinsed several times in sterile water to remove traces of Domestos. Approximately 130g seeds were sown in each bowl. If a larger quantity was sown, then the size of individual roots was reduced and there was an overall lower yield of tissue. The bowls were covered by sterile glass plates (35 x 40cm) held in place by two foldback clips, and a strip of sterile foam rubber placed between the bowl and the glass plate ensured a good seal (Figure 3). The bowls were kept in darkness at 28°C for 72 hours then transferred to continuous light (1,000 lux) at 25°C for a further 64 hours, by which time the primary roots were 8–10cm long.

Maize and barley leaf tissue used in the preparation of nitrate reductase and nitrate reductase inactivators was grown in the following way. Seeds were sown thickly in trays of vermiculite saturated with tap water, and placed in darkness at 28°C. At 24 hour intervals the trays were irrigated with half Hoagland nutrient solution containing 15mM KNO_3. They were transferred to continuous light after 72 hours.

Table 1. Composition of Half Hoagland Nutrient Solution

	g/ litre
Stock solution A	
NaFeEDTA	38.44
Stock solution B	
KH_2PO_4	34.25
$MgSO_4 \cdot 7H_2O$	126.65
$ZnSO_4 \cdot 7H_2O$	0.0555
$CuSO_4 \cdot 5H_2O$	0.0206
H_3BO_3	0.725
$Na_2MoO_4 \cdot 2H_2O$	0.00622
$MnSO_4 \cdot 5H_2O$	0.3904

2ml of each of stock solutions A and B were mixed and diluted to 1 litre with distilled water to give the working solution. KNO_3 was added, at a final concentration of 5mM for growth on agar, or 15mM for growth on vermiculite.

Figure 3. Apparatus for Aseptic Growth of Plants

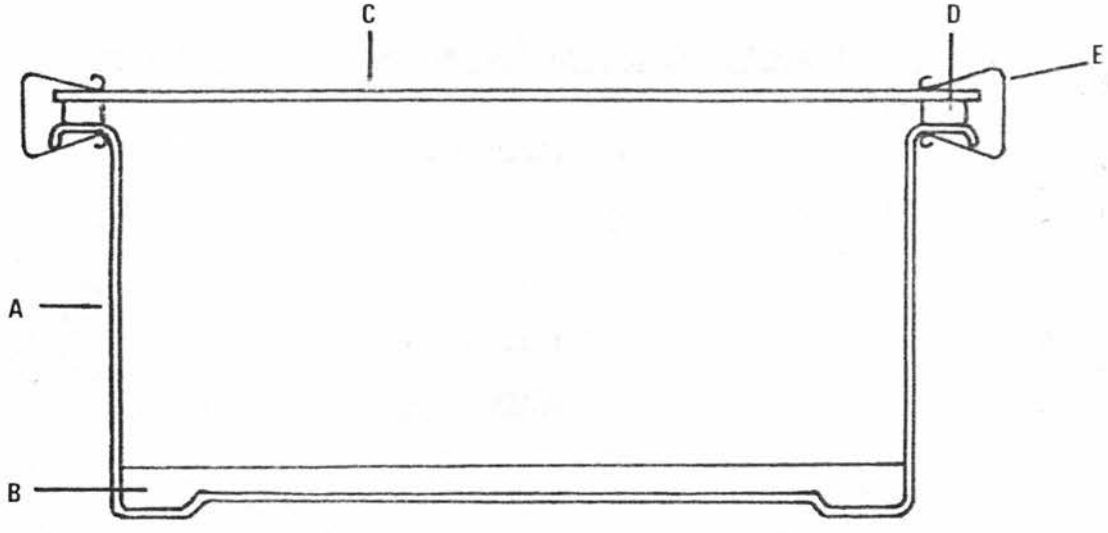
A plastic bowl

B agar

C glass plate

D foam rubber seal

E foldback clip


C
D
E
A
B

PURIFICATION PROCEDURES

All purification steps were carried out at 4°C.

Purification of nitrate reductase

Nitrate reductase was extracted and purified by a slight modification of the method developed for barley nitrate reductase (Small and Wray, 1980; Brown et al., 1981). 6 day old maize shoots were harvested, and homogenized in a mortar and pestle with cold extraction buffer containing 50mM potassium phosphate, 0.1mM EDTA, 10µM FAD and 5mM cysteine, pH 7.5 (Buffer I) (2ml buffer / g tissue). The extract was filtered through a double layer of muslin and centrifuged at 27,000g for 30 minutes, then the fraction precipitated by 0-40% ammonium sulphate was collected and redissolved in Buffer II (Buffer I minus cysteine). This ammonium sulphate fractionation step was carried out by the addition of solid ammonium sulphate to the 27,000g supernatant whilst stirring, and the pH was maintained at 7.5 by the addition of ammonium hydroxide as necessary. After stirring for 15 minutes, precipitated protein was collected by centrifugation at 18,000g for 20 minutes.

The high molecular weight green components were removed by centrifugation for 20 minutes at 40,000 rpm (100,000g) in an MSE Superspeed 65 Mk 1 centrifuge (8 x 25ml rotor), the supernatant was concentrated by 0-40% ammonium sulphate fractionation, and precipitated protein was redissolved in a small volume of Buffer II. This sample (maximum volume 20ml, containing up to 400mg protein) was applied to a Biogel A1.5m column (4.3 x 87cm) equilibrated with Buffer II. Nitrate reductase was eluted with Buffer II and was collected in 18ml fractions. (Remaining traces of the high molecular weight components were eluted in the void volume as a green band which was allowed to travel almost to the bottom of the column before collection of fractions commenced.) Fractions containing nitrate reductase activity were concentrated by 0-40% ammonium sulphate fractionation, and the precipitate was redissolved in 20mM potassium phosphate buffer, pH 7.5, containing 0.1mM EDTA and 10µM FAD (Buffer III). The sample

was usually stored overnight at -70°C at this stage.

Further purification was achieved by application of the nitrate reductase sample to a blue dextran-Sepharose column. (1.8 x 8.5cm) equilibrated with Buffer III. The column was washed with Buffer III until the absorbance at 280nm was less than 0.05 then nitrate reductase was eluted, as a sharp peak, by 5μM NADH contained in Buffer III.

Preparation of partially purified nitrate reductase

In experiments where NADH-cytochrome c reductase activity was not studied, partially purified nitrate reductase was a suitable substrate for the nitrate reductase inactivators and the purification procedure was therefore stopped after the first ammonium sulphate fractionation step. The sample was divided into small aliquots and stored at -70°C until required.

Storage of nitrate reductase

Nitrate reductase samples were dissolved in a small volume of Buffer II or Buffer III, brought to 40% saturation with respect to glycerol, and stored at -70°C. Removal of glycerol prior to use was achieved by ammonium sulphate fractionation using a saturated ammonium sulphate solution, pH 7.5. Protein precipitated in the 0-40% ammonium sulphate fraction was redissolved in Buffer II or Buffer III as required.

Purification of nitrate reductase inactivators

Purification of the inactivators was essentially according to the method of Wallace (1974). Mature roots from 5 day old seedlings were harvested, after discarding the 0-2cm tip region, and homogenized in a mortar and pestle with cold extraction buffer (3ml / g tissue) containing 50mM potassium phosphate, 0.5mM EDTA and 5mM cysteine, pH 7.5. After filtration through a double layer of muslin, the extract was centrifuged at 27,000g for 30 minutes. Ammonium

sulphate fractionation was performed on the supernatant, and the protein precipitated in the 40-70% fraction was resuspended in a small volume of 10mM potassium phosphate buffer, pH 7.0, then dialyzed overnight (17 hours) against 3 changes of 10mM potassium phosphate buffer, pH 7.0, to remove traces of ammonium sulphate.

The pH of the sample was adjusted to 4.0 by the addition of dilute HCl and, after stirring for 15 minutes, precipitated protein was removed by centrifugation at 18,000g for 15 minutes. The supernatant from this step was immediately applied to a column of CM52-cellulose (1.9 x 5.0cm) equilibrated with 10mM acetate buffer, pH 5.0. The column was washed with this buffer until the absorbance of the eluate at 280nm was less than 0.05. Elution of the nitrate reductase inactivators was achieved by passing 240ml 10mM acetate buffer, pH 5.0, containing 50mM NaCl, through the column followed by 200ml 10mM potassium phosphate, pH 8.0. 4ml fractions were collected. Remaining protein was eluted from the column by 0.5M NaOH. Fractions containing nitrate reductase inactivating activity were concentrated by 0-70% ammonium sulphate fractionation and the precipitated protein was redissolved in a small volume of 10mM potassium phosphate, pH 7.0.

Further purification was achieved by Sephadex G200 gel filtration. 2ml samples were applied to a Sephadex G200 column (2.3 x 54.5cm) equilibrated with 10mM potassium phosphate buffer, pH 7.0, and eluted with the same buffer. 2ml fractions were collected and those containing nitrate reductase inactivating activity were pooled, then concentrated by dialysis against glycerol.

Storage of nitrate reductase inactivators

Nitrate reductase inactivator samples were dissolved in a small volume of 10mM potassium phosphate buffer, pH 7.0, brought to 40% saturation with respect to glycerol and stored at -70°C. When required for use, the samples were diluted 100 times in an appropriate buffer.

ENZYME ASSAYS

NADH-nitrate reductase

This was assayed according to the method of Wray and Filner (1970).

A reaction mixture was prepared containing 0.5ml 0.1M potassium phosphate buffer, pH 7.5, 0.1ml 0.1M KNO_3, 0.1ml 1mM NADH, 0.1ml distilled water and 0.2ml enzyme solution. After incubation at 25°C for a suitable time the reaction was stopped and nitrite formation determined by the addition of 1ml 1% (w/v) sulphanilamide in 3M HCl, followed by 1ml 0.02% (w/v) N-(1-naphthyl)ethylenediamine dihydrochloride. The tube contents were mixed, and precipitated protein was sedimented at top speed in a bench centrifuge for 5 minutes.

A blank was prepared in which the 1% sulphanilamide solution was added prior to the enzyme sample. Absorbance of the test sample was read against this blank at 540nm, and the rate of nitrite production estimated by reference to a standard plot of 0-100 nmoles nitrite (Figure 4).

Nitrate reductase inactivating activity

This assay was a modification of the method of Shannon and Wallace (1979).

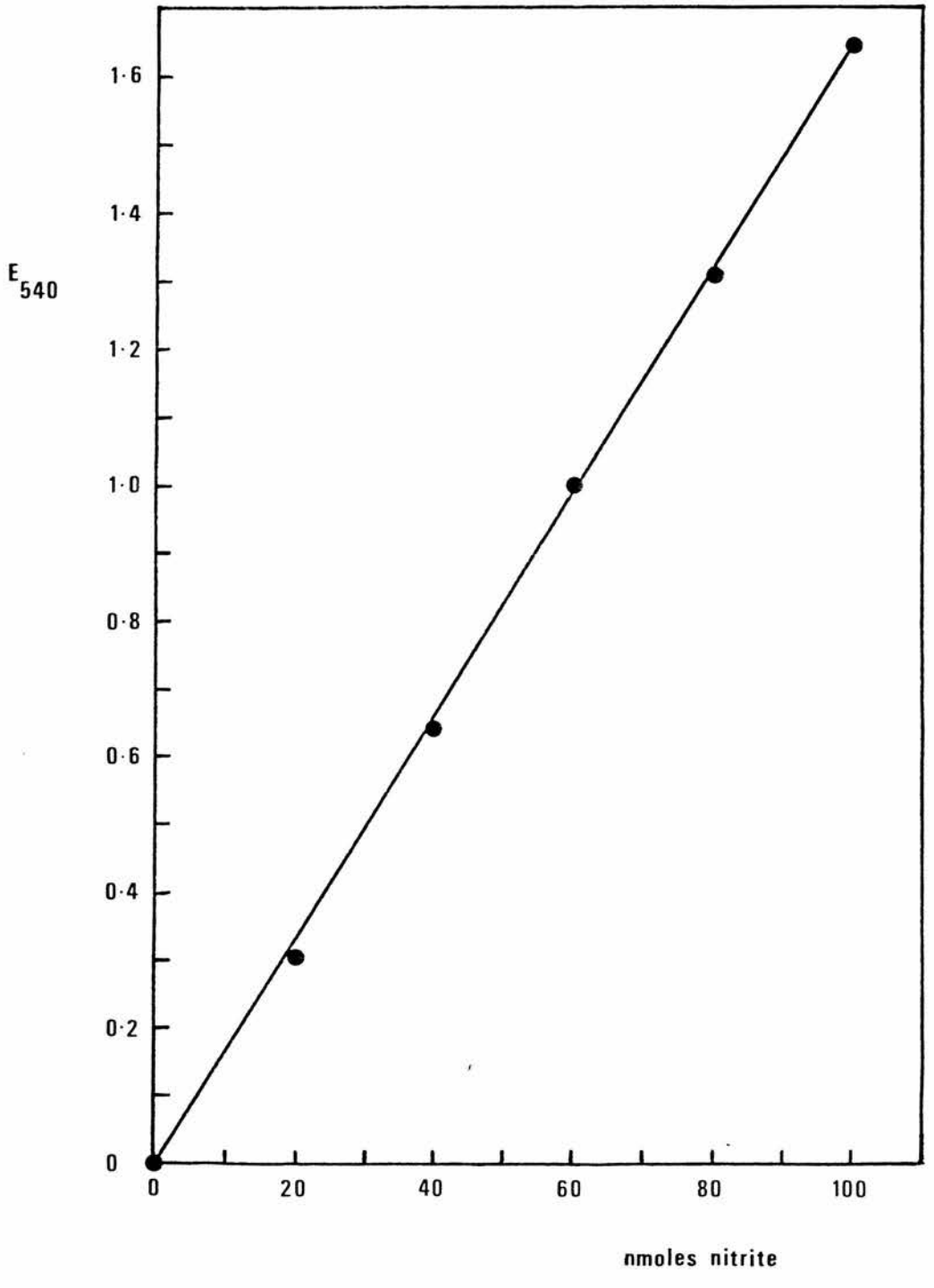
A mixture containing 0.25ml 0.1M potassium phosphate, pH 7.5, 0.05ml partially purified nitrate reductase and 0.1ml nitrate reductase inactivator solution was prepared and incubated at 25°C for 90 minutes. Nitrate reductase activity remaining in the mixture was measured by the addition of 0.375ml 0.1M potassium phosphate, pH 7.5, 0.075ml 0.1M KNO_3, 0.075ml 1mM NADH and 0.075ml distilled water followed by incubation at 25°C for 30 minutes. Nitrite formation was then determined as for NADH-nitrate reductase.

Modifications of this basic method were used in some experiments and details will be given in the Results section where appropriate.

Nitrate reductase inactivating activity was expressed as units of nitrate reductase inactivated per minute at 25°C.

Figure 4. Nitrite Standard Plot

Standard nitrite solutions were prepared and to 1ml of each of these was added 1ml 1% (w/v) sulphanilamide in 3M HCl and 1ml 0.02% (w/v) N-(1-naphthyl)ethylenediamine dihydrochloride. The absorbance was measured at 540nm.


E540
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
0
20
40
60
80
100
nmoles nitrite

NADH-cytochrome c reductase

This was assayed by the method of Wray and Filner (1970).

A reaction mixture was prepared containing 0.2ml 0.1M potassium phosphate buffer, pH 7.5, 0.02ml 2% (w/v) cytochrome c and 0.08ml 1mM NADH. The reaction was started by the addition of enzyme and distilled water to a final volume of 0.4ml, and activity was measured spectrophotometrically by following the rate of increase in absorbance at 550nm.

By reference to the molar extinction coefficient of cytochrome c at 550nm ($2.1 \times 10^4 M^{-1}$) the change in absorbance could be converted to nmoles of cytochrome c reduced using the following equation:

$$\frac{\Delta E_{550}}{0.0525} \times \frac{\text{total volume}}{\text{volume taken} \times t} = \text{nmoles cytochrome c reduced min}^{-1}\text{ fraction}^{-1}.$$

where t = time in minutes.

Cysteine interferes with NADH-cytochrome c reductase activity therefore it is essential that it should be absent from the enzyme solution.

Reduced methyl viologen-nitrate reductase

A reaction mixture containing 0.6ml 0.1M potassium phosphate, pH 7.5, 0.1ml 1mM methyl viologen, 0.1ml 0.1M KNO_3 and 0.1ml enzyme was prepared. Methyl viologen was reduced to its blue form and the reaction started, by the addition of 0.1ml 10mg/ml sodium dithionite in 95mM sodium hydrogen carbonate. After a suitable period of incubation at 25°C the reaction was stopped by vigourous mixing which reoxidised methyl viologen to the colourless form. Nitrite formation was determined in the same way as for NADH-nitrate reductase.

A control tube was prepared in the same way except that the sodium dithionite was omitted.

Catalase

Catalase activity was measured by a modification of the method of Beers and Sizer (1952). A solution containing 100ml 0.1M potassium phosphate, pH 7.5, and 0.4ml 30-volume hydrogen peroxide was prepared. 0.03ml of enzyme was added to 3ml of the above solution and the decrease in absorbance at 240nm was followed using a recording spectrophotometer.

Alcohol dehydrogenase

This was assayed by a modification of the method of Vallee and Hoch (1955). A mixture containing 30ml 0.1M Tris/HCl, pH 8.5, 30ml 3mg/ml NAD, 15ml 2mg/ml dithiothreitol and 30ml 1% ethanol was prepared. 3ml of this mixture and 0.03ml enzyme were placed in a cuvette, and the rate of increase in absorbance at 340nm was measured spectrophotometrically.

Azocasein-degrading activity

This method was based on that of Shannon and Wallace (1979).

0.3ml 10mg/ml azocasein, 0.1ml enzyme and 0.6ml of an appropriate buffer (see Results section) were incubated together at 40°C. After 2 hours the reaction was stopped by the addition of 1ml 5% trichloroacetic acid. Precipitated protein was removed by centrifugation in a bench centrifuge and 0.2ml 10M NaOH was added to the supernatant. The absorbance of the solution was measured at 440nm, against a blank in which the enzyme was replaced by 0.1ml 10mM potassium phosphate, pH 7.0.

Non-enzymic assays

a) Myoglobin was detected by measurement of its absorbance at 415nm.

b) Blue dextran 2000 was detected by its absorbance at 625nm.

ANALYTICAL PROCEDURES

Estimation of Stokes radius of nitrate reductase inactivators

Nitrate reductase inactivator samples were dissolved in 1.5ml 10mM potassium phosphate, pH 7.0, and small aliquots of solid catalase (5.2nm), alcohol dehydrogenase (4.6nm) and myoglobin (2.0nm) were added as reference proteins. This mixture was applied to a column of Sephadex G200 (2.3 x 50cm) equilibrated with 10mM potassium phosphate, pH 7.0, and eluted with the same buffer at a flow rate of approximately 10ml/hr, at 4°C. 2ml fractions were collected. The void volume of the column was determined using blue dextran 2000.

The elution volumes of the reference proteins from Sephadex G200 were related to their Stokes radii using the correlation of Porath (1963) whereby

$$K_d = \frac{V_e - V_o}{V_t - V_g - V_o}$$

K_d is the distribution coefficient; V_e is the elution volume; V_o is the void volume; V_t is the total volume of the column and V_g is the volume occupied by the gel particles (= B x d, where B is the bed volume per gram of dry gel and d is the density of the dry gel).

A linear calibration plot of $K_d^{\frac{1}{3}}$ against Stokes radius is obtained from the reference proteins. The Stokes radius of the protein under investigation could therefore be estimated from this plot once its elution volume was known and the value of $K_d^{\frac{1}{3}}$ had been calculated, using the program described by Small (1980), on a Texas TI 58/59 programmable calculator.

Estimation of sedimentation coefficient by sucrose density centrifugation

Three 25ml polypropylene centrifuge tubes were washed in a hot EDTA solution then rinsed in distilled water and dried. 2%, 4%, 6%, 8%, 10%, 12%, 14%, 16% and 18% (w/v) sucrose solutions containing 0.1M potassium phosphate, 1mM EDTA and 10μM FAD, pH 7.5, were prepared. Starting with the 18% solution

and working in order of decreasing sucrose concentration, 2ml of each solution was carefully pipetted into the centrifuge tubes which were then capped with parafilm and allowed to stand at 4°C for 18–20 hours.

Purified nitrate reductase samples were exposed to nitrate reductase inactivators and then mixed with an appropriate inhibitor, to prevent further inactivation, and small aliquots of solid catalase (11.3S), alcohol dehydrogenase (7.4S) and myoglobin (2.04S) which acted as reference proteins. 0.4ml of this mixture was carefully layered onto each gradient and centrifuged at 95,000g in an MSE Superspeed$_{65}$ Mk I centrifuge (3 x 25ml swing-out rotor) for 24 hours, at 4°C. After centrifugation, each gradient was fractionated by piercing the bottom of the tube and collecting 42 fractions of 15 drops each.

The refractive indices of selected fractions were determined as a means of confirming gradient linearity. Readings obtained from a Bellingham and Stanley Abbé-type refractometer were converted to refractive indices by means of the program described by Small (1980), using a Texas TI 58/59 programmable calculator.

A linear calibration plot of peak fraction number against sedimentation coefficient was obtained from the reference proteins. Using this plot, the sedimentation coefficient of any component in the sample could be estimated from its peak fraction number.

Calculation of molecular weight from Stokes radius and sedimentation coefficient

Calculation of molecular weight was achieved using the equation described by Siegel and Monty (1966):

$$M = \frac{6\pi\eta Nas}{(1 - \bar{\upsilon}\rho)}$$

where M is the molecular weight; η is the viscosity of the medium (assumed to be 1); N is Avogadro's number; a is the Stokes radius, in metres; s is the sedimentation coefficient, in sec^{-1}; $\bar{\upsilon}$ is the partial specific volume (assumed

to be 0.725cm^3/g); and ρ is the density of the medium (assumed to be 1).

The calculation was performed on a Texas TI 58/59 programmable calculator, using a program described by Small (1980).

Polyacrylamide disc-gel electrophoresis

a) Preparation of gels.

A solution of 5% (w/v) acrylamide and 0.4% (w/v) methylene bis-acrylamide in 0.2M Tris/HCl, pH 8.5, was prepared. 20ml of this solution was deaerated, then 0.05ml TEMED and 5mg ammonium persulphate were added. This mixture was quickly pipetted into glass tubes (5 x 75mm) and a few drops of distilled water were layered on top of the gel which was then allowed to polymerize in strong light for about 20 minutes.

A spacer gel was prepared by deaerating 20ml of a solution of 2.5% (w/v) acrylamide and 0.2% (w/v) methylene bis-acrylamide in 0.2M Tris/HCl, pH 6.5, then 0.05ml TEMED and 5mg ammonium persulphate were added. After removal of the distilled water, about 5mm depth of spacer gel was layered on top of the 5.4% gel. A few drops of distilled water were carefully layered on top of this, and the spacer gel was allowed to polymerize in strong light.

b) Electrophoresis.

Nitrate reductase inactivator samples were each mixed with 1 drop glycerol and 1 drop 0.05% bromophenol blue, and 0.05-0.1ml of the mixture was applied to the top of each gel. The lower reservoir of the electrophoresis tank was filled with 0.08M Tris/HCl, pH 8.5, and the upper reservoir (cathode compartment) contained 0.08M Tris/glycine, pH 8.5. Electrophoresis was carried out at 4°C, at 1-2mA/tube, until the bromophenol blue tracking dye was 0.5-1cm from the bottom of the gels.

c) Staining and destaining.

A protein staining solution containing 1.25g Coomassie brilliant blue G, 227ml methanol, 227ml distilled water and 46ml glacial acetic acid was prepared.

Gels were stained in this solution for about 30 minutes then destained by soaking overnight in a solution composed of 250ml methanol, 75ml glacial acetic acid and 675ml distilled water. Several changes of destaining solution were necessary to remove excess stain.

PREPARATION OF BLUE DEXTRAN-SEPHAROSE

Blue dextran-Sepharose was routinely prepared in the laboratory, essentially by the method of Sherrard and Dalling (1979).

3g CNBr-activated Sepharose 4B were swollen in 200ml ice-cold 1mM HCl and washed on a sinter with 500ml 1mM HCl to remove preservatives. 10ml of swollen gel were added to 20ml 0.4M Na_2CO_3, pH 8.0, containing 0.4g blue dextran 2000, and mixed for 18 hours on an end-over-end tumbler, at 4°C. The gel was washed on a sinter with distilled water then suspended in 0.1M Tris/HCl, pH 8.0, at 4°C, for 2 hours, to block unreacted sites on the Sepharose. The gel was washed with 5 cycles of 0.1M sodium citrate, 1M KCl, pH 4.0 and 0.1M borate, 1M KCl, pH 8.0 (50ml of each per 10ml of gel) and was then ready for use.

Between runs the gel was washed with 3M KCl to remove adsorbed protein.

PROTEIN ESTIMATIONS

Protein concentrations were estimated by the method of Bradford (1976). A protein reagent containing 0.01% (w/v) Coomassie brilliant blue G, 4.7% (w/v) ethanol and 8.5% (w/v) phosphoric acid was prepared, and filtered before use. 0.1ml protein solution was added to 5ml protein reagent and mixed by vortexing. After 5 minutes the absorbance at 595nm was read against a blank containing buffer in place of protein solution.

A standard curve of 0–100µg protein was prepared in the same way, from a solution of 1mg/ml BSA.

RESULTS

CHAPTER 1

Purification of Nitrate Reductase and Nitrate Reductase Inactivators

Purification of nitrate reductase

Initially, it was necessary to select the best seedling age for extraction and purification of nitrate reductase therefore the amount of nitrate reductase activity which could be extracted from 5g maize leaf tissue was measured at daily intervals from 5 to 10 days after sowing (Figure 5). Extracts from 5 day old seedlings contained a relatively low amount of nitrate reductase. 6 day old seedlings contained a much larger amount of extractable nitrate reductase activity per gram fresh weight but there was no additional increase in activity in older seedlings, although the total protein content of extracts increased with seedling age so that there was a decrease in specific activity of the nitrate reductase obtainable from seedlings aged 7 or more days old. It was therefore decided to use 6 day old seedlings in routine nitrate reductase extractions.

During homogenization and the initial centrifugation step there was considerable browning of the extract (and an accompanying loss of nitrate reductase activity) which was perhaps due to the oxidation and subsequent polymerization of endogenous phenolic compounds to form tannin-like substances capable of precipitating proteins (Rhodes, 1977). This enzymic browning was partially prevented by the inclusion of 5mM cysteine in the extraction buffer, but it was still necessary to perform ammonium sulphate fractionation as soon as possible in order to remove the influence of phenolic compounds and proteinases and therefore obtain a good yield of nitrate reductase.

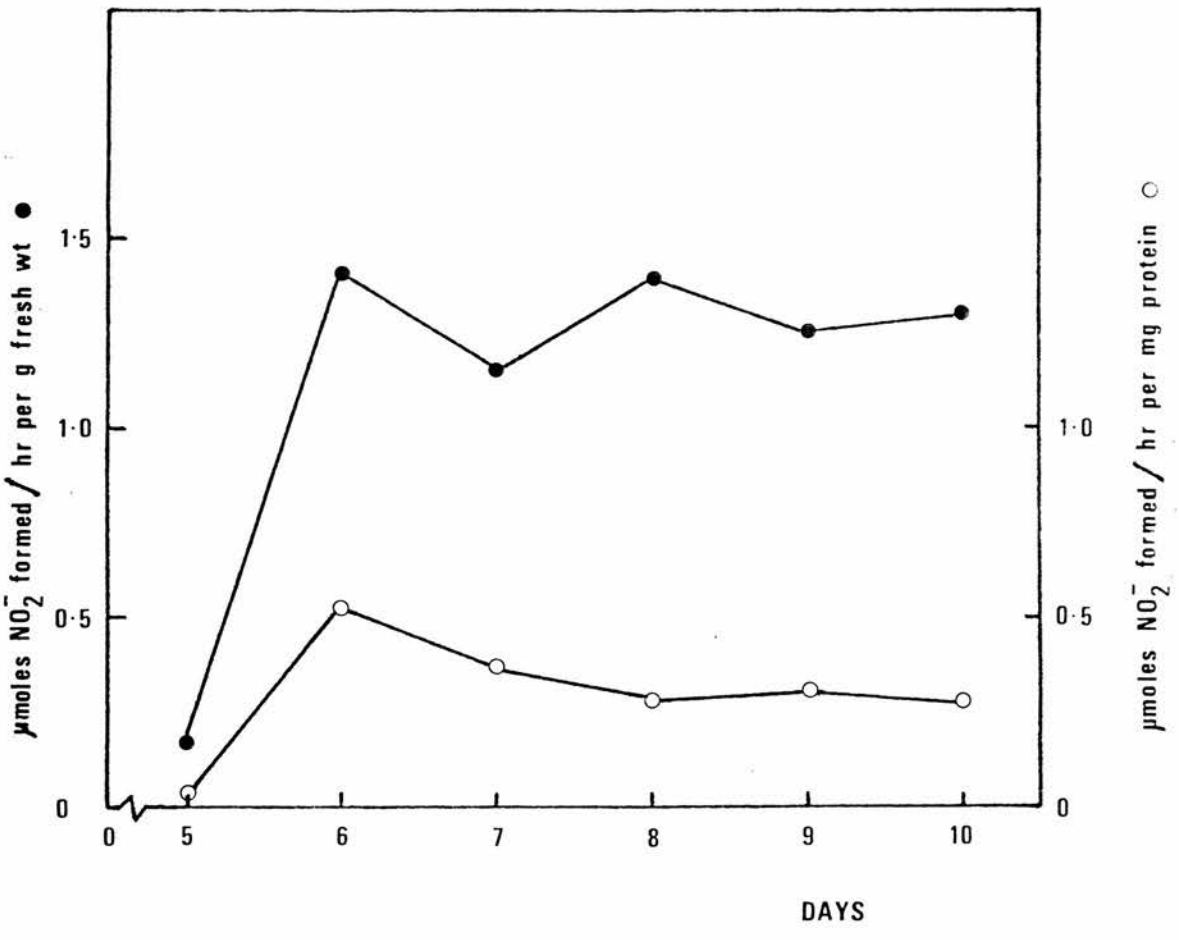
In routine nitrate reductase preparations, 200g maize shoots were extracted according to the procedure described in Methods. Purification was stopped after the initial ammonium sulphate fractionation step when partially purified nitrate reductase was required. This was suitable for use in assaying nitrate reductase inactivating activity in column fractions and in experiments to study the effect of inhibitors on the nitrate reductase inactivators.

However, when the action of the inactivators on the nitrate reductase complex was examined a purer enzyme sample was required, since maize extracts

Figure 5. NADH-Nitrate Reductase Activity Extractable from Maize Seedlings 5-10 Days after Sowing

At daily intervals, 5g samples of maize shoots were harvested and homogenized in 15ml Buffer I, at 4°C. After removal of cell debris by centrifugation, the protein precipitated in the 0-40% ammonium sulphate fraction was collected and redissolved in Buffer I (final volume 4ml) then immediately assayed for NADH-nitrate reductase activity.

Prior to 5 days the primary leaf had not emerged from the coleoptile.


µmoles NO_2^- formed / hr per g fresh wt ●
µmoles NO_2^- formed / hr per mg protein ○
1·5
1·0
0·5
0
0
5
6
7
8
9
10
DAYS

contain large quantities of constitutive high molecular weight (11S) NADH-cytochrome c reductase species (Wallace, 1974, 1975b; Wallace and Johnson, 1978) which must be removed in order to observe changes in the level of nitrate reductase-associated NADH-cytochrome c reductase activity. This separation was achieved by Biogel A1.5m gel filtration when a peak of constitutive NADH-cytochrome c reductase activity was eluted in the void volume along with all of the green components of the extract, and nitrate reductase was eluted subsequently (Figure 6). The largest sample which could be processed by this method contained only 400mg protein and was typically obtained from 90-100g shoots, but samples derived from larger quantities of tissue could be separated on the Biogel A1.5m column provided that excess high molecular weight material was removed. To do this the 0-40% ammonium sulphate fraction was resuspended in a large volume (usually 100ml) of Buffer II to ensure that it was completely dissolved and centrifuged at 100,000g, which removed all but traces of the green components along with much of the constitutive NADH-cytochrome c reductase activity. The supernatant was concentrated by ammonium sulphate fractionation before application to the Biogel A1.5m column (Figure 7).

Further purification was achieved by affinity chromatography on blue dextran-Sepharose (Figure 8). Samples usually contained 20-30mg protein, most of which was not adsorbed onto the gel since blue dextran binds specifically to proteins possessing a dinucleotide fold structure (Thompson et al., 1975). Nitrate reductase was eluted by 5μM NADH and normally only the peak two or three fractions were retained for use in inactivation studies.

A summary of nitrate reductase purification is presented in Table 2.

Purification of nitrate reductase inactivators from maize roots

Maize seedlings were grown in an aseptic environment to avoid contamination of root extracts by fungal nitrate reductase inactivators. Originally seeds were surface sterilized by soaking in 70% ethanol for 3 minutes then in a 40% Domestos solution for 15 minutes, but it was observed that many seeds either

Figure 6. Biogel A1.5m Gel Filtration of the 0-40% Ammonium Sulphate Fraction Derived from 90g 6 Day Old Maize Shoots

90g maize shoots were homogenized in Buffer I at 4°C, filtered through a double layer of muslin and centrifuged at 27,000g, then the protein precipitated in the 0-40% ammonium sulphate fraction (380mg protein) was redissolved in 20ml Buffer II and applied to a Biogel A1.5m column (4.3 x 87cm). Nitrate reductase was eluted with Buffer II. 18ml fractions were collected and assayed for NADH-nitrate reductase (□) and NADH-cytochrome c reductase (●) activities.

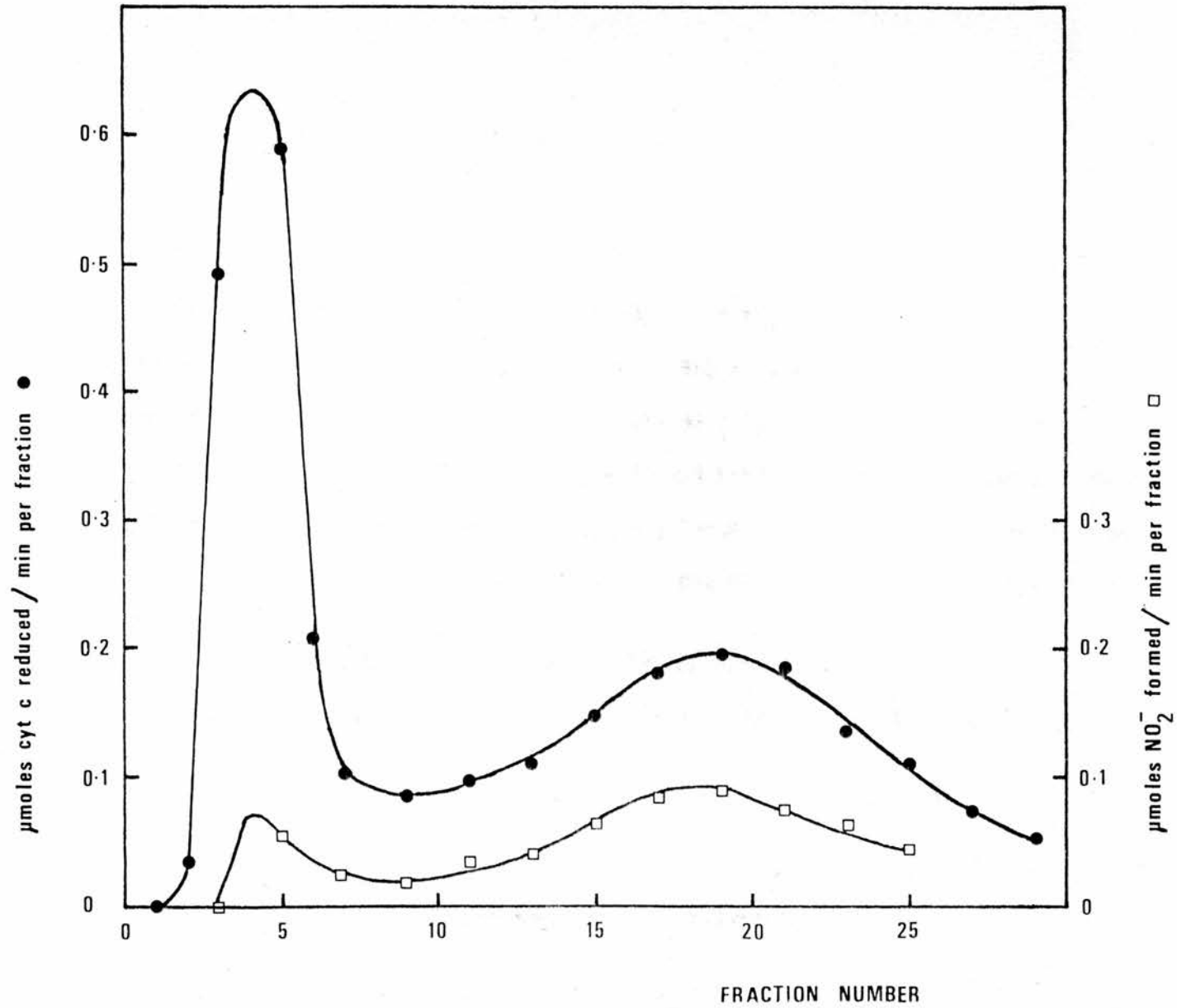

µmoles cyt c reduced / min per fraction ●
µmoles NO_2^- formed / min per fraction □
0·6
0·5
0·4
0·3
0·2
0·1
0
0·3
0·2
0·1
0
0
5
10
15
20
25
FRACTION NUMBER

Figure 7. Biogel A1.5m Gel Filtration of the 0-40% Ammonium Sulphate Fraction Derived from 210g 6 Day Old Maize Shoots, After Centrifugation at 100,000g

The 0-40% ammonium sulphate fraction derived from 210g maize shoots (545mg protein) was centrifuged at 100,000g for 20 minutes. The supernatant, which contained 360mg protein, was concentrated by ammonium sulphate fractionation and applied to a Biogel A1.5m column (4.3 x 87cm). It was eluted with Buffer II and 18 ml fractions were collected then assayed for NADH-nitrate reductase (□) and NADH- cytochrome c reductase (●) activities.

Comparison with Figure 6 shows that a large proportion of the constitutive high molecular weight NADH-cytochrome c reductase activity was removed from the sample during the 100,000g centrifugation step.

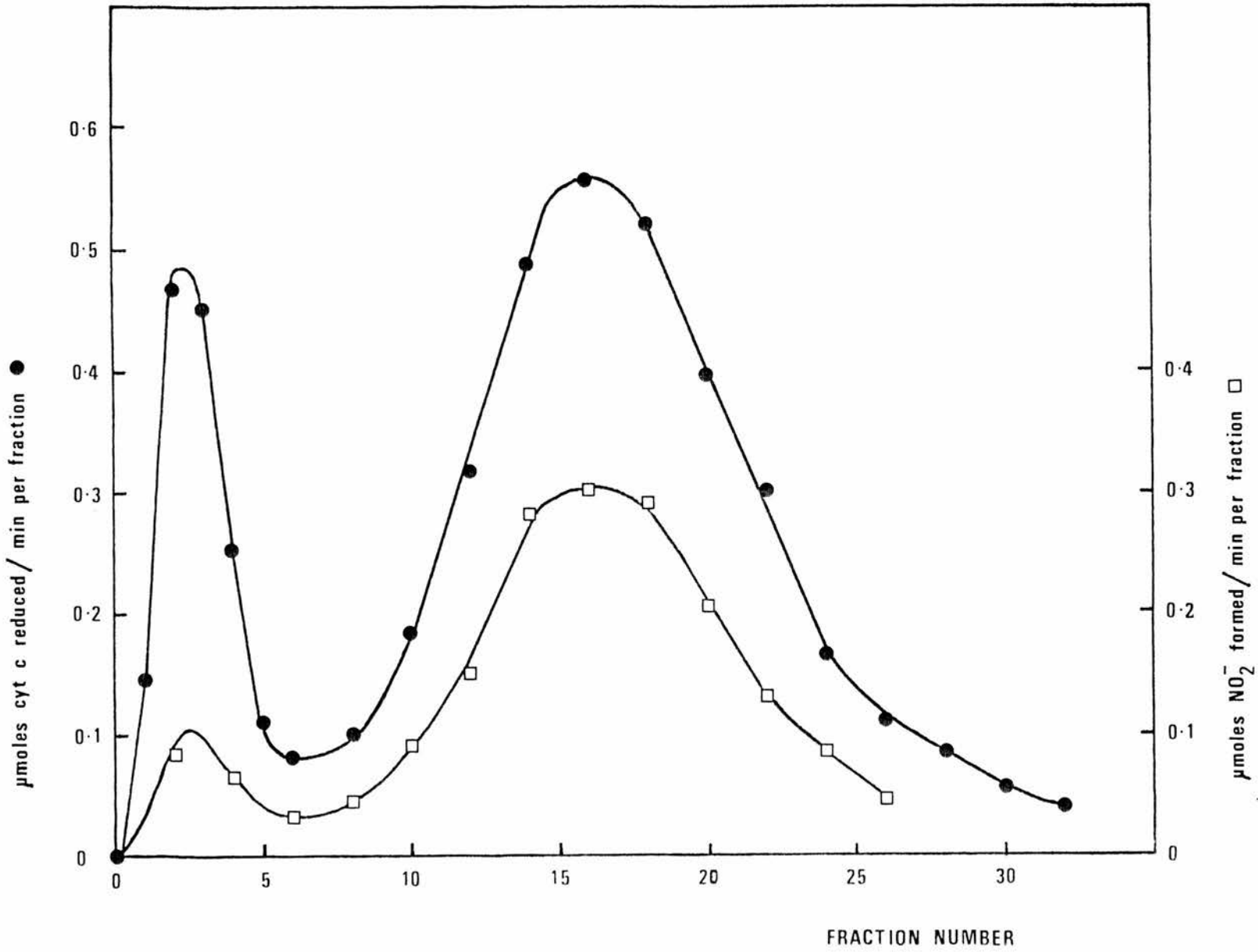

0·6
0·5
0·4
0·3
0·2
0·1
0
µmoles cyt c reduced / min per fraction ●
0·4
0·3
0·2
0·1
0
µmoles NO_2^- formed / min per fraction □
0
5
10
15
20
25
30
FRACTION NUMBER

Figure 8. Elution of Nitrate Reductase from Blue Dextran-Sepharose

170g 6 day old maize shoots were homogenized in Buffer I then nitrate reductase was purified by ammonium sulphate fractionation, centrifugation at 100,000g and Biogel A1.5m gel filtration. The sample (18.9mg protein) was then applied to a blue dextran-Sepharose column (1.8 x 8.5cm) equilibrated with Buffer III and non-adsorbed protein (Δ) was eluted by Buffer III. NADH-nitrate reductase (■) was eluted by 5μM NADH contained in Buffer III and 6ml fractions were collected.

A summary of the purification is presented in Table 2.

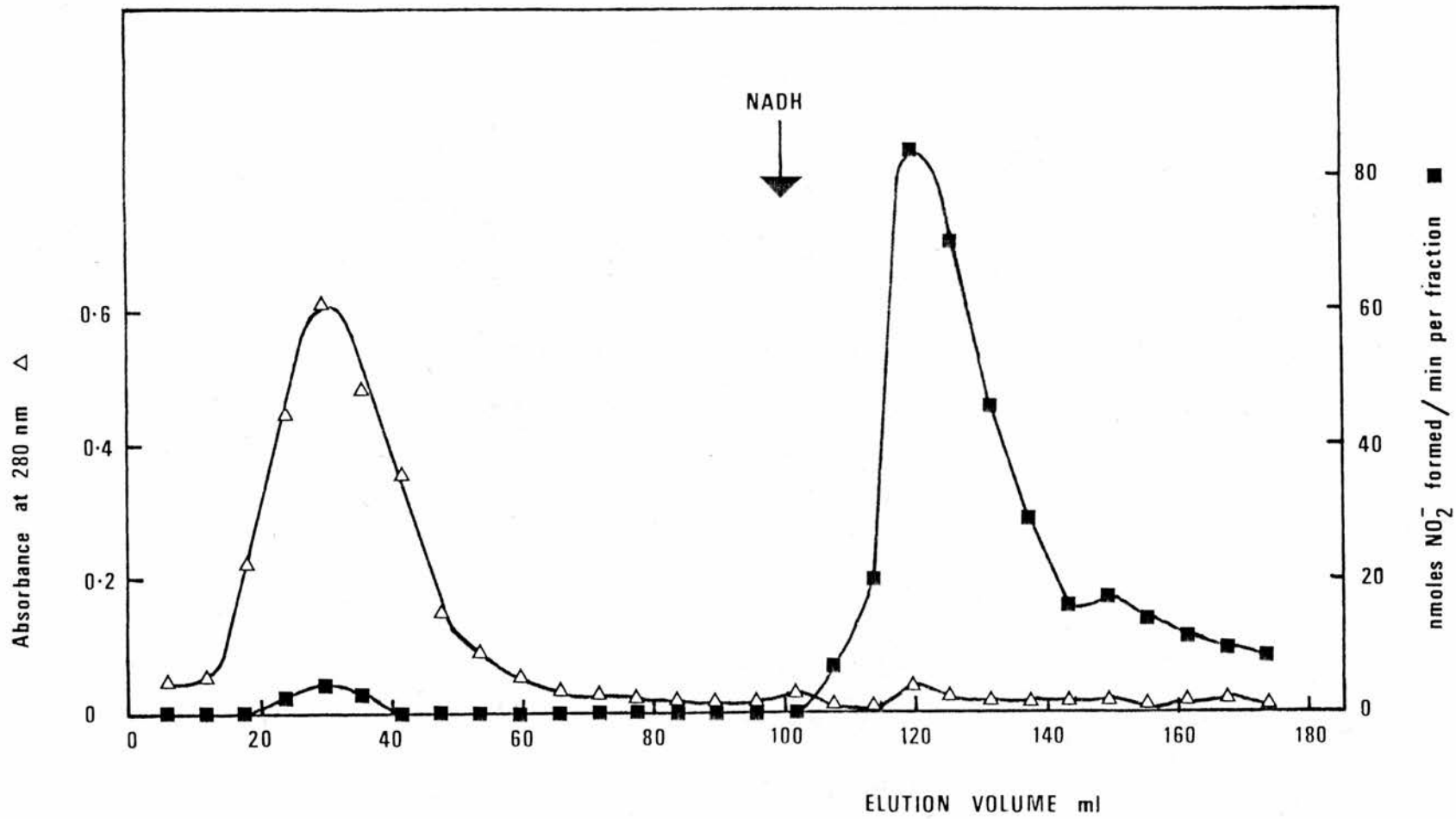

NADH
Absorbance at 280 nm △
nmoles NO_2^- formed / min per fraction ■
0·6
0·4
0·2
0
80
60
40
20
0
0
20
40
60
80
100
120
140
160
180
ELUTION VOLUME ml

Table 2. Purification of Nitrate Reductase from 170g 6 Day Old Maize Shoots

	Protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification factor
Centrifuged extract	867	2.246	0.0026	100	1
0-40% ammonium sulphate fraction	273	1.965	0.0072	87.5	2.8
100,000g supernatant	171	1.757	0.0103	78.2	3.9
0-40% ammonium sulphate fraction	170	1.861	0.0109	82.8	4.2
Biogel A1.5m and 0-40% ammonium sulphate fraction	18.9	0.856	0.0453	38.1	17.5
Blue dextran-Sepharose fraction	0.084	0.231	2.750	10.3	1058

1 unit is defined as 1 μmole nitrite formed per minute at 25°C.

did not grow at all or only produced tiny shoots during the 5 day growth period. It was thought that the sterilization procedure might injure the seed embryo therefore batches of 30 seeds each were subjected to sterilization for different lengths of time then sown in vermiculite and examined after 7 days growth (Table 3).

Lengthening of the Domestos treatment did not appear to decrease the number of seeds capable of growth. In fact the batch subjected to the longest treatment produced the largest number of seedlings but the longer treatments caused retarded germination in a significant proportion of seeds so that many seedlings were stunted and the overall yield of tissue was reduced. As a result of this discovery the sterilization procedure was shortened so that after soaking in 70% ethanol for 3 minutes the seeds were only exposed to 40% Domestos for 10 minutes which was sufficient to prevent fungal growth and yet produced fewer stunted seedlings than the previous method.

Overcrowding of the seeds on agar also produced stunted seedlings. 130g seeds was the largest quantity which could be grown on 1 litre of agar if a good yield of root tissue was to be obtained.

Wallace (1975b, 1978) reported the presence in maize root tips of a component which inhibited nitrate reductase inactivating activity therefore during harvesting the 0-2cm root tip region was discarded and the mature root region was used as the source of nitrate reductase inactivators. Approximately 100g mature root tissue was obtained from 390g seeds and this was the largest quantity which could be processed at one time, the limiting factor being the length of time required for harvesting.

A summary of the purification of nitrate reductase inactivators is presented in Table 4. Nitrate reductase inactivating activity was estimated using a partially purified nitrate reductase sample with an activity of 0.257μmol nitrite formed/mg protein hr^{-1}.

During the extraction and initial centrifugation step, as with nitrate reductase preparations, there was a browning of the extract due to the presence

Table 3. Effect of Sterilization Procedures on Seed Viability and Subsequent Growth

Length of Domestos treatment (minutes)	Number of seeds germinating	Number of stunted seedlings	% of seedlings stunted
Control	25	0	0
0	23	1	4
5	27	2	7
10	24	4	16
15	22	6	27
20	28	8	28

Batches of 30 seeds each were soaked in 70% ethanol for 3 minutes and then in 40% Domestos for 0-20 minutes, rinsed thoroughly in distilled water and grown in pots of vermiculite for 7 days. The control group was untreated.

Table 4. Purification of Nitrate Reductase Inactivators from 92g 5 Day Old Mature Maize Roots

	Protein (mg)	Total activity (units x 10^2)	Specific activity (units/mg x 10^2)	Yield (%)	Purification factor
Centrifuged extract	249	2.90	0.0116	100	1
40-70% ammonium sulphate fraction	175	1.46	0.0083	50.3	0.72
pH 4.0 supernatant	61.7	2.66	0.0431	91.7	3.7
CM52-cellulose nitrate reductase inactivator I fraction	7.0	2.58	0.3685	90	31.8
CM52-cellulose nitrate reductase inactivator II fraction	13.8	0.18	0.0130	6.2[a]	—[b]
Sephadex G200 nitrate reductase inactivator I fraction	2.7	1.83	0.6778	63	58.4

1 unit is defined as 1 unit nitrate reductase inactivated per minute at 25°C.

a - expressed as % of total nitrate reductase inactivating activity in the centrifuged extract; b - not assessed

of phenolic compounds. This was less severe than in leaf extracts and could be partially prevented by 5mM cysteine. However the phenolic compounds inhibited the nitrate reductase used in assaying nitrate reductase inactivating activity and therefore gave a falsely high value for the activity of nitrate reductase inactivators in the centrifuged extract.

The 40-70% ammonium sulphate fraction was dialyzed overnight to remove traces of ammonium sulphate then subjected to acid precipitation, at pH 4.0. As reported by Wallace (1974,1978) there was an increase in nitrate reductase inactivating activity at this stage which was suggested to be due to removal of inhibitors of the nitrate reductase inactivators. The supernatant from this stage typically contained 60-70mg protein and was further purified by ion-exchange chromatography on CM52-cellulose to reveal the presence of two nitrate reductase inactivator species designated inactivators I and II (Figure 9). Nitrate reductase inactivator I was eluted from the column by 10mM acetate, pH 5.0, containing 50mM NaCl, and inactivator II was eluted by 10mM potassium phosphate, pH 8.0. No other peaks of nitrate reductase inactivating activity were detected.

At this stage the inactivators were stored at -70°C until required. Nitrate reductase inactivator I was further purified by Sephadex G200 gel filtration, and a single peak of nitrate reductase inactivating activity was obtained (Figure 10).

Purification of nitrate reductase inactivators from maize leaves

Nitrate reductase inactivators were reported to be present in the leaves of 23 day old maize seedlings (Wallace, 1975a). In an attempt to determine whether the inactivators could be detected in the leaves of younger seedlings, the primary and secondary leaves of 10 day old seedlings were harvested and homogenized in cold 50mM potassium phosphate buffer, pH 7.5, containing 0.5mM EDTA and 5mM cysteine (3ml/g tissue). Purification of nitrate reductase inactivating activity was carried out in the same way as for that from root

Figure 9. Elution of Nitrate Reductase Inactivators from CM52-Cellulose

The 40-70% ammonium sulphate fraction derived from 92g 5 day old mature root tissue was dialyzed overnight then subjected to acid precipitation at pH 4.0. The supernatant from this step (61.7mg protein) was immediately applied to a CM52-cellulose column (1.9 x 5.0cm) equilibrated with 10mM acetate, pH5.0. Unadsorbed protein was eluted with 10mM acetate, pH 5.0, then nitrate reductase inactivator I was eluted by 10mM acetate, containing 50mM NaCl, and inactivator II was eluted by 10mM potassium phosphate, pH 8.0.

Inset: Nitrate reductase inactivating activity was assayed using a partially purified nitrate reductase sample with an activity of 0.175 µmol nitrite formed/mg protein hr^{-1} and containing 8mg protein/ml.

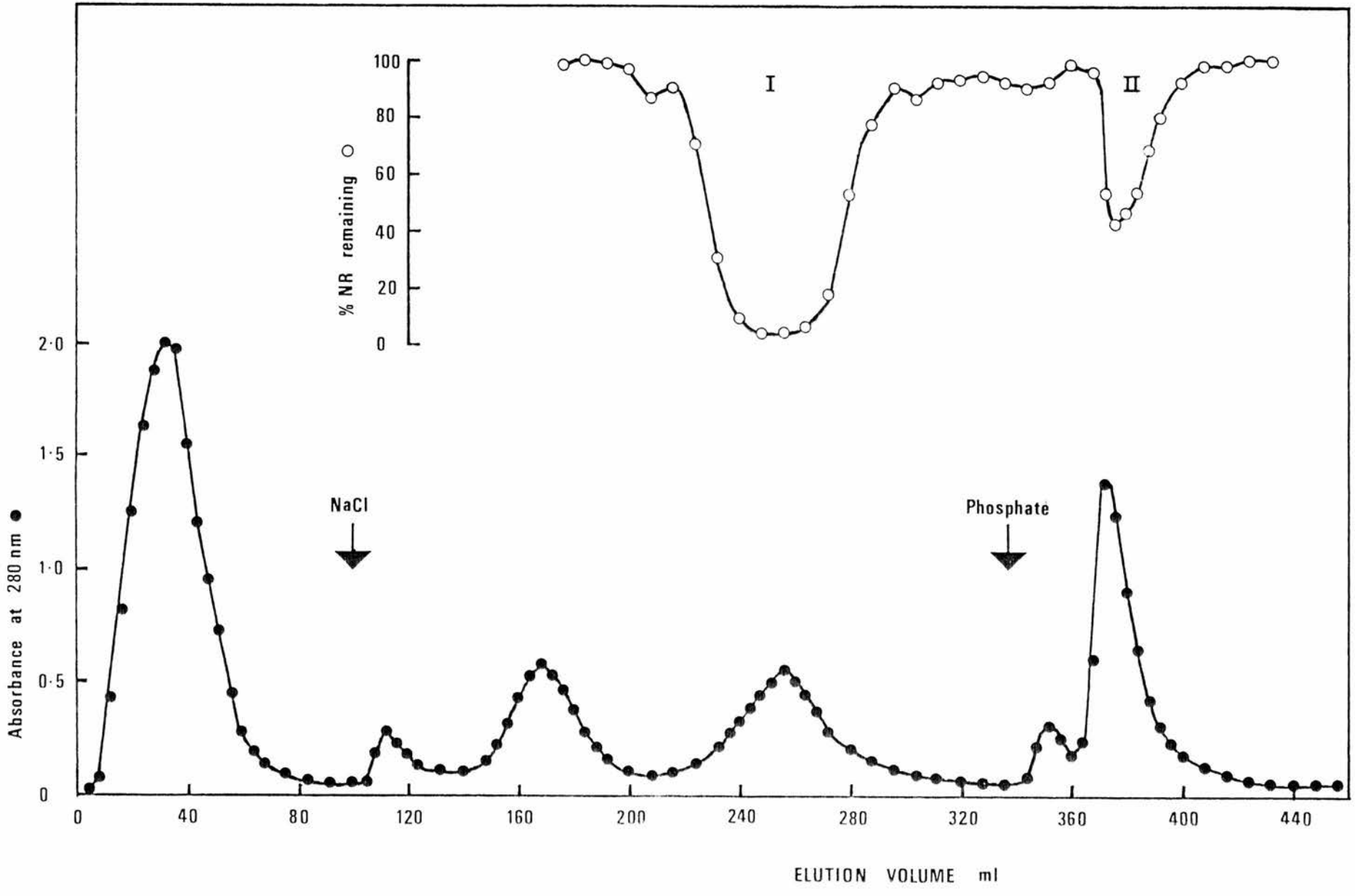
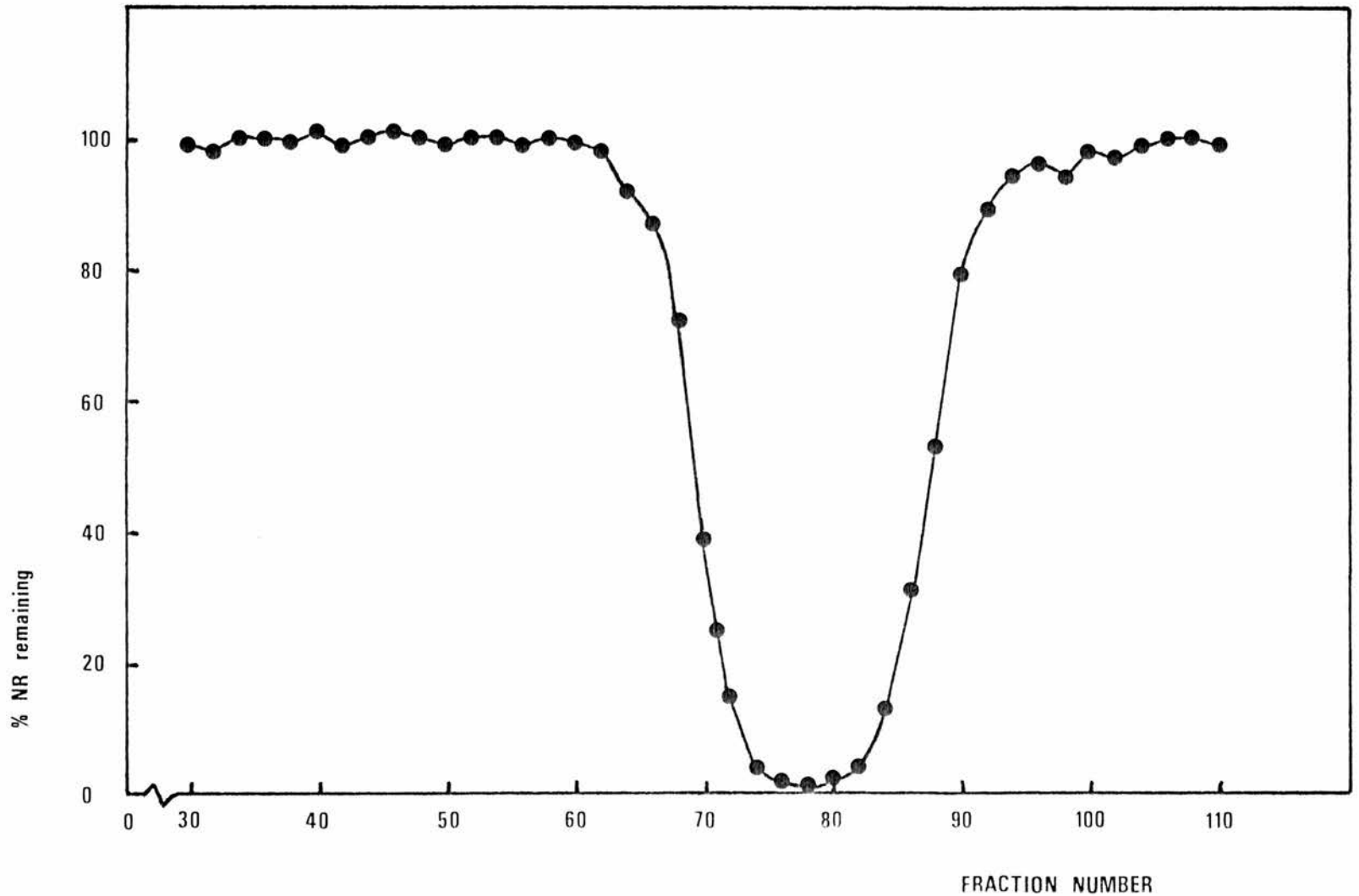

100
80
60
40
20
0
% NR remaining ○
I
II
2·0
1·5
1·0
0·5
0
Absorbance at 280 nm ●
NaCl
Phosphate
0
40
80
120
160
200
240
280
320
360
400
440
ELUTION VOLUME ml

Figure 10. Elution of Nitrate Reductase Inactivator I from Sephadex G200

After elution from CM52-cellulose, nitrate reductase inactivator I (7mg protein) was concentrated by 0-70% ammonium sulphate fractionation. Precipitated protein was redissolved in 2ml 10mM potassium phosphate, pH 7.0, and applied to a Sephadex G200 column (2.3 x 54.5cm) equilibrated with the same buffer. The sample was eluted with 10mM potassium phosphate, pH 7.0, and 2ml fractions were collected.

Nitrate reductase inactivating activity was assayed using a partially purified nitrate reductase sample containing 11mg protein/ml and with an activity of 0.176 μmol nitrite formed/mg protein hr^{-1}.


100
80
60
40
20
0
% NR remaining
0
30
40
50
60
70
80
90
100
110
FRACTION NUMBER

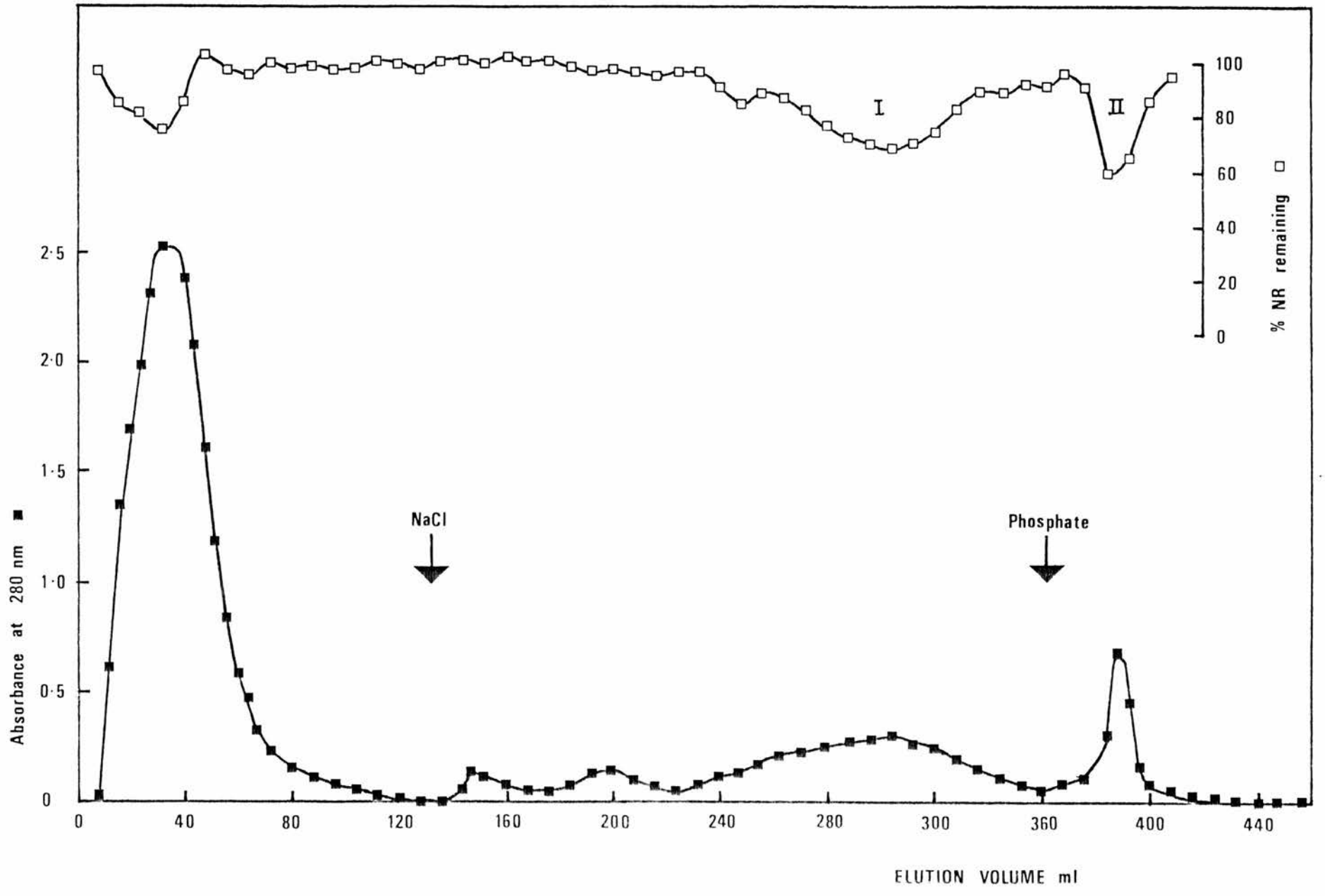
tissue ie. filtration and centrifugation of the crude extract, dialysis of the 40–70% ammonium sulphate fraction, pH 4.0 precipitation and ion-exchange chromatography on CM52-cellulose (Figure 11).

The activity of the nitrate reductase inactivators from leaf tissue was very low compared to that obtained from an equivalent quantity of 5 day old mature root tissue, but both leaf (Figure 11) and root (Figure 9) extracts seemed to contain essentially similar components.

Figure 11. Elution of Maize Leaf Nitrate Reductase Inactivators from CM-Cellulose

75g 10 day old primary and secondary maize leaves were homogenized in 225ml cold 50mM potassium phosphate, pH 7.5, containing 0.5mM EDTA and 5mM cysteine, and the 40-70% ammonium sulphate fraction was dialyzed overnight prior to pH 4.0 precipitation. The supernatant from this step (100mg protein) was applied to a CM52-cellulose column (1.9 x 5.0cm) equilibrated with 10mM acetate, pH 5.0, and unadsorbed protein was washed from the column with this buffer. One peak of nitrate reductase inactivating activity was eluted by 10mM acetate, pH 5.0, containing 50mM NaCl, and another was eluted by 10mM potassium phosphate, pH 8.0. Nitrate reductase inactivating activity was also present in the initial peak of unadsorbed protein due to overloading of the column. 4ml fractions were collected.

Inset: Nitrate reductase inactivating activity was assayed using a partially purified nitrate reductase sample containing 5mg protein/ml and with an initial activity of 0.246 μmol nitrite formed/mg protein hr^{-1}.


I
II
100
80
60
40
20
0
% NR remaining □
2·5
2·0
1·5
1·0
0·5
0
Absorbance at 280 nm ■
NaCl
Phosphate
0
40
80
120
160
200
240
280
300
360
400
440
ELUTION VOLUME ml

CHAPTER 2

Characterization of Nitrate Reductase Inactivator I

Azocasein-degrading properties

Since both proteolytic and non-proteolytic nitrate reductase inactivators have been reported (see Introduction), the ability of nitrate reductase inactivator I to degrade azocasein was studied as a means of determining whether it was a proteinase. The results of this work (Figure 12) showed that inactivator I was a proteinase and that the pH optimum for azocasein degradation was at pH 9.5.

Purity of nitrate reductase inactivator I preparations

Nitrate reductase inactivator I was analysed by polyacrylamide disc-gel electrophoresis both before and after the Sephadex G200 gel filtration step (Figure 13 and Table 5) as a means of assessing its purity. Gels of the sample eluted from CM-cellulose contained five distinct protein bands and a broad intensely stained region which appeared to contain at least three components. After gel filtration the sample contained six protein bands since the broad band at R_f values 0.704-0.817 had been resolved into four components and three other bands were missing (Figure 13). The Sephadex G200 gel filtration step brought about a 2-fold increase in the purity of the nitrate reductase inactivator I preparation (Table 4). Nitrate reductase inactivator I eluted from CM-cellulose will be referred to as partially purified nitrate reductase inactivator I and samples which were further purified by gel filtration will be referred to as purified nitrate reductase inactivator I.

It was of interest to determine which of the components in preparations of inactivator I (Figure 13) were responsible for nitrate reductase inactivation and azocasein degradation. Therefore polyacrylamide disc-gel electrophoresis was performed on purified and partially purified nitrate reductase inactivator I samples (4 gels per sample). After electrophoresis two gels per sample were stained for protein while the remaining gels were sliced into 5mm sections, eluted into 10mM potassium phosphate buffer, pH 7.0, at room temperature and assayed for azocasein degradation and nitrate reductase inactivating activity

Figure 12. Influence of pH on Azocasein-Degrading Activity of Nitrate Reductase Inactivator I

Azocasein-degrading activity of a nitrate reductase inactivator I sample (purified by Sephadex G200 gel filtration and containing 49µg protein/ml) was assayed in the pH range 5-10.5, using 0.1M acetate buffer (●), 0.1M potassium phosphate buffer (▲) or 0.1M glycine/NaOH buffer (■) as appropriate.

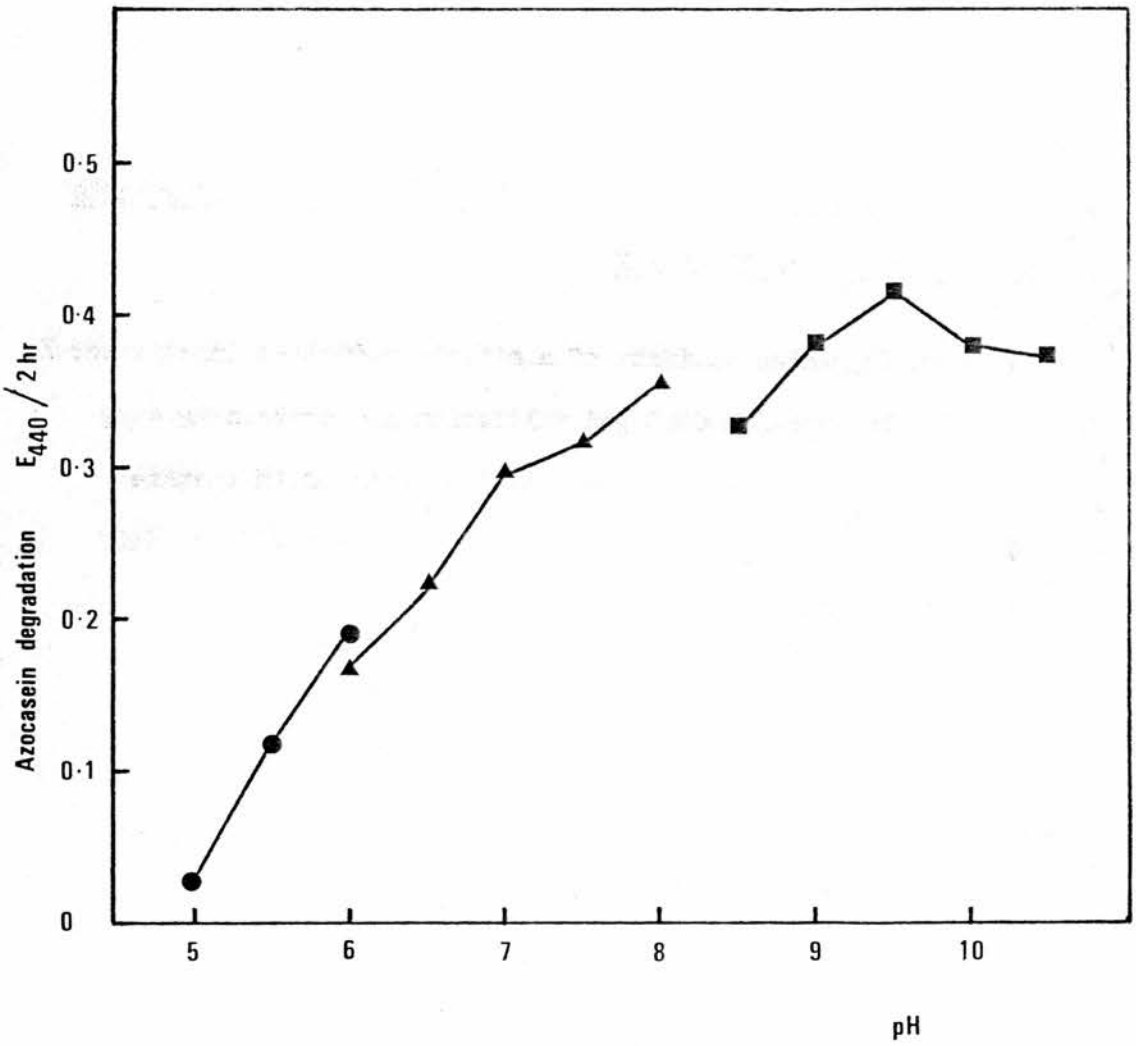
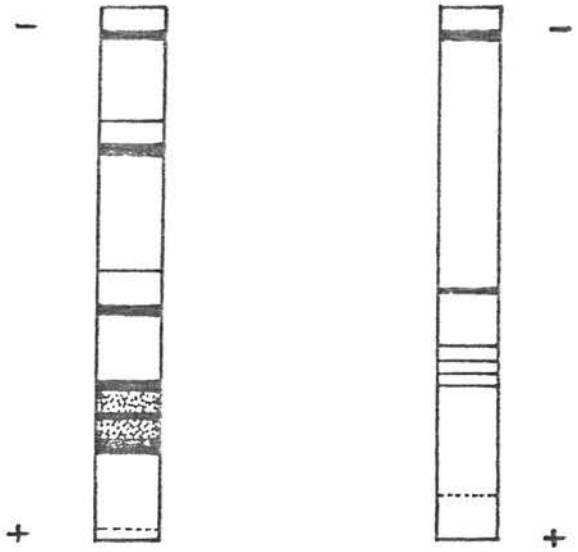

Azocasein degradation E_{440} / 2 hr
0·5
0·4
0·3
0·2
0·1
0
5
6
7
8
9
10
pH

Figure 13. Polyacrylamide Disc-Gel Electrophoretic Analysis of a Nitrate Reductase Inactivator I Preparation Before and After Sephadex G200 Gel Filtration

Samples of nitrate reductase inactivator I eluted from CM52-cellulose (A, 85μg protein/gel) or further purified by Sephadex G200 gel filtration (B, 27μg protein/gel) were subjected to polyacrylamide disc-gel electrophoresis and stained for protein. The dotted area in gel A represents an intensely stained region in which it was impossible to determine the exact number of protein bands present, while the broken line close to the bottom of each gel shows the distance of migration of the bromophenol blue tracking dye.

The R_f values of the protein bands are shown in Table 5.



A B

Table 5. R_f Values of Components Present in a Preparation of Nitrate Reductase Inactivator I Before and After Sephadex G200 Gel Filtration

Before gel filtration	After gel filtration
0.056	0.055
0.211	
0.268	
0.493	
0.563	0.562
	0.703
0.704–0.817	0.734
	0.758
	0.781

Nitrate reductase inactivator I was analysed by polyacrylamide disc-gel electrophoresis after elution from CM-cellulose and also after further purification by Sephadex G200 gel filtration (Figure 13). The R_f values of the protein bands observed are shown above.

(Figure 14). Most of the azocasein-degrading and nitrate reductase inactivating activity was present in the top two sections of the gels, and seemed to correspond to the position of a protein band with an R_f value of 0.055. There appeared to be a small amount of nitrate reductase inactivating activity in some other gel sections but this result was not reproducible and therefore was not considered to indicate the presence of other nitrate reductase inactivating proteins in the inactivator I preparations.

Inhibition of nitrate reductase inactivator I

The effect of several inhibitors on the nitrate reductase inactivating activity of inactivator I was studied as a means of classifying the proteinase (Table 6). It was completely inhibited by PMSF, an inhibitor of serine proteinases, but not by the chelating agents EDTA and 1,10-phenanthroline or by leupeptin, an inhibitor of those trypsin-like serine and cysteine proteinases which cleave peptide bonds at the carboxyl side of arginine and lysine residues. It was therefore concluded that nitrate reductase inactivator I was a serine proteinase which did not resemble trypsin.

The exogenous proteins BSA and casein have been reported to stabilize nitrate reductase in crude extracts from many species and some workers suggested that this effect was due to protection of nitrate reductase from proteolytic enzymes (Schrader et al., 1974; Purvis et al., 1976; Sherrard and Dalling, 1978; Brown et al., 1981). However, although 2% casein completely inhibited nitrate reductase inactivator I, 3% BSA was without effect. The stabilizing effect of casein on nitrate reductase activity in crude extracts seems likely to be due to inhibition of inactivator I while BSA might act on different proteinases.

Estimation of molecular weight of nitrate reductase inactivator I

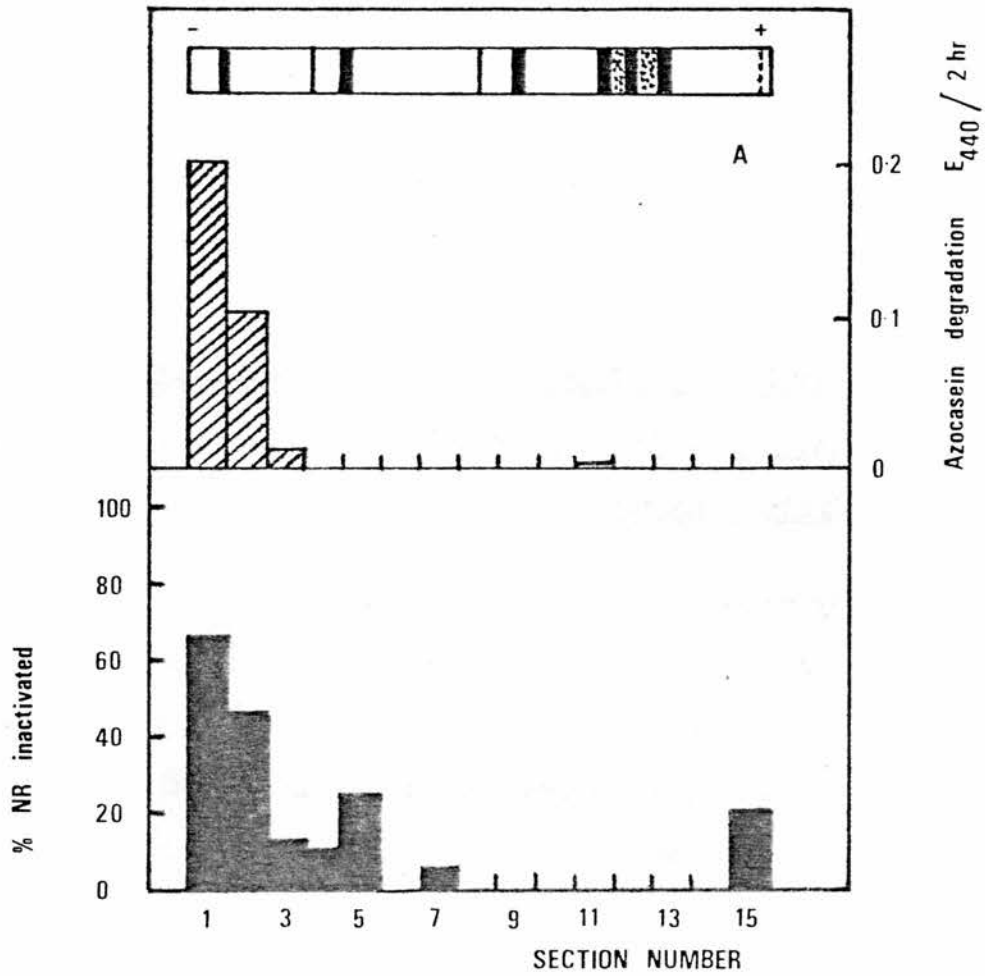
Molecular weight was estimated by the method of Siegel and Monty (1966) whereby both Stokes radius and sedimentation coefficient data are taken into account, thus compensating for errors caused by the shape of non-spherical

Figure 14. Identification of the Nitrate Reductase Inactivating Protein After Polyacrylamide Disc-Gel Electrophoresis of a Nitrate Reductase Inactivator I Preparation

Partially purified (A) and purified (B) nitrate reductase inactivator I samples (85μg and 27μg protein respectively) were subjected to polyacrylamide disc-gel electrophoresis then the gels were either stained for protein (inset) or cut into 5mm sections which were each eluted into 0.2ml 10mM potassium phosphate, pH 7.0, at room temperature. The resulting eluates were assayed for nitrate reductase inactivating and azocasein-degrading activities.

Nitrate reductase inactivating activity was measured by incubating 0.05ml 10mM potassium phosphate, pH 7.0, 0.05ml gel eluate and 0.1ml partially purified nitrate reductase (0.25mg protein) at 25°C for 45 minutes. This mixture was then used as the enzyme in a 15 minute NADH-nitrate reductase assay, and compared with a control containing 10mM potassium phosphate, pH 7.0, in place of eluate.

Azocasein-degrading activity was determined by incubating 0.3ml 10mg/ml azocasein, 0.65ml 0.1M glycine/NaOH buffer, pH 9.5, and 0.05ml gel eluate at 40°C for 2 hours, then the reaction was stopped and absorbance at 440nm measured as described in Methods.


-
+
A
0·2
0·1
0
Azocasein degradation E_{440} / 2 hr
100
80
60
40
20
0
% NR inactivated
1
3
5
7
9
11
13
15
SECTION NUMBER

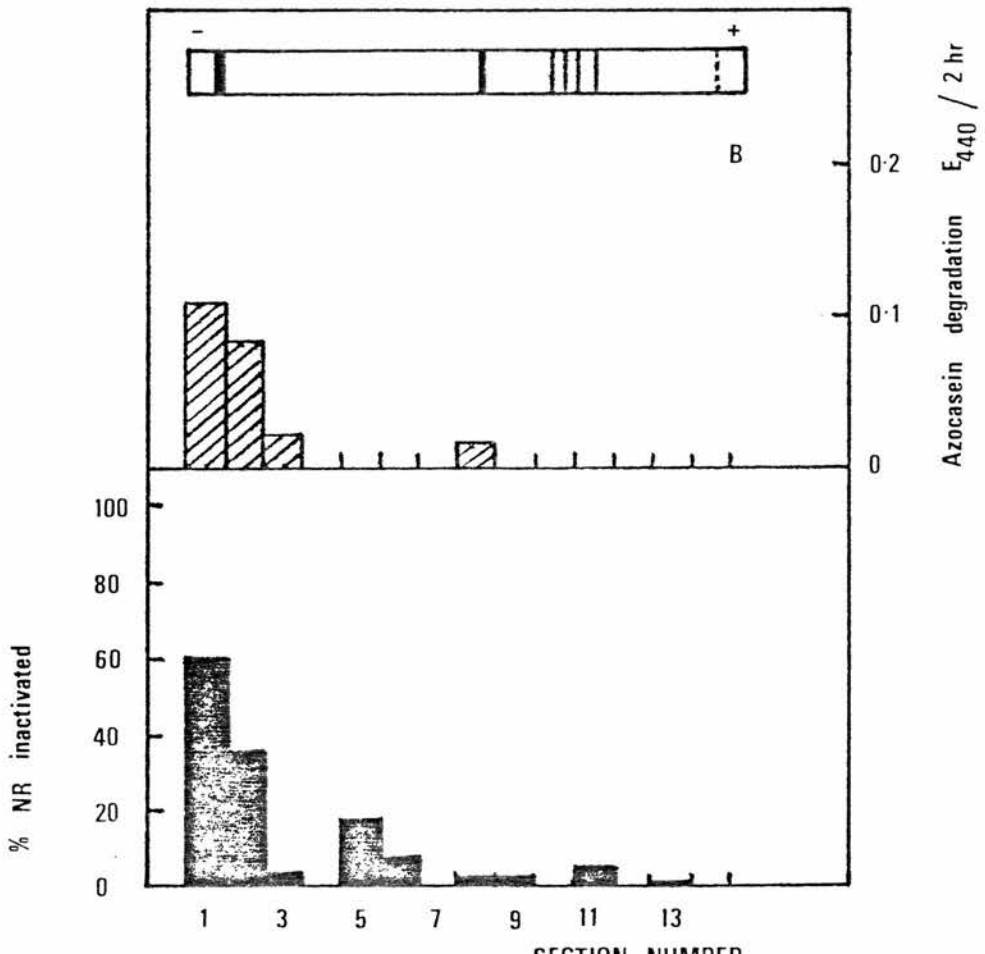

-
+
B
0·2
0·1
0
Azocasein degradation E_{440} / 2 hr
100
80
60
40
20
0
% NR inactivated
1
3
5
7
9
11
13
SECTION NUMBER

Table 6. Effect of Inhibitors on the Activity of Nitrate Reductase Inactivator I

Inhibitor	Concentration	% Inhibition
BSA	3%	0
Casein	1%	85
	2%	100
EDTA	10mM	4
Leupeptin	10µM	0
1,10-phenanthroline	1mM	0
PMSF	0.25mM	100

Nitrate reductase inactivating activity was assayed in the presence of each inhibitor, except leupeptin, using an incubation mixture containing 3.5ml 0.1M potassium phosphate buffer, pH 7.5, plus or minus inhibitor and 1ml partially purified nitrate reductase inactivator I (0.14mg protein). 0.5ml partially purified nitrate reductase (4mg protein) was added and the mixture was incubated at 25°C for 60 minutes then assayed for residual NADH-nitrate reductase activity. The percentage inhibition of nitrate reductase inactivator I was calculated by reference to controls containing 1ml 0.1M potassium phosphate buffer, pH 7.5, in place of nitrate reductase inactivator I.

25mM PMSF and 100mM 1,10-phenanthroline were prepared in isopropanol then 0.05ml PMSF, 1,10-phenanthroline or isopropanol was added to the relevant incubation mixtures in place of 0.05ml buffer.

Leupeptin was tested using duplicate tubes containing 0.25ml 0.1M potassium phosphate, pH 7.5, 0.1ml partially purified nitrate reductase inactivator I (0.025mg protein), 4µl leupeptin and 0.05ml partially purified nitrate reductase (0.4mg protein). After incubation for 90 minutes at 25°C residual NADH-nitrate reductase activity was assayed.

proteins.

The Stokes radius of nitrate reductase inactivator I was estimated by Sephadex G200 gel filtration (Figure 15) and values of 2.93 nm, 2.90nm and 2.87 nm were obtained.

Sucrose density gradient centrifugation was used to estimate the sedimentation coefficient of nitrate reductase inactivator I (Figure 16). Samples were diluted so that they contained less than 2% glycerol and would therefore float on the 2% sucrose present at the top of each gradient. In each of three experiments the sedimentation coefficient was estimated to be 4.7S.

Using a Stokes radius of 2.90nm and a sedimentation coefficient of 4.7S, the molecular weight of nitrate reductase inactivator I was calculated to be 56,200.

Figure 15. Estimation of the Stokes Radius of Nitrate Reductase Inactivator I by Sephadex G200 Gel Filtration

Purified nitrate reductase inactivator I (2.7mg protein) dissolved in 1.5ml 10mM potassium phosphate, pH 7.0, was mixed with catalase (C), alcohol dehydrogenase (A) and myoglobin (M), applied to a Sephadex G200 column (2.3 x 50cm) and eluted with 10mM potassium phosphate buffer, pH 7.0.

Nitrate reductase inactivating activity was measured by incubating 0.35ml column eluate with 0.05ml partially purified nitrate reductase (0.35mg protein) at 25°C for 90 minutes, then residual NADH-nitrate reductase activity was determined as described in Methods, and compared with a control containing 10mM potassium phosphate, pH 7.0, in place of column eluate.

Inset: Calibration plot of $K_d^{\frac{1}{3}}$ (calculated from the elution volume of each protein peak) against Stokes radius. The arrow denotes the position of nitrate reductase inactivator I.

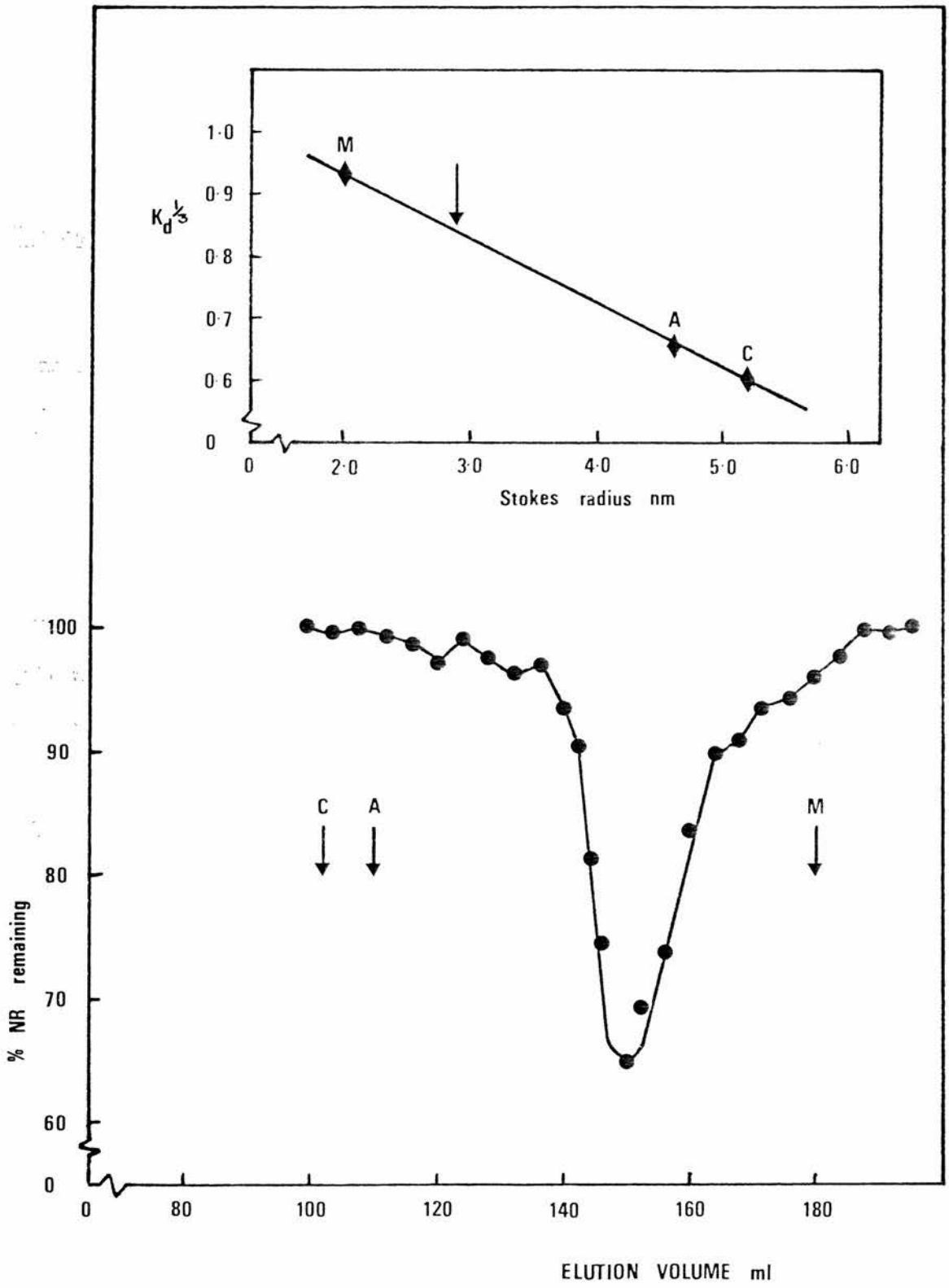
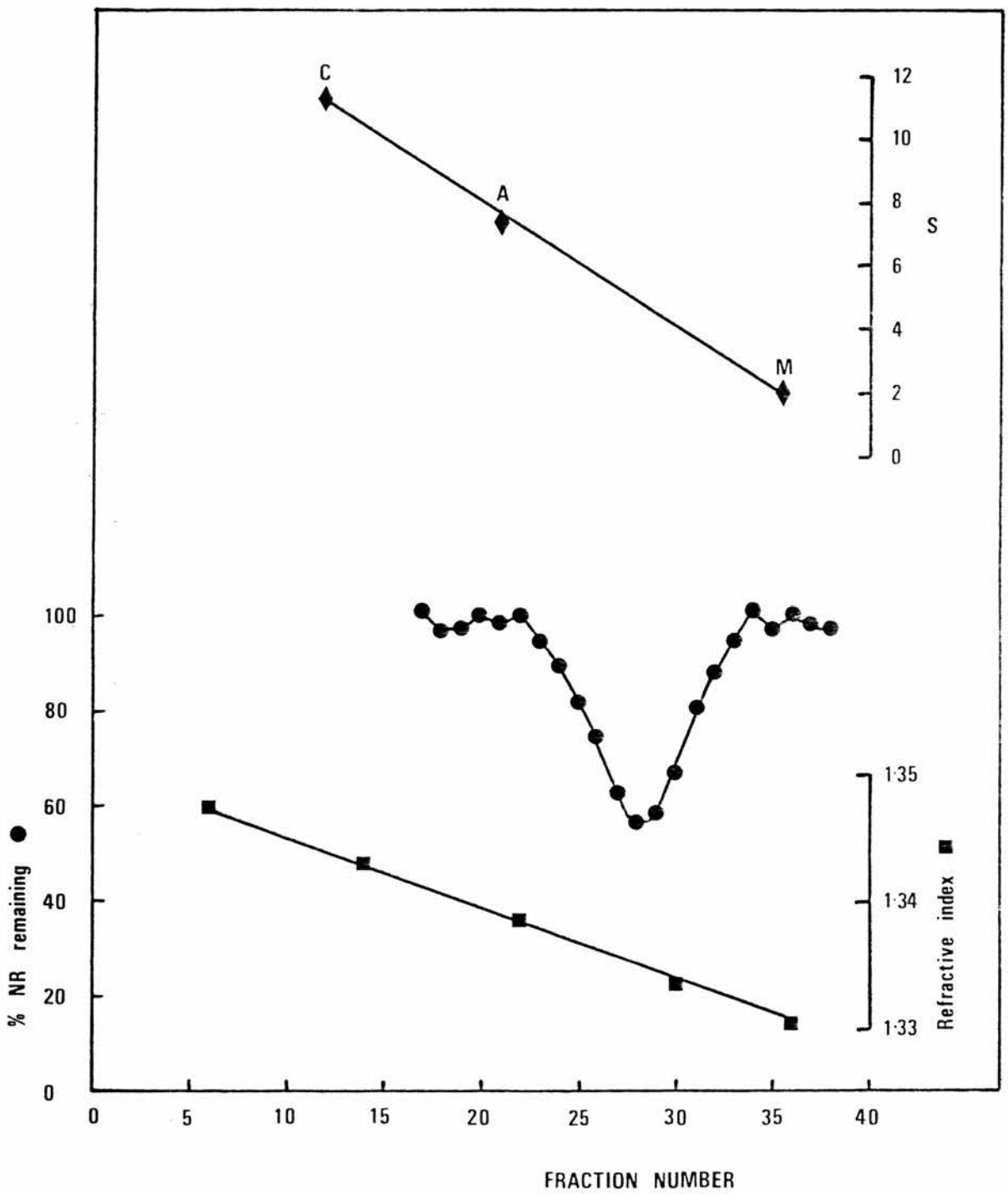

1·0
0·9
0·8
0·7
0·6
0
$K_d^{1/3}$
M
A
C
0
2·0
3·0
4·0
5·0
6·0
Stokes radius nm
100
90
80
70
60
0
% NR remaining
C
A
M
0
80
100
120
140
160
180
ELUTION VOLUME ml

Figure 16. Sucrose Density Gradient Centrifugation of Nitrate Reductase Inactivator I

Partially purified nitrate reductase inactivator I dissolved in 0.1M potassium phosphate, pH 7.5, was mixed with small amounts of solid catalase, alcohol dehydrogenase and myoglobin then 0.4ml aliquots (0.05mg inactivator I) were applied to 2-18% sucrose gradients. After centrifugation and fractionation of the gradients, nitrate reductase inactivating activity was measured by incubating 0.2ml gradient fraction, 0.15ml 0.1M potassium phosphate, pH 7.5, and 0.05ml partially purified nitrate reductase (0.15mg protein) at 25°C for 2 hours. This mixture was immediately assayed for residual NADH-nitrate reductase activity and compared with a control containing 0.1M potassium phosphate, pH 7.5, in place of gradient fraction.

C, A and M denote the respective positions of catalase, alcohol dehydrogenase and myoglobin after centrifugation.


C
A
M
12
10
8
6
4
2
0
S
100
80
60
40
20
0
% NR remaining ●
1·35
1·34
1·33
Refractive index ■
0
5
10
15
20
25
30
35
40
FRACTION NUMBER

CHAPTER 3

Inactivation of Nitrate Reductase by Nitrate Reductase Inactivator I

Time course of inactivation of the activities of nitrate reductase

The time course of nitrate reductase inactivation was studied as a means of determining which of the activities of the nitrate reductase complex was most sensitive to nitrate reductase inactivator I and therefore the probable site of attack by the inactivator.

From the results presented in Figure 17 it can be seen that NADH-nitrate reductase was inactivated most rapidly, closely followed by NADH-cytochrome c reductase activity, while MVH-nitrate reductase activity was inactivated at a slower rate. If nitrate reductase was exposed to inactivator I for a sufficiently long time, all of the activities of the nitrate reductase complex were eventually inactivated.

Analysis of inactivated nitrate reductase by sucrose density gradient centrifugation

After inactivation for 30 minutes, aliquots were removed from the incubation mixtures used in the time course experiment and analysed by sucrose density gradient centrifugation (Figure 18). The maize nitrate reductase profile (Figure 18a) was similar to that obtained previously for barley nitrate reductase (Small and Wray, 1979, 1980) in that it consisted of a large peak of NADH-cytochrome c reductase activity coincident with the peaks of NADH-nitrate reductase and MVH-nitrate reductase activity which therefore represented the nitrate reductase complex, and in addition there was a smaller peak of NADH-cytochrome c reductase activity sedimenting in the 3-4S region of the gradient. In 13 experiments, the maize nitrate reductase complex was found to have a sedimentation coefficient of 7.7 $\pm$ 0.1S, which is identical to the value obtained for barley nitrate reductase (Small and Wray, 1979, 1980).

The inactivated nitrate reductase sample (Figure 18b) contained very little NADH-nitrate reductase activity relative to that in the control (Figure 18a), and the NADH-cytochrome c reductase activity was also greatly reduced. There appeared to be a small peak of NADH-cytochrome c reductase activity sedimenting

Figure 17. Time Course of Inactivation of Nitrate Reductase by Nitrate Reductase Inactivator I

5ml purified nitrate reductase (20μg protein) was incubated at 25°C with 1ml of Buffer III (closed symbols) or with 1ml purified nitrate reductase inactivator I (17μg protein) dissolved in Buffer III (open symbols). At selected times 0.5ml aliquots were removed from the mixtures, added to 5μl 50mM PMSF, and immediately assayed for residual NADH-nitrate reductase (A), MVH-nitrate reductase (B) and NADH-cytochrome c reductase (C) activities.

When this data was plotted on semi-log graph paper, the half-lives of NADH-nitrate reductase, MVH-nitrate reductase and NADH-cytochrome c reductase activities were 7.5, 28.5 and 12 minutes respectively in the presence of inactivator I, compared with half-lives of 70, 149 and 153 minutes respectively in the control sample.

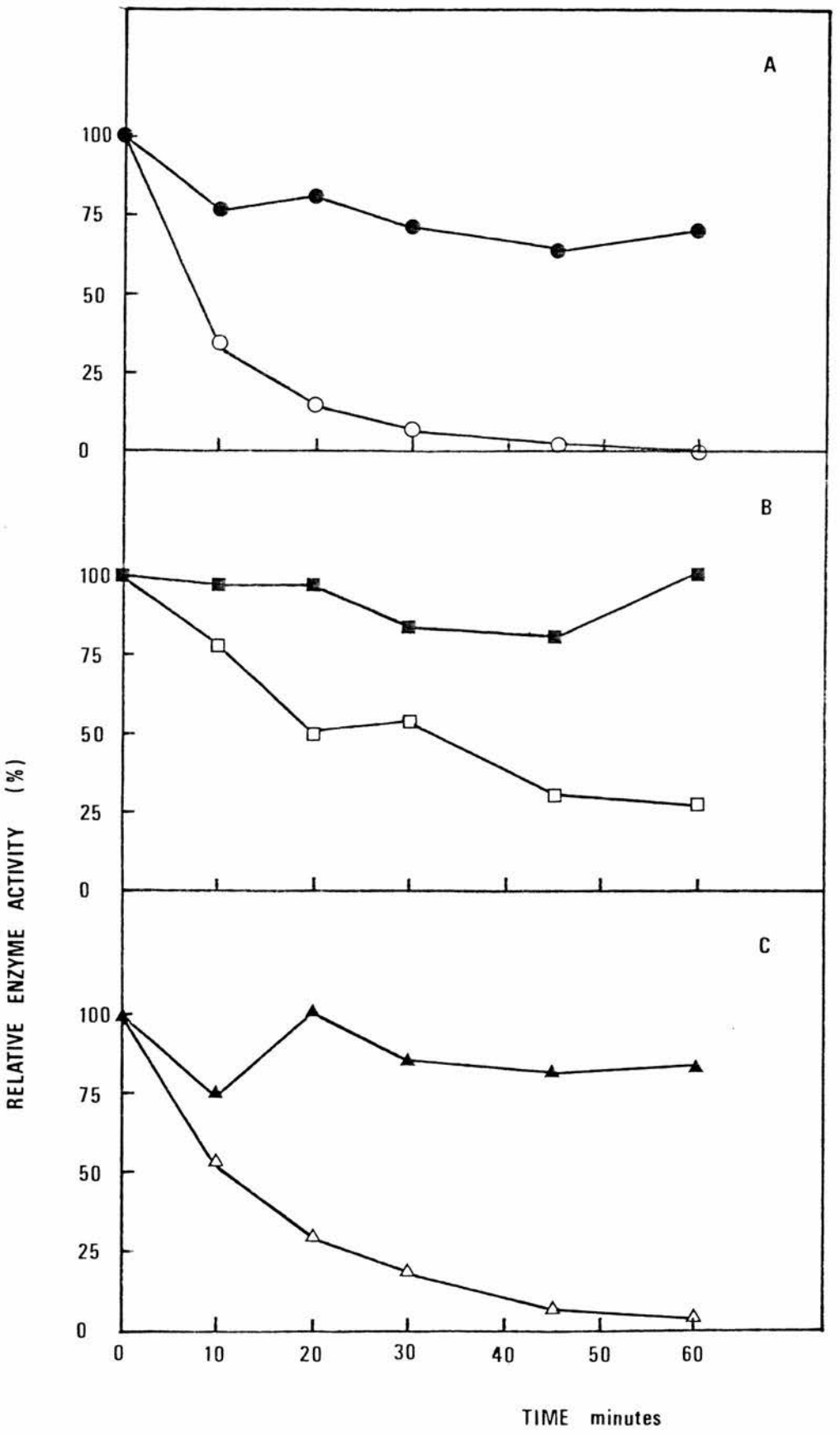
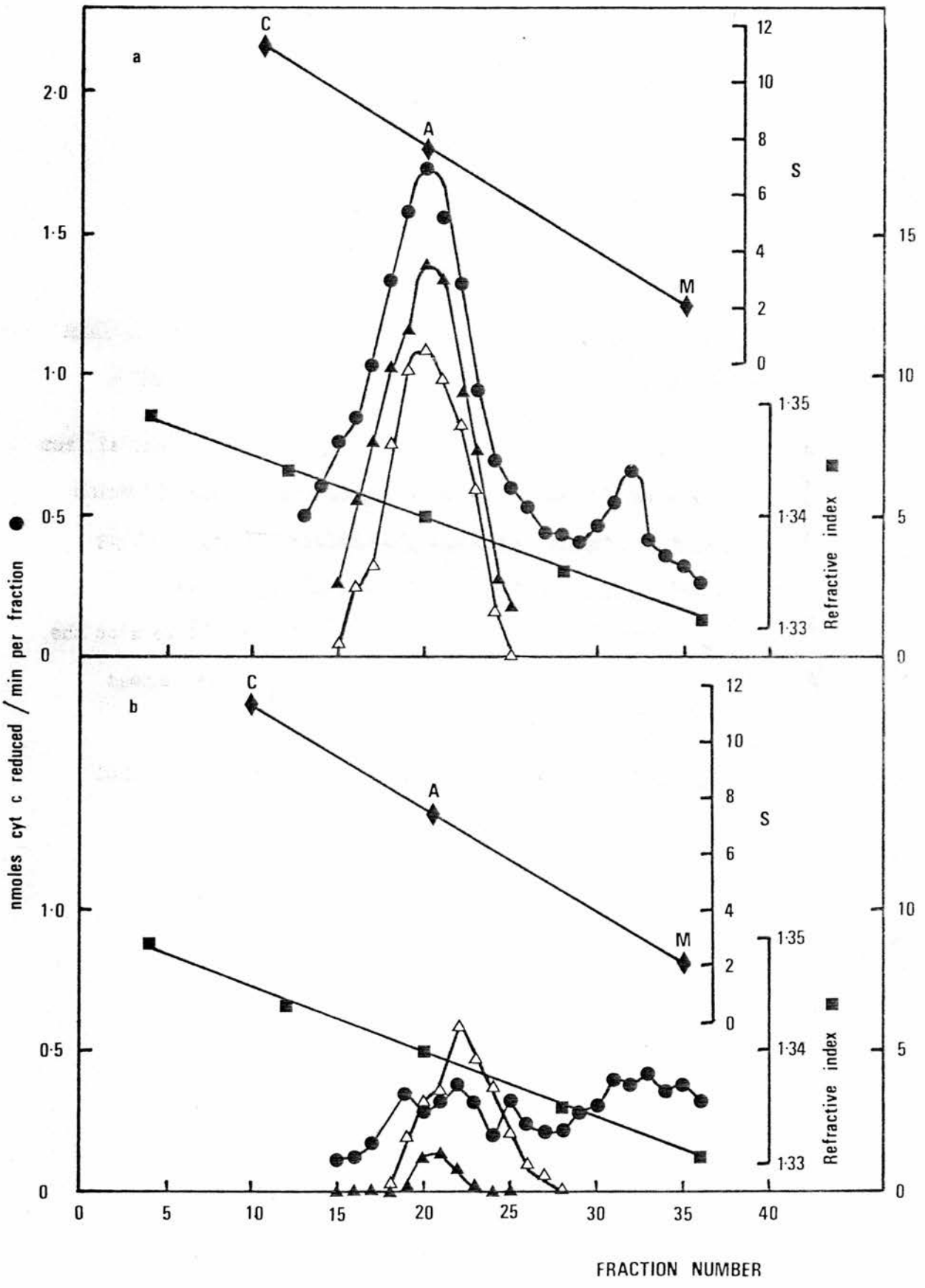

A
B
C
100
75
50
25
0
0
10
20
30
40
50
60
RELATIVE ENZYME ACTIVITY (%)
TIME minutes

Figure 18. Sucrose Density Gradient Analysis of Nitrate Reductase Before and After Inactivation by Nitrate Reductase Inactivator I

After incubation at 25°C for 30 minutes, an aliquot was removed from each of the samples used in the time course experiment (Figure 17) which contained 20µg purified nitrate reductase plus Buffer III (a), or 20µg purified nitrate reductase plus 17µg purified nitrate reductase inactivator I (b). PMSF was added (final concentration 0.5mM) to stop the inactivation reaction then the samples were analysed on 2-18% sucrose gradients.

C, A and M denote the respective positions of catalase, alcohol dehydrogenase and myoglobin after centrifugation.


a
b
C
A
M
S
Refractive index
nmoles cyt c reduced / min per fraction ●
nmoles NO_2^- formed / hr per fraction (NADH-NR ▲ ; MVH-NR △)
FRACTION NUMBER

at 6.8S, coincident with a peak of MVH-nitrate reductase activity, and this 6.8S species might represent a product of the limited proteolysis of nitrate reductase by nitrate reductase inactivator I.

The results of this experiment were difficult to interpret since so little activity remained in the inactivated sample. Therefore the experiment was repeated using a smaller amount of nitrate reductase inactivator I so that even after 90 minutes at 25°C, 17% of the initial NADH-nitrate reductase activity remained in the sample (Figures 19 and 20).

The control nitrate reductase profile (Figure 19) again consisted of a large peak of NADH-cytochrome c reductase activity coincident with the NADH-nitrate reductase and MVH-nitrate reductase activities at 7.7S, and a smaller peak of NADH-cytochrome c reductase activity in the 3S region. After inactivation for 30 minutes (Figure 20a), the MVH-nitrate reductase peak had an asymmetrical profile with greatest activity at about 7.2S but there was no evidence for the production of smaller MVH-nitrate reductase species in the 3-4S region at this stage, or after inactivation for a further 60 minutes (Figure 20b). Further evidence for the formation of a species only slightly smaller than the nitrate reductase complex was provided after inactivation for 90 minutes (Figure 20b) since the asymmetrical peak of NADH-nitrate reductase had greatest activity at a slightly lower S value (7.2S) than the normal nitrate reductase complex.

Figure 19. Sucrose Density Gradient Analysis of Nitrate Reductase

5ml purified nitrate reductase (20µg protein) was incubated with 1ml Buffer III at 25°C for 30 minutes then 60µl 50mM PMSF was added and the sample was analysed by sucrose density gradient centrifugation. This represents the control for the inactivated samples shown in Figure 20.

C, A and M denote the respective positions of catalase, alcohol dehydrogenase and myoglobin after centrifugation.

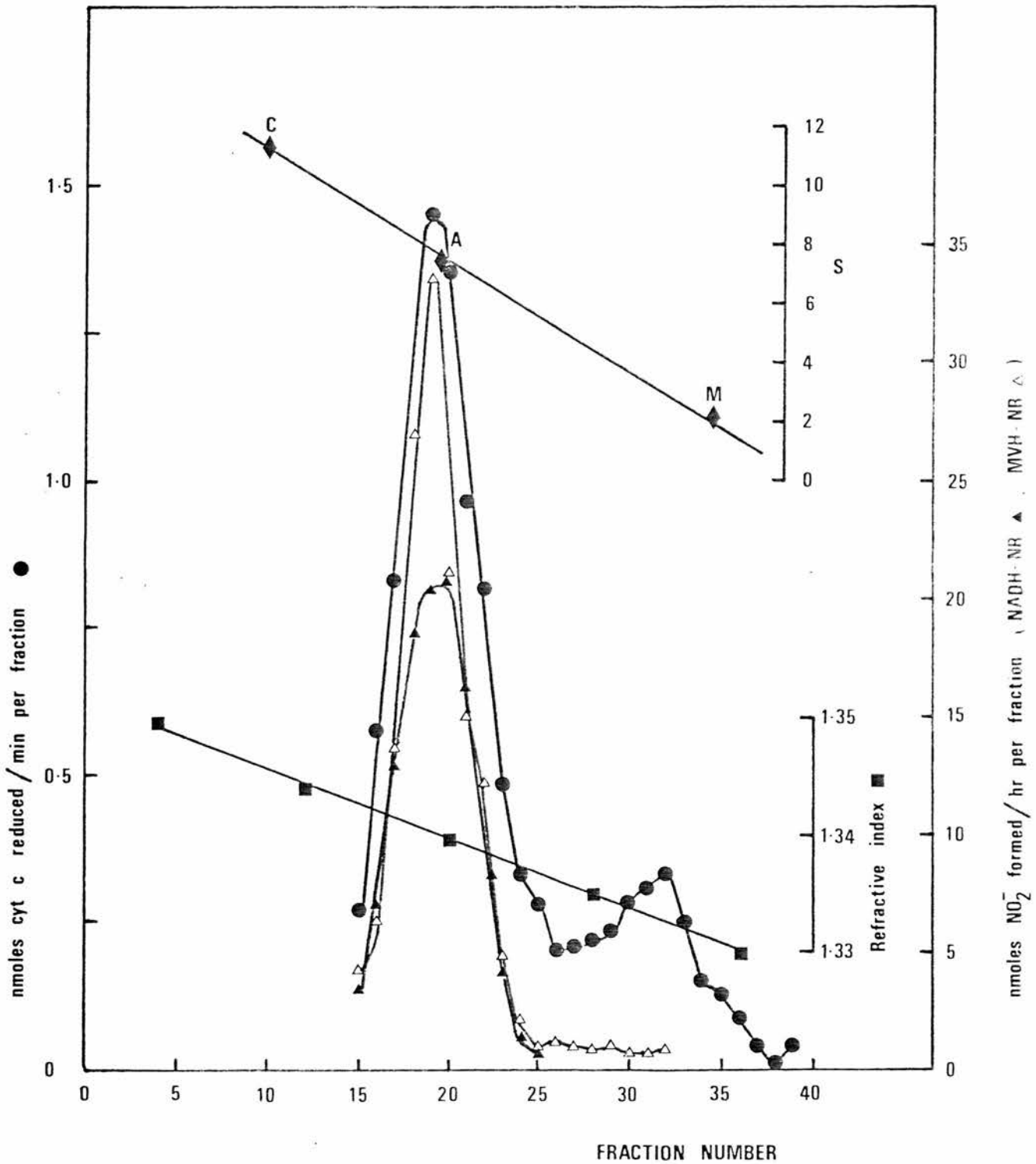
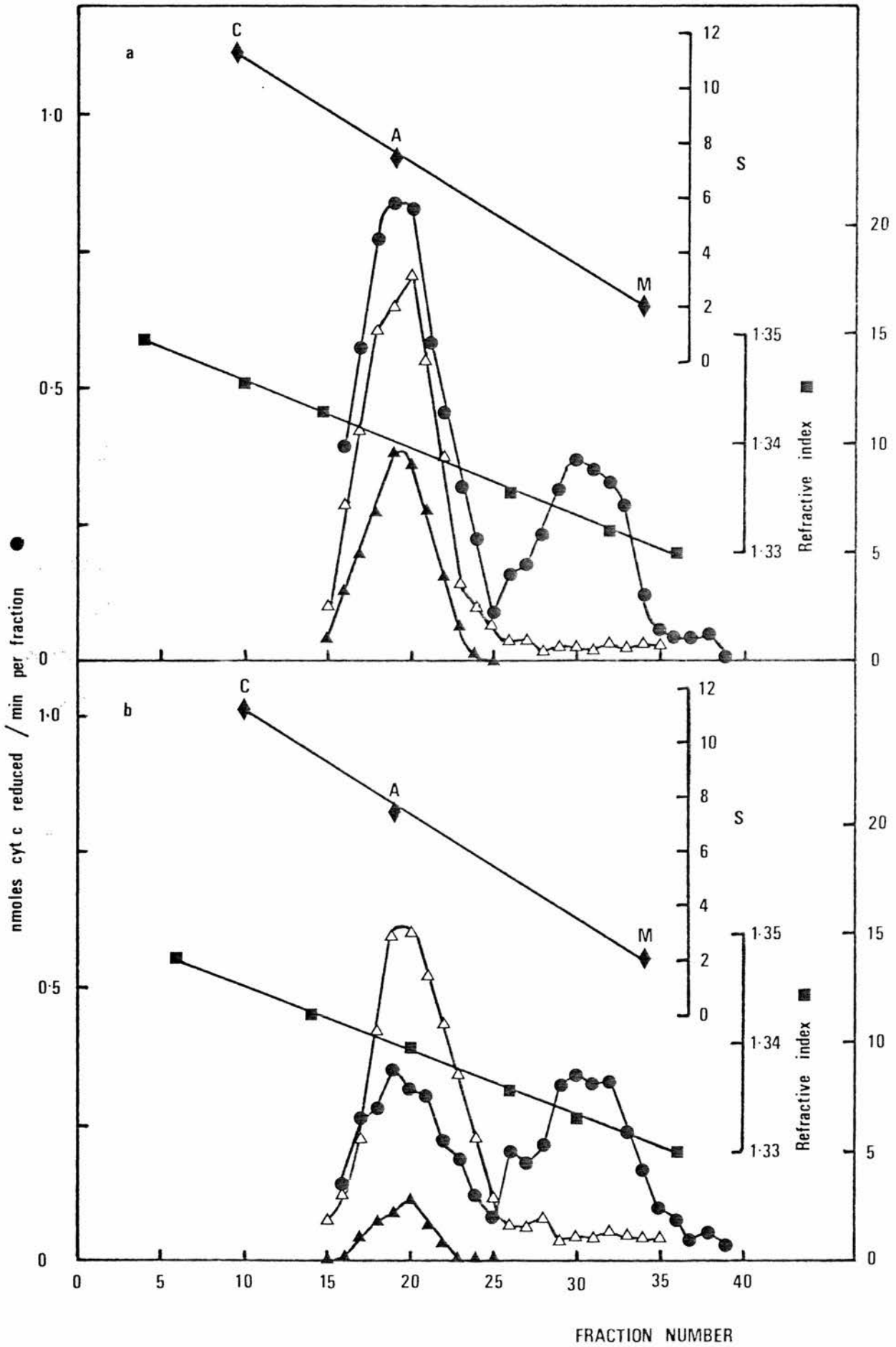

C
A
M
S
12
10
8
6
4
2
0
1·5
1·0
0·5
0
35
30
25
20
15
10
5
0
1·35
1·34
1·33
Refractive index
nmoles cyt c reduced / min per fraction
nmoles NO_2^- formed / hr per fraction (NADH-NR ▲, MVH-NR △)
0
5
10
15
20
25
30
35
40
FRACTION NUMBER

Figure 20. Sucrose Density Gradient Analysis of Nitrate Reductase After Partial Inactivation by Nitrate Reductase Inactivator I

5ml purified nitrate reductase (20µg protein) and 1ml purified nitrate reductase inactivator I (14µg protein) were incubated together at 25°C. After 30 minutes (a) and 90 minutes (b) 0.4ml aliquots were removed and added to 4µl 50mM PMSF to prevent further inactivation, then analysed by sucrose density gradient centrifugation.

The control sample is shown in Figure 19.

C, A and M denote the respective positions of the reference proteins catalase, alcohol dehydrogenase and myoglobin after centrifugation.


a
b
C
A
M
S
Refractive index
nmoles cyt c reduced / min per fraction ●
nmoles NO_2^- formed / hr per fraction (NADH-NR ▲ ; MVH-NR △)
FRACTION NUMBER

CHAPTER 4

Partial Characterization of Nitrate Reductase Inactivator II

Nitrate reductase inactivator II had not been reported prior to this work. It was separated from nitrate reductase inactivator I by the CM-cellulose chromatography step and the purification factor could not be estimated since it was impossible to assess the proportion of nitrate reductase inactivating activity in the crude extract which was due to nitrate reductase inactivator II (Table 4). In the experiments described below, nitrate reductase inactivator II eluted from CM-cellulose was used without further purification but it is likely that the preparation was very impure since its specific activity was low (Table 4). There was not time to assess the purity of nitrate reductase inactivator II by polyacrylamide disc-gel electrophoresis.

Azocasein-degrading properties

The effect of nitrate reductase inactivator II on azocasein was studied in the pH range 5-10.5 (azocasein was insoluble below pH 5.0). No significant azocasein degradation was observed (Figure 21) but this was insufficient evidence to discount the fact that inactivator II might be a proteinase. The low readings obtained in the pH range 8.5-10.5 may have been due to incomplete sedimentation of azocasein in the centrifugation step following addition of trichloroacetic acid, since any particles remaining in suspension could have dissolved in the 10M sodium hydroxide to give the observed absorbances.

Inhibition of nitrate reductase inactivator II

The effect of several inhibitors on the activity of nitrate reductase inactivator II was studied as a means of confirming that inactivator II differed from inactivator I. Nitrate reductase inactivator II was inhibited by the chelating agents EDTA and 1,10-phenanthroline, but not by BSA, casein or leupeptin, while PMSF caused very slight inhibition (Table 7). This was in marked contrast to the results obtained for inactivator I (Table 6) and demonstrated that the two inactivators were very different.

Figure 21. Effect of pH on the Ability of Nitrate Reductase Inactivator II to Degrade Azocasein

Nitrate reductase inactivator II (0.6mg protein/ml) was assayed for azocasein-degrading activity in the pH range 5-10.5, using 0.1M acetate buffer (●), 0.1M potassium phosphate buffer (▲) or 0.1M glycine/NaOH buffer (■) as appropriate.

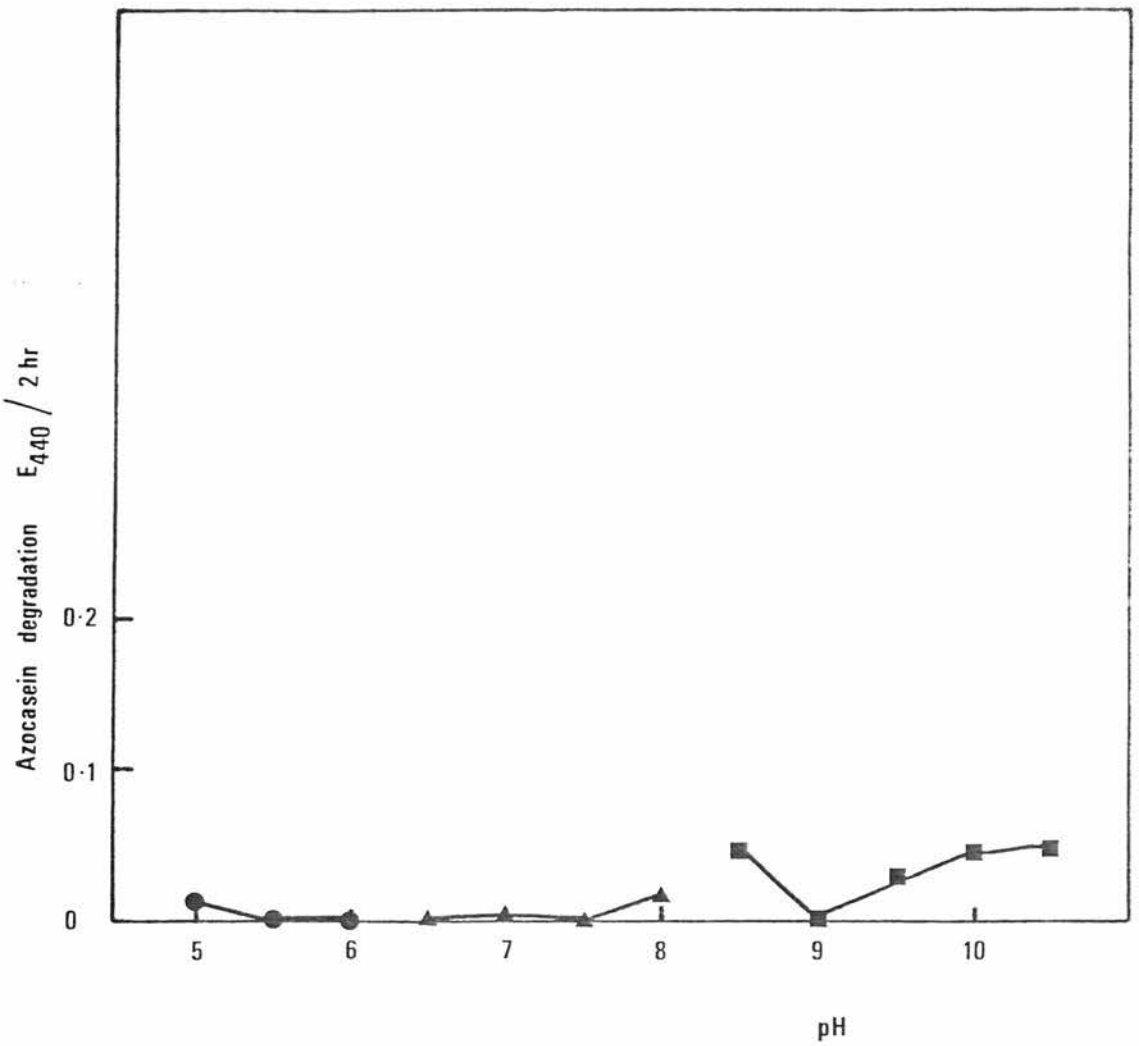

Azocasein degradation E_{440} / 2 hr
0·2
0·1
0
5
6
7
8
9
10
pH

Table 7. Effect of Inhibitors on the Activity of Nitrate Reductase Inactivator II

Inhibitor	Concentration	% Inhibition
BSA	3%	0
Casein	2%	0
EDTA	10mM	60
Leupeptin	10μM	0
1,10-phenanthroline	1mM	67
PMSF	0.25mM	13

Nitrate reductase inactivating activity was measured in the presence and absence of each inhibitor by the method described in Table 6, using nitrate reductase inactivator II containing 0.76mg protein/ml and partially purified nitrate reductase containing 8mg protein/ml.

Sedimentation coefficient of nitrate reductase inactivator II

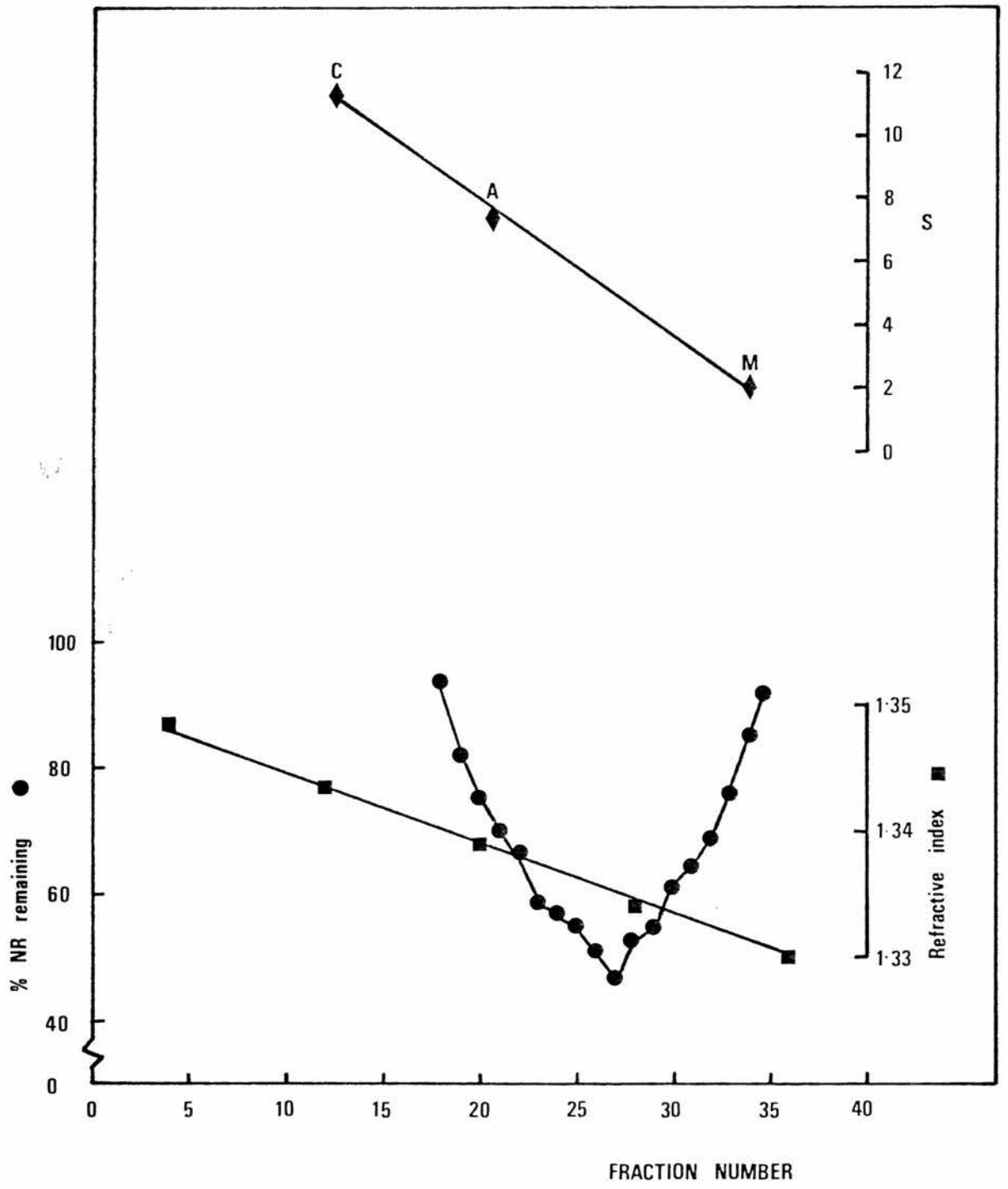
Sucrose density gradient analysis of nitrate reductase inactivator II resulted in the detection of a single peak of nitrate reductase inactivating activity (Figure 22) which in three experiments was found to have a sedimentation coefficient of 4.6S, 4.7S or 5.2S, giving an average value of 4.8 S. The peak of activity was rather broad, although the reference proteins produced sharp peaks, and this may indicate that the preparation contained inactivator molecules which were heterogeneous with respect to size.

It was not possible to estimate the molecular weight of nitrate reductase inactivator II since there was insufficient time to measure the Stokes radius.

Figure 22. Sucrose Density Gradient Analysis of Nitrate Reductase Inactivator II

Glycerol was removed from a nitrate reductase inactivator II sample by 0-70% ammonium sulphate fractionation then precipitated protein was redissolved in 0.1M potassium phosphate buffer, pH 7.5, and 0.4ml aliquots (0.9mg protein) were analysed by sucrose density gradient centrifugation. After fractionation of the gradients, nitrate reductase inactivating activity was assayed by the method described in Figure 16.

C, A and M denote the respective positions of catalase, alcohol dehydrogenase and myoglobin after centrifugation.


C
A
M
12
10
8
6
4
2
0
S
100
80
60
40
0
% NR remaining ●
1·35
1·34
1·33
Refractive index ■
0
5
10
15
20
25
30
35
40
FRACTION NUMBER

CHAPTER 5

Inactivation of Nitrate Reductase by Nitrate Reductase Inactivator II

Time course of inactivation of nitrate reductase by nitrate reductase inactivator II

In the presence of nitrate reductase inactivator II, NADH-nitrate reductase was inactivated rapidly, closely followed by NADH-cytochrome c reductase activity (Figure 23). In contrast MVH-nitrate reductase activity appeared to be unaffected by nitrate reductase inactivator II, and a similar result was also obtained in three other experiments.

Sucrose density gradient analysis of inactivated nitrate reductase

Purified nitrate reductase was analysed by sucrose density gradient centrifugation both before and after inactivation by nitrate reductase inactivator II (Figures 24 and 25) in an attempt to determine whether inactivator II acted in a different way to inactivator I. The control nitrate reductase profile (Figure 24) was similar to those obtained previously (Figures 18 and 19) in that it consisted of a large peak of NADH-cytochrome c reductase activity coincident with peaks of MVH-nitrate reductase and NADH-nitrate reductase at 7.7S, and a much smaller peak of NADH-cytochrome c reductase activity in the 3-4S region of the gradient.

Inactivation for 30 minutes (Figure 25a) resulted in a significant decrease in all of the activities of the 7.7S nitrate reductase complex, but there was no evidence to suggest the formation of smaller enzymically-active species, although after inactivation for a further 60 minutes (Figure 25b) the MVH-nitrate reductase peak was slightly asymmetrical with a shoulder of activity at 6.8S. The lowest MVH-nitrate reductase activity in this experiment was recorded in the sample inactivated for 30 minutes (Figure 25a) but this was due to the fact that the percentage of activity lost during centrifugation was not the same in each gradient, and therefore the data could not be used to quantitate nitrate reductase inactivation.

When this experiment was repeated, the control nitrate reductase profile (Figure 26) contained a larger amount of NADH-cytochrome c reductase activity in

Figure 23. Time Course of Inactivation of Nitrate Reductase by Nitrate Reductase Inactivator II

5ml purified nitrate reductase (15μg protein) was incubated at 25°C with 1ml Buffer III (closed symbols) or 1ml nitrate reductase inactivator II (120μg protein) dissolved in Buffer III (open symbols). At the times indicated 0.5ml aliquots were withdrawn, added to 5μl 200mM 1,10-phenanthroline to inhibit further inactivation, then immediately assayed for residual NADH-nitrate reductase (A), MVH-nitrate reductase (B) and NADH-cytochrome c reductase (C) activities.

When this data was plotted on semi-log graph paper, the half-lives of NADH-nitrate reductase, MVH-nitrate reductase and NADH-cytochrome c reductase activities were 17, 119 and 24.5 minutes respectively in the presence of nitrate reductase inactivator II, compared with half-lives of 94, 118 and 70 minutes respectively in the control sample.

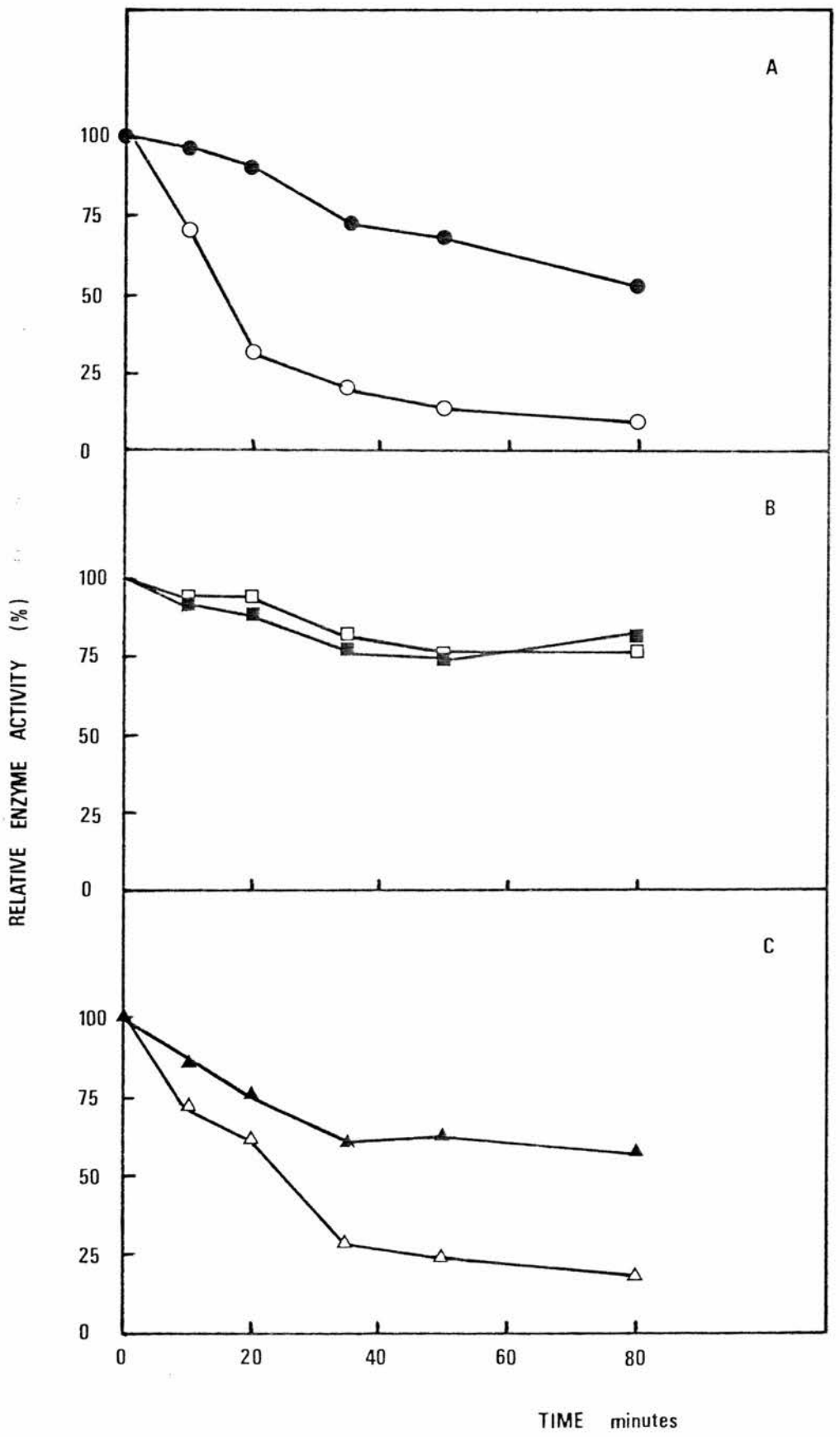

A
B
C
100
75
50
25
0
0
20
40
60
80
RELATIVE ENZYME ACTIVITY (%)
TIME minutes

Figure 24. Sucrose Density Gradient Analysis of Nitrate Reductase

7ml purified nitrate reductase (25µg protein) was incubated with 1ml Buffer III for 30 minutes, at 25°C, then 1,10-phenanthroline was added (to a final concentration of 2mM) and the sample was analysed by sucrose density gradient centrifugation. This represents the control for the inactivated samples shown in Figure 25.

C, A and M denote the respective positions of catalase, alcohol dehydrogenase and myoglobin after centrifugation.

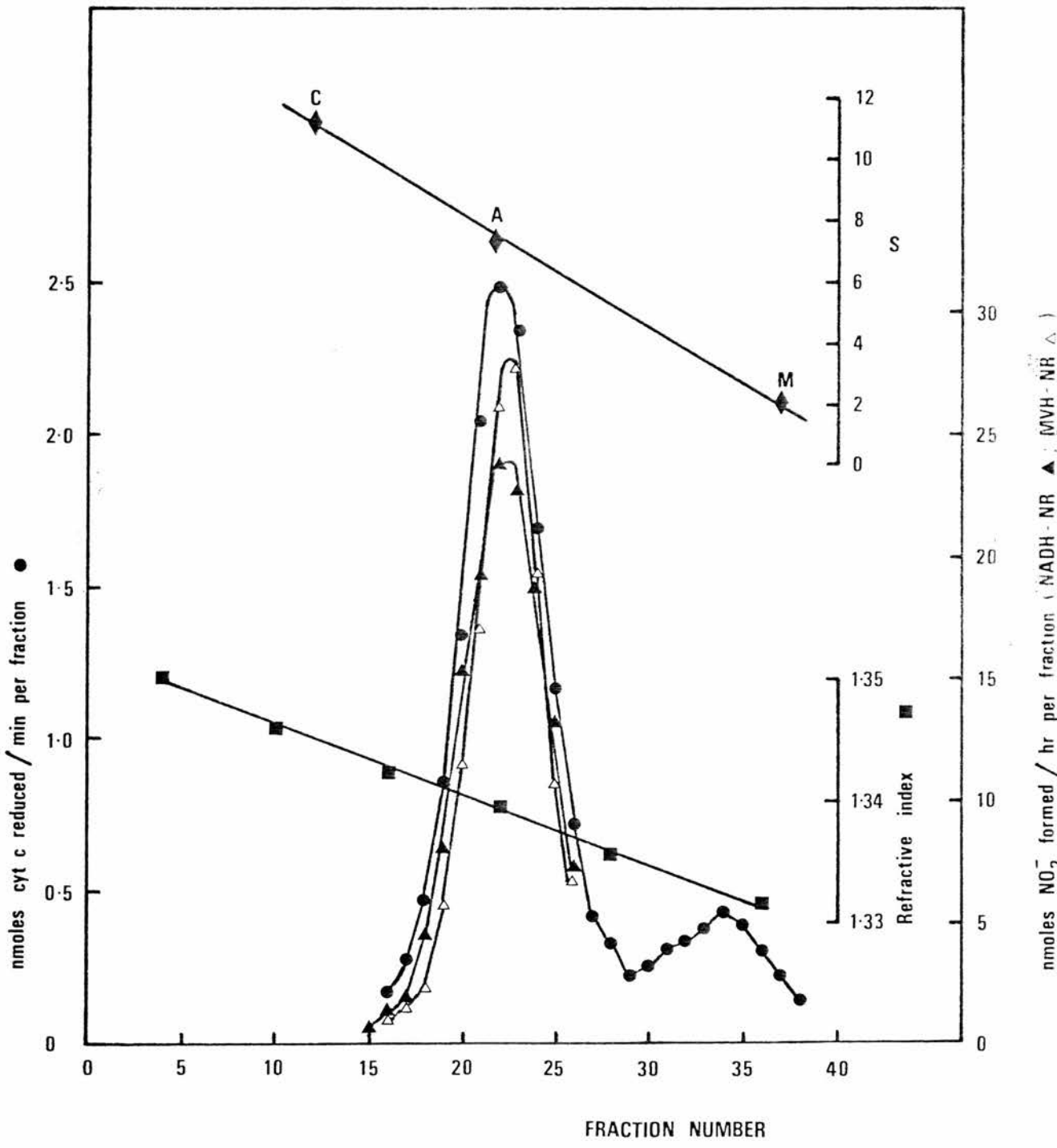

C
A
M
S
12
10
8
6
4
2
0
2·5
2·0
1·5
1·0
0·5
0
30
25
20
15
10
5
0
1·35
1·34
1·33
Refractive index
0
5
10
15
20
25
30
35
40
FRACTION NUMBER
nmoles cyt c reduced / min per fraction ●
nmoles NO_2^- formed / hr per fraction (NADH-NR ▲; MVH-NR △)

Figure 25. Sucrose Density Gradient Analysis of Nitrate Reductase After Partial Inactivation by Nitrate Reductase Inactivator II

7ml purified nitrate reductase (25µg protein) and 1.5ml nitrate reductase inactivator II (180µg protein) dissolved in Buffer III were incubated together at 25°C. After 30 minutes (a) and 90 minutes (b) 0.4ml aliquots were removed from the mixture, added to 4µl 200mM 1,10-phenanthroline to inhibit further inactivation, then subjected to analysis by sucrose density gradient centrifugation. The control sample is shown in Figure 24.

C, A and M denote the respective positions of the reference proteins catalase, alcohol dehydrogenase and myoglobin after centrifugation.

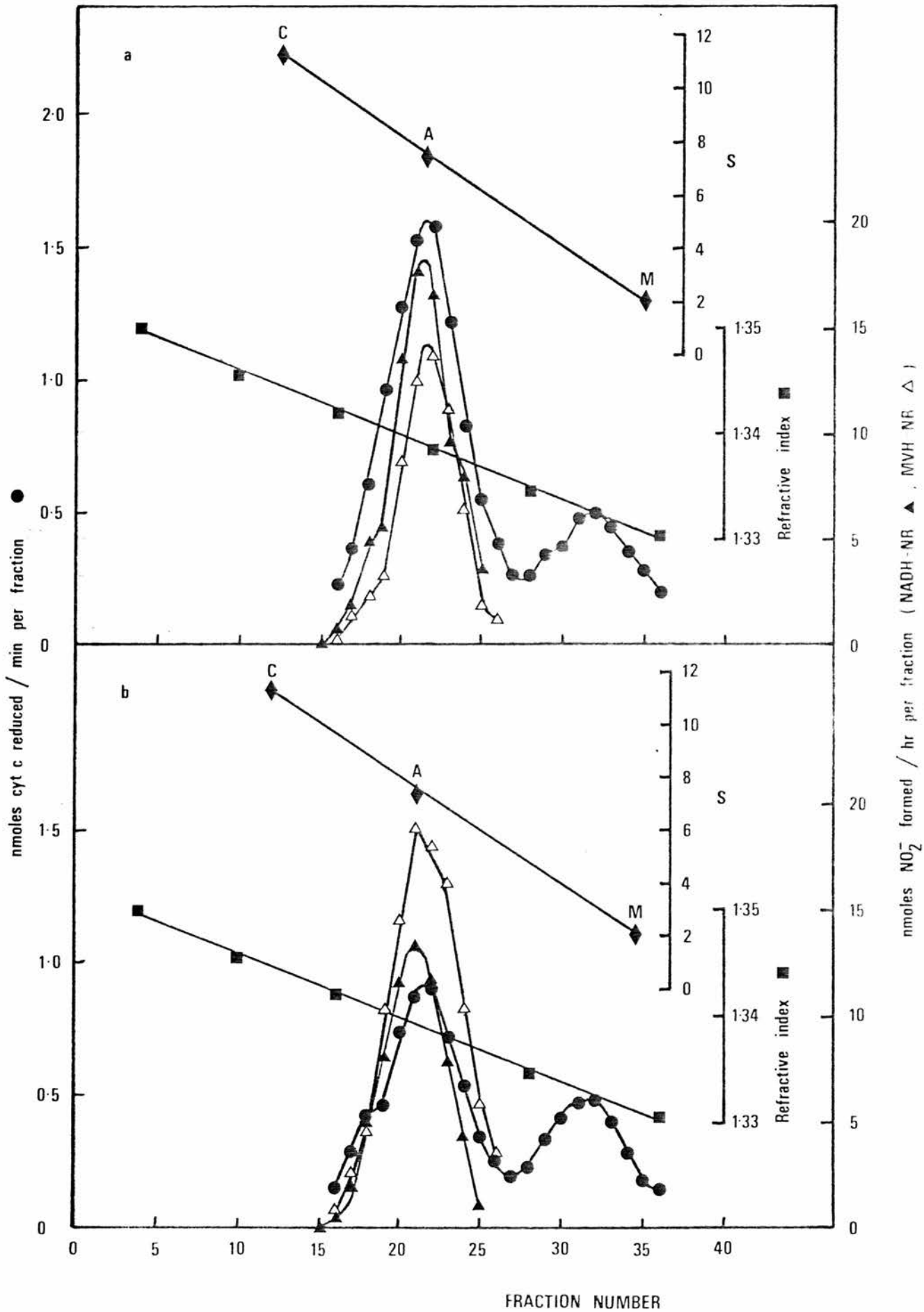
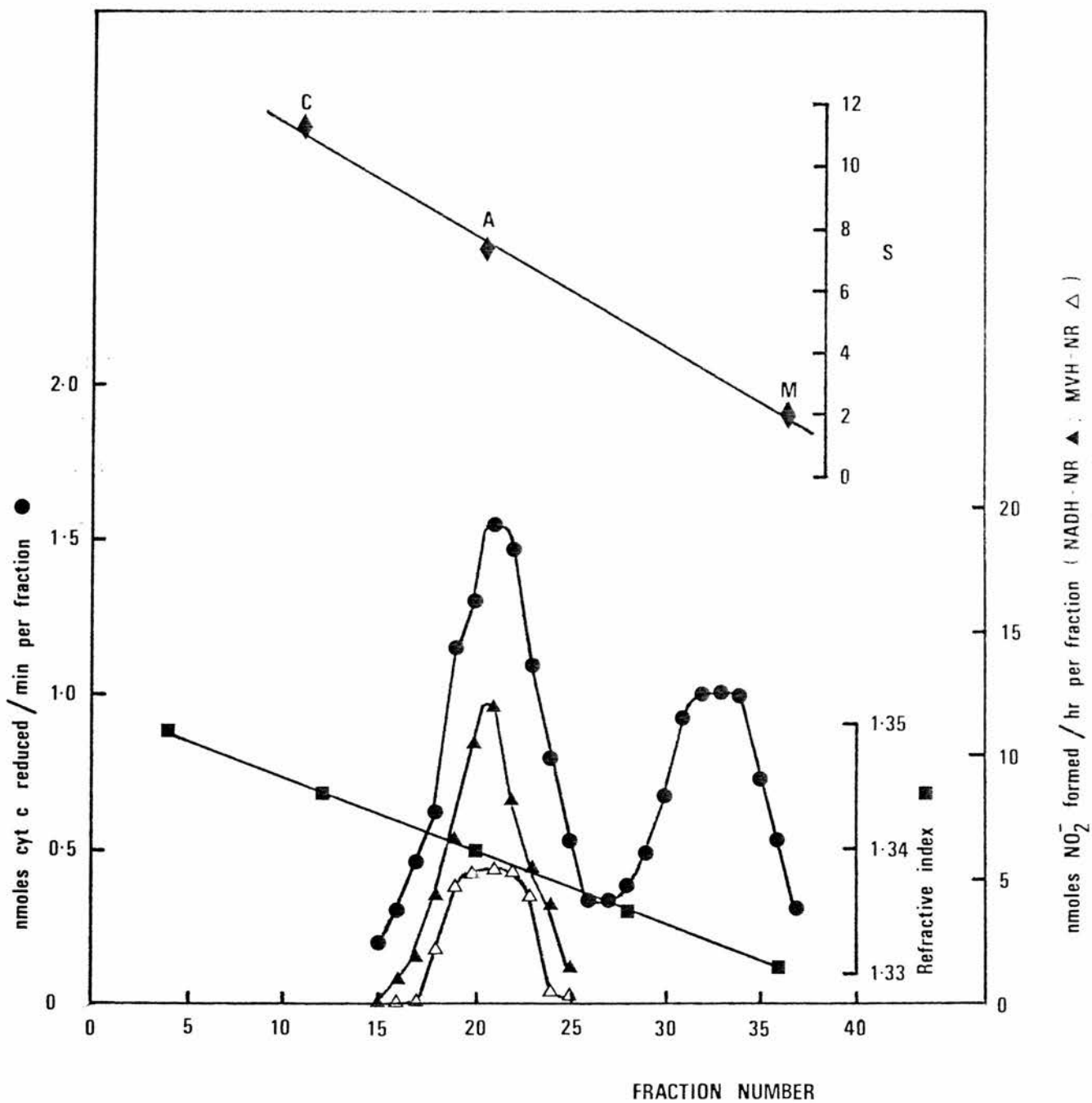

a
b
C
A
M
S
Refractive index
nmoles cyt c reduced / min per fraction ●
nmoles NO_2^- formed / hr per fraction (NADH-NR ▲, MVH NR △)
FRACTION NUMBER

Figure 26. Sucrose Density Gradient Analysis of Nitrate Reductase

5ml purified nitrate reductase (15µg protein) was incubated with 1ml Buffer III for 30 minutes at 25°C, then 60µl 200mM 1,10-phenanthroline was added and the sample was analysed by sucrose density gradient centrifugation. This represents the control for the inactivated samples shown in Figure 27.

C, A and M denote the respective positions of catalase, alcohol dehydrogenase and myoglobin after centrifugation.


C
A
M
12
10
8
6
4
2
0
S
2·0
1·5
1·0
0·5
0
nmoles cyt c reduced / min per fraction ●
1·35
1·34
1·33
Refractive index ■
20
15
10
5
0
nmoles NO_2^- formed / hr per fraction (NADH-NR ▲ ; MVH-NR △)
0
5
10
15
20
25
30
35
40
FRACTION NUMBER

the 3-4S region of the gradient than had been observed previously, and this may have been due to partial breakdown of nitrate reductase during the purification procedure. All activities of the control sample were low relative to those of the inactivated samples (Figure 27a, b) and this was again due to the percentage of activity lost during centrifugation being different in each gradient. In both inactivated samples (Figure 27) there was a peak of MVH-nitrate reductase activity at 6.8S in addition to a peak at 7.7S, but it was not possible to say whether the 6.8S species was also present in the control and therefore present in the sample at the start of the experiment or whether it was formed by the action of inactivator II on nitrate reductase.

It is difficult to draw any conclusion from the results given above due to the problems caused by the differential activity loss during centrifugation. It is interesting to note however that NADH-cytochrome c reductase activity was much more stable during the centrifugation step than were NADH-nitrate reductase and MVH-nitrate reductase activities, so that 60-70% of the applied NADH-cytochrome c reductase activity remained after centrifugation while there was usually only 20-30% of NADH-nitrate reductase and MVH-nitrate reductase activity remaining. This may have been due to a conformational change taking place in the nitrate-reducing region of the nitrate reductase complex during centrifugation of both the control and inactivated samples.

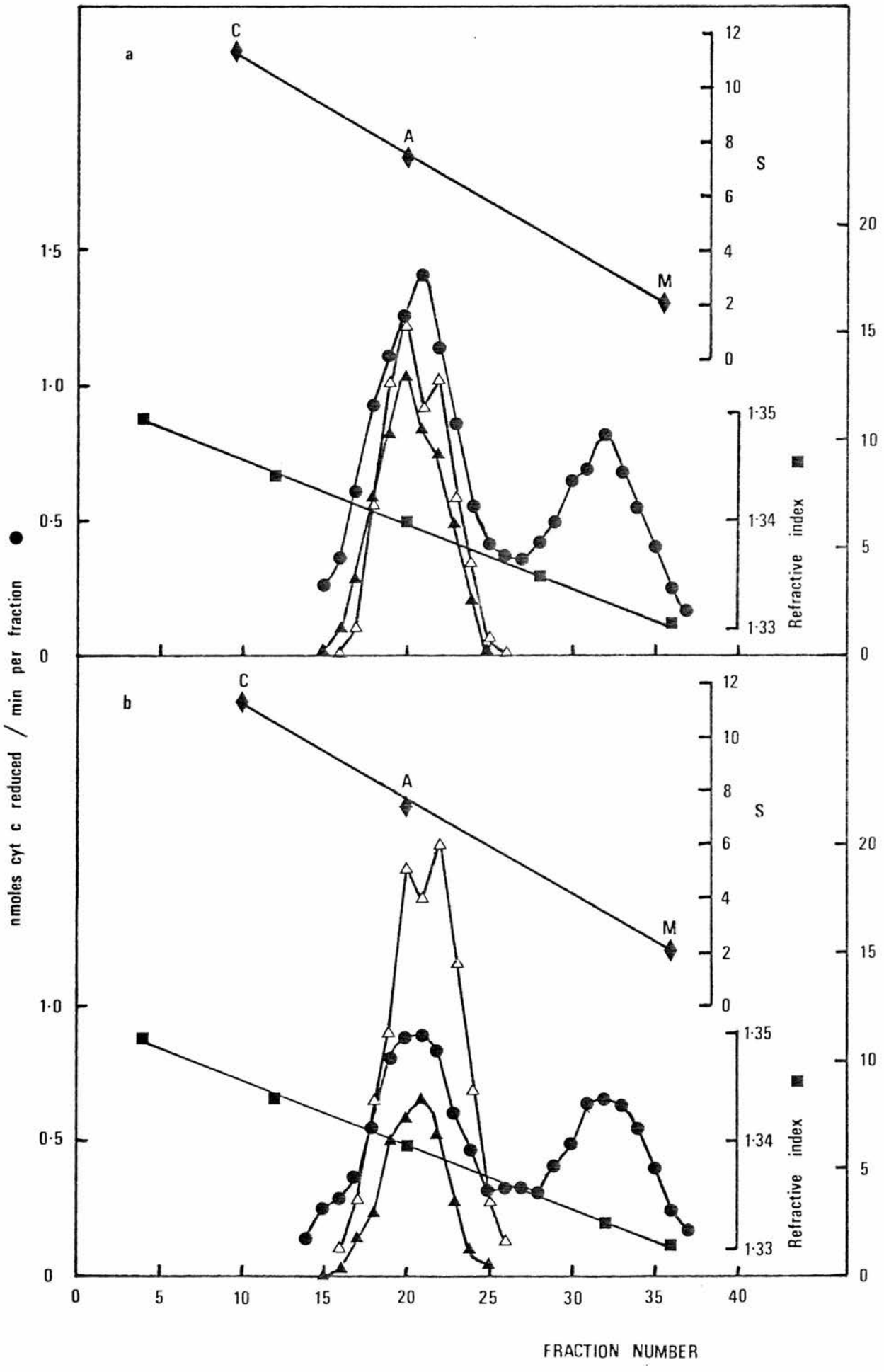
Despite this, it would seem that nitrate reductase inactivator II inactivates nitrate reductase without causing production of smaller enzymically-active species, and since MVH-nitrate reductase activity is virtually unaffected, the inactivator possibly acts only on the NADH-oxidising region of the nitrate reductase complex.

Figure 27. Sucrose Density Gradient Analysis of Nitrate Reductase After Exposure to Nitrate Reductase Inactivator II

5ml purified nitrate reductase (15µg protein) was incubated with 1ml nitrate reductase inactivator II (120µg protein) at 25°C. After 30 minutes (a) and 90 minutes (b), 0.4ml aliquots were removed, mixed with 4µl 200mM 1,10-phenanthroline and analysed by sucrose density gradient centrifugation.

The control sample is shown in Figure 26.

C, A and M denote the positions of catalase, alcohol dehydrogenase and myoglobin after centrifugation.


a
b
C
A
M
S
Refractive index
nmoles cyt c reduced / min per fraction ●
nmoles NO_2^- formed / hr per fraction (NADH-NR ▲, MVH-NR △)
FRACTION NUMBER

CHAPTER 6

Barley Nitrate Reductase Inactivator

Age-dependent breakdown of nitrate reductase into smaller NADH-cytochrome c reductase species in barley leaf extracts (Brown et al., 1981) was a major interest in this laboratory, and since this breakdown was prevented by 3% BSA and retarded by PMSF it had been suggested that the phenomenon was due to proteolytic degradation of the nitrate reductase complex. The following brief study was undertaken to determine whether nitrate reductase inactivators were present in barley seedlings and whether these inactivators were responsible for degradation of barley nitrate reductase to yield smaller NADH-cytochrome c reductase species.

Isolation of a nitrate reductase inactivator from barley roots

Nitrate reductase inactivating activity from 5 day old barley roots was purified by the method previously used for maize nitrate reductase inactivators. Ion-exchange chromatography on CM-cellulose (Figure 28) revealed that the barley preparation contained fewer components than a similar maize root preparation (Figure 9) since two protein peaks which were eluted from maize samples by 10mM acetate buffer, pH5.0, containing 50mM NaCl, were completely absent from barley preparations. Only one peak of barley nitrate reductase inactivating activity was detected and this was eluted by 10mM potassium phosphate buffer, pH 8.0.

Purification of barley nitrate reductase inactivator from leaf tissue

Having demonstrated the existence of a nitrate reductase inactivator in barley roots, the next step was to find out whether an inactivator could also be detected in leaf tissue. Since breakdown of barley nitrate reductase was greatest in older tissue (Brown et al., 1981) an attempt was made to purify the inactivator from 7 day old barley leaves. The extract was treated in the same manner as the root extract described above, and on elution from CM-cellulose (Figure 29) one peak of nitrate reductase inactivating activity was found, eluted

Figure 28. Elution of Barley Root Nitrate Reductase Inactivator from CM-Cellulose

50g 5 day old barley roots were homogenized in 150ml cold 50mM potassium phosphate buffer, pH 7.5, containing 0.5mM EDTA and 5mM cysteine, then the protein precipitated in the 40-70% ammonium sulphate fraction was redissolved in a small volume of buffer, dialyzed overnight and subjected to pH 4.0 precipitation. The supernatant from this step (7mg protein) was applied to a CM52-cellulose column (1.9 x 5.0cm) equilibrated with 10mM acetate buffer, pH 5.0. The column was washed with the same buffer then with 10mM acetate, containing 50mM NaCl, and the barley nitrate reductase inactivator was subsequently eluted by 10mM potassium phosphate, pH 8.0. 4ml fractions were collected.

Inset: Nitrate reductase inactivating activity was assayed using a partially purified maize nitrate reductase sample with an initial activity of 0.217μmol nitrite formed/mg protein hr^{-1} and containing 6 mg protein/ml.

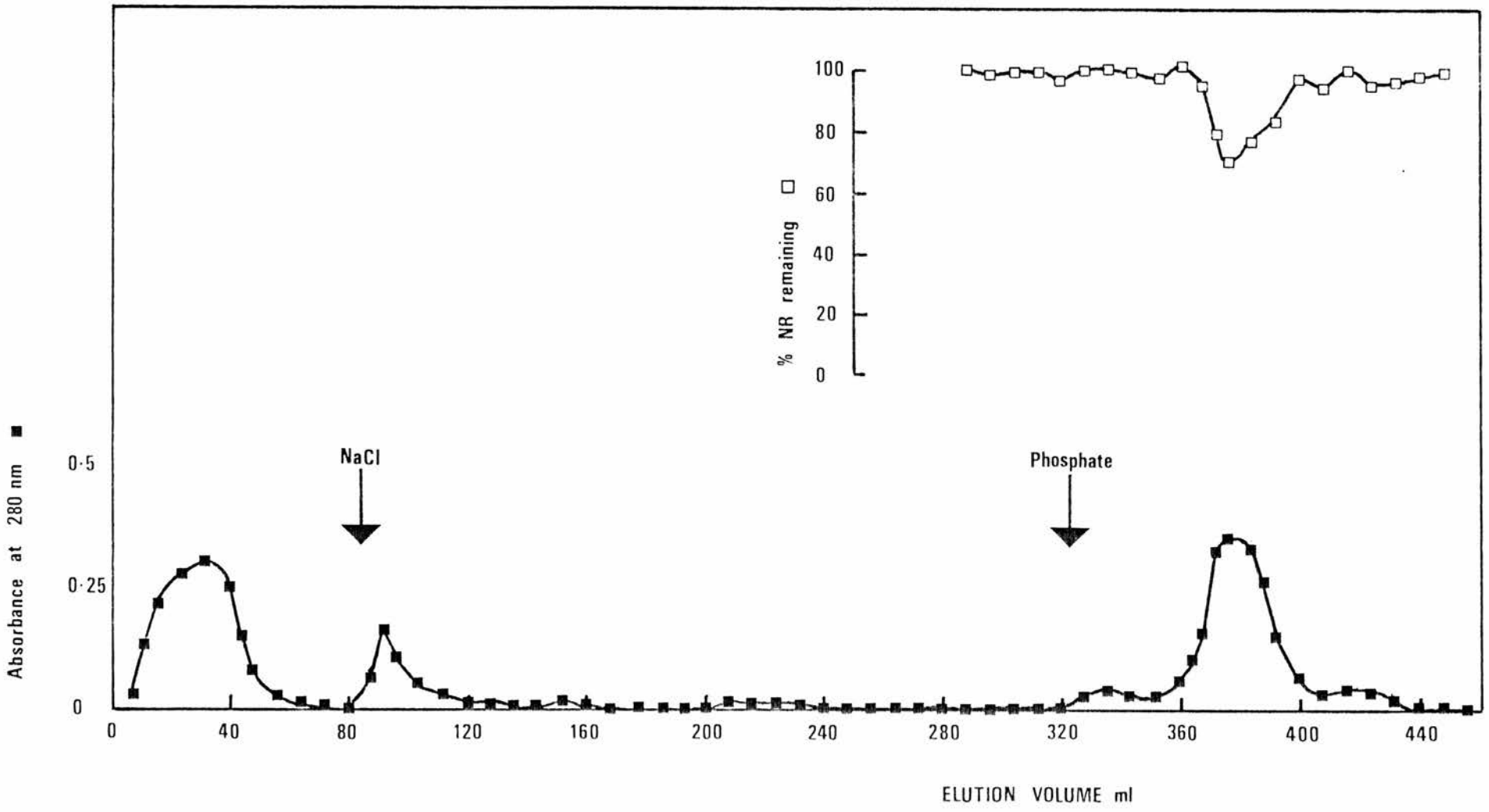
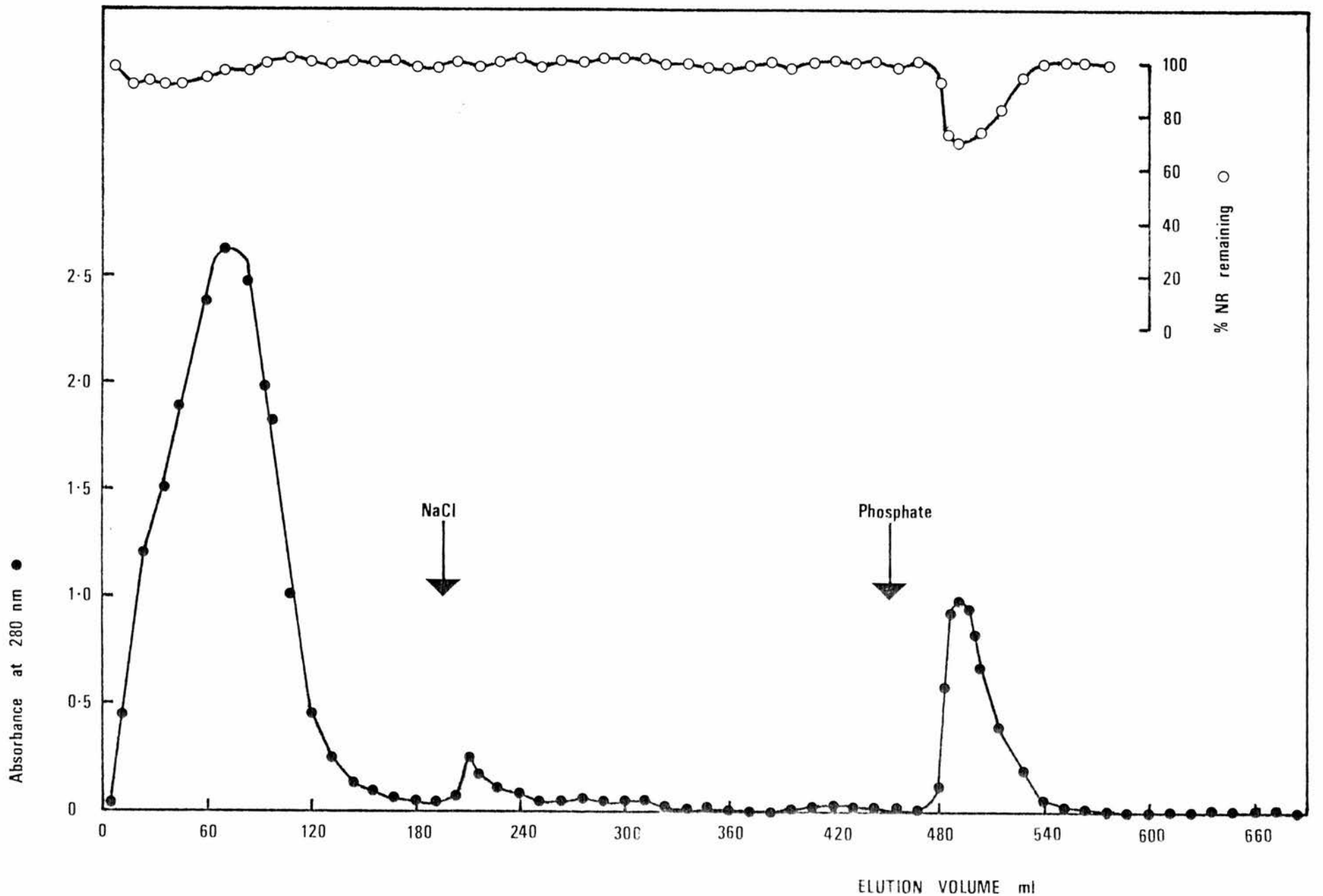

100
80
60
40
20
0
% NR remaining □
NaCl
Phosphate
0·5
0·25
0
Absorbance at 280 nm ■
0
40
80
120
160
200
240
280
320
360
400
440
ELUTION VOLUME ml

Figure 29. Elution of Barley Leaf Nitrate Reductase Inactivator from CM-Cellulose

150g 7 day old barley leaves were homogenized in 450ml cold 50mM potassium phosphate buffer, pH 7.5, containing 0.5mM EDTA and 5mM cysteine. After filtration and centrifugation, the protein precipitated in the 40-70% ammonium sulphate fraction was resuspended in a small volume of 10mM potassium phosphate, pH 7.0, dialyzed overnight then subjected to acid precipitation at pH 4.0. The supernatant from this step (188mg protein) was immediately applied to a CM52-cellulose column (1.9 x 5.0cm) equilibrated with 10mM acetate buffer, pH 5.0, and the column was washed with 10mM acetate, pH 5.0, then with 10mM acetate, pH 5.0, containing 50mM NaCl. Barley leaf nitrate reductase inactivator was eluted by 10mM potassium phosphate, pH 8.0, and 4ml fractions were collected.

Inset: Nitrate reductase inactivating activity was assayed using a partially purified barley nitrate reductase sample with an initial activity of 0.48μmol nitrite formed/ml hr^{-1}.


100
80
60
40
20
0
% NR remaining ○
2·5
2·0
1·5
1·0
0·5
0
Absorbance at 280 nm ●
NaCl
Phosphate
0
60
120
180
240
300
360
420
480
540
600
660
ELUTION VOLUME ml

by 10mM potassium phosphate buffer, pH 8.0. The elution profile of the leaf tissue sample was similar to that of the root nitrate reductase inactivator preparation (Figure 28). The CM-cellulose column had been slightly overloaded and therefore the initial peak of unadsorbed protein also contained a small amount of nitrate reductase inactivating activity.

Inhibition of barley nitrate reductase inactivator

The effect of various inhibitors on barley leaf nitrate reductase inactivator was studied as a means of comparing it with the two maize nitrate reductase inactivators described above. Maize nitrate reductase was used as the substrate in these experiments since no barley nitrate reductase was available at the time that the work was performed. Barley leaf nitrate reductase inactivator was found to be inhibited by EDTA and 1,10-phenanthroline, and to a lesser extent by leupeptin, but its activity was not affected by BSA, casein or PMSF (Table 8). It therefore resembled maize nitrate reductase inactivator II which was inhibited by EDTA and 1,10-phenanthroline, but not by BSA, casein or leupeptin (Table 7).

Sucrose density gradient analysis of inactivated barley nitrate reductase

Sucrose density gradient analysis of barley nitrate reductase revealed the presence of two peaks of NADH-cytochrome c reductase activity sedimenting at 3S and 7.7S, the latter coinciding with a peak of NADH-nitrate reductase activity and therefore representing the nitrate reductase complex (Figure 30a). After exposure to barley nitrate reductase inactivator for 60 minutes (Figure 30b) the NADH-cytochrome c reductase and NADH-nitrate reductase activities of the nitrate reductase complex were reduced, while the activity of the 3S NADH-cytochrome c reductase species seemed to be unchanged. No data was obtained for MVH-nitrate reductase activity, but the results in Figure 30 clearly indicate that barley nitrate reductase inactivator did not cause degradation of nitrate reductase to smaller NADH-cytochrome c reductase species.

Table 8. Effect of Inhibitors on the Activity of Barley Nitrate Reductase Inactivator

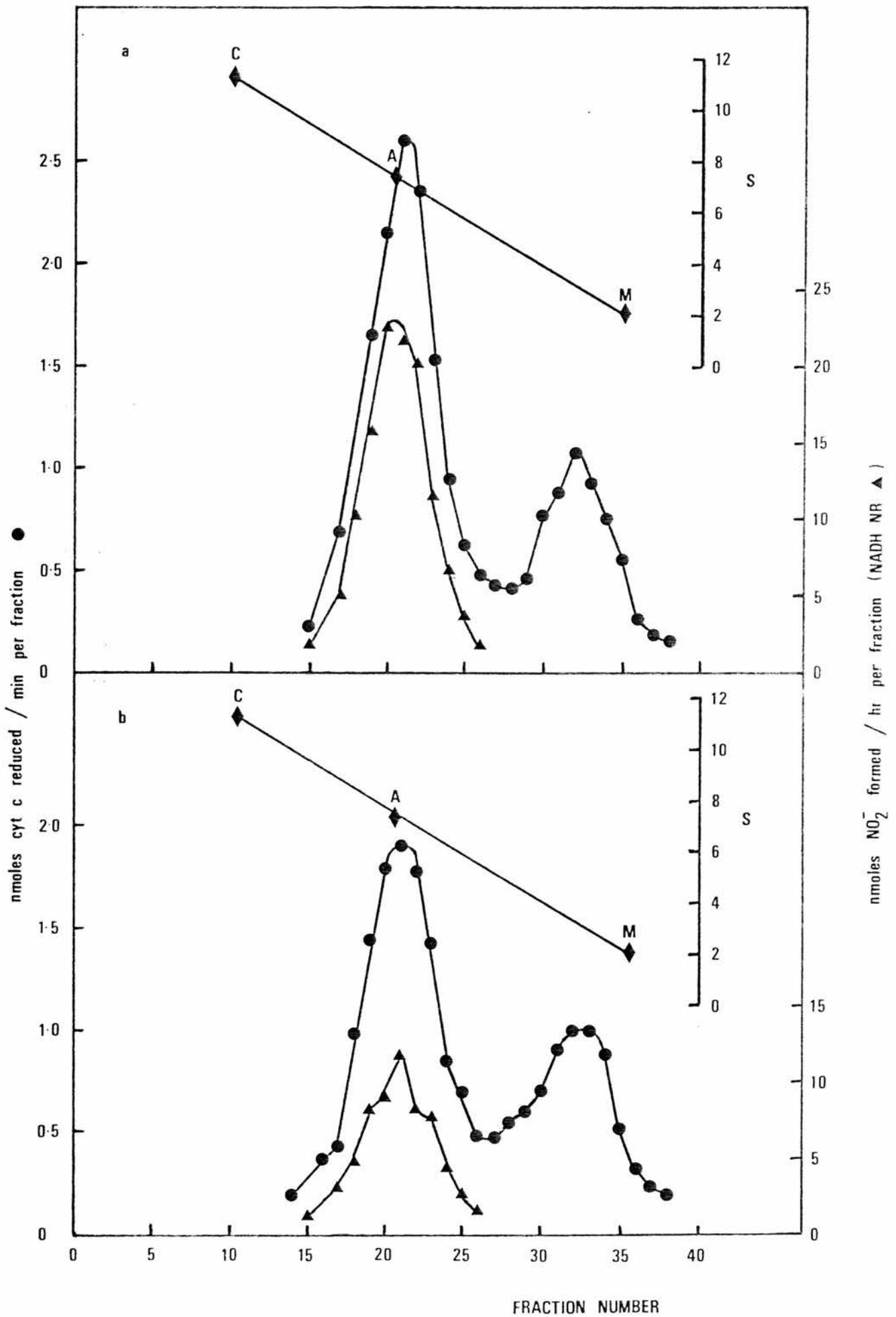
Inhibitor	Concentration	% Inhibition
BSA	3%	0
Casein	2%	0
EDTA	10mM	46
Leupeptin	10μM	19
1,10-phenanthroline	1mM	50
PMSF	0.25mM	0

Nitrate reductase inactivating activity, in the presence and absence of each inhibitor, was measured by the method described in Table 6, using partially purified maize nitrate reductase containing 11mg protein/ml, and barley nitrate reductase inactivator containing 0.4mg protein/ml.

Figure 30. Sucrose Density Gradient Analysis of Barley Nitrate Reductase Before and After Exposure to the Barley Leaf Nitrate Reductase Inactivator

1ml purified barley nitrate reductase was mixed with 280µl barley leaf nitrate reductase inactivator dissolved in Buffer III (224µg protein) then 20µl 200mM 1,10-phenanthroline was added either immediately (a) or after incubation for 60 minutes at 25°C (b) to inhibit nitrate reductase inactivation. 0.4ml aliquots of each sample were analysed on 2-18% sucrose gradients.

C, A and M denote the respective positions of catalase, alcohol dehydrogenase and myoglobin after centrifugation.


a
b
C
A
M
S
nmoles cyt c reduced / min per fraction ●
nmoles NO_2^- formed / hr per fraction (NADH NR ▲)
FRACTION NUMBER

DISCUSSION

Nitrate reductase in barley leaf extracts became increasingly unstable as seedlings aged. This instability had been found to correlate with an increased breakdown of the nitrate reductase complex to smaller NADH-cytochrome c reductase species (Brown et al., 1981) and might have been brought about by attack from endogenous proteinases. A proteolytic nitrate reductase inactivator isolated from mature maize root extracts (Wallace, 1973b, 1974, 1975b, 1978) was shown to inactivate the activities of the nitrate reductase complex at different rates but it seemed that the NADH-cytochrome c reductase component was the main site of attack (Wallace, 1975b). In the work reported in this thesis, an attempt was made to characterize the mechanism of nitrate reductase inactivation by two maize nitrate reductase inactivators and a barley nitrate reductase inactivator using sucrose density gradient data to look for evidence of the release of smaller enzymically-active fragments from the nitrate reductase complex during the inactivation process.

Nitrate reductase is a large molecule which in higher plants is thought to consist of two subunits of molecular weight 100,000 (Kuo et al., 1980a; Small and Wray, 1980) and a molybdenum-containing component of approximate molecular weight 1,000. Each subunit is proposed to consist of several independently folded domains linked together by exposed hinge regions which would be sensitive to proteinase attack. FAD and cytochrome b_{557} are thought to be located in separate domains while another domain is involved in binding the molybdenum-containing component (Figure 31; Brown et al., 1981; Wray and Kirk, 1981). The appearance of 3.1S (molecular weight 40,000) and 3.8S (molecular weight 61,000) NADH-cytochrome c reductase species in barley nitrate reductase extracts might be due to proteolytic cleavage at the hinge regions so that the 3.1S species would contain the FAD domain and the NADH-binding site while the 3.8S species may contain both the FAD and cytochrome b_{557} domains. Recently the 3.1S NADH-cytochrome c reductase species has been purified to homogeneity and found to be completely free from haem (J.L.Wray, personal communication). Species of 5.6S and 6.8S have also been observed in barley extracts (Wray et al.,

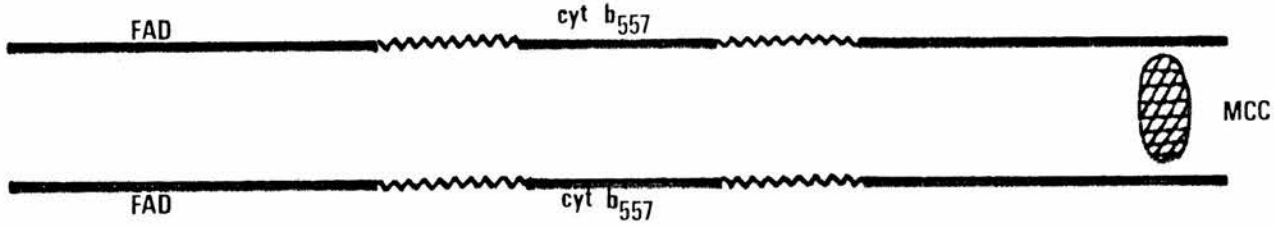
Figure 31. Proposed Structure of Nitrate Reductase in Higher Plants

The FAD and MCC-binding domains are envisaged as having molecular weights of 40,000 each, while the cytochrome b_{557} domain would have a molecular weight of 20,000, resulting in an overall subunit size of 100,000.

Key: MCC molybdenum-containing component


interdomain hinge region


domain


FAD
cyt b_{557}
MCC
FAD
cyt b_{557}

1979; Brown et al., 1981) and since the 6.8S species has both NADH-nitrate reductase and NADH-cytochrome c reductase activities it may represent the remainder of the 7.7S nitrate reductase complex after removal of one FAD domain.

Spinach nitrate reductase preparations have been shown to contain 3.6S and 8.1S NADH-cytochrome c reductase species, the latter of which also had NADH-nitrate reductase activity and therefore represented the nitrate reductase complex, and if plants were grown in the absence of molybdenum then two additional species of 5.5S and 6.9S were also observed (Notton et al., 1976). These species seem to correspond to the NADH-cytochrome c reductase species in barley. Maize nitrate reductase extracts also contained 7.7S and 6.8S species which exhibited NADH-nitrate reductase, NADH-cytochrome c reductase and MVH-nitrate reductase activities while a smaller NADH-cytochrome c reductase species sedimented in the 3-4S region (Figures 18, 20, 27). Small (4S) NADH-cytochrome c reductase species have been observed to form from the 7.6S tobacco nitrate reductase complex during affinity chromatography, although this was thought to be due to dissociation of nitrate reductase subunits rather than specific proteolytic attack (Mendel and Müller, 1980). From this data, it seems likely that the higher plant nitrate reductases will all prove to have very similar structures.

Most of the nitrate reductase inactivating activity in the mature root of 5 day old maize seedlings was due to nitrate reductase inactivator I, but it was difficult to estimate the total nitrate reductase inactivating activity of the crude extract because of interference by other substances which also inhibited nitrate reductase therefore the purification factors reported in Table 4 are erroneously low. A more accurate assessment of nitrate reductase inactivator I activity could have been obtained by measuring azocasein-degrading activity, but nitrate reductase inactivator II activity could not have been assessed in this way.

Nitrate reductase inactivator I and the nitrate reductase inactivating enzyme described by Wallace (1973b, 1974, 1975a,b, 1978) behaved similarly

during CM-cellulose chromatography (Figure 9; Wallace, 1974) and both were able to degrade azocasein (Figure 12; Wallace, 1978). Both proteinases were inhibited by PMSF and casein (Table 6; Wallace, 1974, 1975a) but the inactivating enzyme was also inhibited by EDTA and 1,10-phenanthroline (Wallace, 1974) which had no effect on nitrate reductase inactivator I activity (Table 6). BSA and leupeptin were unable to inhibit inactivator I (Table 6) but the nitrate reductase inactivating enzyme had not been tested with these substances. This data suggests that nitrate reductase inactivator I and the inactivating enzyme, which was shown to be the main proteinase of the maize root (Shannon and Wallace, 1979), are identical. The only major difference between them is in the effect of EDTA and 1,10-phenanthroline but this could have been due to contamination of Wallace's preparation by another nitrate reductase inactivator, such as inactivator II.

The molecular weight of nitrate reductase inactivator I was calculated to be 56,200 but could be slightly larger since the estimated Stokes radius of 2.9nm (Figure 15) may be somewhat low due to adsorption of inactivator I to Sephadex G200 in the presence of a low ionic strength buffer (Yamaya et al., 1980a). However, this value confirms previous molecular weight estimates of 44,000 (Wallace, 1974) and 54,000 (Shannon and Wallace, 1979) although it is slightly smaller than the values of 66,000 and 75,000 obtained for maize inactivator I by Yamaya et al. (1980a).

Polyacrylamide disc-gel electrophoresis of nitrate reductase inactivator I (Figure 14) demonstrated that azocasein degradation and nitrate reductase inactivation were properties of a single protein band which, at pH 8.5, had an R_f value of 0.055. The purified nitrate reductase inactivator I preparation contained several contaminants, none of which seemed to possess nitrate reductase inactivating activity and since all had R_f values greater than 0.5 (Table 5) pure nitrate reductase inactivator I could be prepared by the inclusion of an electrophoretic step in the purification procedure.

Maize nitrate reductase inactivator I was able to inactivate all activities

of the maize nitrate reductase complex (Figure 17) although it was not possible to obtain reliable results for $FMNH_2$-nitrate reductase activity since the traces of glycerol present in inactivator I samples interfered with $FMNH_2$-nitrate reductase assays (Kuo et al., 1980a). NADH-nitrate reductase and NADH-cytochrome c reductase activities were inactivated much more rapidly than MVH-nitrate reductase activity (Figure 17) confirming the results of Wallace (1975b), who suggested that the NADH-cytochrome c reductase component of the maize nitrate reductase complex was the main site of attack by maize nitrate reductase inactivator I. However, inactivation of nitrate reductase from Chlorella vulgaris by maize nitrate reductase inactivator I differed since it resulted in activation of MVH-nitrate reductase activity although NADH-nitrate reductase and NADH-cytochrome c reductase activities were inactivated (Yamaya et al., 1980b).

Wallace (1974) proposed that inactivator I inactivated nitrate reductase by proteolysis. Recently evidence was obtained for the limited proteolytic cleavage of Chlorella vulgaris nitrate reductase by maize nitrate reductase inactivator I since complete inactivation resulted in a change in the electrophoretic mobility of both the major protein band and MVH-nitrate reductase activity (Yamaya et al., 1980b) suggesting the formation of a smaller MVH-nitrate reductase species. However Chlorella vulgaris nitrate reductase has a significantly different structure from the higher plant nitrate reductases since it is thought to consist of three subunits arranged in a globular shape (Giri and Ramadoss, 1979) while nitrate reductase from higher plants is considered to be an asymmetrical cigar-shaped molecule (Notton et al., 1976; Small and Wray, 1980) containing two subunits (Kuo et al., 1980a; Small and Wray, 1980). Recently it has also been shown by immunological means that nitrate reductase from Chlorella vulgaris is significantly different to that from maize (Smarelli and Campbell, 1981) and it is therefore reasonable to expect that the effect of maize nitrate reductase inactivator I on maize nitrate reductase might be very different from its effect on Chlorella vulgaris nitrate reductase.

In the work described here, sucrose density gradient centrifugation was

used to look for evidence of limited proteolysis during the inactivation of maize nitrate reductase by maize nitrate reductase inactivator I, but the data could not be used to quantitate nitrate reductase inactivation due to inconsistent recovery of activity from the gradients. An approximately 6.8S species possessing NADH-nitrate reductase, NADH-cytochrome c reductase and MVH-nitrate reductase activities appeared during nitrate reductase inactivation (Figures 18, 19 and 20) and may represent a product of the initial cleavage of the nitrate reductase complex. This species did not accumulate and therefore it is likely that nitrate reductase inactivator I attacks maize nitrate reductase at more than one site to eventually produce small enzymically-inert fragments which could not be detected by the methods used. This contrasts with the results obtained with Chlorella vulgaris nitrate reductase (Yamaya et al., 1980b) since maize nitrate reductase inactivator I did not seem able to inactivate the smaller MVH-nitrate reductase species which appeared during inactivation of Chlorella nitrate reductase.

The 6.8S species (Figures 18 and 20) might be formed by the removal from the maize nitrate reductase complex of one, approximately 3S, FAD-containing domain, which would be expected to exhibit NADH-cytochrome c reductase activity. However it has already been shown that NADH-cytochrome c reductase activity is rapidly inactivated by maize nitrate reductase inactivator I (Figure 17) therefore if such a 3S species was released during nitrate reductase inactivation it was quickly degraded to inactive fragments.

Maize nitrate reductase inactivator II was separated from inactivator I by CM-cellulose chromatography (Figure 9) and represented a previously uncharacterized species. It differed from nitrate reductase inactivator I in its response to inhibitors since it was inhibited by EDTA and 1,10-phenanthroline, and only very slightly by PMSF, but not by casein, BSA or leupeptin (Table 7). Recently a macromolecular component capable of inactivating nitrate reductase has been observed in maize extracts (Shannon and Wallace, 1979; Wallace and Shannon, 1981) which was inhibited by EDTA and 1,10-phenanthroline but not by

casein and may therefore be equivalent to nitrate reductase inactivator II. Maize inactivator II also differed from inactivator I in being unable to degrade azocasein (Figure 21) but this was insufficient evidence to rule out the possibility that inactivator II might be a proteinase, since a proteinase which degraded casein and haemoglobin but not azocasein has been observed in maize roots (Shannon and Wallace, 1979; Wallace and Shannon, 1981) thus stressing the importance of testing suspected proteinases against a range of substrates. Nitrate reductase inactivator II might demonstrate proteolytic activity if it was tested against a suitable substrate.

The nitrate reductase inactivator II preparation had a very low specific activity (Table 4) and was probably very impure but there was insufficient time to attempt further purification. Sucrose density gradient analysis of nitrate reductase inactivator II revealed a relatively broad peak of activity (Figure 22) therefore the preparation may have contained more than one nitrate reductase inactivating species, particularly since Shannon and Wallace (1979) fractionated a corresponding preparation by Sephadex G200 gel filtration to reveal the presence of two peaks of nitrate reductase inactivating activity. Inactivator II had a sedimentation coefficient of 4.8 S (Figure 22) and was therefore of a similar size to the 4.7S nitrate reductase inactivator I (Figure 16).

Nitrate reductase inactivator II inactivated the NADH-nitrate reductase and NADH-cytochrome c reductase activities of the maize nitrate reductase complex but not the MVH-nitrate reductase activity (Figure 23). Sucrose density gradient analysis of maize nitrate reductase after inactivation by inactivator II was difficult to interpret since the percentage of activity lost during centrifugation was not the same in each gradient, but the data confirmed the existence of 7.7S and 6.8S species possessing NADH-nitrate reductase, NADH-cytochrome c reductase and MVH-nitrate reductase activities (Figures 24-27). Although the MVH-nitrate reductase activity data was very erratic, it seemed to agree with the data obtained from the time course of nitrate reductase inactivation (Figure 23) in suggesting that MVH-nitrate reductase is not inactivated by nitrate reductase

inactivator II, therefore inactivator II might act only on the NADH-oxidising region of the nitrate reductase complex and not affect the nitrate-reducing region.

Maize nitrate reductase inactivator II resembled a rice nitrate reductase inactivating factor (Kadam et al., 1974; Yamaya and Ohira, 1977, 1978a; Yamaya et al., 1980a) since both reacted similarly to inhibitors and had a similar effect on the activities of nitrate reductase, but the rice factor was significantly larger in size, with a molecular weight of 150,000-200,000 (Yamaya and Ohira, 1977; Leong and Shen, 1980; Yamaya et al., 1980a). The rice nitrate reductase inactivating factor was thought to be a nitrate reductase-binding protein since it had no proteolytic activity towards casein, BSA, rice nitrate reductase (Yamaya and Ohira, 1977) azocasein or Chlorella vulgaris nitrate reductase (Yamaya et al., 1980a,b) and its effect on rice nitrate reductase was completely reversed by NADH (Yamaya and Ohira, 1978b). Binding of the rice factor to nitrate reductase would increase the size of the nitrate reductase complex and therefore its electrophoretic mobility would be expected to decrease. However electrophoretic analysis of rice nitrate reductase after inactivation by the rice nitrate reductase inactivating factor showed that the mobility of the major protein band and MVH-nitrate reductase activity was unchanged (Yamaya et al., 1980b) which would tend to suggest that the rice factor is not in fact a nitrate reductase-binding protein. The rice nitrate reductase inactivating factor also inactivated nitrate reductase from maize and Chlorella vulgaris but this effect could not be reversed by NADH (Yamaya et al., 1980a,b) possibly due to structural differences between rice nitrate reductase and that from maize and Chlorella vulgaris, and this would seem to be additional evidence indicating that the rice factor is not a binding protein. The effect of NADH on maize nitrate reductase inactivator II was not tested.

Nitrate reductase inactivator II did not appear to bind to nitrate reductase since sucrose density gradient analysis of inactivated maize nitrate reductase (Figures 25 and 27) failed to produce evidence for the existence of a

species larger than the 7.7S nitrate reductase complex.

A nitrate reductase inactivator was also observed in barley seedlings and was similar to maize nitrate reductase inactivator II in that it was eluted from CM-cellulose under the same conditions (Figures 9, 28, 29) and was inhibited by EDTA and 1,10-phenanthroline but not by casein or BSA (Table 8). In contrast to maize inactivator II it was completely unaffected by PMSF and was slightly inhibited by leupeptin. The barley nitrate reductase inactivator was able to inactivate maize nitrate reductase (Figure 28) in addition to barley nitrate reductase. No data was available for the time course of inactivation of barley nitrate reductase by the barley inactivator, but sucrose density gradient analysis of inactivated barley nitrate reductase (Figure 30) did not produce evidence for the formation of smaller enzymically-active species during the inactivation process.

Miller and Huffaker (1980a, b, 1981, 1982) have recently reported the presence of a cysteine proteinase in extracts of barley leaves which was inhibited by leupeptin and represented 85% of the total proteolytic activity present in the extracts. Leupeptin stabilized nitrate reductase activity in barley leaf extracts (Wray and Kirk, 1981) and prevented breakdown of the nitrate reductase complex to the smaller NADH-cytochrome c reductase species observed by Brown et al., (1981). It is therefore possible that the barley cysteine proteinase (Miller and Huffaker, 1981) may be responsible for this nitrate reductase breakdown, particularly since it has recently been observed to act by limited proteolysis (Miller and Huffaker, 1982). Although the barley nitrate reductase inactivator was partially inhibited by leupeptin (Table 8) it was probably very impure and the preparation may have contained several proteinases therefore the failure of sucrose density gradient analysis of inactivated barley nitrate reductase to produce evidence for the breakdown to smaller NADH-cytochrome c reductase species is not conclusive (Figure 30). If the inactivator preparation contained the cysteine proteinase, most of its activity would have decayed during purification since the proteinase is reported to be very unstable in the

absence of reduced sulphydryl reagents (Miller and Huffaker, 1981) and any small NADH-cytochrome c reductases which formed in its presence were probably further degraded by other proteinases. Although the work of Wray and Kirk (1981) suggests that the cysteine proteinase is responsible for the observed nitrate reductase breakdown, the effect of the isolated proteinase on nitrate reductase will need to be determined in order to verify this theory.

BSA has been reported to stabilize nitrate reductase activity (Schrader et al., 1974; Schrader and Vogelzang, 1974; Purvis et al., 1976; Brown et al., 1981) and to prevent conversion of barley nitrate reductase to smaller NADH-cytochrome c reductase species (Brown et al., 1981). It has been speculated that BSA might act as an alternative substrate for a nitrate reductase-degrading proteinase (Schrader et al., 1974; Brown et al., 1981) but in the work described above there was no evidence to suggest that BSA inhibited either of the maize nitrate reductase inactivators (Tables 6 and 7) or the barley nitrate reductase inactivator (Table 8). BSA was also unable to inhibit the rice nitrate reductase inactivating factor (Kadam et al., 1974; Leong and Shen, 1980) although it inhibited a nitrate reductase inactivating factor from wheat leaves (Sherrard et al., 1979a). BSA did not protect Chlorella vulgaris nitrate reductase from inactivation by trypsin (Yamaya et al., 1980b) and could not prevent hydrolysis of ribulose-1,5-bisphosphate carboxylase by two barley leaf proteinases (Miller and Huffaker, 1981) therefore it would seem that the protective effect of BSA towards nitrate reductase activity is not due to protection from proteolysis. It has also been shown that BSA is unlikely to prevent dissociation of the nitrate reductase complex merely by increasing the protein concentration since the stability of barley nitrate reductase in crude extracts was not decreased by dilution (Brown et al., 1981). BSA did not increase the amount of nitrate reductase extracted from tissue nor did it increase the stability of nitrate reductase from cotton cotyledons, indicating that the factors responsible for nitrate reductase instability in vitro were not inhibited by BSA, which appeared instead merely to enhance nitrate reductase activity (Purvis et al., 1976). It

has been suggested that BSA acts by protecting nitrate reductase from inhibition by phenolic compounds (Purvis et al., 1976) and this does not conflict with the results described here.

Recent work with barley nitrate reductase (Kuo et al., 1980b) has shown that it is more stable at high pH and that, at pH 8.5, nitrate reductase of a high specific activity could be extracted from barley leaf tissue. This was possibly due to inhibition of the cysteine proteinase (Miller and Huffaker, 1981) which was unstable above pH 7.5, and evidence has now been obtained which suggests that the purification procedure is important in determining the specific activity of the nitrate reductase preparation. Using the purification procedure described in Methods, an electrophoretically homogeneous barley nitrate reductase sample was prepared and when this was analysed by SDS-polyacrylamide gel electrophoresis it was found that the major protein band had a molecular weight of only 60,000. However if nitrate reductase was purified in the presence of leupeptin then the molecular weight of the major protein band was 100,000 (J.L.Wray, personal communication) indicating that nitrate reductase prepared in the absence of leupeptin had been attacked by endogenous proteinases. The purified maize nitrate reductase used here had a low specific activity (Table 2) and may have been partially degraded even before exposure to the nitrate reductase inactivators so that it might have been more susceptible to attack by the inactivators. The nitrate reductase inactivators were more active towards purified nitrate reductase than towards partially purified nitrate reductase therefore non-nitrate reductase protein may have been able to interfere with the inactivation reaction.

In addition to the nitrate reductase inactivators reported above, a non-protein inhibitor of nitrate reductase has been detected in boiled maize root extracts (Wallace, 1975a; Aslam, 1977) although there was no evidence for the existence of this heat inducible inhibitor in maize leaf extracts (Aslam, 1977). It seems likely that each plant species will be found to contain several nitrate reductase inactivators since at least three exist in maize, two in Neurospora

crassa (Sorger et al., 1978) yeast (Wallace, 1978) and possibly also in barley, although only one nitrate reductase inactivator has so far been found in wheat (Sherrard et al., 1979a, b) soybean (Jolly and Tolbert, 1978) and rice (Yamaya and Ohira, 1977). Some nitrate reductase inactivators have been shown to be proteinases while others are thought to be non-proteolytic, but the inactivators are also divided into two groups by the fact that some inactivate the NADH-oxidising region of nitrate reductase (maize nitrate reductase inactivators I and II, barley, rice (Yamaya and Ohira, 1977, 1978a) and Neurospora (Sorger et al., 1978) inactivators) while others inactivate the nitrate-reducing region of the complex (wheat (Sherrard et al., 1979a, b) and soybean (Jolly and Tolbert, 1978) nitrate reductase inactivators).

Many proteinases have specific inhibitors which serve to prevent unwanted proteolysis (Laskowski and Kato, 1980) and a similar situation seems also to exist for the nitrate reductase inactivators since several authors have reported the existence of inhibitors. There was an apparent activation of maize nitrate reductase inactivating activity during the purification procedure (Table 4; Wallace, 1974, 1978) which was thought to be due to removal of an inhibitor which was heat-stable and thus resembled a nitrate reductase-stabilizing factor from cotton cotyledons (Purvis and Tischler, 1976; Purvis et al., 1976, 1980), an inhibitor of yeast proteinase B (Saheki et al., 1974) and an inhibitor of the Neurospora tryptophan synthase inactivator (Yu et al., 1973). (The tryptophan synthase inactivator has properties similar to nitrate reductase inactivator I and therefore may also be able to inactivate nitrate reductase).

A nitrate reductase activator which inhibited both maize nitrate reductase inactivator I and the rice nitrate reductase inactivating factor was obtained from the 80% ethanol-soluble fraction of maize scutellum (Yamaya and Oaks, 1980; Yamaya et al., 1980b). It activated NADH-nitrate reductase and NADH-cytochrome c reductase activities, although it had a slightly inhibitory effect on $FMNH_2$-nitrate reductase and MVH-nitrate reductase activities, and therefore acted on the same region of the nitrate reductase complex as the maize and rice

inactivators. The activator was not affected by trypsin and therefore seemed to be a non-protein substance (Yamaya and Oaks, 1980). A thermostable inhibitor of nitrate reductase inactivators has also been observed in Neurospora (Sorger et al., 1978) while two protein-like nitrate reductase stabilizing factors in wheat inhibited the wheat nitrate reductase inactivator (Sherrard et al., 1979a). The nitrate reductase stabilizer protein present in extracts of young cotton cotyledons seemed to be non-functional in vivo since nitrate reductase activity decayed rapidly in young cotyledons (Purvis et al., 1976, 1980). It was thought to function by preventing dissociation of the nitrate reductase subunits rather than by inhibiting an inactivator, so that the increased lability of nitrate reductase in extracts of older cotyledons was thought to be due to the disappearance of the stabilizer rather than the appearance of a specific inactivator (Purvis et al., 1980). A nitrate reductase stimulator was also observed in soybean leaves (Jolly and Tolbert, 1978). With the exception of the wheat and cotton nitrate reductase stabilizers, all inhibitors of nitrate reductase inactivating activity appear to be non-protein substances and there may be similarities between the inhibitors from different species.

In conclusion, three nitrate reductase inactivators have been described in this work. Maize nitrate reductase inactivator I was shown to be a serine proteinase but maize inactivator II and the barley nitrate reductase inactivator might also prove to be proteinases if tested against suitable substrates. These three inactivators preferentially inactivated the NADH-oxidising portion of the nitrate reductase complex, and therefore did not generate small (3-4S) NADH-cytochrome c reductase species such as those observed in barley extracts (Small and Wray, 1980; Brown et al., 1981). Maize nitrate reductase inactivator I seemed to initially form a 6.8S NADH-cytochrome c reductase species, but this was then subject to further proteolysis, as described above.

A great deal of work remains to be done before the in vitro mechanism of nitrate reductase inactivation will become clear. The most obvious areas for further study are:

i) To determine whether other nitrate reductase inactivators are present in barley extracts.

ii) Isolation and characterization of these inactivators.

iii) Isolation of the barley cysteine proteinase (Miller and Huffaker, 1981) and determination of its effect on nitrate reductase.

iv) Further purification and characterization of maize nitrate reductase inactivator II.

v) Determination of the properties of the non-protein inhibitors of nitrate reductase inactivators.

Although all of the nitrate reductase inactivators reported to date appear to be located in the cytoplasm, they may be physically separated from nitrate reductase *in vivo* so that the observed nitrate reductase inactivation *in vitro* may be an artifact of the extraction procedure. The precise role of the nitrate reductase inactivators *in vivo* has yet to be determined.

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