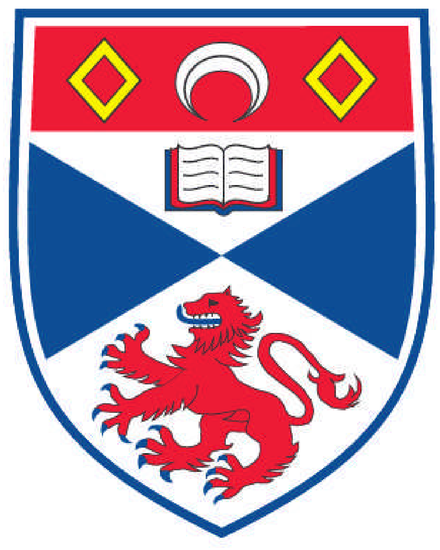
ISOLATION AND CHARACTERISATION OF CHLORATE RESISTANT MUTANTS OF BARLEY

Barbara Steven

A Thesis Submitted for the Degree of PhD at the University of St. Andrews



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ISOLATION AND CHARACTERISATION OF CHLORATE RESISTANT MUTANTS OF BARLEY

by

BARBARA STEVEN

A thesis submitted to the University of St Andrews in application for the degree of Doctor of Philosophy

August 1986

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DECLARATION

I, Barbara Steven, hereby certify that this thesis has been composed by myself, that it is a record of my own work, and that it has not been accepted in partial or complete fulfilment of any other degree or professional qualification.

August 1986

Certificate

I hereby declare that Barbara Steven has spent nine terms in research work under my direction and that she has fulfilled the condition of Ordinance No. 16 (St Andrews), and that she is qualified to submit this thesis for the degree of Doctor of Philosophy.

ACADEMIC RECORD

I was admitted to the Faculty of Science of the University of St Andrews under Ordinance General No. 12 on 1 October 1982 and as a candidate for the degree of PhD on 1 October 1982.

August 1986

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ABSTRACT

The object of this study has been to characterise barley mutants which lack the functional nitrate reductase (NR) enzyme system. In the long term it is hoped that such studies will lead to improved nitrate utilisation and ultimately to improved quality barley protein. The progeny of nine chlorate resistant selections, in the barley cultivars Maris Mink and Golden Promise, were studied. Four (R9201, R11301, R12202 and R12801) lacked NADH-NR and FMNH2-NR activities, the rest had the NR+ phenotype. None of the four were nitrate uptake mutants since they all possesed wild type or greater levels of nitrate. R9201, R11301 and the previously characterised R9401 (Bright **et al**, 1983) were not molybdenum uptake or Mo-accumulation mutants.

R9201, R11301 and R12202 lacked xanthine dehydrogenase (XDH) (an enzyme which contains the same molybdenum-containing cofactor, MoCo) activity suggesting that these lines have defective MoCo's, whilst R12801 possessed XDH activity indicating that it might have defective apoprotein subunits.

These four lines are similar to R9401 since they lack NR activity and are unlike other previously selected barley NR mutants (Kleinhofs **et al**, 1980) which are leaky and possess up to 5% of the wild type (cv. Steptoe) **in vitro** NR activity.

R9201 and R11301, like R9401, were all caused by single recessive nuclear gene mutations.

The MoCo mutants, R9201, R9401, R11301 and R12202, could be divided into two groups on the basis of i) allelism, ii) presence or absence of wild type levels of dimeric NR and iii) the ability of their extracted MoCo's to reconstitute NADPH-NR in an extract of **N. crassa nit1** mutant (which supplies NR monomers) in the presence of excess molybdate.

R11301 is not allelic to R9201, whilst R9201 and R9401 are allelic. R9401 is also thought to be allelic to Az 34, a barley MoCo mutant isolated and characterised by Kleinhofs **et al** (1980). Az 34 has been designated **nar2a** and it is proposed that the allelic R9401 and R9201 should be classified as **nar2b** and **nar2c** respectively. It is possible that R11301 is either allelic with one of the other barley MoCo lines, **nar3, nar4** or it is defective in a different MoCo gene. The same is also true for R12202.

R11301 was shown to possess inactive NR dimer at wild type concentrations, whilst R9201, R9401 and R12202 had little or no NR (inactive dimer) present under these conditions. R12801 possessed no dimer.

The MoCo extracted from R11301 was able to reconstitute the same level of NADPH-NR in the **nit1** extract as MoCo extracted from the wild types. R9201, R9401 and R12202 lacked this ability.

AUTHORS NOTES

1. Throughout this thesis italics have been replaced by bold type to denote specific genes and organisms.
2. Through the limitations imposed by the use of the word processing package for the Commodore 8096 computer, the following chemical formulae are represented in this thesis as follows:

correct formula	**representation**
$CaCl_2.2H_2O$	CaCl2.2H2O
$CaCl_2.6H_2O$	CaCl2.6H2O
$Ca(NO_3)_2$	Ca(NO3)2
$^{36}ClO_3^-$	36ClO3-
^{60}Co	60Co
$CuCl_2$	CuCl2
$CuSO_4$	CuSO4
$CuSO_4.5H_2O$	CuSO4.5H2O
$FeCl_3.6H_2O$	FeCl3.6H2O
$Fe_4:S_4$	Fe4:S4
$FMNH_2$	FMNH2
H_2O	H2O
H_3BO_3	H3BO3
H_2SO_4	H2SO4
$KClO_3$	KClO3
KH_2PO_4	KH2PO4
KNO_2	KNO2
KNO_3	KNO3
$Mg(NO_3)_2.5H_2O$	Mg(NO3)2.5H2O
$MgSO_4$	MgSO4
$MgSO_4.7H_2O$	MgSO4.7H2O
$MnCl.4H_2O$	MnCl.4H2O
$MnSO_4$	MnSO4

correct formula	representation
$MnSO_4.5H_2O$	MnSO4.5H2O
$NaHClO_3$	NaHClO3
Na_2MoO_4	Na2MoO4
$NaNO_3$	NaNO3
NH_4+	NH4+
$^{13}NH_4+$	13NH4+
NO_2-	NO2-
$^{13}NO_2-$	13NO2-
NO_3-	NO3-
$^{13}NO_3-$	13NO3-
$^{15}NO_3-$	15NO3-
NH_4Cl	NH4Cl
$(NH_4)_2SO_4$	(NH4)2SO4
$(NH_4)_6.Mo_7O_{24}.4H_2O$	(NH4)6.Mo7O24.4H2O
$ZnSO_4$	ZnSO4
$ZnSO_4.7H_2O$	ZnSO4.7H2O

ABBREVIATIONS

BSA	Bovine serum albumin
BVH	Benzyl Viologen (reduced)
CR	Cytochrome c reductase
CRM	Cross-reacting material
DEAE-cellulose	Diethyl amino ethyl-cellulose
DCPIP	2,6-dichlorophenol indophenol
ELISA	Enzyme-linked immunoassay
EMS	Ethylmethane sulphonate
ENU	N-ethyl-N-nitrosourea
F1	first generation after cross
F2	second generation after cross
FAD	Flavine adenine dinucleotide
FMNH2	Flavine mononucleotide (reduced)
GOGAT	Glutamine synthase
GS	Glutamate synthetase
Imas	maximum initial rate of ion uptake
Km	Michaelis constant
Leupeptin	Acetyl-L-leucyl-L-leucyl-L-arginal
M1	Mutagenised generation
M2	first generation arising from self fertilisation of M1
M3	next generation arising from self fertilisation of M2
M4	next generation arising from self fertilisation of M3
MMNG	N-methyl-N'-nitro-N-nitrosoguanidine
MoCo	Molybdenum-containing cofactor
NAD+	Nicotinamide adenine dinucleotide (oxidised)
NADH	Nicotinamide adenine dinucleotide (reduced)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NED	N(1-napthyl)-ethylene diamine hydrochloride
NR	Nitrate reductase
NiR	Nitrite reductase
MSO	Methionine sulphoximine
Nitroblue tetrazolium	Diphenyl-3,3'-(3,3'-dimethoxy-4,4'-diphenylene) ditetrazolium chloride
PAGE	Polyacrylamide gel electrophoresis
pHMB	para-Hydroxymercuribenzoate
PMSF	Phenyl methyl sulphonyl fluoride
SDS	Sodium dodecyl sulphate
Szechrome NAS	Diphenylamine sulphonic acid chromogene
TEMED	N,N,N',N'-tetramethyl-ethylene diamine ethane
XDH	Xanthine dehydrogenase

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CHAPTER 1

INTRODUCTION

The principal routes of nitrogen into organic form in biological systems are by the fixation of atmospheric nitrogen or the assimilation of nitrate.

Burns and Hardy (1975) calculated the overall biological fixation of molecular nitrogen to be 2×10^2 megatons annually. Guerrero **et al** (1981) estimated that 2×10^4 megatons of inorganic nitrogen is assimilated annually by plants on the basis of the relative C and N content of plants and the CO2 levels fixed annually by plants. Taking these two estimates it is clear that about 100-fold more inorganic nitrogen is assimilated than fixed. Therefore, the major path of inorganic nitrogen in to the biosphere is through nitrate assimilation (Dunn-Coleman **et al**, 1984). In terms of human consumption, Evans (1975) estimates that two thirds of edible dry matter and half the protein produced in the world is contributed by cereals. The production of ammonium and nitrate based fertilisers is energetically expensive. At least 1 megaton of N-fertiliser is added to crops in the UK each year and empirical evidence suggests that 50% of this is leached out (Wilkinson and Greene, 1982). With these points in mind the relevance of understanding the physiology, biochemistry and molecular biology of nitrate assimilation in crop plants becomes clear. One long term aim of this area of research must be to improve the efficiency of nitrate assimilation in crop plants.

Often, in order to get a better understanding of metabolic pathways, mutant organisms are characterised for the following reasons:

i) A number of different gene products are usually involved and this

technique can define which specific gene directly affects each step.

ii) Regulatory mutants can also be isolated allowing a clearer understanding of the mechanisms of regulation.

iii) This perturbation of the normal pathway will have effects on other linked pathways, allowing the physiology to be studied.

(iv) The genetic analysis of these selections can be achieved to give a better understanding of the interactions of different alleles and genes. A good example of this type of approach is given for the nitrate reductase found in **Aspergillus nidulans** by Cove (1979).

(v) Biochemical anaylsis of the nitrate reductase enzymes of these mutants gives a more precise understanding of what is important for the formation of an active nitrate reductase. This can be illustrated with the work carried out on the **Neurospora crassa** (Dunn-Coleman **et al,** 1984) and **Nicotiana plumbaginifolia** (Mendel **et al,** 1986) nitrate reductase systems.

Barley is a convenient species to study since there is a large pool of knowledge concerning many aspects of the physiology of nitrate assimilation and nitrate reductase. For genetic analyses barley has a distinct advantage as it is diploid and self-fertile. This allows the second generation (M2) after mutagenesis to be screened for recessive mutations. Cross pollination is easy, with high seed setting rates, and at least two generations may be grown, under artificial conditions, a year (Briggs, 1978).

1. THE NITRATE ASSIMILATION PATHWAY

Three biochemical steps are involved in the reduction of nitrate to ammonium; nitrate uptake, reduction of nitrate to nitrite, reduction of nitrite to ammonium (figure 1). The ammonium then is incorporated into the alpha-amino position of glutamate via glutamine synthetase and glutamate synthase. This amine group is then transferred by transamination to other amino acids and N compounds. The ability to assimilate nitrate is restricted to plants, algae, fungi and some bacteria (cyanobacteria, photosynthetic bacteria and chemoergonic bacteria).

1.1 Uptake

Nitrate is accumulated across the root plasmalemma membrane in an energy dependent, saturable (Rao and Rains, 1976a), inducible (Huffaker and Rains, 1978) process. These are the characteristics of a carrier system (Rao and Rains, 1976b, Jackson **et al** , 1973). Other passive or non-saturable components also occur (Huffaker and Rains, 1978). These uptake carriers can be quantified in an analogous manner to enzymes using Michaelis-Menten parameters. A measure of the affinity (Km) for the transported ion and the maximum rate (Imax) of inward movement can be assessed by using the ion and its analogues. Nitrate is also accumulated in vacuoles and remobilised from them (Beevers and Hageman, 1969; Aslam **et al**, 1976) to be exported through the xylem and taken up by leaf cells for reduction or sequestration into vacuoles (Aslam **et al**, 1976; Ferrari **et al**, 1973). The regulation of the processes of

Figure 1

Pathway of nitrate assimilation in higher plants

The following numbers indicate:

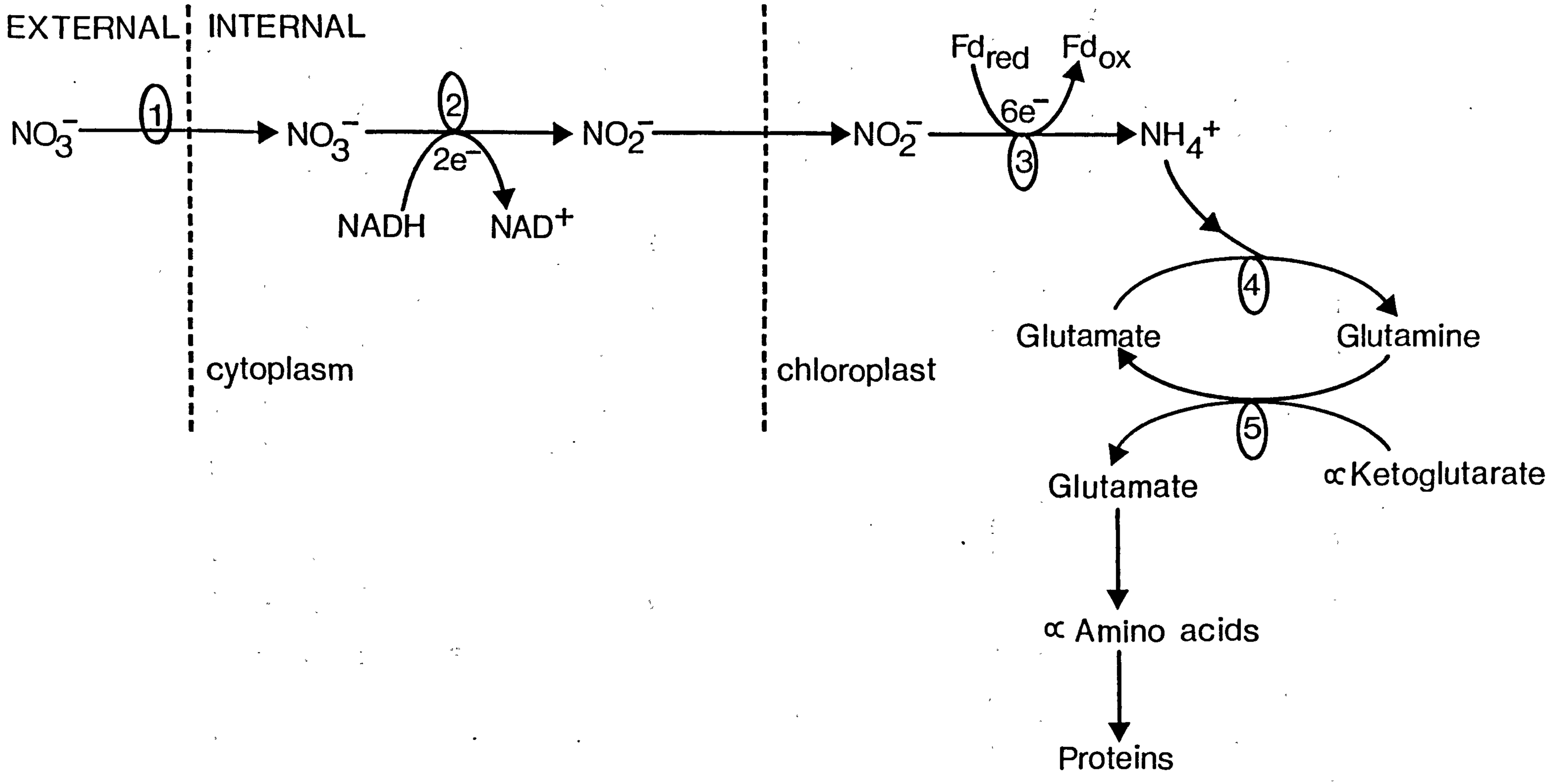
1 = NO3- uptake

2 = nitrate reductase

3 = nitrite reductase

4 = glutamine synthetase (GS)

5 = glutamate synthase (GOGAT)


EXTERNAL
INTERNAL
NO_3^-
1
NO_3^-
2
$2e^-$
NADH
NAD^+
NO_2^-
NO_2^-
Fd_{red}
Fd_{ox}
$6e^-$
3
NH_4^+
4
Glutamate
Glutamine
5
Glutamate
∝ Ketoglutarate
∝ Amino acids
Proteins
cytoplasm
chloroplast

uptake, transport, reduction and storage are largely unknown. Higher plant mutants with impaired nitrate uptake systems would provide a handle with which to start identifying the genes directly involved in these steps.

1.2 Nitrate reductase

Nitrate reductase (NR) converts nitrate to nitrite in a two electron step (figure 1). Nitrate reductase will be discussed in further detail in Section 2.

1.3 Nitrite Reductase

Nitrite reductase (NiR) in a six electron step converts nitrite to ammonium (figure 1).

The assimilatory nitrite reductase (EC.1.7.7.1) of higher plants, green algae and red algae is a chloroplastic enzyme which uses ferredoxin as the physiological electron donor (Guerrero **et al**, 1981).

This enzyme has been purified from squash (Hucklesby **et al**, 1976), spinach (Vega and Kamin, 1977), barley (Ida **et al**, 1974) and wheat (Small and Gray, 1984). NiR from these sources are monomers with molecular weights of approximately 60 kilodaltons (kDa). Nitrite reductase possesses an unusual iron-tetrahydroporphyrin, sirohaem (Scott **et al**, 1978). Iron-sulphur centres (Fe4S4) are also a feature of nitrite reductase (Guerrero **et al**, 1981).

1.4 Glutamate synthase cycle

This cycle involves the sequential action of glutamine synthetase (GS EC.6.3.1.2) and glutamate synthase (GOGAT) as shown in figure 2. The glutamine synthase (GS/GOGAT) cycle also reassimilates the ammonium released during photorespiration (see review Wallsgrove **et al**, 1983).

There are two isoenzymes of glutamine synthetase (GS) found in leaves of **Glycine max, Hordeum vulgare, Oryza sativa, Pisum sativum** and **Phaseolus vulgare** (see review Miflin and Lea, 1982). One, GS1, is found in the cytosol, and the other, GS2, is located in the chloroplast (Lara **et al**, 1984). In **P. vulgare** nodules there are two isoforms of glutamine synthetase; one, GSn1, is nodule-specific; while the other, GSn2, is identical to the leaf cytosolic form (GS1) and is also found in the roots (Lara **et al**, 1984). The different isoforms are made up of different combinations of electrophoretically distinct polypetides (Lara **et al**, 1984).

It has been shown that GS2 (chloroplastic) is developmentally regulated during the greening process of etiolated **Triticum aestivum** leaves, whilst GS1 is not (Tobin **et al**, 1985). The absence in the root, nodules and embryo leaves of the polypeptides which form GS2 implies that this GS2 isoform could be encoded for by a gene (or genes) that is only expressed in mature leaves. The chloroplastic (GS2) isoform is a multimeric enzyme (Lara **et al**, 1984, McNally **et al**, 1984). It is not clear at present whether these constituent polypeptides are encoded by several genes or whether they arise from post-transcriptional modifications.

Figure 2

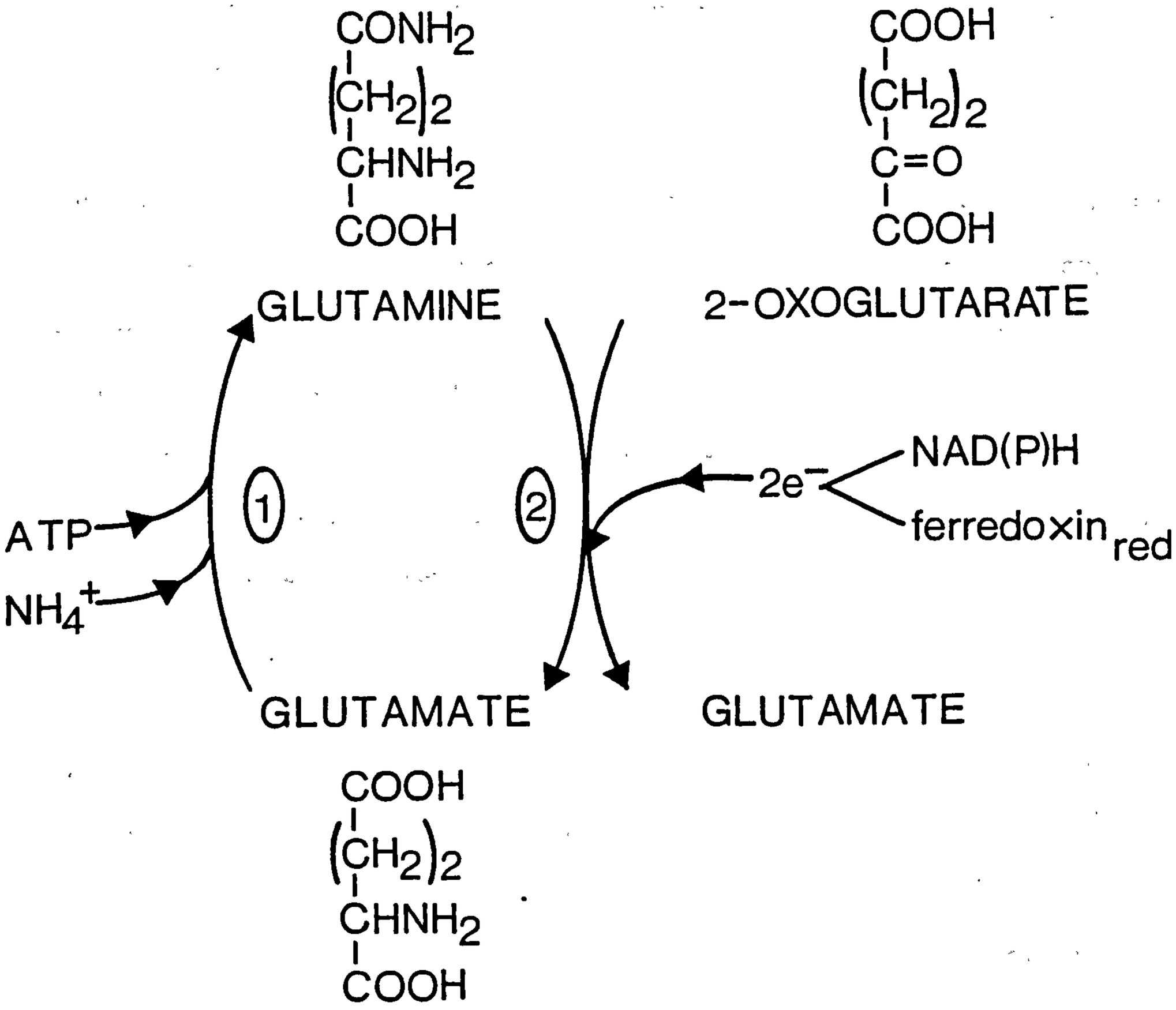
The glutamate synthase (GS/GOGAT) cycle

The following numbers indicate:

1 = glutamine synthetase (GS)

2 = glutamate synthase (GOGAT)

(from Wallsgrove **et al**, 1983)


GLUTAMINE
2-OXOGLUTARATE
ATP
NH_4^+
1
2
$2e^-$
NAD(P)H
ferredoxin red
GLUTAMATE
GLUTAMATE

The glutamine synthetase system in higher plants is therefore amenable to study at the molecular level. It would increase the understanding of the nitrate reductase enzyme if similar molecular questions could be asked.

Glutamate synthase (GOGAT) exists in higher plants in two forms, one utilising NAD(P)H as reductant (EC.1.4.7.14) and the other requiring reduced ferredoxin (EC. 1.4.7.1). The ferredoxin-dependent enzyme occurs in shoot and root tissue from a wide selection of higher plants, cyanobacteria and green algae (Stewart **et al**, 1980). The NAD(P)H-dependent glutamate synthase has been found in all non-green tissues studied (Stewart **et al**, 1980).

1.5 The importance of nitrate reductase in nitrate assimilation

The level of NR activity in plants influences the flow of nitrogen through the nitrate assimilation pathway.

As can be concluded from the following facts, its is possible that nitrate reductase is the rate limiting step.

i) Whilst nitrate may accumulate to high levels in plant tissues, nitrite and ammonium do not (Spencer, 1958; Hageman **et al**, 1967).

ii) Extractable NR activity and NR protein are induced in most nitrate assimilating organisms (see reviews by Beevers and Hageman, 1969; Oaks and Hirel, 1985).

iii) Ammoniumaccumulates in cases where GS is inhibited by the chemical methionine sulphoximine (MSO) (Arima, 1979; Arima and Kumazawa, 1979) or genetic means (Oaks and Hirel, 1985).

iv) In maize (Zieserl **et al**, 1963), Populus (Dykstra, 1974) and Triticale (Singh **et al**, 1976) increased nitrate reductase correlated with increased plant growth.

v) In wheat (Eilrich and Hageman, 1973; Johnson **et al**, 1976) and maize (Deckard **et al**, 1973) nitrate reductase activity has been correlated with grain protein and nitrogen content.

However, it is also possible that although NR is highly regulated and affected by many factors (see Section 7), it is not the rate limiting step of this pathway, but is rather one of several control points each modifying the flux of nitrate in response to different signals from other areas of metabolism. This flux of nitrogen is thought to be in balance with both carbon and sulphur metabolism within the organism (Huffaker and Rains, 1978; Aslam and Oaks, 1975; Reuveny **et al**, 1980). Evidence supporting this includes the work reported by Oh **et al** (1980). They showed that the total N and percentage grain protein in barley NR deficient plants (possessing low levels of **in vitro** NR activity throughout growth) can utilise nitrate as an N source and that NR activity is not closely associated with percentage grain protein (Oh **et al**, 1980).

Reed and Hageman (1980) showed that nitrate flux in **Z. mays** was not associated with the levels of leaf NR activity, again supporting the second theory.

It is clear that nitrate reductase is a key enzyme in the pathway whether or not it is the rate limiting step of nitrate assimilation.

The isolation of NR minus mutants would allow a closer examination of the role of NR and would lead to the analysis of the interactions of the genes controlling the levels of NR activity, to give a better understanding of the regulation of NR at the molecular level.

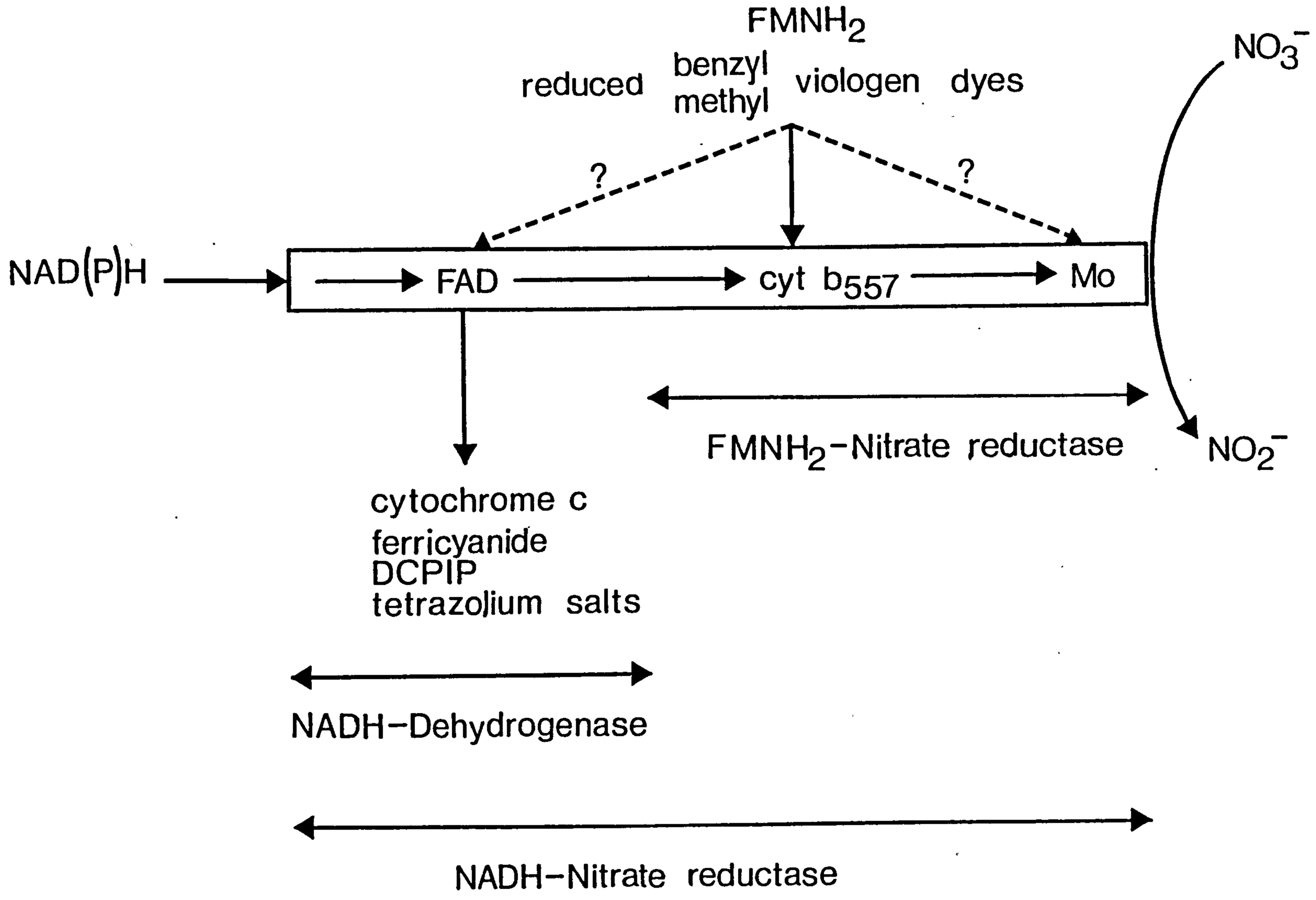
2 THE ASSIMILATORY NITRATE REDUCTASE FROM HIGHER PLANTS

Nitrate is reduced to nitrite by nitrate reductase. There are two distinct forms of nitrate reductase in higher plants, one which uses NADH specifically as an electron donor, whilst the other is a bispecific enzyme which can utilise NADPH or NADH. The presence of the bispecific enzyme, the relative proportions of the two isoforms and their regulation differ between species (Heath-Pagliuso **et al**, 1984). The most abundant form of nitrate reductase in higher plants is the NADH specific enzyme (EC.1.6.6.1). The best characterised enzymes are those from barley (**Hordeum vulgare**), spinach (**Spinacae oleracea**), squash (**Cucurbita maxima**) and tobacco (**Nicotiana tabacum**).

The assimilatory nitrate reductases are soluble, electron transferring molybdoproteins of 150 to 250 kDa molecular weight (Notton **et al**, 1977; Mendel and Müller, 1980; Small and Wray, 1980, Kuo **et al**, 1982; Redinbaugh and Campbell, 1985) (table 1). Enzymically active higher plant NR consists of two apoprotein subunits and one or two low molecular weight molybdenum-containing co-factors (MoCo). Redinbaugh and Campbell (1985) suggest that two MoCo are present in active NR from

Figure 3

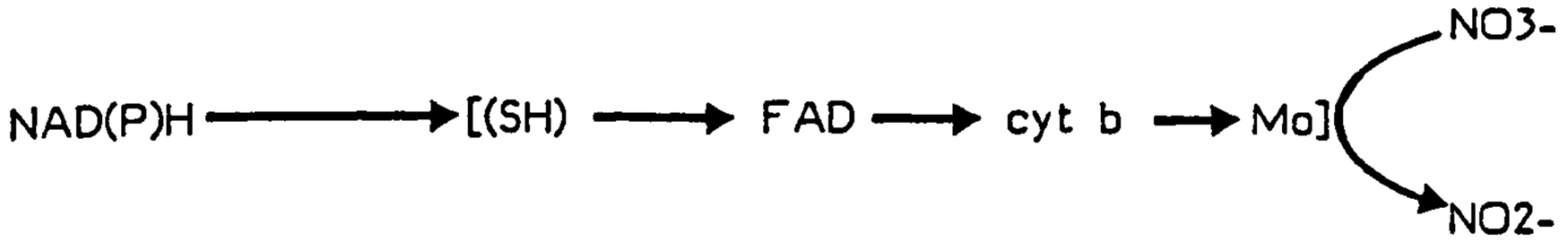
In vitro activities of nitrate reductase


FMNH2
reduced benzyl methyl viologen dyes
NO3−
?
?
NAD(P)H
FAD
cyt b557
Mo
FMNH2-Nitrate reductase
NO2−
cytochrome c
ferricyanide
DCPIP
tetrazolium salts
NADH-Dehydrogenase
NADH-Nitrate reductase

squash since a purified sample was found to contain approximately 0.7 mol molybdenum/mol of subunit. It is also known that molybdenum is easily lost from molybdenum-containing enzymes on purification (Coughlan, 1980). One MoCo per subunit is also the situation found for the algal NR of **C. vulgaris** (Howard and Solomonson, 1982). The fungal NR from **N. crassa** appears to be dimer sharing one MoCo (Pan and Nason, 1978).

2.1 The NR reaction

The generally accepted sequence of the electron transport chain within the enzyme is given below:



from Garrett and Amy (1978)

In the plant nitrate is reduced to nitrite at the molybdenum reaction site, which is separated from the pyridine nucleotide oxidation site by the haem group, cytochrome b557, and FAD (Campbell and Smarrelli, 1978).

A sulphydryl group required for pyridine nucleotide oxidation has been implicated by the inhibition of the pyridine nucleotide linked nitrate reductase by p-hydroxymercuribenzoate (pHMB) (Garrett and Nason, 1969; Schrader **et al**, 1968).

The oxidation of the pyridine nucleotide is followed by reduction of the flavin region of the enzyme. The flavin found in all higher plant NR is FAD (Hewitt, 1975; Hewitt and Notton, 1980; Mendel and Müller, 1979; Campbell and Wray, 1983; Redinbaugh and Campbell, 1985). The higher plant FAD is less tightly bound to the enzyme when compared to that found in **Chlorella** (Ullrich, 1983).

The cytochrome b associated with NR activity was first identified as b557 in **N. crassa** (Garrett and Nason, 1969) and later confirmed for the nitrate reductase from **H. vulgare** (Somers et al, 1982), **N. tabacum** (Mendel and Müller, 1980) and **S. oleracae** (Notton **et al**, 1977). This cytochrome b557 was also detected in algal NR (Solomonson **et al**, 1975; Dela Rosa **et al**, 1981) and yeast NR (Guerrero and Gutierrez, 1977), again indicating the similarity of the basic components of NR from different phylogenic sources. Notton and Hewitt (1971) provided direct evidence for the presence of molybdenum in higher plant NR using radioactive molybdenum.

2.2 In vitro NR activities

Under **in vitro** conditions nitrate reductase has two additional types of non-physiological activities (as well as the physiological reduction of nitrate); a dehydrogenase (diaphorase) activity (in the presence of reduced pyridine nucleotides) which is independent of nitrate reduction (Wray and Filner, 1970) and a reduction of nitrate with reducing power generated from reduced violegen dyes or flavins (Creswell **et al**, 1965; Schrader **et al**, 1968; Wray and Filner, 1970) (figure 3).

Altered levels of activity and inhibition of these partial activities have been useful in defining the sequence of the components within the electron transport chain. The diaphorase activity (usually measured as cytochrome c reductase (CR) activity) is pyridine nucleotide linked, and at the proximal end of the electron transport chain, it mediates the reduction of electron acceptors such as cytochromes c (Wray and Filner, 1970), ferricyanide (Pateman **et al**, 1967) or dichlorophenolindophenol (DCPIP) (Nicholas and Nason, 1954) (figure 3).

Cyanide, a metal binding agent, inhibits only the reactions involving the molybdenum moiety, and has no effect on the dehydrogenase activity (characterised by NADH-cytochrome c reductase, NADH-CR, activity) of nitrate reductase (Garrett and Nason, 1969; Hewitt and Notton, 1980). This is also true after treatment with azide, or organic chelating agents resulting in the inhibition of all NR activities excluding NADH-CR activity (Smarrelli and Campbell, 1979). It is well established that the activities associated with molybdenum are at the distal end of the electron transport chain and that the terminal electron donor is the molybdenum within MoCo (Hewitt and Notton, 1980). The NADH-CR activity can be independent of the reduction of nitrate since electrons are not passing through the molybdenum utilising section of the electron transport chain (figure 3). The NADH-CR and molybdenum requiring moieties participate sequentially in the transfer of electrons from NAD(P)H to nitrate (Losada and Guerrero, 1979). The molybdenum-containing cofactor (MoCo) is discussed in greater detail in Section 5.

3. LOCATION OF HIGHER PLANT NITRATE REDUCTASE

Nitrate reductase and the NR protein is extrachloroplastic in higher plants (Ritenour **et al**, 1967). Both Ritenour **et al** (1967) and Wallsgrove **et al** (1979) used centrifugation to separate the subcellular fractions of green leaf tissue (**in Z. mays** and **P. sativum** respectively) to show that whilst nitrite reductase activity was found wholly in intact chloroplasts, NR activity was non-chloroplastic and probably not associated with any cell membrane (Wallsgrove **et al**, 1979). Vaughn **et al** (1984) used immunofluorescence to confirm this for soybean cotyledons. Chloroplastic nitrite reductase is closely linked to photosynthesis since it requires reduced ferredoxins to provide electrons (Vega and Kamin, 1977).

4. PHYSIOLOGICAL ELECTRON DONORS FOR NITRATE REDUCTASE

Assimilatory nitrate reductases can be split into two classes depending on their specificity for the electron donor; 1) ferredoxin-dependent and 2) pyridine nucleotide-dependent. There are substantial differences between the two classes.

4.1 Ferredoxin-NR

Ferredoxin dependent NR species are found in cyanobacteria (Hattori, 1970; Manzano **et al**, 1976; Ortega **et al**, 1976) and photosynthetic bacteria (Alef and Klemme, 1979, Malofeera **et al**, 1975). The NR enzymes of photosynthetic bacteria are tightly bound to photosynthetic

membranes (Ortega **et al**, 1976).

4.2 Pyridine Nucleotide-NR

There are three subclasses of pyridine nucleotide dependent NR; NADH specific (EC.1.6.6.1) NADPH specific, (EC.1.6.6.3) and NAD(P)H bispecific, (EC.1.6.6.2).

4.2.1 NADH-specific NR

This was discussed in Section 2 and will be described in greater detail in Section 6.

4.2.2 NADPH specific NR

The NADPH specific NR is found in all fungi that reduce nitrate (Garrett and Amy, 1978). It has been characterised from **Aspergillus nidulans** (MacDonald and Coddington, 1974), **Neurospora crassa** (Pan and Nason, 1978) and **Penicillium chrysogenum** (Renosto **et al**, 1981).

NADPH-NR activity has also been found in some green algae including **Dunaliella tertiolecta** (LeClaire and Grant, 1972) and **Chlamydomonas reinhardii** (Sosa and Cardenas, 1977).

4.2.3 NAD(P)H bispecific NR

Higher plant bispecific NR has been briefly discussed in Section 2. The bispecific NAD(P)H NR has been identified in most green algae, excluding **Chlorella vulgaris** (Ahmed and Spiller, 1976; Diez **et al**, 1979; Solomonson, 1979) and including both yeasts **Rhodotorula glutinis** (Guerrero **et al**, 1977) and **Torulopsis nitratophila** (Rivas **et al**, 1973) as the most prevalent form of nitrate reductase. In rice (**Oryza sativa**) a chloramphenicol induced bispecific NAD(P)H-NR (which

glutinis (Guerrero **et al**, 1977) and **Torulopsis nitratophila** (Rivas **et al**, 1973) as the most prevalent form of nitrate reductase. In rice (**Oryza sativa**) a chloramphenicol induced bispecific NAD(P)H-NR (which was independent of the nitrate induced NADH-NR) was also found (Shen, 1972; Shen **et al**, 1976), suggesting that rice seedlings have both NAD(P)H and NADH NR activities (Shen **et al**, 1976). Soybean leaves also contain both forms of higher plant NR (Campbell, 1976).

Bispecificity is thought to reside in a single enzyme since both activities (NADH-NR and NADPH-NR) are found in the same fraction after affinity chromatography on Blue Dextran Sepharose (Campbell, 1976; Shen **et al**, 1976) or from DEAE-cellulose (Jolly **et al**, 1976), both have the same pH optimum (Dailey **et al**, 1982b; Campbell, 1976) and have the same rate of decay (Kleinhofs pers. comm.). These bispecific enzymes may be smaller than their NADH specific counterparts (Redinbaugh and Campbell, 1981). The NAD(P)H bispecific NR found in soybean has a lower affinity for nitrate, with a Km value of 4.5 mM, than its NADH specific NR (0.11 mM). The presence of bispecific NR has also been reported in the **narla** mutant of barley although it was not detected in the wild type, cv. Steptoe (Dailey **et al**, 1982b). Amongst the higher plants, the tropical legume **Erythrina senegalensis** is unique as it possesses only the bispecific NAD(P)H-NR enzyme (Stewart and Orebamjo, 1979).

The two types of higher plant NR enzymes show different induction (Shen **et al**, 1976) and developmental (Oriheul-Iranzo and Campbell, 1980) patterns.

5. THE MOLYBDENUM CONTAINING COFACTOR OF NR

5.1 Structure

The existence of a dialysable, molybdenum-containing cofactor (MoCo) common to NR and xanthine dehydrogenase (XDH) was first postulated by Pateman **et al** (1964) after studying the pleiotrophic loss of NR and XDH from a series of **A. nidulans** mutants. They further suggested that this common cofactor was synthesised and regulated by the products of at least five independent cnx genes (Pateman **et al**, 1964).

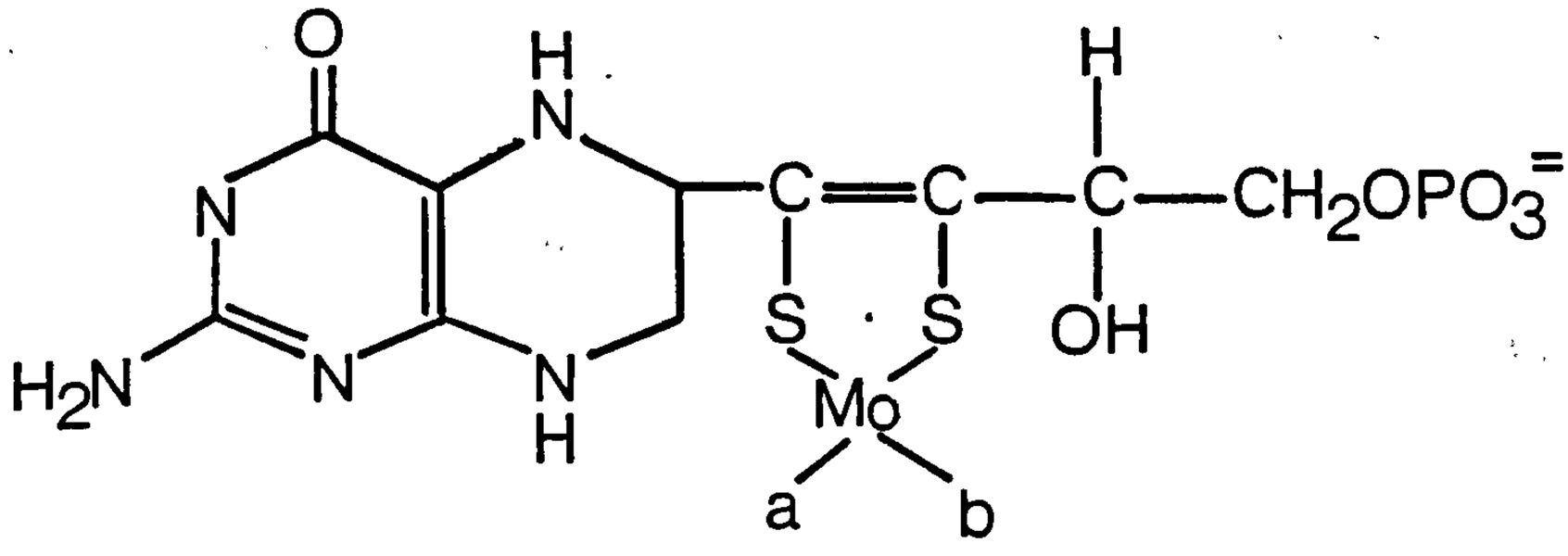
Shah and Brill (1977) and Pienkos **et al** (1977b) found that the cofactor from component I of the molybdoenzyme nitrogenase contains both iron and molybdenum and is significantly different from the NR molybdenum-containing cofactor (MoCo). The other molybdoenzymes, xanthine oxidase, sulphite oxidase, aldehyde oxidase and formate dehydrogenase obtained from diverse bacterial, fungal, plant and animal sources all possess the same molybdenum-containing cofactor as NR (MoCo) (Johnson, 1980). MoCo has a molecular weight of between 1000 (Tachiki and Nason, 1982) and 2000 Da (Johnson, 1980) which is dialysable and very sensitive to oxygen (Lee **et al**, 1974, Amy and Rajagopalan, 1979). In many organisms MoCo is apparently synthesised in excess of its requirement as a prosthetic group for the various molybdoenzymes (Johnson, 1980). This form of 'free' MoCo has been found as part of a macromolecular complex, in **E. coli** this was a 40 kDa carrier molecule (Johnson **et al**, 1977) and in **Clostridium thermoaceticum** a 60 kDa protein was involved (Ljungdahl and Andreesen, 1975). Although the nature of the carrier protein seems to vary from organism to organism

Figure 4

Proposed structure of MoCo

Where a and b indicate molybdenum ligand binding sites which are supplied by the apoprotein subunits.

(Johnson et al, 1984)


O
H
N
N
N
N
N
H
H
C
C
C
H
CH2OPO3=
S
S
OH
Mo
a
b
H2N

it would seem to have the same function of protection against oxygen and also possibly the aqueous environment (Johnson, 1980). This association must also allow access to MoCo by the receptor enzyme and permit the transfer of MoCo to the enzyme. The instability of MoCo has prevented an accurate determination of its molecular weight and structure. However, the following details have been discovered.

The molybdenum is chelated to an unusual phosphorylated pterin structure (figure 4) (Johnson **et al**) 1980; Johnson **et al,** 1984). Although Johnson **et al** (1984) proposed this model for the stucture of MoCo, the length of the side-chain on the pterin structure, the oxidation state of molybdenum and the liganding of sulphur to the molybdenum are still in debate (Kleinhofs **et al,** 1985). X-ray absorption studies suggest that the molybdenum in MoCo from various sources is surrounded by sulphur and oxo-groups (Cramer **et al,** 1981). Nitrate reductase appears to have two oxo-groups as the terminal ligands at positions a and b (figure 4).

There is also a possibility of the presence of a short peptide component in MoCo since MacDonald and Cove (1974) discovered a temperature sensitive **cnxH** mutant with a thermolabile NR activity in **A. nidulans.** This peptide has not been identified.

5.2 Function

MoCo is non-covalently bound to the apoprotein and appears to have two functions, namely catalytic reduction of nitrate and mediating the assembly of the subunits to form dimeric NR (Ketchum **et al,** 1970; Nason **et al,** 1970; Mendel and Müller, 1980).

MoCo is essential for nitrate reduction, but is not required for the NR-associated CR activity, as can be seen with MoCo defective mutants (MacDonald **et al,** 1974; Mendel and Müller, 1980). These two activities (catalytic and assembly) seem to reside in different regions of MoCo since some mutations in **N. tabacum** (Mendel and Müller, 1980; Alikulov and Mendel, 1984) and **Arabidopsis thaliana** (Braaksma and Freenstra, 1982b) allow dimerisation whilst others do not. This is also true for the MoCo of the fungi, **N. crassa** (Ketchum **et al,** 1970) and **A. nidulans** (MacDonald **et al,** 1974). Kramer **et al** (1984) showed that the molybdopterin part of MoCo was defective in the **N. crassa nit1** mutant which does not form dimers.

Chlorella vulgaris grown without added molybdenum produced an inactive 'demolybdo' NR which was of the same molecular weight as the native holoenzyme. This could be activated both **in vivo** and **in vitro** by the addition of molybdate (Gewitz **et al,** 1981; Ramadoss **et al** 1981). Mendel and Müller (1980) found that dimerisation is independent of the presence of molybdenum within the MoCo. This direct evidence supports the previous work by Wray and Filner (1970), who showed that NR containing tungsten instead of molybdenum was an inactive dimer.

Sulphydryl groups on the apoprotein subunits and MoCo are important for the dimerisation of NR subunits (Alikulov and Mendel, 1984). Wahl **et al** (1984) confirmed this by using the thiol-blocker, N-ethylmaleimide to prevent the dimerisation of the NR subunits in the presence of exogenous active MoCo.

Table 1

Molecular weights and dimensions of NR

References

1. Amy and Garrett (1974)
2. Guerrero and Gutierrez (1977)
3. Dela Rosa **et al** (1980)
4. Dela Rosa **et al** (1981)
5. Solomonson **et al** (1985)
6. Howard and Solomonson (1982)
7. Giri and Ramadoss (1979)
8. Renosto **et al** (1981)
9. Horner (1983)
10. Garrett and Nason (1969)
11. MacDonald and Coddington (1974)
12. Minagawa and Yoshimoto (1982)
13. Downey and Focht (1974)
14. Cooley and Tomsett (1985)
15. Notton **et al** (1977)
16. Nakagawa **et al** (1985)
17. Redinbaugh and Campbell (1985)
18. Mendel and Müller (1980)
19. Campbell and Wray (1983)
20. Small and Wray (1980)
21. Kuo **et al** (1982)

Table 1
Molecular weights and dimensions of NR

Species	total	subunit	(n)	S-value	radius (nm)	f/f'	Ref
DIATOMS							
T. pseudonana	330			10.5	7.7	1.69	1
YEAST							
R. glutinis	230	118	(2)	7.9	7.05		2
ALGAE							
A. braunii	460	58	(8)	10.9	9.8	1.92	3
	467	59	(8)				4
C. vulgaris	356	100	(3)	9.7	8.9		5
	360/180	90	(2-4)		8.1	1.52	6
	280	90	(3)	10.0		1.45	7
FUNGI							
P. chrysogenum	199	97-98	(2)	7.4	6.3		8
N. crassa	228	145	(2)				9
	228	115	(2)	8.0	7.0	2.4	10
A. nidulans	190			7.6	6.3	1.7	11
	180	59		7.6	5.6	1.51	12
	196	49	(4)				13
	190-180	91	(2)				14
HIGHER PLANTS							
S. oleracea	197			8.1	6.0	1.55	15
	207	114	(2)	10.5	6.3		16
C. maxima	230	115	(2)				17
N. tabacum	200	90	(2)	7.6			18
H. vulgare	205	103	(2)				19
	203			7.7	6.4	1.65	20
	221	100	(2)	7.9	6.8		21

6. HIGHER PLANT NITRATE REDUCTASE

6.1 Physical and kinetic parameters

The apparent Km for nitrate in higher plant NR ranges from 0.07 mM for the maize enzyme (Campbell and Smarrelli, 1978) to 4.5 mM for the NAD(P)H-NR of soybean, using NADPH as the donor (Campbell, 1976) (table 2). The apparent Km (NO3-) values for NR of fungi (Minagawa and Yoshimoto, 1982; Garrett and Nason, 1969) and yeast (Guerrero and Gutierrez, 1977) also fall within this range (table 2).

The highest specific activity quoted for purified spinach NR is 130 µmol NO3- reduced/min/mg protein (Nakagawa **et al**, 1985). This value compares well with the specific activities obtained for fungi and yeast (table 2), but is significantly higher than that reported for NR from other higher plants (table 2). This might reflect the problems experienced in extracting active higher plant nitrate reductases (Nakagawa **et al**, 1985).

Stokes radii range from 6.0 nm for spinach NR (Notton **et al**, 1977) to 6.8 nm for barley (Kuo **et al**, 1982) and are much smaller than the algal values. The Stokes radii of the fungal NRs seem to vary more than those from higher plants (table 1). The frictional ratios of the NR species given in table 1 lie between 1.45 and 1.92. It is noteworthy that values around 1.5 are obtained from **A. nidulans, C. vulgaris** and **S. oleracea** which are indicative of a spherical shape (Minagawa and Yoshimoto, 1982). For the first two species at least, there are suggestions of tetrameric and trimeric forms of NR (Downey and Focht,

Table 2

Physical parameters of NR

References

1. Amy and Garrett (1974)
2. Guerrero and Gutierrez (1977)
3. Dela Rosa **et al** (1980)
4. Dela Rosa **et al** (1981)
5. Renosto **et al** (1981)
6. Minagawa and Yoshimoto (1982)
7. Garrett and Nason (1969)
8. Nakagawa **et al** (1985)
9. Mendel and Müller (1980)
10. Campbell and Wray (1983)
11. Kuo **et al** (1980)
12. Dailey **et al** (1982a)
13. Campbell (1976)
14. Redinbaugh and Campbell (1985)
15. Campbell and Smarrelli (1978)

Table 2
Physical parameters of NR

Species	Apparent Km (donor) µM	Km (NO3-) mM	pH Optimum	Specific Activity nmol/ min/mg Protein	Ref
DIATOMS					
T. pseudonana	15	0.062	7-8	2,050	1
YEAST					
R. glutinis	160 NADH	0.45 NADH		177,800	2
	20 NADPH	0.125 NADPH		177,800	2
ALGAE					
A. braunii				61,250	3
				80,000	4
FUNGI					
P. chrysogenum	10			225,000	5
A. nidulans	45	0.2	7-8	15,500	6
N. crassa	62	0.2	7-8	129,500	7
HIGHER PLANTS					
S. oleracea				130,000	8
N. tabacum	7.5	0.2	7.5	1,400	9
H. vulgare					
cv. Golden Promise				6,480	10
cv. Steptoe		0.24 NADH	7.5	800	11
		NAD(P)H	6-6.5		12
G. max NADH	8	0.11	6.5	360	13
NAD(P)H	3.9 NADH 1.5 NADPH	4.5	6.5	650	13
C. maxima				95,000	14
C. pepo			7.5	1,900	15
Z. mays	3 NADH	0.07	7.5	6,900	15

1974; Giri and Ramadoss, 1979; Howard and Solomonson, 1982), which might account for a more spherical shape than the NR from **Ankistrodesmus braunii** with a Stokes radius of 1.92 (table 1).

The implications from the Stokes radii and frictional ratios are that the higher plant and fungal NR enzymes are cigar-shaped, which would describe spheres of radii 5 to 7 nm if left to rotate through a viscous solution. Algal NR enzymes are of similar shape but are of greater length describing spheres of 8 to 10 nm in radius under the same conditions. The pH optima for higher plant NADH-NR enzymes are either pH 6.5 for **G. max** (and NAD(P)H-NR), or pH 7.5 for **H. vulgare, Z. mays** and **C. pepo** (table 2). The pH optima for fungal and diatom NR enzymes were broader, covering the pH range of 7-8 (table 2).

6.2 Molecular weights

Higher plant NRs have molecular weights ranging from 197 kDa for spinach (Notton **et al,** 1977) to the value given for squash of 230 kDa (Redinbaugh and Campbell, 1985). In comparison, the algal nitrate reductases have molecular weights of 356 kDa (Solomonson **et al,** 1975) and 467 kDa (Dela Rosa **et al,** 1981) for **C. vulgaris** and **A. braunii** respectively. Whilst NR from the fungus **A. nidulans** has a molecular weight of 180-190 kDa (Downey and Focht, 1974; Minagawa and Yoshimoto, 1982; MacDonald and Coddington, 1974) the fungal NR from **P. chrysogenum** and **N. crassa** have molecular weights of around 200 kDa (table 1).

The sedimentation coefficients for higher plant NR range from 7.6 S for

tobacco (Mendel and Müller, 1980) to 8.1 S for spinach (Notton **et al,** 1977). These are much smaller than the algal S values (table 1). The S values of the fungal enzymes are very similar to those found in higher plants (table 1).

One of the major problems in determining the molecular weight subunit sizes of NR from higher plants has been the interpretation of the multiple protein bands found on sucrose density gradients, polyacrylamide gels (native and denaturing) and gel filtration. Spinach nitrate reductase when treated with sodium dodecyl sulphate (SDS) and electrophoresed shows clear multiple bands of 37 kDa, 73 kDa, and 120 kDa. However, when subjected to sucrose density gradients, the calculated fragment sizes were 45 kDa, 85 kDa, 120 kDa and 152 kDa (Notton **et al,** 1979). In tobacco there is a similar situation (Mendel and Müller, 1980), two major and two minor bands were found with molecular weights between 40 and 70 kDa on denaturing gel electrophoresis. In barley it seems to be more complicated still. Five bands are found in an SDS gel after electrophoresis of a highly homogeneous preparation of the NR subunit (110 kDa, 60 kDa, 41 kDa, 35 kDa and 25 kDa) (Kuo **et al,** 1982). Only the 110 kDa fragment had diaphorase (NADH-CR) activity. Kuo **et al** (1982) suggested that the fragments of molecular weights less than 100 kDa were contaminants which were present throughout the purification; they show no homology with the NR monomer when subjected to tryptic cleavage and mapping of fragments. However, these band sizes are very similar to those found by Small and Wray (1980) and Campbell and Wray (1983) which are as follows; 105-103 kDa (monomer), 59-61 kDa, 38-40 kDa, 20-28 kDa.

Campbell and Wray (1983) put forward the hypothesis that the fragments which they identified after SDS electrophoresis were specific sizes of proteolytic degradation products of the NR macromolecule and were not (under their conditions) contaminants. They (Campbell and Wray, 1983) argued that the extra purification step of gel-filtration was used (unlike Kuo **et al**, 1982) which would have excluded contaminating species of 59 kDa and 38 kDa, and therefore the contaminants described by Kuo et al (1982) are unlikely to be the same proteins as those described by Campbell and Wray (1983) in their fragmentation pattern.

Wray and Kirk (1981) proposed that some 'hinge' regions between the various functional domains were more sensitive to proteolytic attack. Finlayson (1984) argues that the functionally intact, independently folded regions of the polypeptide chains are held together by flexible, loosely structured hinge regions which are highly susceptible to proteinase attack. This theory is supported by earlier work which indicated that the FAD and cytochrome b557 regions, in proteins other than NR, were separated by a region of hyper-sensitivity to proteolytic attack (Jacq and Lederer, 1974). The region containing cytochrome b557 with the FAD function is known to be distinct from the molybdenum containing region (Johnson and Rajagopalan, 1977; Southerland and Rajagopalan, 1978).

6.3 Subunit composition of NR

The higher plant NR enzymes which have been purified all seem to be homodimers (table 1). Purified algal nitrate reductase possesses two

or four subunits (giving total molecular weights of 180 kDa and 360 kDa respectively) depending on the concentration of NR (Howard and Solomonson, 1982). The NR from **N. crassa** has been purified and characterised as a homodimer with subunits of 145 kDa (Horner, 1983). The subunit composition of **A. nidulans** NR was less well defined with reports of tetrameric NR consisting of subunits of various molecular weights (table 1) ranging from 49 kDa to 54 kDa (MacDonald and Coddington, 1974; Minagawa and Yoshimoto, 1982; Steiner and Downey, 1982). Cooley and Tomsett (1985) have recently used immunoprecipitation (using monoclonal antibodies) to speed up the extensive purification procedure of **A. nidulans** nitrate reductase. They isolated a single 91 kDa polypeptide from various constitutive and nitrate inducible NR mutants and suggest that this is the subunit size of **A. nidulans** (Cooley and Tomsett, 1985). Before the inclusion of proteinase inhibitors was standard practise the subunit composition was difficult to determine due to the instability of the NR enzyme whilst purification took place (see below).

6.4 Stability

Nitrate reductase is unstable **in vitro.** If either casein or BSA is included in the extraction buffer this reduces proteolytic decay increasing the half life for NR activity in a crude extract (from 6 day old barley) to 64 minutes (Wray and Kirk, 1981). It was found that if 10 μM leupeptin was added in addition to casein or BSA, the half life of the barley NR was increased to 470 minutes. However, the serine proteinase inhibitor, phenyl methyl sulphonyl fluoride (PMSF) had very little effect (Wray and Kirk, 1981). Leupeptin is the generic name of

a group of compounds found in the culture filtrate of Streptomyces species (Aoyagi **et al,** 1969) which are propionyl (or acetyl)-L-leucyl-L-leucyl-L-argininal and their analogues, where leucine is replaced by isoleucine or valine (Aoyagi **et al,** 1969; Kondo **et al,** 1969). Leupeptin competitively inhibits trypsin-like enzymes (Kondo **et al,** 1969). Two types of endoproteinases have been identified in barley primary leaves, a serine proteinase and a thiol endoproteinase (Miller and Huffaker, 1980a and b). Their serine proteinase was unlikely to be the cause of these smaller CR species. As well as maintaining the activity of nitrate reductase, leupeptin also slowed down the rate of fragment formation (Kirk and Wray, 1981). Since barley nitrate reductase, when treated with trypsin, gave very similar size fragments (Brown **et al,** 1981) it was concluded that it was likely that the thiol endoproteinase was mainly responsible for degradation of NADH-NR to smaller NADH-CR species in primary leaf extracts.

7. REGULATION OF HIGHER PLANT NITRATE REDUCTASE

7.1 Substrate induction

The extractable NR activity is increased by feeding plants with nitrate. This induction is a well documented process (see Srivastava, 1980, and Beevers and Hageman, 1969). In some cases considerable amounts of enzyme are found in the absence of nitrate.

This 'constitutive' level of nitrate reductase activity is found in barley roots (Smith and Thompson, 1971), tobacco cells (Müller and Grafe, 1978) and cotton seedlings (Radin, 1974). For example, in both cultured cells and various tissues of tobacco there is up to 20% of the fully induced levels of nitrate reductase when grown on a reduced nitrogen source such as ammonium succinate or amino acids (Müller and Grafe, 1978; Mendel and Müller, 1979; Heimer and Riklis, 1979).

The increase of NR activity in the presence of nitrate could be due to a) **de novo** synthesis of NR protein, b) activation of inactive precursor or c) increased stability of the existing enzyme. Inhibitors of protein and RNA synthesis block NR induction in several higher plants, supporting the theory of **de novo** synthesis of protein as the mechanism responsible for increased NR activity (Beevers and Hageman, 1969; Hewitt, 1975; Oaks **et al**, 1972).

The increase in NR activity in **N. crassa** (Amy and Garrett, 1979; Amy and Garrett, 1980) and in cucumber (Ulitzsch and Shiemann, 1984) was also caused by **de novo** synthesis.

Using polyclonal antibodies, it was found that the increase in nitrate reductase activity under induced conditions was due to **de novo** protein synthesis since there was a parallel increased in cross reacting material (CRM) (Schiemann and Müller, 1985). Had there been no increase in CRM on induction, this would have suggested that the 'induction' of activity was due to the activation of a preformed inactive precursor. When induced barley plants were transferred to a

nitrate-free nutrient solution, both NR activity and CRM levels decreased (Somers **et al,** 1983b). This suggests that proteolysis of nitrate reductase might also be an important regulatory mechanism. It was found that the loss of nitrate reductase activity in **N. crassa** due to nitrogen starvation or presence of ammonium was caused by repression of enzyme synthesis and continued protein degradation (Amy and Garrett, 1980; Sorger **et al,** 1974). There has been no evidence in the literature of increased stability of existing nitrate reductase in the presence of nitrate.

Only in **Chlorella** has the increase in NR activity been shown to be due to activation of a preformed inactive precursor (Sherman **et al,** 1983).

Nitrate reductase activity can be affected by plant age (Brown **et al,** 1981), light (intensity and duration), temperature, ammonia and amino acids, hormones, water stress and O2/CO2 ratios (see reviews by Srivastava, 1980; Beevers and Hageman, 1969).

Although the effects of various environmental and nutritional factors on NR activity are well documented in many systems, the exact mechanism, at the molecular level, are undefined. It is clear, however, that the enzyme is sensitive to various factors and that all possible mechanisms for regulation are so intertwined that mutants and gene probes are necessary to make much further progress.

8. GENETICS AND MOLECULAR GENETICS OF E. COLI NR

E. coli possess a dissimilatory nitrate reductase of 100 kDa molecular weight. This is made up of four A subunits (142 kDa), four B subunits (60 kDa) and eight C subunits (20 kDa). Subunit A is thought to possess the NR catalytic site; the function of subunit B is unclear and subunit C seems to contain the cytochrome b557 which is specific to nitrate reductase (Stewart and MacGregor, 1982). Despite these differences from the eukaryotic NR, the MoCo from **E. coli** is functionally similar to that found in all the systems described above. It was shown to restore NR activity to **N. crassa nit1** extracts (Amy and Rajagopalan, 1979). Since the manipulation and interpretation of **E. coli** genes is relatively straightforward, a large amount of work has been done to clarify the genetics of **E. coli** NR.

The **chlC** gene is thought to code for at least part of the apoprotein structure since DeMoss (1978) found temperature sensitive **chlC** mutants and MacGregor (1975) showed that a **chlC** mutant NR protein was more unstable **in vivo** than the NR protein from the wild type. Stewart and MacGregor (1982) studied the **chlC** mutants further and found that the **chlC** locus encompassed five genes, **narG, narH, narI, narK** and **narL**. Mutants in all of these genes possessed wild type levels of MoCo activity. **narG, narH** and **narI** lacked one or more of the NR polypeptides and **narL** mutants had altered nitrate mediated regulation (Stewart and MacGregor, 1982). The functions of the **narL** and **narK** gene products are unclear.

Five other **chl** genes, **chlA, chlB, chlD, chlG** and **chlE,** were identified; these were concerned with the formation of an active MoCo.

chlB is likely to code for an associated factor responsible for the insertion of MoCo into the appropriate enzyme (Miller and Amy, 1983). The gene product, designated FA, is not required for activity but is needed for the association of the E. **coli** NR apoprotein subunits (Riviere **et al**, 1975).

Sperl and DeMoss (1975) showed that **chlD** mutants, when grown on high levels of molybdate, recovered NR activity. However, if a **chlD** mutant is extracted in the presence of excess molybdate, there is no recovery of NR activity. The **chlD** mutant was shown to contain inactive holoenzyme macromolecules (Sperl and DeMoss, 1975). The MoCo activity of various **chlD** mutants (caused by insertions) depended on the concentration of molybdate present in the growth medium (Sperl and DeMoss, 1975). Miller and Amy (1983) suggested that **chlD** strains produce a cofactor which is unstable at low concentrations of molybdate since **chlD** mutants have wild type levels of molybdate present (Glaser and DeMoss, 1971). **chlG** mutants could also be partially reversed by growth on high levels of molybdate in the medium (del Campillo-Campbell and Campbell, 1982). Grown in the absence of molybdate no MoCo activity was detected, but the levels of demolybdo-MoCo found were comparable to the wild type (Miller and Amy, 1983). From these data Miller and Amy (1983) concluded that the demolybdo-MoCo synthesis was normal in the **chlG** mutants. They suggested that the **chlG** gene may be involved in the transporting and processing of molybdenum. Therefore,

both **chlD** and **chlG** seem to be concerned with the transport, processing and insertion of molybdenum into demolybdo-MoCo to allow the formation of active NR macromolecules.

chlA and **chlE** are both thought to be involved with the formation of the phosphorylated pterin structure since mutants of these loci had no detectable levels of MoCo under any of the growth conditions used (Miller and Amy, 1983).

The molecular characterisation of these genes has been started. The **chlA** (Taylor **et al**, 1983; Giordano **et al**, 1980), **chlC** (Barr and Palm-Nicholls, 1981), **chlB**, **chlD**, **chlE** and **chlG** (Kleinhofs **et al**, 1985) genes have all been cloned. The **E. coli chlC** (one of the NR structural genes) has been cloned in a 4.8 kb Hind III fragment by Barr and Palm-Nicholls (1981). Since multiple copies of the gene did not lead to overproduction of NR, the authors suggest that either the availability of the other subunits limits the enzyme activity or that normal control processes prevent overproduction. Barr and Palm-Nicholls (1981) also suggest that possibly two genes are encoded in this 4.8 kb fragment since one of the **chlC** mutants was not complemented by the cloned DNA. Earlier Orth **et al** (1980) isolated an **E. coli** NR apoprotein mutant, **chlI**, which was tightly linked to the other apoprotein structural gene **chlC**. A more detailed study (Bonnefoy-Orth **et al**, 1981) showed that these two genes were effectively under the control of the same operator-promotor region. It is possible that **chlI** is the second gene thought to be on the 4.8 kb fragment of DNA cloned by Barr and Palm-Nicholls (1981).

9. MOLYBDENUM PROCESSING AND STORAGE PROTEINS

Pienkos **et al** (1977a) found a Mo-storage protein in **A. vinelandii.** This fits in with work carried out later in **Chlamydomonas pasteuranium** as described below.

A total of four molybdenum processing proteins have been identified (Hinton and Mortenson, 1985a). These were designated as the molybdenum binding-storage protein (MoB-SP), a 30 kDa molybdoprotein (Mop), a low molecular weight species and a high molecular weight species (Hinton and Mortenson, 1985a). Following the incorporation of 99Mo under pulse-chase conditions the molybdenum seemed to follow the following route: i) membranes ii) a low molecular species iii) MoB-SP and Mop iv) into the enzyme formate dehydrogenase and the MoFe protein of nitrogenase (Hinton and Mortenson, 1985b). The MoB-SP, 30 kDa Mop and low molecular weight species seem to be likely candidates for the molybdenum processing for its eventual incorporation into the molybdoenzymes, formate dehydrogenase and nitrogenase as their appearance in pulse-chase experiments was either simultaneous with, or just before, the detection of formate dehydrogenase.

10. GENETICS OF NITRATE ASSIMILATION

The use of cell lines or whole plants which have defective nitrate reductase have been extremely useful in determining both the biochemistry and genetics of the nitrate reductase enzyme and

regulation of NR in fungi, algae and higher plant systems.

11. FUNGAL MUTANTS

Fungal nitrate assimilation mutants are well studied due to the ease of selection and genetic analysis. As a result of this a large body of information has been collected, particularly from **Aspergillus nidulans, Neurospora crassa** and **Penicillium chrysogenum.**

The plant NR enzymes show many similarities to the fungal systems, therefore, useful comparisons can be made.

Growth on chlorate (Tomsett and Garrett, 1980; Birkett and Rowlands, 1981) and nitrate non-utilisation (Cove and Pateman, 1969; Pateman **et al,** 1964) have both been used to isolate mutants from **A. nidulans** and **P. chrysogenum.** Using these screens it is possible to isolate six classes of mutants; namely 1) NR apoprotein, 2) NR cofactor (MoCo), 3) nitrite reductase, 4) nitrate uptake, 5) regulatory and 6) other undefined mutants.

11.1 NR apoprotein mutants (structural gene mutations)

A single class of apoprotein mutants have been isolated from **A. nidulans** (MacDonald and Coddington, 1974), **N. crassa** (Dunn-Coleman **et al,** 1984) and **P. chrysogenum** (Birkett and Rowlands, 1981) and were designated **niaD, nit3** , and **nia** respectively.

MacDonald and Coddington (1974) characterised several A. nidulans **niaD** temperature sensitive mutants which could not grow on nitrate, but could survive on nitrite. The nitrate reductase protein of **niaD** was more thermolabile than that of the wild type (MacDonald and Coddington, 1974). This indicates that the **niaD** mutants were caused by mutations in the structural gene, since increased temperature sensitivity implies an alteration in the primary amino acid sequence of a protein. The apoprotein mutants isolated from individual fungal species were all allelic. This suggests that in fungal systems, NR seems to have a single structural gene.

11.2 NR cofactor (MoCo) mutants

In A. nidulans seven MoCo complementation groups and five-genetic loci (**cnxABC** , **cnxE**, **cnxF**, **cnxG** and **cnxH**) were identified (Cove, 1979) by their inability to grow on nitrate or hypoxanthine. Since they all lacked NR and xanthine dehydrogenase activity, Pateman **et al** (1964) suggested that these mutants were defective in a common molybdenum-containing cofactor (MoCo). Scazzocchio **et al** (1973) showed that the xanthine dehydrogenase activity of A. **nidulans** was due to two enzymes, purine hydroxylase I and purine hydroxylase II. Both purine hydroxylases (I and II) contained molybdenum, so it was suggested that both used MoCo (Scazzocchio **et al**, 1973).

Four classes of MoCo mutants (designated **nit1**, **nit7**, **nit8** and **nit9**) in N. **crassa** (Dunn-Coleman **et al**, 1984) and six complementation groups with four different genetic loci (designated **cnxABC**, **cnxE**, **cnxF** and **cnxG**) in P. **chrysogenum** have also been identified due to their lack of

NR and xanthine dehydrogenase activities. Garrett and Cove (1976) found that the cohomogenisation of **niaD** mycelia (structural gene mutation) with the mycelia from the strains from the **cnxA , cnxE , cnxF, cnxH** classes, gave NADPH-NR activity indicating that these strains had mutations in different genes from **niaD** mutants.

Crosses between the **A. nidulans** mutants showed that **cnxA,B** and C are closely linked. The **cnxA,B** and C mutants show an overlapping complementation pattern; such that **cnxB** mutants fail to complement one another and also fail to complement **cnxA** and **cnxC** mutants, but **cnxA** mutants complement **cnxC** mutants. The **cnxABC** locus is thought to be either a single gene which has three intracistronic complementation groups or two sequential genes **cnxA** and **cnxC** (the **cnxB** mutants covering part of both **cnxA** and **cnxC**) (Cove, 1979). The **N. crassa nit9** mutant lines fall into three complementing groups in a manner similar to that of the **cnxA,B** and C mutants of **A. nidulans** (Dunn-Coleman **et al**, 1984). The **P. chrysogenum** mutants from the classes **cnxA,B** and C also gave the same unusual complementation patterns (Birkett and Rowlands, 1981). Despite this genetic similarity between the **nit9** (**N. crassa**) and **cnxABC** (**A. nidulans**) mutants, the **nit9** mutants are more likely to be analogous to the **A. nidulans cnxE** mutants since the NR and xanthine dehydrogenase from the **nit9** and **cnxE** mutants could be partially repaired by growth on excess molybdate (Arst **et al**, 1970; Dunn-Coleman, 1984). Arst **et al**, 1970) suggested that the defect of **cnxE** mutants was connected with the insertion of molybdenum into the cofactor. The **cnxE** mutants from **P. chrysogenum** were also analogous to **A. nidulans cnxE** mutants.

Temperature sensitive strains of A. **nidulans** **cnxE**, **cnxF** and **cnxH** were isolated by MacDonald and Cove (1974). However, only the enzyme isolated from some **cnxH** mutants were also temperature sensitive. As discussed in Section 5, it is possible that **cnxH** might encode a short peptide found in MoCo. It is not clear whether any of the MoCo defective lines from **N. crassa** or **P. chrysogenum** are equivalent to the A. **nidulans** **cnxH** mutants.

The functions of the A. **nidulans** **cnxF**, and **cnxG**; **N. crassa** **nit1**, **nit7**, **nit8** and **nit9**; and **P. chrysogenum** **cnxABC**, **cnxE**, **cnxF** and **cnxG** gene products are unclear.

11.3 Nitrite reductase mutants

One nitrite reductase structural gene has been identified in A. **nidulans** (niiA), **N. crassa** (**nit6**) and **P. chrysogenum** (nii). The **niiA** and **niaD** genes in A. **nidulans** (Pateman and Cove, 1967) and the **nii** and **nia** genes in **P. chrysogenum** (Birkett and Rowlands, 1981) are contigious, whilst the **nit3** and **nit6** genes in **N. crassa** are on different linkage groups (Dunn-Coleman **et al**, 1984). Birkett and Rowlands (1981) isolated some **niinia** mutants in **P. chrysogenum** which had deletions covering part of both the **nii** and **nia** loci.

11.4 Uptake mutants

A. **nidulans** uptake mutants, designated **crnA**, were isolated as chlorate resistant strains, but these strains could be grown on nitrate and hypoxanthine (unlike the NR mutants) (Tomsett and Cove, 1979). No similar mutants have been described for **N. crassa** or **P. chrysogenum**.

11.5 Regulatory mutants

11.5.1. cnxJ

Arst et al (1982) successfully isolated two new alleles of the **cnxJ** gene in **A. nidulans.** These mutants have lower levels of MoCo than the wild type. They suggest that the **cnxJ** gene has a regulatory role in MoCo production. No equivalent genes have been identified in **N. crassa** or **P. chrysogenum.**

11.5.2. nirA

The **A. nidulans nirA** gene product is thought to play a positive role in the presence of nitrate for the induction of nitrate and nitrite reductase (Cove, 1979). The **nit4/nit5** gene product is also required for the induction of nitrate reductase activity by nitrate (Tomsett and Garrett, 1980) in **N. crassa.** The analogous gene has not been identified in **P. chrysogenum.**

11.5.3 areA

The **A. nidulans areA** and **N. crassa nit2** gene products are thought to be responsible for nitrogen metabolite repression of nitrate assimilation and other pathways of nitrogen aquisition (Cove, 1979; Tomsett and Garrett, 1980).

11.6 Other mutants

Chlorate resistance screens would have selected some, but not all, of the pppA mutants (lack the enzyme transketolase from the pentose phosphate pathway, Hankinson, 1974) and the pppB mutants (defective in the induction of the pentose phosphate pathway enzymes, Hankinson, 1974), from A. nidulans.

12 HIGHER PLANT MUTANTS

Four screening methods have been used to select for nitrate assimilation mutants, these are: chlorate resistance (Bright **et al**, 1983; Müller and Grafe, 1978; Steffen and Scheider, 1984; Ryan **et al**, 1983; Nelson **et al**, 1983; Marton **et al**, 1982a), low **in vivo** levels of NR activity (Kleinhofs **et al**, 1978), nitrate utilisation (King and Khanna, 1980) and auxotrophy (Strauss **et al**, 1981; Fankhauser **et al**, 1984). These screens have been carried out after a variety of mutagenic treatments on seeds, callus tissue or protoplasts. Partial enzymic activities, sedimentation coefficients, growth on high levels of molybdate, MoCo activity and the genetics of these selections have been studied to achieve a better understanding of the higher plant NR in comparison with the earlier fungal work. Despite this effort in a wide range of plants, some of the fungal mutant classes had not been identified. At the start of this project there were no clear cases of nitrate uptake or regulatory mutants.

12.1 Confirmed NR apoprotein mutants (structural gene mutations)

Confirmed NR apoprotein mutants have been isolated from **Nicotiana tabacum** (designated **nia**), **Nicotiana plumbaginifolia** (designated **NA**) and **Hordeum vulgare** (designated **narl**).

12.1.1 N. tabacum

An extensive study of the genetics of NR mutants of **N. tabacum** has been carried out by Müller's group (Gatersleben, DDR). Müller and Grafe (1978) used N-ethyl-N-nitrosourea to mutagenise amphihaploid (n=24) cell suspensions of **N. tabacum** L. var. Gatersleben and subsequently screened for chlorate resistance. Thirty six lines were chlorate resistant, NR minus, but retained xanthine dehydrogenase activity, suggesting structural gene mutations, and so were designated **nia** (using the terminology given to similar mutants in **A. nidulans**). This was supported further by the evidence that these mutants had lost the CR activity associated with the NR subunits and had lost all the partial activities (NADH-NR,FMNH2-NR, reduced benzyl viologen-NR (BVH-NR)) associated with the apoprotein subunits of NR. There was one exception to this generalization, **nia95** , which retained inducible FMNH2-NR and BVH-NR activities despite having lost the CR activity (Mendel and Müller, 1979). The differing patterns of partial enzyme activities found in extracts of **nia** mutants (Mendel and Müller, 1979) could be explained if the structural gene had been affected at different points. These mutants were assayed for the presence of NR cross reacting material (CRM). It was found that **nia** mutants generally possessed less NR CRM than the wild type (Shiemann and Müller, 1985), indicating that the **nia** mutants possessed less NR apoprotein subunit protein than

the wild type.

In order to study the genetics of these genes in a classical fashion, Müller (1983) managed to regenerate only 15 of the 36 **nia** lines into amphidiploid plants. The crosses between these different lines showed that they were all allelic and that the amphidiploid plants obtained were homozygous for two unlinked recessive nuclear genes. These two loci were designated **nia1** and **nia2** and were unlinked (Mendel, 1983). **N. tabacum** (2n=48) is thought to have arisen by hybridisation of two diploid species, **N. sylvestris** (2n=24) and **N. tomentosiformis** (2n=24) (Gray et al, 1974), suggesting that **N. tabacum** has a duplicate arrangement of genetic information. Several of the characteristics of **N. tabacum** are known to be controlled by duplicate genes and functional diploidisation has also been observed (Smith, 1968). Mendel (1983) suggests that one **nia** gene locus comes from each parental type within **N. tabacum.** This could be the reason for the low frequency of recovered NR minus mutants (approximately 10^{-7}) for **N. tabacum** compared to the numbers for the haploid species **N. plumbaginifolia** (10^{-5}) (Marton **et al**, 1982a).

In contrast to barley, **N. tabacum** exhibits partially constitutive expression of NR. When grown on ammonium succinate or amino acids as a source of nitrogen and in the absence of nitrate, both cultured cells (Müller and Grafe, 1978) and various tissues of whole tobacco plants (Müller, 1983) develop up to 20% of the fully induced level of nitrate reductase activity. Only at the young seedling stage did the NR activity level correlate with the number (1-4) of **nia+** (wild type)

alleles in the various plants resulting from the backcross and test cross programme undertaken by Müller (1983). This suggests that NR synthesis is constitutive initially and that during development this expression changes to being regulatory (Müller, 1983).

12.1.1 N. plumbaginifolia

Marton **et al** (1982a) isolated 29 chlorate resistant NR minus cell lines of the haploid (n=10) **N. plumbaginifolia.** Of nine lines further characterised, five possessed xanthine dehydrogenase and were allelic as shown by failure to complement in somatic hybrids (Marton **et al,** 1982b). These were designated **NA. In vitro** complementation between NA lines and the **N. tabacum** structural gene mutant **nia63** were unsuccessful suggesting that the **NA** lines could also be caused by structural gene mutations (Marton **et al,** 1982a).

Negrutiu **et al** (1983) isolated 26 chlorate resistant NR minus lines from haploid protoplasts of **N. plumbaginifolia** var. Viviani. All still possessed xanthine dehydrogenase and were classified as structural gene mutants. Dirks **et al** (1985) managed to regenerate the cell lines into plants and found that the defects were due to mutations in single recessive nuclear genes.

12.1.3 H. vulgare

Barley mutants deficient in NR were first isolated by screening the M2 population (after treatment with azide) for low (less than 10% of the wild type) levels of **in vivo** NR activity (Warner **et al,** 1977). Originally Kleinhofs **et al** (1978) had used chlorate to screen the M2

generation for loss of NR activity. Although chlorate resistant plants were selected, all attempts to grow these plants failed, suggesting that these defects were lethal (Kleinhofs **et al**, 1978).

Nine nitrate reductase deficient lines when tested for allelism by crossing, were all found to be allelic to one another. The genes were designated **narla - narli.** All the mutants had low but detectable levels of **in vitro** NR in leaves using NADH-NR as the electron donor (Kleinhofs **et al**, 1980). The **narl** gene is nuclear and codominant, that is, the F1 plants from a cross between **narl** mutants and wild type plants gave NR activities which were intermediate between the wild type and the mutant values (Kleinhofs **et al**, 1980). It was identified as the structural gene using the following pieces of evidence; the various allelic **narl** mutants displayed differences in the amount of nitrate inducible CR activity (associated with the apoprotein subunits), all mutants except **narlh** lack nitrate inducible FMNH2-NR activity (associated with the distal end of the electron transport chain) and NR cross reacting material (CRM) from the **narl** mutants correlated highly with the levels of NR associated partial activities (Kuo **et al**, 1981; Somers et al, 1983a); the **narld** CR species possessed a single altered amino acid when compared to the wild type using Cleveland mapping (Kuo **et al**, 1984). All these results point to a series of different mutations occurring at different sites within a structural gene. Narayanan **et al** (1984) produced further evidence for this when no NR activity was found on coincubating free MoCo from xanthine oxidase with extracts of any of the **narl** alleles. When the mutants, **narla** and **narlb**

(possessing on average only 10% of the wild type NR activity when

compared to the wild type at various stages of growth), were grown to maturity with nitrate as the sole nitrogen source, they provided as much dry weight and reduced nitrogen per plant as the wild type. This was also the case when sterile embryos were grown aseptically (Warner and Kleinhofs, 1981). However, when grown under field conditions, the grain yield was lower than that of the wild type (Oh **et al**, 1980). This suggests that plants possessing less than 10% of the wild type NR levels in leaves can grow on nitrate almost as well as the wild type plants. It is possible that this 10% NR activity is caused either by a leaky mutation at the **nar1** gene or by a second nitrate reductase enzyme which is NAD(P)H-bispecific (Dailey **et al**, 1982a and b). This bispecific NR is not detected in the wild type plants.

Shumny and Tokarev (1982) isolated a chlorate resistant, NR deficient line, Xno 29, from the barley cultivar Winer, which was allelic to **nar1** (Kleinhofs **et al**, 1983).

12.2 Putative NR apoprotein mutants

These have been isolated and partially characterised in **Chlamydomonas reinhardii** (Nichols **et al**, 1978), **Arabidopsis thaliana** (Braaksma and Feenstra, 1982b), **Pisum sativum** (Warner **et al**, 1982; Feenstra and Jacobsen, 1980; Jacobsen **et al**, 1984), and **Petunia hybrida** (Steffen and Schieder, 1984).

12.2.1 C. reinhardii

The C. **reinhardii nitA**, mutant seems to be similar to **niaD (nit3)**, of

A. nidulans (N. crassa). However, the C. reinhardii **nitA** mutant does not take up or accumulate nitrate, whereas the **nit3** and **niaD** mutants of N. crassa and A. nidulans do.

12.2.2 A. thaliana

Two complementation groups, **chl-2** and **chl-3** have lowered levels of nitrate reductase but normal levels of xanthine dehydrogenase activity. It was tentatively suggested by Braaksma and Feenstra (1982b) that either or both could be structural NR genes. However, as Wray (1985) points out, these mutants are not well characterised.

12.3 NR cofactor (MoCo) mutants

12.3.1 Criteria for distinguishing mutants caused by mutations in different MoCo genes

Genetic analyses of the selections is the clearest and least ambiguous method of classifying the MoCo mutants. This can be achieved at the whole plant level if the plants are fertile (Kleinhofs **et al**, 1980), or by using protoplast fusion if the plants are infertile or when using cell lines which are difficult to regenerate (Grafe and Müller, 1983), Glimelius **et al**, 1978, Marton **et al**, 1982b). As was shown with the fungal systems there is one class of MoCo mutants which, when supplied with excess molybdate, can grow in the presence of nitrate and the NR and xanthine dehydrogenase activities are partially restored (Arst **et al**, 1970; Mendel and Müller, 1985).

The best characterised MoCo mutants have been isolated in **Nicotiana tabacum, Nicotiana plumbaginifolia** and **Hordeum vulgare.**

12.3.2 N. tabacum

Müller and Grafe (1978) isolated four chlorate-resistant lines which lacked NR and xanthine dehydrogenase activity, but retained CR activity associated with the apoprotein subunits. They were isolated from **N. tabacum** L. var. Gatersleben in three independent experiments. These lines were designated **cnx** since they seemed to be analogous to the **cnx** mutants from **A. nidulans.** It was impossible to test these lines for allelism by crossing since Grafe and Müller (1983) were unable to regenerate the cell lines into whole plants. To overcome this problem somatic hybridisation was used. All combinations of **nia15** and the **cnx** lines used resulted in colonies able to grow on nitrate as the sole nitrogen source and possessing nitrate reductase activity (Grafe and Müller, 1983). The **cnx** + **cnx** combinations gave no NR activity and could not grow on nitrate as the sole nitrogen source. From this Grafe and Müller (1983) argued that the **cnx** mutants were all recessive and allelic with each other and non-allelic to the **nia15** or any other **nia** line. These **cnx** mutants occured in amphihaploid cells and the **nia** mutants have been shown to carry two unlinked mutations which affect the duplicate structural genes for the NR apoprotein. Grafe and Müller (1983) have therefore suggested that these **cnx** mutants are also double mutants in the two (duplicate) genes **cnxA1** and **cnxA2.** Buchanan and Wray (1982) isolated another four chlorate resistant, NR minus lines from amphihaploid cell lines of **N. tabacum** L. var. Xanthi. All lacked xanthine dehydrogenase activity and were unable to grow on

nitrate as the sole nitrogen source. It also proved impossible to regenerate these **cnx** lines, so again genetic analysis depended upon somatic hybridisation. It was found by Xuan **et al** (1983) that these four **cnx** lines failed to complement each other but did produce NR activity when combined with a **nia** mutant, suggesting these **cnx** lines contained allelic nuclear recessive mutations.

The cohomogenisation of two **cnx** lines or a **cnx** and **nia** line gave further backing to this suggestion (Mendel **et al,** 1984). The **cnxA** mutants isolated by Müller and Grafe (1978) were partially repairable **in vivo** by growth on high molybdate medium (Mendel and Müller, 1985) suggesting that **cnxA** from **N. tabacum** is analogous to **cnxE** from **A. nidulans.** The **cnx** mutants isolated by Buchanan and Wray (1982) were not repaired by growth on high levels of molybdate and this was not due to a defect in molybdate uptake or transport (Mendel **et al,** 1984). These mutants were therefore designated **cnxB.**

12.3.3 N. plumbaginifolia

Marton **et al** (1982a) isolated four NR minus lines which lacked xanthine dehydrogenase activity and were designated **NX.** The regeneration of the plants proved to be impossible and therefore somatic hybridisation was used to discover the relationship between these lines. The four lines belonged to three complementation groups (**NX1** and **NX9** ; **NX21**; **NX24**) (Marton **et al,** 1982b). Complementation analysis between the respective **N. tabacum** and **N. plumbaginifolia cnx** mutants allowed the **NX1** and **NX9**; **NX24** and **NX21** mutant loci to be described as **cnxA, cnxB** and **cnxC** respectively (Xuan **et al,** 1983). The **cnxA** mutants were shown to

possess wild type levels of the dimeric NR enzyme (inactive with respect to reduction of nitrate, but exhibiting the CR activity associated with the subunits). The **cnxB** and **cnxC** mutants possessed only monomers (Mendel et al, 1986). The **cnxA** mutants, like the **cnxA** mutants from **N. tabacum,** could grow on nitrate when excess molybdenum was provided and both the NR and xanthine dehydrogenase activities were partially restored (Mendel **et al,** 1986).

Negrutiu **et al** (1983) and Dirks **et al** (1985) also isolated four lines and found, by using both somatic hybridisation and genetic crosses, that there were also three complementation groups **CNX20** and **CNX82; CNX27 ; CNX103.** All of these mutations were single gene recessive mutations. **CNX20** and **CNX82** were identified as belonging to the **cnxA** group (analogous to **cnxA** from **N. tabacum).** **CNX27** was assigned to class **cnxB** since it complemented with **NX1** and **NX21**, but not with **NX24.**

The fourth line **CNX103** displayed complementation with **cnxA** mutants (**CNX20**), **cnxB** mutants (**CNX27**) and **cnxC** mutants (**CNX21**), indicating that **CNX103** belonged to a different complementation group, designated **cnxD** (Dirks **et al,** 1985).

12.3.4 H. vulgare

Kleinhofs **et al** (1980) also isolated a MoCo mutant in barley, this was designated **nar2a** since it was non-allelic to the apoprotein, **nar1** mutants. The mutant **(nar2a)** was isolated on the basis of low **in vivo** NR assays from the shoot tips of the M2 seeds arising from the self fertilisation of azide treated seeds. The progeny of this selection were leaky with respect to NR activity and possessed on average 50% of the wild type levels and low levels of xanthine dehydrogenase activity. It is a poor source of functional MoCo in the reconstitution of NR activity from a barley apoprotein extract (Narayanan **et al**, 1984).

Tokarev and Shumny (1981) isolated two chlorate resistant selections possessing low levels of NR and xanthine dehydrogenase activities and therefore classified as leaky MoCo mutants. These mutants were allelic to each other (Tokarev and Shumny, 1981) and non-allelic to **nar2a**, therefore classified as **nar3a** and **nar3b** (Kleinhofs **et al**, 1983). Kleinhofs et al (1985) have recently identified a further MoCo mutant designated **nar4**, which is non-allelic to both **nar2** and **nar3** plants. This suggests that at least two MoCo genes in barley have been identified.

All these barley mutants (excluding **nar4**) had low but detectable NR activity and the ability to grow on nitrate in contrast to the work by Bright **et al** (1983). Bright **et al** (1983) were able to maintain a number of chlorate resistant seedlings. One of these, R9401, contained a conditional lethal mutation causing the loss of NR and xanthine dehydrogenase activity.

The **nar3** mutation can be partially repaired by high levels of molybdate (Kleinhofs **et al,** 1985) making it equivalent to the **cnxE** of **A. nidulans** and **cnxA** of **N. tabacum.** The **nar2a** mutant cannot be repaired in this manner and therefore corresponds to a different locus concerned with MoCo synthesis. The **nar2** and **nar3** mutants possess some assembled subunits since they have a 7.7 S CR species when subjected to density gradient analyses (Narayanan **et al,** 1983).

12.4 Less well characterised cofactor (MoCo) mutants

There are many reports of plant MoCo mutants for which there is less information available. These include mutants of **Chlamydomonas reinhardii** (Nichols **et al,** 1978; Nichols and Syrett, 1978; Fernandez and Cardenas, 1982), **Physcomitrella patens** (Dunlop, 1982), **Hyoscyamus muticus** (Strauss **et al,** 1981; Fankhauser **et al,** 1984), **Datura innoxia** (King and Khanna, 1980), **Pisum sativum** (Warner **et al,** 1982; Feenstra **et al,** 1982) and **Arabidopsis thaliana** (Oostindier-Braaksma and Feenstra, 1973; Braaksma and Feenstra, 1979; Braaksma and Feenstra, 1982a,b and c).

12.4.1 C. reinhardii

Nichols and Syrett (1978) generated chlorate resistant mutants using UV light. These NR minus mutants were classified into three groups designated, **nitA, nitB** and **nitC** (Nichols **et al,** 1978). They suggested that **nitC** was analogous to the **cnx** mutant strains of **A. nidulans** since it cannot grow on hypoxanthine.

The **nitB** locus is tentatively suggested to have a regulatory role since, despite appearing to have no nitrate reductase, it can take up and assimilate nitrate.

Sosa **et al** (1978) generated NR minus mutants in **C. reinhardii** using N-methyl-N'-nitro-nitrosoguanidine (MNNG). Fernandez and Cardenas (1982) analysed these mutants for their partial activities and **in vitro** complementation patterns. Three lines, 102, 104 and 307 were equivalent to the **cnx** mutants in **A. nidulans** (Fernandez and Cardenas, 1982).

12.4.2 P. patens

After an initial isolation of three chlorate resistant mutants, **nat1**, **nat2** and **nat3** by Ashton and Cove (1977), a further 34 nitrate non-utilising mutants were isolated (Dunlop, 1982). A total of 27 of these new mutants were further characterised, 19 did not grow at all on nitrate, 8 showed leaky growth. Of the 19, 12 were characterised as possible cofactor mutants (designated **cnx** since they lacked both NR and xanthine dehydrogenase). All 12 had constitutive nitrite reductase. Using protoplast fusion for complementation studies, three classes of **cnx** mutants were identified. One of these appears to be repairable by molybdate suggesting that it is analogous to **cnxE** from A. nidulans.

12.4.3 H. muticus

Five chlorate resistant cell lines of **H. muticus** were isolated from (MNNG) mutagenised haploid protoplasts screened with chlorate (Strauss **et al**, 1981; Fankhauser **et al**, 1984). Unusually for the isolation of

NR mutants, these lines were isolated after initial growth on enriched media as part of an attempt to produce a broad spectrum of auxotrophs.

Four of the five cell lines were unable to grow on nitrate as sole nitrogen source, had normal nitrate uptake and lacked both NR and xanthine dehydrogenase activities. All four possessed CR and nitrite reductase activities and, when supplied with free MoCo, recovered NR activity. Complementation studies subdivided these tentative **cnx** type mutants in to two classes; the lines MA2 and I2D12 were molybdate repairable **in vivo,** suggesting defects in an analogous gene to **cnxE** of **A. nidulans;** whilst the other two lines could not be repaired by molybdate (Fankhauser **et al,** 1984).

12.4.4 D. innoxia

A possible MoCo mutant has been isolated in **D. innoxia** by King and Khanna (1980). From a haploid cell culture the cell line, NR1, was selected. It was unable to grow on nitrate as sole nitrogen source and lacked NR activity. The NR associated CR activity was, however, retained and the defect could be repaired by exogenous molybdate suggesting that NR1 could be analogous to the **cnxE** mutant of **A. nidulans.**

12.4.5 P. sativum

A mutant, A300, was isolated from the M2 population (after azide treatment of the M1) on the basis of low levels of **in vivo** NR activity. It retained 20% of the wild type NR activity but lacked xanthine dehydrogenase activity, suggesting that it was a cofactor mutant. It

was designated **nar2.**

Warner **et al** (1982) showed that **narl** (the proposed apoprotein) mutants and **nar2** mutants were non-allelic, and the F1 hybrid plants between each mutant and the wild type (Juneau) had intermediate levels of NR activity, suggesting incomplete dominance of the mutant alleles. The F2 segregation patterns indicated that nitrate reductase deficiency was controlled by single nuclear genes in each case.

12.4.6 A. thaliana

Two mutants, B25 and B73, were isolated using chlorate screening (Oostindier-Braaksma and Feenstra, 1973). Both had lowered nitrate reductase and xanthine dehydrogenase activity, indicating possible equivalence to the **cnx** mutants of **A. nidulans** (Braaksma and Feenstra, 1982a and b). The damaged NR of B73 seemed to be repaired by high levels of exogenous molybdate, suggesting that this mutant is analogous to **cnxE** from **A. nidulans.**

12.5 Nitrate uptake mutants

There has been only one higher plant nitrate uptake mutant described (Doddema and Telkamp, 1979). A total of 51 chlorate resistant selections in **A. thaliana** were found to be caused by allelic mutations at the locus designated **chl-1.** They had a defective nitrate uptake system (Doddema and Telkamp, 1979).

12.6 Regulatory mutants

There have been no reports of any definite regulatory mutants isolated in higher plants. A possible regulatory mutant has been identified in **Glycine max** (Nelson **et al**, 1983; Ryan **et al**, 1983). **G. max** possesses two types of NR activity, nitrate inducible NR, and a constitutive NR activity found when the plants were grown in the presence of urea as the nitrogen source (Lahav **et al**, 1976). Soybean mutants lacking this constitutive NR activity have been isolated by Nelson **et al** (1983) and Ryan **et al** (1983). The M2 generation, after treatment of the M1 with various mutagens, were subjected to a chlorate screen, followed by an **in vivo** NR assay. Three lines LNR-2, LNR-3 and LNR-4 were selected in this manner, they were found to be allelic and designated **nr1** (Nelson **et al**, 1984). These mutant genes were recessive and nuclear encoded. When grown on nitrate, these lines had approximately 50% of the wild type NR activity. However, when grown on urea, the mutants had no detectable nitrate reductase. Recently the constitutive NR activity was found to consist of two species (Robin **et al**, 1985; Streit **et al**, 1985). One form was the NADH-specific NR and the other was immunologically identical to the bispecific NAD(P)H-NR described by Jolly **et al** (1976). Since the loss of constitutive NR activity was caused by a single gene mutation, Streit **et al** (1985) argues that the **nr1** mutation may be situated in the regulatory gene for the constitutive NRs. Another possibility is that, similar to the situation found in **P. vulgare** glutamine synthetase, isoforms of NR are present which are made up of different combinations of subunits and the **nr1** gene codes one of for the subunits which form the constitutive NR.

The application of molecular techniques to the questions raised by these mutants would help to distinguish between the two possibilities.

In summary, at the start of this project the situation with barley mutants was as follows:-

1. Kleinhofs **et al** (1978) isolated chlorate resistant plants from azide treated M2 seedlings. However, all died on transplanting.
2. **In vivo** NR assays were then used on the same source of M2 seedlings to isolate plants with low levels of NR activity. All except one of the NR deficient plants isolated in this fashion were apoprotein mutants. There were two possible reasons for the lack of greater numbers of MoCo mutants isolated, a) all the non-leaky MoCo mutants died under the growth conditions available after rescue from the screening procedure or, b) azide as a mutagenic agent was not causing mutagenesis in the MoCo genes which are essential for the production of MoCo. It might therefore be a good idea to mutagenise barley seeds with a mutagenic agent which is known to use a different mode of action from that of azide.
3. Of all the barley chlorate resistant selections, one, R9401, is fully resistant, it has less than 1% of the wild type NR activity and cannot grow with nitrate as the nitrogen source (Bright **et al**, 1983). The other MoCo mutants **nar2a**, **nar3a** and **nar3b** were only partially resistant to chlorate and possessed less than 5% of the wild type activity. The **nar4** mutant had not been characterised.
4. The apoprotein mutants were all allelic (Kleinhofs **et al**, 1980; Kleinhofs **et al**, 1985).

Therefore, the following questions were asked at the start of this project:-

1. Can more NR mutants be isolated which affect different genes to those already described for barley?
2. How are these mutants related to each other, to the known defects in **narl,** 2 and 3 of barley and other known mutants?
3. Does the MoCo of barley have both functions ascribed to it in other species?
4. Is barley MoCo formed by an analogous, multistep process as is described for **E. coli** and suggested for **N. tabacum**?
5. Is chlorate resistance due to a defect in the nitrate uptake system or lack of NR?

I have attempted to answer these questions in the following chapters.

CHAPTER 2

MATERIALS AND METHODS

A. MATERIALS

Chemicals

The following were obtained from the Sigma Chemical Company (London); Alcohol dehydrogenase (Yeast); Bovine serum albumin (crystallised and lyophilised); Catalase (bovine liver); Coomassie Brilliant Blue G250; Cysteine (free base); FAD (Grade III); FMN (sodium salt, synthetic); Glutathione (reduced); Hypoxanthine; Methyl viologen; Myoglobin (whale skeletal muscle type III); NAD+ (Yeast, Grade III); NADH (Yeast, Grade III); NADPH (Type I); Nitroblue tetrazolium (Grade III); Phenazine methosulphate; Xanthine oxidase (buttermilk, Grade I). Acrylamide (electrophoresis grade), N-N'-methylene bis-acrylamide (electrophoresis grade) and toluene-3,4-dithiol were obtained from British Drug Houses Ltd. Cytochrome c (horse heart, salt free) was obtained from BCL. Szechrome NAS was obtained from Polysciences Inc, Warrington, PA 18976. Leupeptin was obtained from Peptide Institute Inc, Osaka, Japan. All other chemicals were of the highest grade and from the usual commercial sources.

Growth Materials

Vermiculite was obtained from Hoben Davies Ltd, Newcastle, Staffordshire. Eff compost was obtained from Eff Products Ltd, Glaziers Lane, Normandy, Guildford, Surrey.

B. METHODS

I SELECTION OF CHLORATE RESISTANT PLANTS

The starting materials for screening were M2 (second generation after mutagenesis) seeds of barley cultivars, Golden Promise and Maris Mink provided by Dr. S. W. J. Bright. Mutagenesis had been carried out with sodium azide (Kleinhofs **et al**, 1978) or irradiation with gamma rays or fast neutrons.

1.1 Mutagenised seed stocks

Two batches of barley, cv. Golden Promise, had been treated with 15 and 18 krad of gamma radiation respectively. The mutagenised (M1) seed was planted in a field plot, grown to maturity and allowed to self fertilize forming the M2 generation. These seeds were bulk harvested, cleaned, air dried and stored at room temperature until required.

A further two batches of barley cultivars Golden Promise and Maris Mink had been treated with 1 mM sodium azide a pH 3 for two hours (Warner **et al**, 1977) after presoaking for 18 hours at 5° C in water. The azide was rinsed off the seeds by washing with running tap water for 30 minutes. Seeds were then air dried, sown, and then treated in a similar fashion to the seeds treated with gamma radiation or fast neutrons.

1.2 Selection of chlorate resistant M2 seedlings

Forty grams of M2 seed were placed on a 1 cm layer of Hemmington grit (2-3 mm diameter) in each of four 21 x 35 cm seed trays and were

covered with a further 2 cm of grit. The trays were put into a larger tray and subirrigated to a depth of 0.5-1 cm with a solution of either 8 mM KNO3 plus 2 mM Mg(NO3)2. 6H20 or 4 mM (NH4)2SO4. The seeds were allowed to germinate and grow under the following conditions; an 18 hour photoperiod of 245 $\mu Em^{-2} s^{-1}$ supplied by tungsten and fluorescent lights; constant temperatures of 20° C and 16° C in the light and dark respectively. On the fourth or fifth day (depending on the cultivar) the seed trays were drained and the nutrient solution replaced, to the same depth, with 5, 10 or 15 mM KC103. After 9 days of this treatment the seedlings which were green and taller than the scorched background were rescued.

1.3 Nomenclature of selected plants and progeny

Every selected plant was identified by the letter R or K followed by a four or five figure number. The first two or three figures coded for the screen number and the final two identified the selected plant. For example, R35103 is the third selected plant from screen number R351. If this plant, R35103, was crossed with Maris Mink the F1 progeny would be identified as F1 [R35103 x Maris Mink]. If one of these F1 plants, maybe the second out of the packet of F1 seeds, was self-fertilised the F2 progeny would be described as F2 [R35103 x Maris Mink] 2.

1.4 Growth of selected M2 seedlings

The chlorate resistant seedlings rescued from the seed trays were grown, with their roots in the dark (to reduce algal growth in the nutrient solution), hydroponically under the following conditions; an 18 hour photoperiod of 100 $\mu Em^{-2} s^{-1}$ supplied

by tungsten and fluorescent lights; constant temperatures of 20° C and 16° C for light and dark periods respectively and a relative humidity of 80% throughout. Separate, constantly aerated containers were used for each plant. For the first 7 days the roots were placed in demineralised water to remove chlorate from the root system. Then the basic nutrient solution (table 3) was supplied once a week. Glutamine was added twice a week as the nitrogen source to give a final concentration of 1 mM. Demineralised water was added as required to replace water lost due to evaporation.

These plants were tested for their NR status, using the **in vivo** NR assay, following an 18 hour induction period, if NR minus they were grown to maturity. Genetic crosses were carried out between these selected plants and their parental cultivars (Maris Mink and Golden Promise). The mutants were also allowed to self-pollinate.

2. PLANT GROWTH CONDITIONS

2.1 For biochemical analyses.

The seeds were sown individually in 21 x 35 cm seed trays, subdivided into 12 numbered pots containing vermiculite. The trays were initially watered with tap water and then placed in the dark at 28° C, to germinate and grow for two days. After this the seedlings were placed at 26° C in continous light (30 or 70 $\mu Em^{-2} s^{-1}$) supplied by three Grolux lamps.

Table 3

Composition of the basic nutrient solution for hydroponic growth of selected plants

	µM	mg/l
MgSo4.7H2O	675	365
CaCl2.6H2O	1246	273
K2HPO4	1320	230
NaFe EDTA	12.8	47
KCl	90.5	6.75
H3BO3	121.3	7.5
MnSO4	28.1	4.25
ZnSO4	4.2	0.675
(NH4)6.Mo7O24.4H2O	0.5	0.675
CuSO4.5H2O	2.4	0.6
H2SO4	103	5.2
MES	5000	

pH 6.0

Every 24 hours the plants were watered with half strength Hoaglands Solution (table 4). Plants were induced with half strength Hoaglands Solution containing 15 mM KNO3 for 18 hours prior to assaying enzyme activities.

2.2 For genetic analyses

For experimental details see Chapter 5.

3. PLANT EXTRACTION FOR STUDIES ON CRUDE EXTRACTS

Leaf tissue was ground, in a 3:1 or 5:1 (v/w) ratio, to homogeneity in ice-cold NR Extraction Buffer (50 mM potassium phosphate, pH 7.5 containing 10 µM FAD and 0.1 mM EDTA with freshly added 3% (w/v) BSA and 1 mM cysteine) with a pre-chilled mortar and pestle. The brei was centrifuged at 4° C for 20 minutes at 38,000 g. The supernatant was used as a source of crude enzyme (Small and Wray, 1980).

4. IDENTIFICATION OF NR MINUS PLANTS IN A SEGREGATING POPULATION

Two methods were used to determine whether individual plants were lacking in nitrate reductase activity.

4.1 In vivo nitrate reductase

In vivo levels of nitrate reductase were assayed in 4-6 day old seedlings by incubating 10-20 mg (2-3 mm sliced segments) of leaf tip

Table 4

Composition of half strength Hoaglands solution

	nM	mg/l
NaFe EDTA	210	38.44
KH2PO4	500	34.25
MgSO4.7H2O	1030	126.65
ZnSO4.5H2O	0.4	0.063
MnSO4.5H2O	85.4	0.021
H3BO3	23.4	0.725
Na2MoO4.7H2O	0.05	0.006

pH 6.5

in 3 ml of 0.1 M potassium phosphate buffer (pH 7.5) containing 0.1 M KNO3 and 2% propan-1-ol. This was degassed under reduced pressure and flushed with oxygen-free nitrogen twice. The tubes were stoppered and incubated for 2 hours in the dark (to inhibit nitrite reductase activity) at 25° C. An 0.1 ml aliquot was removed and added to 2.9 ml distilled water and mixed well. A 1 ml aliquot of this mixture was added to 1 ml 1% sulphanilamide/3N HCl and 1 ml 0.02% N(1-naphthyl)-ethylene diamine dihydrochloride (NED) to produce a pink colour which is proportional to the amount of nitrite formed. After mixing, the test tubes were left to stand for 10 minutes at room temperature for maximum colour development and then were centrifuged at 4,000 g for 5 minutes at room temperature. The absorbance of the supernatant was measured at 540 nm in either a Unicam SP500 series 2 or a Cecil CE272 spectrophotometer, against a blank containing buffer and no leaf material.

4.2 Qualitative in vitro nitrate reductase

The NR status of each induced plant was checked after 5 days (growth conditions given in Section 2). 10-20 mg samples of individual leaf tips were ground up with a chilled pestle and mortar in 0.9 ml ice-cold NR Extraction Buffer (see Section 3). To start the reaction, a 0.1 ml volume containing 0.1 mM NADH and 0.01 M KNO3, was added. This was incubated for 30 minutes at 25° C and the reaction was then stopped by the addition of 1 ml 1% sulphanilamide/3N HCl and 1 ml 0.02% NED. The tubes were shaken and then left for 10 minutes to allow the colour to develop fully. Those tubes with no pink colour in the supernatant, indicating a lack of nitrite, were classed as coming from NR minus

plants.

5. STANDARD ENZYME-ASSAYS

5.1 In vitro NADH-nitrate reductase (NADH-NR)

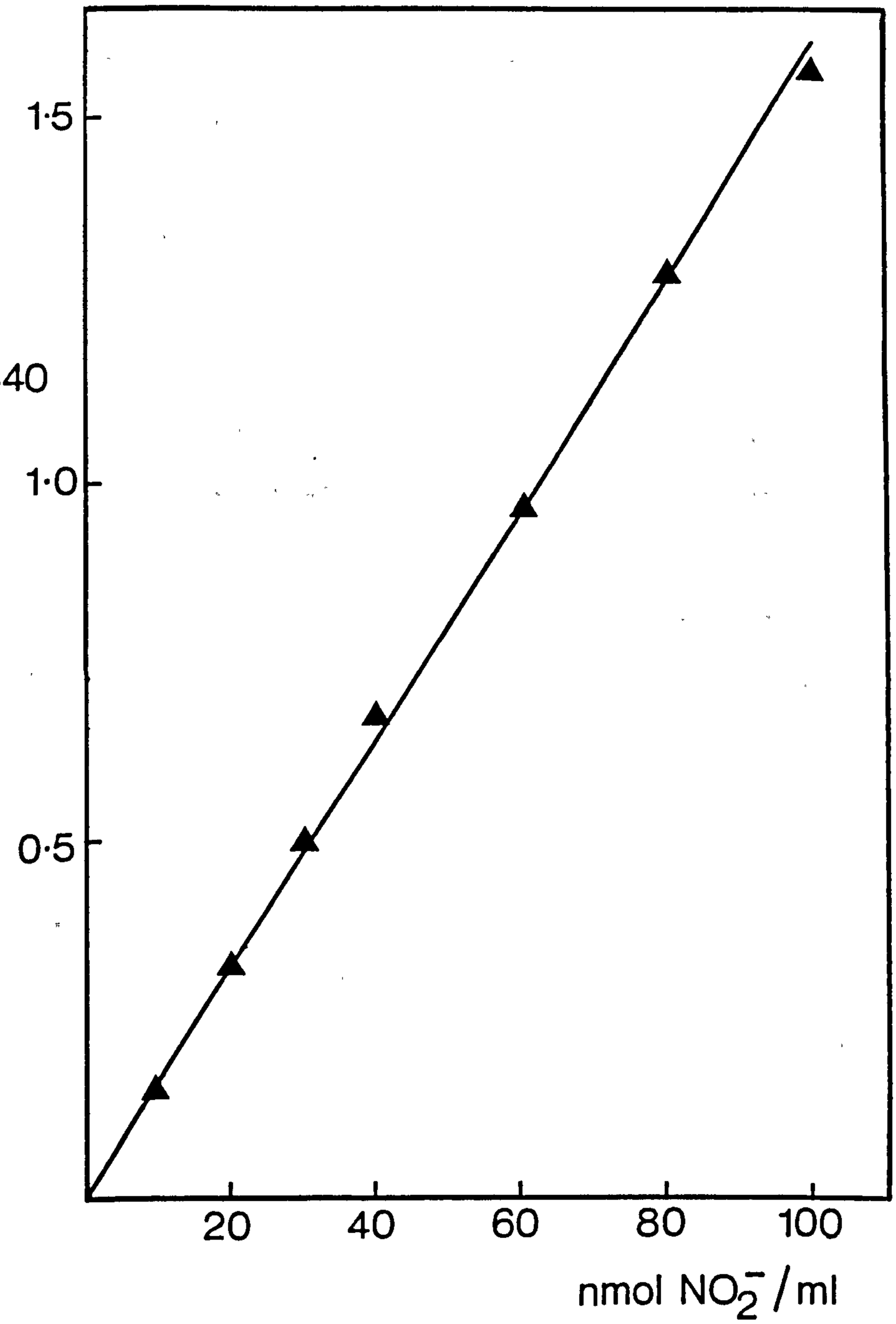
The method of Wray and Filner (1970), which measures the amount of nitrite produced, was used. Centrifuge tubes of 0.05 M potassium phosphate buffer (pH 7.5) containing 0.01 M KNO3, and 0.1 mM NADH, were made up in a final volume of 0.8 ml. Controls contained no NADH. The reaction was started by addition of up to 0.2 ml of crude enzyme extract and then incubated at 25° C for an appropriate length of time (15-30 minutes). To stop the reaction, 1 ml aliquots of 1% (w/v) sulphanilamide/3N HCl and 0.02% (w/v) NED were added. The colour was developed over 10 minutes by standing at room temperature after whirlimixing. The test tubes were then centrifuged at 4,000 g for 5 minutes at room temperature. The absorbance of supernatant was measured at 540 nm in a spectrophotometer.

The absorbance was then converted into µmol nitrite produced/incubation period by comparing with a standard curve (figure 5). These curves were arrived at by preparing 0 to 100 µM KNO_2 stock solutions, 1 ml aliquots of these were added to test tubes containing 1 ml 1 % sulphanilamide and 1 ml 0.02% NED. When assaying fractions from a sucrose density gradient, 0.1 ml aliquots were incubated for 2 hours at 25° C in the usual NADH-NR assay mixture.

Figure 5

Nitrite standard curve

This plot shows the relationship between nitrite concentration in standard nitrite solutions and absorbance at 540 nm after addition of 1 ml 1% sulphanilamide/3N HCl and 1 ml 0.02% NED as described in the text (Chapter1, Section 5).


1·5
1·0
0·5
A_{540}
20
40
60
80
100
nmol NO_2^- / ml

5.2 In vitro NADPH-nitrate reductase (NADPH-NR)

Exactly the same method was used as for NADH-NR activity except that the 0.1 mM NADH was replaced by 0.1 mM NADPH.

5.3 In vitro FMNH2-nitrate reductase (FMNH2-NR)

The method described by Wray and Filner (1970), in which FMNH2 is utilised to reduce nitrate, was used as outlined below. FMN is supplied and is reduced to FMNH2 by the addition of sodium dithionite.

Centrifuge tubes containing: 0.4 M potassium phosphate buffer (pH 7.5), 0.6 mM FMN and 0.01 M KNO3 were made up to give a final volume of 0.8 ml.

A volume of up to 0.1 ml crude enzyme extract was added. The tubes were evacuated and flushed with oxygen-free nitrogen twice. To start the reaction 0.1 ml of 95 mM sodium dithionite was added. The dithionite reduces FMN to a pale straw colour (FMNH2). The tubes were then stoppered and incubated for 15-30 minutes at 25° C. The reaction was stopped by whirlimixing to reoxidise the FMNH2. This was followed by addition of 1 ml of 1% sulphanilamide/3N HCl and 1 ml 0.02% NED. After whirlimixing, the test tubes were left to stand at room temperature for 10 minutes and then centrifuged at 4,000 g for 5 minutes at room temperature. The absorbance of the supernatant was measured at 540 nm in a spectrophotometer. Control tubes contained buffer but no sodium dithionite.

5.4 In vitro reduced methyl viologen-nitrite reductase (NiR)

The method used was that according to Wray and Filner (1970). Methyl viologen was supplied to nitrite reductase to allow the reduction of nitrite to take place, the loss of nitrite was measured. 0.5 ml aliquots of a solution containing; 0.3 M potassium phosphate buffer (pH 7.5), 2 mM KNO2 and 1 mM methyl viologen were added to centrifuge tubes, and made up to a final volume of 0.8 ml with distilled water and crude enzyme extract. The tubes were degassed under pressure and flushed with oxygen-free nitrogen twice. The reaction was initiated by the addition of 0.2 ml of 0.29 M sodium dithionite. The tubes were stoppered and gently shaken to reduce the methyl viologen, giving a dark blue colour. The centrifuge tubes were incubated for 15-30 minutes at 25° C. The reaction was stopped by vigorous whirlimixing. An 0.1 ml aliquot was removed and added to 2.9 ml distilled water. 1 ml of the diluted reaction mixture was added to 1 ml 1% sulphanilamide/3N HCl and 1 ml 0.02% NED. The tubes were centrifuged at 4,000 g for 5 minutes after being left to stand at room temperature for 10 minutes to aid full colour development. The absorbance of the supernatant was determined at 540 nm and compared to controls which were treated in exactly the same manner except that the controls contained no dithionite.

5.5 In vitro NADH-cytochrome c reductase (NADH-CR)

This activity was determined by the method of Wray and Filner (1970). A 0.3 ml aliquot of a solution of 0.05 M potassium phosphate buffer (pH 7.5) containing; 0.4% cytochrome c and 0.2 mM NADH was placed in a glass microcuvette. The reaction was started by addition of enzyme

extract and distilled water to a final volume of 0.4 ml. The cuvette was inverted three or four times and placed in a spectrophotometer linked to a chart recorder. The increase in absorbance at 550 nm with time was measured for 3-4 minutes.

For the sucrose density gradient fractions, 50-100 µl were assayed for 7 minutes. Four fractions were assayed simultaneously using an SP505 programme controller to record the absorbance at 550 nm every 30 seconds for each cuvette sequentially. The changes in absorbance at 550 nm/minute were converted to µmol cytochrome c reduced/min/ml using the 1 mM extinction coefficient of 29.5 (CRC Handbook of Biochemistry, 1970).

5.6 Xanthine dehydrogenase assay (XDH)

Polyacrylamide disc gels were used and stained for xanthine dehydrogenase activity by the method of Mendel and Müller (1976).

5.6.1 Preparation of 5 and 7% polyacrylamide disc gels

Stock solutions of 0.1 M sodium pyrophosphate (pH 8.0) and the same buffer containing 14% acrylamide with 0.364% methylene bisacrylamide were made up and stored in dark bottles at 4° C. The acrylamide-methylene bis-acrylamide solution was warmed to room temperature before use to ensure that the acrylamide was in solution. The acrylamide-methylene bis-acrylamide solution was diluted to 5 or 7% (with respect to acrylamide) by addition of 0.1 M sodium pyrophosphate (pH 8.0) to a final volume of 40 ml. 50 µl aliquot of N-N-N'-N'-tetra-methyl-1,2-diamino ethane (TEMED) was added.

After this, the solution was degassed, 10 mg solid ammonium persulphate was added and thoroughly mixed in. The gel solution was then pippetted into glass tubes (4.5 dia x 80 mm) previously sealed with Nescofilm at one end. Distilled water was gently layered on top to prevent inhibition of polymerisation by air.

After polymerisation the seal was removed and the gels were electrophoresed for 1 hour at 2 mA per tube using 0.05 M Tris (containing 1.5 mM EDTA, titrated to pH 8.3 with solid boric acid) as both reservoir buffers.

5.6.2 Application of sample

A crude extract (5:1 (v/w) ratio) was prepared in NR Extraction Buffer (Section 3). Sucrose was added to saturation point in order to increase the density and so allow for a more concentrated application of sample. Bromophenol Blue (0.05% (w/v) in 10% sucrose, pH 6.8) was added to the top, cathode, reservoir until the buffer appeared to be a very pale blue. An 80 µl sample of crude extract was applied to the top of two gels to allow the indentification of hypoxanthine-(substrate)-dependent dehydrogenase activity.

5.6.3 Electrophoresis

The gels were run at a constant current of 5 mA per tube for 3-4 hours until the Bromophenol Blue reached the end of the gels.

5.6.4 Staining for xanthine dehydrogenase activity

The gels were extracted from their tubes using a jet from a syringe, of

distilled water containing Triton X-100 detergent. They were stained in darkness for 1 hour with a solution of 0.1 M sodium pyrophosphate (pH 8.0) containing; 0.1 mM nitroblue tetrazolium, 0.3 mM phenazine methosulphate, 0.1 mM NAD+ and 2.0 mM hypoxanthine. The NAD+ and phenazine methosulphate can be used by any dehydrogenase enzyme present in the gel if the correct substrate is also present. The NADH formed from this reaction can then reduce nitroblue tetrazolium to a formazan which forms a dark purple colour in the area of the gel where the enzyme is present (Siri-Venugopal and Adiga, 1980). To ensure that only hypoxanthine-dependent dehydrogenases are being stained a second disc gel for each sample was stained in the same solution lacking hypoxanthine. All were destained over night in distilled water, again in the dark, as the colour was unstable in the light.

6. STANDARD NON-ENZYMATIC DETERMINATIONS

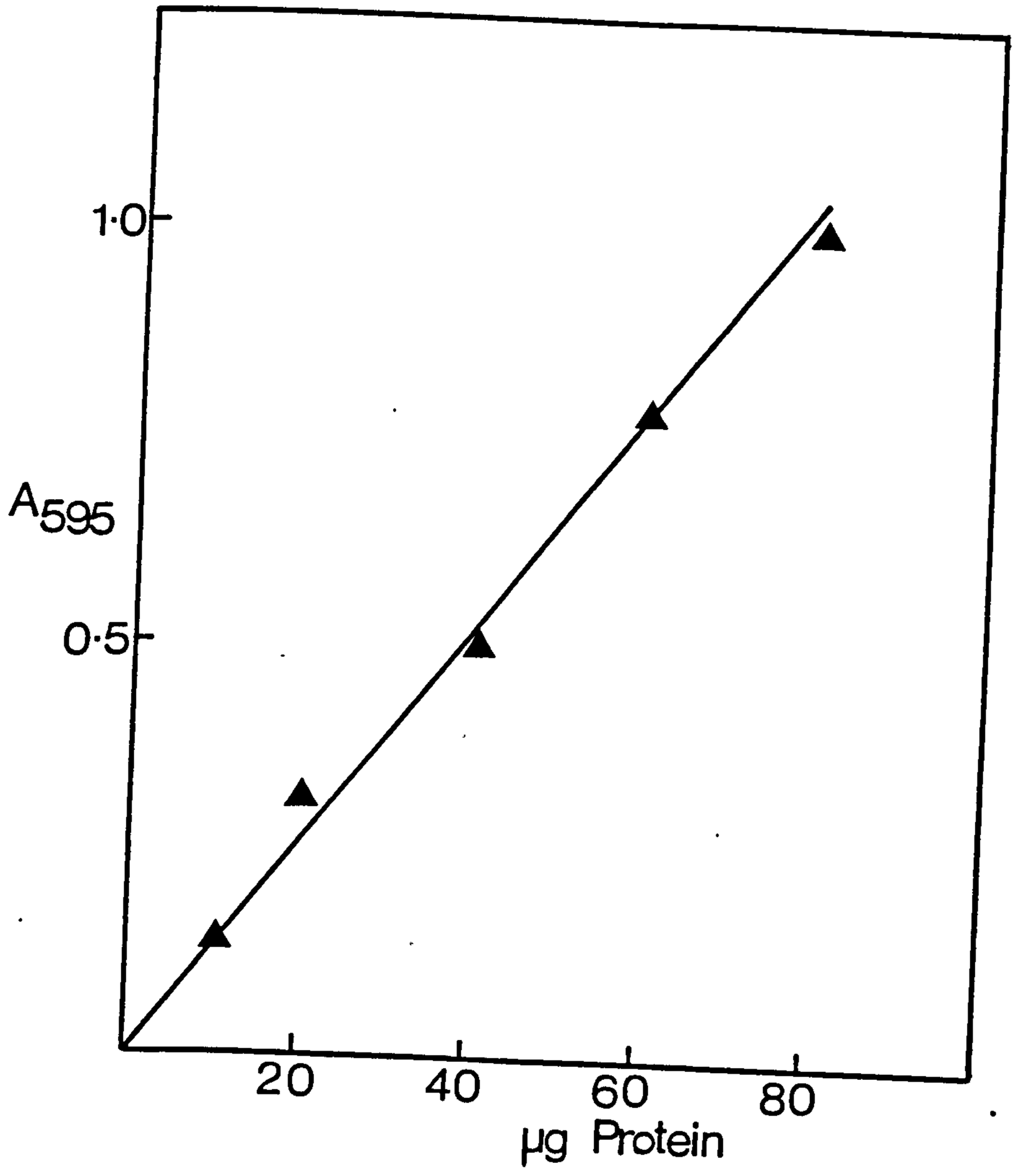
6.1 Protein estimation

To determine the protein concentration in extracts, excluding those containing additional BSA, Bradford's (1976) method was used. A stock solution (Bradford's Reagent) was made up containing 100 mg Coomassie Brilliant Blue G-250 in 50 ml 95% ethanol. When this had dissolved, 100 ml 85% (v/v) phosphoric acid was added and made up to a final volume of 1 l with distilled water. This was filtered through Whatman No 1 filter paper and stored for up to 1 month at 4° C. A standard curve was obtained for each set of protein determinations using 0-80 mg BSA made up in 0.5 ml aliquots, of the buffer in which the sample was prepared, (figure 6) as a standard protein.

Figure 6

Protein standard curve

This plot shows the relationship between protein concentration in standard solutions of BSA and absorbance at 595 nm after addition of the Bradford protein reagent as described in the text (Chapter 2, Section 6).


1·0
0·5
A595
20
40
60
80
µg Protein

6.2 Nitrate determination

6.2.1 Preparation of stock solution

Equal volumes of concentrated phosphoric acid and sulphuric acid (both Analar grade) were mixed and left to stand in a Winchester bottle for two weeks. The contaminating nitrate in the acids decreased over this period. When required, 5 g diphenylamine sulphonic acid (Szechrome NAS reagent) was added to 1 l of the acid mixture in a sealed dark screw-cap bottle. This was stirred until all the solid and liberated gas were dissolved.

6.2.2 Measurement of nitrate concentration

A 0.5 ml sample of crude extract, usually diluted 50-100 fold in NR Extraction Buffer (Section 3), was added to 5 ml of the reagent. The test tube was sealed with Nescofilm and inverted several times to mix the two layers. The mixture was left to stand at room temperature for 30 minutes to allow for maximum colour development, and then the absorbance of the solution was read at 570 nm on a spectrophotometer. This colour was stable for up to an hour. The increase in absorbance with nitrate was linear up to 100 nmol and was compared to a standard curve using KNO_3 dissolved in the same buffer (figure 7).

6.3 Molybdenum determination

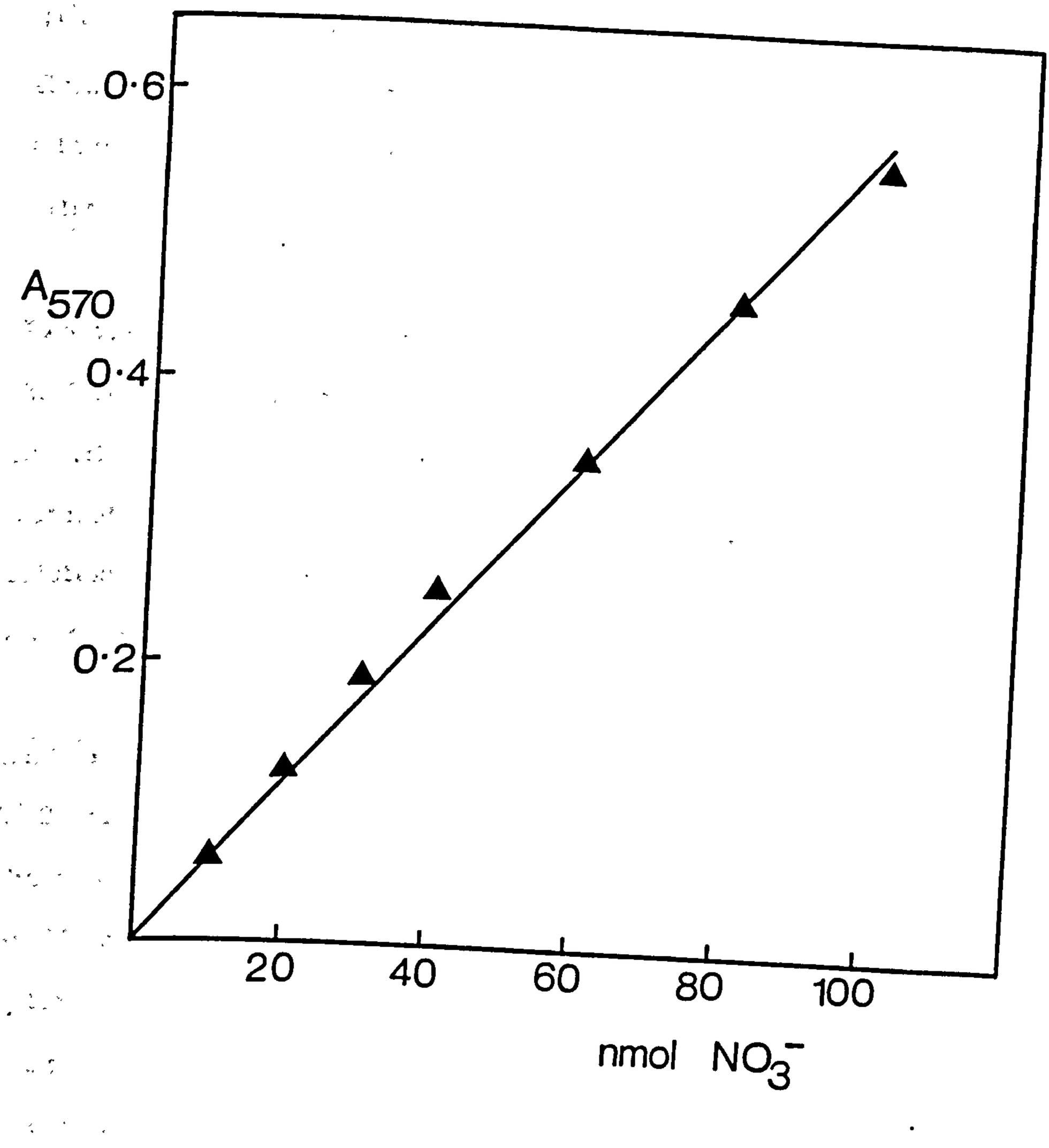
6.3.1 Plant growth

Plants were grown as described in Section 2 for 7 days and induced using half strength Hoaglands Solution containing 15 mM KNO_3 for 48 hours prior to extraction. Qualitative **in vitro** NR assays (Section 4) were performed on individual 7 day old plants in order to

Figure 7

Nitrate standard curve

This plot shows the relationship between nitrate concentration in standard nitrate solutions and the absorbance at 570 nm after the addition of the Szechrome NAS reagent as described in the text (Chapter 2, Section 6).


0·6
0·4
0·2
A_{570}
20
40
60
80
100
nmol NO_3^-

identify NR+ and NR minus plants.

6.3.2 Extraction

The molybdenum content of the mutants was assayed by the method of Cardenas and Mortenson (1974). This involves formation of Mo(VI) dithiol complexes in strong (8N) sulphuric acid in the cold and its extraction into isoamyl acetate.

Two stock solutions were made up:

A: 9mM NaOH containing 13 mM toluene-3,4-dithiol

B: 1:2 (v/v) ratio of ß mercaptoethanol to glacial acetic acid. The mercaptoethanol/glacial acetic acid mixture was added dropwise to Solution A until permanent opalescent turbidity occurred. This mixture was called the dithiol reagent.

6.3.3 Hydrolysis of sample

A 2 ml aliquot of 36N H_2SO_4 was added to 2 g fresh plant material (shoot and root) and boiled at about 320° C in a solid heating block for 30 minutes. A 3 ml volume of 30% (v/v) H_2O_2 was then added and the mixture boiled for approximately 10 minutes to remove the dark colour. A 2 ml aliquot of this solution was added to 4 ml H_2O and 3 ml of the dithiol reagent. The solution was vortexed after the addition of the dithiol and left to stand for 15 minutes at room temperature. 3 ml of isoamyl acetate was added and the tube was vortexed extensively for 5 minutes, then left to stand at room temperature. Phase separation was complete after about 15 minutes and the absorption of the organic phase was read at 680 nm against a blank which contained

isoamyl acetate. A calibration curve (figure 8) was obtained by measuring the molybdenum in the organic phase obtained by vortexing together 2 ml 36N H_2SO_4, 4 ml H_2O (containing 0-60 nmol sodium molybdate), 3 ml dithiol reagent and 3 ml isoamyl acetate and following the same procedure as that used for the determination of molybdate in the samples. The absorbance of a control containing no sodium molybdate was subtracted from all measurements of absorbance at 680 nm.

7. LEVELS OF ENZYME ACTIVITIES IN THE PROGENY OF SELECTIONS

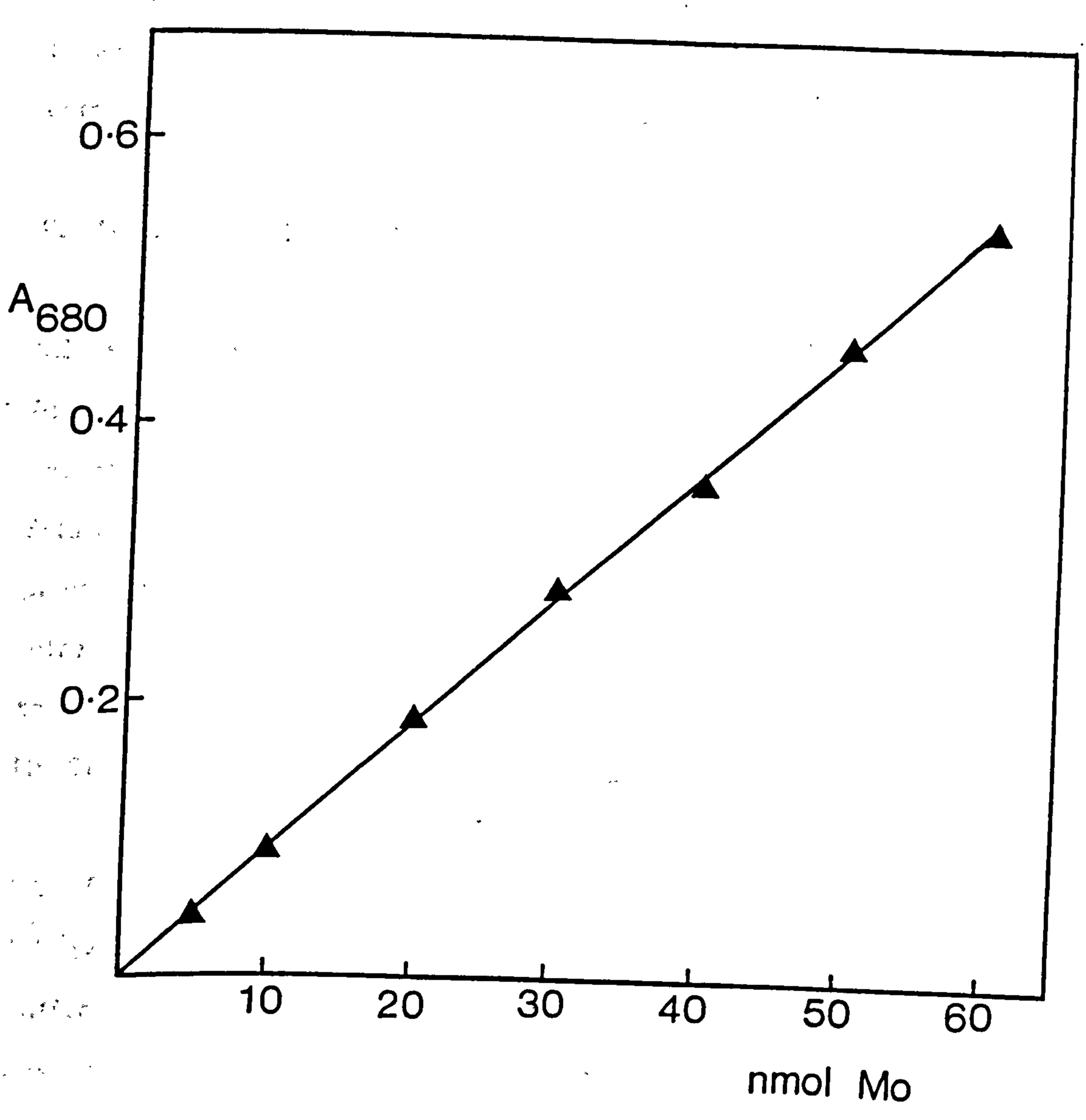
In vitro nitrate reductase (NADH-NR and $FMNH_2$-NR), xanthine dehydrogenase, NADH-CR, nitrite reductase activities and nitrate determinations were carried out on crude extracts of 9 day old induced shoot tissue from the M3 and M4 generations of selected plants. A group of 30-60 seeds of segregating M4 seedlings of R9201 and R11301 were sown and grown as described in Section 2, with an 18 hour period of induction using half strength Hoaglands Solution containing 15 mM KNO_3, prior to extraction. M3 seedlings of R12202, R12801, R13202, K1803, K1901, K2301 and K2401, arising from self fertilisation of the M2, were treated in the same fashion.

The seedlings were assayed when 6 days old for **in vivo** levels of NR (Section 4), after an 18 hour induction period, to classify individual seedlings into NR+ and NR minus plants.

Figure 8

Molybdenum standard curve

This plot shows the relationship between molybdenum concentration in standard solutions of sodium molybdate after addition of a dithiol reagent (Cardenas and Mortenson, 1974) as described in the text (Chapter 2, Section 6).


0·6
0·4
0·2
A_{680}
10
20
30
40
50
60
nmol Mo

It was necessary to water the plants with half strength Hoaglands Solution between the two induction periods since the toxic effects (leaf withering and death) on the NR minus plants were apparent after 3 consecutive days on nitrate. Individual 9 day old plants were extracted in a 5:1 ratio (v/w) in NR Extraction Buffer (Section 3).

8. SUCROSE DENSITY GRADIENTS

8.1 Plant growth

Plants were grown for 6 days under the conditions given in Section 2 except that the seedlings were induced with half strength Hoaglands Solution containing 15 mM KNO3, 48 hours prior to extraction. NR minus plants were identified using either the **in vivo** NR or qualitative **in vitro** NR assays (Section 4) 24 hours before extraction and application to the gradients. The segregating M4 generations of R9201, R11301 and R9401 and the M3 generations of R12202 and R12801 were used.

8.2 Extraction

3:1 (v/w) extractions were made of 6 day old tissue in NR Extraction Buffer (Section 3), containing 10 µM leupeptin. This crude extract was assayed for levels of **in vitro** NADH-NR activity. Microgramme quantities of solid myoglobin, alcohol dehydrogenase and catalase (S values of 2.04, 7.4 and 11.3 respectively) were added to the extract to act as internal standards.

8.3 Preparation of gradients

A continuous 18 ml 20-4% sucrose gradient was made by overlaying 3.6 ml aliquots of 20, 16, 12, 8 and 4% sucrose solutions in a 23 ml polypropylene centrifuge tube (MSE 34411-138). The sucrose solutions were made up in NR Extraction Buffer (Section 3) containing 10 µM leupeptin. The leupeptin was required to stabilise the nitrate reductase during the long centrifugation. Three gradients were prepared 18 hours prior to centrifugation to allow the zones to form a continuous gradient.

8.4 Sample application to gradients

0.4 ml aliquots of crude extracts containing protein standards, were gently layered on to the surface of each gradient. All three gradients were balanced against each other.

8.5 Centrifugation

Three gradients were placed in an MSA 30.25 rotor (3 x 23 ml swing out) and centrifuged at 94,000 g (38,000 rpm) for 20 to 22 hours at 6° C in an MSE Superspeed 65 Preparative Ultracentrifuge.

8.6 Fractionation of gradient

The gradients were fractionated from the bottom using an MSE Tube Piercer, collecting about 45 fractions of 0.4 ml aliquots. The fractions were numbered and stored at 4° C. All fractions were assayed for NADH-CR activity; fractions 10-32 were assayed for NADH-NR activity: and the relevant fractions were assayed for the protein standards.

8.7 Protein standard assays for gradients

8.7.1 Myoglobin

The absorbance of total fractions were read, in a microcuvette at 415 nm in a spectrophotometer, prior to any other assay of the gradient.

8.7.2 Alcohol dehydrogenase

A solution of 27 mM Tris/HCl (pH 8.5) containing 1 mM NAD+ and 54 mM ethanol was prepared. 3 ml aliquots were placed in a quartz cuvette and 20 µl fraction volumes were added. The contents of the cuvette were mixed by inversion and rapidly placed in a spectrophotometer linked to a chart recorder. The increase in absorbance at 340 nm, due to the formation of NADH, was measured for periods of up to 5 minutes.

8.7.3 Catalase

0.4 ml of 30 vol hydrogen peroxide was added to 100 ml 0.1 M potassium phosphate buffer, pH 7.5. A 3 ml aliquot was placed in a quartz cuvette, 30 µl was added from the fractions to this solution, the mixture shaken and the decrease in the absorbance at 240 nm, due to the decrease in hydrogen peroxide concentration, was measured using a spectrophotometer linked to a chart recorder.

8.8 Linearity of gradient

The linearity of the sucrose gradient was measured using an Abbé Refractometer. The values recorded, measured in sugar units, were converted to refractive index by the use of tables (supplied with refractometer by Bellingham and Stanley, London).

9. GENETICS

See Chapter 5 for experimental details.

10. REPAIR OF NITRATE REDUCTASE AND XANTHINE DEHYDROGENASE ACTIVITIES BY GROWTH ON HIGH LEVELS OF MOLYBDATE

Seeds, usually 30, were sown and grown according to Section 2. When the seedlings were 5 days old, they were assayed for **in vivo** NR activity (Section 4) after 18 hours prior induction with half strength Hoaglands Solution containing 15 mM KNO3. On the basis of this assay, the plants were then divided into two sets; one set was watered with half strength Hoaglands Solution containing 15 mM KNO3, the other set with half strength Hoaglands Solution containing 15 mM KNO3 and 2 mM sodium molybdate. Both sets had NR+ and NR minus plants present in the segregating M4 generations of R9201, R11301 and R9401, used for this study. After 18 hours of these treatments, **in vitro** NADH-NR assays (Section 5) were carried out on half of the plants in each set to determine the effect of molybdate on NR minus plants. There was not sufficient material from individual plants to allow both NR and xanthine dehydrogenase activities to be measured. Therefore, the remaining plants were watered in the same manner and their **in vitro** NADH-NR and xanthine dehydrogenase activities were measured the following day. This allowed two measurements of **in vitro** NR activity to be taken after the addition of excess molybdate.

11. RECONSTITUTION OF NR ACTIVITY TO DETERMINE MoCo ACTIVITY OF THE MUTANTS

The ability of mutant MoCo to reconstitute fungal **nit1** NR activity was studied by extracting the MoCo, employing Mendel's (1983) heating method, and incubating with **N. crassa nit1** extract, in the presence of excess molybdate. The assay was optimised for barley by Mendel et al (1985).

The segregating M4 generations of R9201, R11301 and R9401, and the M3 generations from R12202 and R12801 were used.

11.1 Neurospora crassa nit1 subculture

N. crassa nit1 was routinely subcultured on to sterile, solid (1.5% agar), Fries Medium (table 5) containing 31 mM NH_4Cl as sole nitrogen source and grown at 25-30° C for three days. It was stored at 4° C until required.

11.2 Induction of N. crassa nit1

500 ml of Fries medium in a 1 l flask, containing ammonium chloride and no agar, was innoculated with conidiospores rinsed from the surface of the subculture with 10 ml of medium. This innoculum was shaken vigorously (2,000 rpm) in a Status Mark 5 Orbital Shaker for 40 hours at 25° C. The mycelia were recovered by filtration, washed three times in warm distilled water, filtered and finally blotted as dry as possible using hand pressure.

Table 5

Composition of Fries Complete Medium

	µM	mg/l
Sodium tartrate	10800	2480
*NH4Cl	31410	1680
KH2PO4	294	400
MgSO4.7H2O	810	200
NaCl	680	40
CaCl.2H2O	480	52.8
Sucrose	17530	6000
Sodium tetraborate	251.7	0.096
Na2MoO4	262.2	0.054
ZnSO4	4.4	0.704
FeCl3.6H2O	2.8	0.768
CuCl2	2.0	0.274
MnCl.4H2O	293.1	0.058
CuSO4.5H2O	1.5	0.508
Biotin	18.5	4.5

*NH4Cl was replaced by NaNO3 in Fries NR Induction Medium

The mycelial mat was added to a 500 ml flask containing 250 ml sterile Fries Induction Medium (Fries Complete Medium with 32 mM NaNO3 replacing NH4Cl) see table 5. The flask was shaken on the Orbital Shaker, under the same conditions, for a further 5 hours. After this induction period the mycelia were filtered and washed thoroughly with distilled water, to help remove any excess nitrate. The mycelial mat was then blotted extensively until dry.

11.3 N. crassa nit1 extraction

The mycelial mat was peeled into small fragments and ground up in liquid nitrogen with a pestle and mortar. The fine powder was transferred into a centrifuge tube containing Breakage Buffer (0.1 M potassium phosphate pH 7.2, containing 1% (w/v) NaCl and 5 mM EDTA) in a 3:1 (v/w) ratio. This slurry was mixed with a glass rod and centrifuged at 20,000 g for 30 minutes at 4° C. The supernatant was collected, frozen in 1 ml aliquots (in liquid nitrogen) and stored at -20° C until required.

11.4 MoCo extraction from plant material

Plant tissue was extracted in a 10:1 (v/w) ratio in 50 mM potassium phosphate buffer pH 7.5, containing 5 mM reduced glutathione and 10 mM sodium molybdate. This extract was centrifuged at 20,000 g for 20 minutes at 4° C. The supernatant was collected and an aliquot, usually 50 µl, removed for protein determination. The supernatant was evacuated, flushed twice with oxygen-free nitrogen, incubated at 80° C for 90 seconds and then rapidly cooled on ice, this caused the loss of NR activity and protein denaturation.

11.5 MoCo content assay

50 µl of the MoCo supernatant was added to 100 µl of **N. crassa nit1** extract with 5 µl of 20 µM NADPH in a centrifuge tube. Tubes were stoppered and incubated for 30 minutes at 25° C after two rounds of evacuation and flushing with oxygen-free nitrogen. After addition of 50 µl 0.1 M KNO3 and 100 µl 0.1 mM FAD, the tubes were evacuated and flushed as previously. Activity of the reconstituted nitrate reductase was then assayed by the further incubation for 30 minutes at 25° C. The reaction was stopped by plunging the tubes into a boiling water bath for 5 minutes. After cooling, 0.5 ml of both 1% sulphanilamide/3N HCl and 0.02% NED were added. The tubes were centrifuged at 4,000 g for 5 minutes at room temperature to remove precipitated protein. The absorbance at 540 nm was measured. This was compared to a standard curve (figure 9) based on the following: 50 µl of MoCo extraction buffer; 100 µl of **nit1** extraction buffer containing 1 µmol NADPH; 100 µl of 0.1 mM FAD; 50 µl of (0-100 nmol) KNO solution. To this, 0.5 ml 1% sulphanilamide/3N HCl and 0.5 ml 0.02% NED were added. The absorbance was measured at 540 nm and compared to blanks in which the MoCo extract was replaced with the same buffer lacking any plant material.

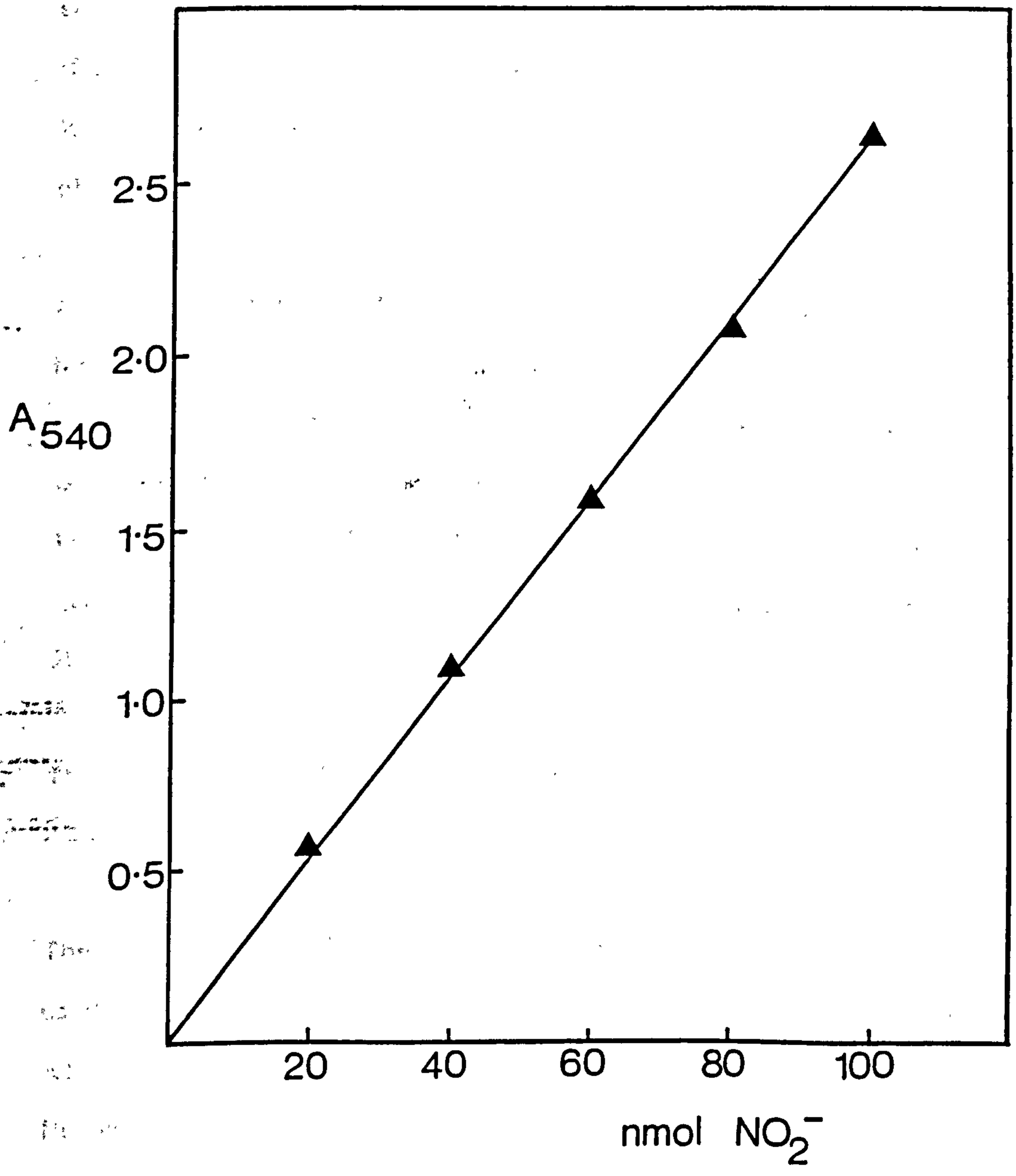
12. RECONSTITUTION OF NADH-NR ACTIVITY IN EXTRACTS OF R12801 USING MoCo SUPPLIED BY XANTHINE OXIDASE

Preliminary experiments to see whether R12801 could complement free MoCo to form an active NR were attempted using the following two methods:

Figure 9

Nitrite standard curve employed to estimate MoCo content

This plot shows the relationship between nitrite concentration in standard nitrite solutions and the absorbance at 540 nm after following the procedure given in the text (Chapter 2, Section 11).


2·5
2·0
A_{540}
1·5
1·0
0·5
20
40
60
80
100
nmol NO_2^-

1. R12801 was extracted, in a 3:1 (v/w) ratio, under exactly the same conditions used to extract **nit1** (see above) apoprotein. The 'free' MoCo was supplied by xanthine oxidase (0.30 Units of Sigma X-1875) which was heat treated at 80° C for 90 seconds, as for plant MoCo release.

A 100 µl aliquot of R12801 extract was incubated with 50 µl MoCo extract from xanthine oxidase in the presence of 1 µmol NADH in a final volume of 155 µl. This was evacuated, twice flushed with oxygen-free nitrogen and incubated for 30 minutes at 25° C. The reconstructed NR activity was determined by the addition of 100 µl 0.1 mM FAD and 50 µl 0.1 M KNO3 and incubated for a further 30 minutes at 25° C.

2. The method was that used by Narayanan **et al** (1984) with slight modifications.

The extraction buffer for R12801 was the usual extraction buffer used for crude extracts of NR. A 3:1 (v/w) extraction was made of R12801 and a 0.7 ml aliquot of this slurry was added to a nitrogen flushed and cooled microfuge tube. To this 0.3 ml of heat treated (80° C for 90 seconds) MoCo from xanthine oxidase was added. This was microfuged at 4° C for 20 minutes after evacuating and flushing with nitrogen. 300 µl aliquots of the supernatant were added to 0.7 ml of the **in vitro** NADH-NR reaction mixture (containing 50 µmol potassium phosphate pH 7.5, 10 µmol KNO3, 0.1 µmol NADH). After a 30 minute incubation period the reaction

was stopped and the amount of nitrite produced was determined by the addition of 1 ml of 1% sulphanilamide/3N HCl and 1 ml 0.02% NED. The absorption at 540 nm was measured as usual.

13. UPTAKE KINETICS

These experiments were performed in collaboration with Dr. M. Drew, then at Letcombe Laboratory.

13.1 Plant Growth

The seed from the M4 generations of R9401 were imbibed at 20° C in the dark for 2 days and then transferred to a stainless steel mesh suspended over a well aerated nutrient solution containing (M); $Ca(NO_3)_2$ (0.15), KNO_3 (0.5), KH_2PO_4 (0.1), $MgSO_4$ (0.15), $NaNO_3$ (0.2) with added iron and micronutrients. After 2 days of this treatment they were transferred to a growth room (16 hour photoperiod of 500 $\mu Em^{-2}s^{-1}$ at 20° C). This growth on nitrate for 4 days allowed the selection of NR minus plants (8 days from the start of the experiment) on the basis of bleached tips and withering of the leaves. These plants were shown to possess no **in vitro** NR activity in either roots or shoots in a preliminary experiment. Since prolonged exposure to nitrate is toxic (Bright pers. comm.) the NR+ and NR minus plants were then transferred to a dilute nutrient solution containing (M); $CaCl_2$ (1.0), KCl (1.0), $(NH_4)_2SO_4$ (0.5, $MgSO_4$ (1.0), KH_2PO_4 (1.0) with iron and micronutrients as previously (NH_4^+ was the main nitrogen source). Thirteen days after imbibition (12 hours prior

to measuring influx) addition of KNO3 was made to all cultures to give a concentration of 1.0 M KNO3 to fully induce NR activity and any nitrate transport mechanism.

13.2 Formation of 13NO3-

The Variable Energy Cyclotron at the Atomic Energy Research Establishment, Harwell was used to generate 13NO3-. Pure water was bombarded with a beam of protons under the supervision of Dr. H. E. Sims. 13NO3- (half life 9.96 minutes) is the main product under well oxygenated conditions (Tiedje **et al**, 1981), 5-15% contamination is caused by 13NO2- and 13NH4+. These impurities were removed by boiling the irradiated water under alkaline and then acid conditions before adjusting to pH 6.0 with NaHCO3. Aliquots of this were added to volumes of the nutrient solution containing the appropriate concentration of 'cold' nitrate prior to measuring the uptake by the roots of intact plants. Fluorine-18 is also a contaminant, this like 13NO3-, is a positron emitter but has a much longer half life (110 minutes). This contaminant was accounted for by recounting the samples after an hour and correcting the gamma count taken earlier.

13.3 Experimental treatment of plants

Before the experiment began the plants were removed from the nutrient solution and rinsed for 2 minutes in nitrate-free 'buffer' (0.5 M KH2PO4 and 0.5 M CaSO4 adjusted to pH 6.0 with KOH). Uptake of 13NO3- over a 10 minute period in vigorously aerated media was measured at four concentrations of nitrate (1, 4, 20, 100 mM) made up in the 'buffer'.

At the end of this uptake period roots were rinsed twice with two lots of 'buffer' for 1 minute periods. This was done to remove any isotope adhering to the surface of the roots. The moisture was then removed from the roots by blotting and the intact plant was inserted into a polyethylene container ready for gamma counting. The uptake solutions were also counted. The roots and shoots were weighed and subjected to an **in vivo** NR assay similar to that described previously. This experiment was subdivided into two runs due to the short half life of 13N. All nitrate concentrations and plant phenotypes were present in both runs. A total of 40 plants were assessed for their uptake parameters.

13.4 Radioactive counting

Although 13N is a positron (β+) emitter, it is possible to measure the secondary product, gamma radiation (of 1.02 MeV) by normal gamma spectroscopy. This gamma radiation is caused by annihilation events and can be measured without the need to add scintillants etc. The polyethylene pots were counted for 1 minute in a NaI crystal scintillation counter. These samples were recounted after an hour to correct for the contribution made by 18F.

14. COMPARISON OF IN VITRO NADH AND NADPH NITRATE REDUCTASE ACTIVITY

The standard **in vitro** NADH-NR assays (Section 5) were carried out on extracts (Section 3) of 7 day old shoots and roots (grown as in Section 2) after a 48 hour induction period (using 15 mM KNO_3 in half strength

Hoaglands Solution). The NR+ and NR minus plants were identified before extraction by an **in vivo** assay (Section 3).

The NADPH assays (Section 5) were carried out on the same extractions. The M4 segregating populations of R9201, R11301 and R9401 were used, as were the M3 populations of R12801 and R13202.

CHAPTER 3

PRIMARY SELECTIONS AND BIOCHEMICAL CHARACTERISATION OF THEIR PROGENY

1. INTRODUCTION

At the start of the project a number of barley plants (R9201, R11301, R12202, R12801 and R13202) had been selected at Rothamsted Experimental Station from M2 seeds grown in the presence of chlorate (table 6). Azide was used in all these cases to create the original mutation(s) in the M1 seed. Azide is thought to damage DNA by alkylation (Owais **et al**, 1983), leading to point mutations (Kleinhofs and Smith, 1976). Warner et al (1977) used azide to enhance the rate of mutational events in barley seeds and employed an **in vivo** NR assay to select NR deficient plants. They selected 10 NR deficient plants from 10,000 seedlings, 9 of whose progeny were characterised as apoprotein mutants and were all allelic (designated **nar1**), whilst the progeny of the other plant was characterised as a cofactor (MoCo) mutant, designated **nar2** (Kleinhofs **et al**, 1980). Kleinhofs **et al** (1978) also screened a further 10,000 M2 barley seedlings (treated with azide as M1 seeds) and found 6 chlorate resistant plants. However, all attempts to transplant and propogate these plants failed. All were lethal (Kleinhofs **et al**, 1978). Bright **et al** (1983) screened 260,000 M2 barley seeds (the M1 seeds were treated with azide) using chlorate and isolated a single conditional lethal MoCo mutant. Ethylmethane sulphonate (EMS), another mutagen causing point mutations, has been used to mutagenise barley seeds (Tokarev and Shumny, 1977). They screened about 13,000 M2 seedlings for partial resistance to chlorate and identified three NR deficient lines, one was allelic to the **nar1** mutants, the other two were MoCo mutants, designated **nar3a** and **nar3b** (Shumny and Tokarev, 1981) since they were allelic to each other but non-allelic with the **nar2a** mutant

(Kleinhofs **et al,** 1983). It is clear that very few genetic loci concerned with NR have been identified when azide or other point mutagens were used, therefore it was decided to use gamma radiation to mutagenise barley seeds in an attempt to widen the spectrum of mutants available, since it is known that different mutagens produce different varieties of mutants (Stadler, 1928). Gamma radiation causes deletion mutations (Auerbach, 1976) and double and single strand breaks (Ward, 1975). Since gamma radiation has a different effect on DNA it is more likely that different mutations will occur, possibly allowing the identification of new classes of NR mutants to those characterised already.

The mutants in nitrogen metabolism produced in higher plants so far have been caused by treatment with the following mutagenic agents; azide (Bright **et al,** 1983; Warner **et al,** 1977); N-methyl-N'-nitro-N-nitrosoguanidine (Strauss **et al,** 1981; Fankhauser **et al,** 1984; Lazar **et al,** 1983); N-ethyl-N-nitrosourea (Müller and Grafe, 1978; Evola, 1983a; Marton **et al,** 1982a and 1982b); ethylmethane sulphonate (Buchanan and Wray, 1982; Xuan **et al,** 1983); and X-rays (Steffen and Schieder, 1984).

2. SELECTIONS FROM M2 SEEDLINGS AFTER GAMMA RADIATION MUTAGENESIS

Owing to the ease of identification, chlorophyll mutants (pale green to albinos), which are assumed to be caused by single gene mutations, are often used as a rough guide to measure the effectiveness of various

Figure 10

A comparison of Golden Promise and Maris Mink seedlings after growth in the presence of chlorate for 4 days

Seedlings were grown under the conditions described for selection given in Chapter 2, Section 1.

Golden Promise
Mink
Chlorate
5mM

Table 6
The M2 seed batches from which the selections were isolated

M2 seed batch	Chlorophyll* mutants/100 M2	Mutagenic treatment	Selections
M2/81/9	1.3	gamma radiation 18 krad	K2401 K1901 K2301
M2/81/8	1.4	gamma radiation 15 krad	K1803
M2/78/3	5.7	Azide 10 mM	R9401 R9201
M2/78/7	6.4	Azide 10 mM	R11301
M2/81/1	3.5	Azide 5 mM	R12202 R12801 R13202

* Chlorophyll mutants per 100 germinated M2 seeds. These data were kindly supplied by Dr. S. W. J. Bright.

mutagenic treatments to increase the number of mutations in a given population of plants. The M2 generation of gamma irradiated seeds (M2/81/9 and M2/81/8) gave similar numbers of chlorphyll mutants per 100 germinated seedlings to those found in the M2 population after azide treatment (M2/78/3, M2/78/7 and M2/81/1) (table 6). This suggests that gamma irradiation is as effective as azide in its ability to cause mutations at these single loci.

I set up chlorate screens using gamma irradiated M2 seedlings from the cultivar Golden Promise. Previous selections were taken from azide treated M2 seedlings from the cultivar Maris Mink. It seemed as if the mutagenised Golden Promise seedlings were more sensitive to chlorate as they died more quickly than the mutagenised Maris Mink seedlings. This was investigated by growing non-mutagenised Golden Promise and Maris Mink seedlings under the chlorate screen conditions supplying 5, 10 or 15 mM chlorate. As can be seen in figure 10, the Maris Mink seedlings were further developed and slightly less damaged than the Golden Promise seedlings when subjected to 4 days of 5 mM chlorate treatment. For this reason 5 mM chlorate was used, with one exception, to distinguish between possible nitrogen metabolism mutants and the non-chlorate resistant plants. Exposing the Golden Promise M2 seedlings to 4 rather than 5 days of chlorate treatment (as was usual for Maris Mink M2 seedlings) gave a greater chance of survival to partially chlorate resistant selections on transfer to the hydroponic system (Chapter 2, Section 1) since there was less chlorate damage. It is possible that the developmental stage of the seedling is important in the level of toxicity shown by the seedling when grown in the presence of chlorate.

From two M2 populations of Golden Promise treated with either 15 or 10 krad gamma radiation, a total of 48,000 seeds were screened. The seedlings were initially subirrigated with 10 mM nitrate for 5 days, followed by subirrigation with 5 or 10 mM chlorate for 4 days (Chapter 2, Section 1).

A total of 10 plants were selected from these screens as being less scorched than the other seedlings when grown in the presence of chlorate. These seedlings were rescued from the screens and grown hydroponically with glutamine as the source of reduced nitrogen, according to the method in Chapter 2, Section 1.

Of these 10 selections only one, K2401, survived to maturity. K2401 was selected from M2 progeny of Golden Promise seeds treated with 18 krad gamma radiation (table 6). This selection was allowed to self-fertilise to produce an M3 generation. Plants from the M3 generation were analysed biochemically.

3. CHARACTERISATION OF THE PROGENY OF SELECTED PLANTS

3.1 Selections available

As described above, K2401 was available in the M3 generation for biochemical characterisation. A further 9 selections, K1803, K1901, K1901, K2301, R9201, R11301, R12202, R12801 and R13202, isolated by Mr. P. B. Norbury and Dr. S. W. J. Bright (table 6) were also available for characterisation. R9401, another selection, had been isolated by the

same method and shown to be a conditional lethal NR cofactor mutant (Bright **et al**, 1983). This was included in some experiments as a negative control.

3.2 Background of the selections available for characterisation

The selection of K2401 has been described above. The details of seed batches, mutagenic treatment, pretreatment and chlorate concentration used to select, K2401, K1803, K1901, K2301, R9201, R11301, R12202, R12801 and R13202 are found in tables 6 and 7. K1901, K2301 and R9201 were all selected using chlorate after a pretreatment with ammonium ions rather than nitrate.

3.3 Generations used for characterisation

The self-fertilised M3 generations of K2401, K1803, K1901, K2301, R12202, R12801 and R13202 and the M4 segregating populations (after a backcross in the M2) of R9201 and R11301 were used for these characterisation experiments.

Preliminary **in vivo** NR assays were used to identify the NR+ and NR minus phenotype plants in R9201 and R11301 lines after backcrosses with the wild type. This produced a similar situation to that observed for R9401 segregation after a similar backcross (Bright **et al**, 1983).

K2401, K1803, K1901 and K2301 were all green and showed wild type growth on compost containing nitrate. The 'K' series of selection all set seed, giving rise to the M3. The selected plant R13202 (and its progeny) was pale green and grew more slowly than the wild type plants

Figure 11

R11301 NR+ and NR minus plants from the segregating M4 generations

NR+ and NR minus plants were grown for a period of 4 weeks in Eff compost, which contains nitrate.

Growth conditions were 12 hour photoperiod of 150 $\mu Em^{-2}s^{-1}$; 80% relative humidity; 18° C in the photoperiod and 15° C in the dark.

but still produced selfed seeds, giving rise to the M3.

The NR minus plants in the M4 generations of R9201 and R11301 died in compost containing nitrate (eg figure 11). These NR minus plants showed a characteristic phenotype; after one to two weeks the primary leaves began to show signs of withering and this was followed by death of the seedling after a further two weeks growth. On the other hand, the NR+ plants from the M4 populations of R9201 and R11301 did not show these symptoms, grew to maturity and set many seeds.

R12202 could not grow on nitrate as the sole nitrogen source. This was not due to nitrogen starvation since these plants did not show the typical N-starvation feature of pink pigmentation at the base of the shoot, but died instead.

R12801 could grow in the presence of nitrate as the main nitrogen source. When grown in the presence of either ammonium ions or glutamine (as the nitrogen source) in liquid culture R12801 plants seemed to be more prone than the wild types to fungal infections. R12801 seedlings were initially pale green but each leaf darkened with age (see Section 5). However, R12801 plants reached maturity slowly in compost, several months after wild type plants grown under the same conditions (figure 12).

Figure 12

A comparison of the growth of R12801 with that of Maris Mink

The seedlings were grown on compost under the conditions given in Chapter 2, Section 2.1.

M. MINK
67 DAY OLD
12801
67 DAY OLD

4. BIOCHEMICAL CHARACTERISATION

Using the methods described in Chapter 2, Sections 5 and 6, the **in vitro** activities associated with nitrate reductase were assayed in: wild type, Golden Promise and Maris Mink plants; K2401, K1803, K1901, K2301, and R13202 plants; R9201, R11301, R12202, and R12801 plants. For R9201 and R11301 both the NR+ and NR minus plants from the segregating M4 generation were assayed. The results are summarised in tables 8 and 9 and figures 13 to 17.

4.1 Characterisation of wild types

T-tests were used to determine whether there were significant differences in the levels of enzyme activities between the NR minus and NR+ and wild type plants in the following Sections (see Appendix 1).

4.1.1 Xanthine dehydrogenase activity

Extracts of both Maris Mink and Golden Promise gave a purple staining, hypoxanthine-dependent dehydrogenase band (Mendel and Müller, 1976) when subjected to polyacrylamide gel electrophoresis.

4.1.2 In vitro NADH-NR activity

Maris Mink seedlings had an NADH-NR activity of 2.7 µmol NO_2^- produced/h/gfw, with a standard error of 0.2 µmol/h/gfw from a total of 27 (n) samples (table 8 and figure 13). The average value of NADH-NR activity in Golden Promise was 2.0 ± 0.2 µmol/h/gfw calculated from 30 independent assays (table 8 and figure 13). A t-test showed that these mean values of NADH-NR activities found in extracts of Maris

Table 8

Activities associated with nitrate reductase in the progeny of chlorate resistant selections and wild types

Leaves of 9 day old nitrate induced plants were assayed as described in Chapter 2, Section 7.

The number of individual plants assayed (in duplicate) for each activity is given in brackets below the mean and standard error.

The numbers i, ii, iii, iv, v and vi indicate:

(i) xanthine dehydrogenase presence detected by gel assay

(ii) nitrate content (µmol/gfw)

(iii) nitrate reductase (µmol nitrate reduced/h/gfw)

(iv) nitrate reductase (µmol nitrate reduced/h/gfw)

(v) nitrite reductase (µmol nitrite reduced/h/gfw)

(vi) cytochrome c reductase (µmol cytochrome c reduced/h/gfw)

Values were tested for significance at the 95% confidence limit by Students t-test (Appendix 1). The letters a, b, c signify:

a - not significantly different from the wild type

b - significantly greater than the wild type

c - significantly lower than the wild type.

nd- not determined

Table 8

Genotype	XDH(i)	Nitrate content (ii) µmol/gfw	Enzyme Activity (µmol/h/gfw) NADH-NR (iii)	FMNH2-NR (iv)	NiR (v)	NADH-CR (vi)
Golden Promise	+	54.5 ± 5.8 (30)	2.0 ± 0.2 (30)	1.9 ± 0.2 (24)	46.0 ± 3.0 (33)	0.451 ± 0.044 (32)
Maris Mink	+	62.7 ± 2.2 (24)	2.7 ± 0.2 (27)	2.4 ± 0.2 (28)	44.7 ± 3.8 (28)	0.489 ± 0.029 (24)
Combined wild type	+	58.0 ± 3.4 (54)	-	2.2 ± 0.1 (54)	45.4 ± 2.4 (61)	0.467 ± 0.028 (56)
K2401	+	46.1 ± 3.1 a (6)	2.0 ± 0.2 a (6)	2.9 ± 0.2 a (6)	65.0 ± 4.1 b (6)	0.424 ± 0.024 a (6)
K1803	+	56.5 ± 5.0 a (5)	2.2 ± 0.2 a (6)	n.d.	62.0 ± 6.5 b (6)	0.306 ± 0.044 a (6)
K1901	+	29.0 ± 6.7 c (4)	3.6 ± 0.3 b (4)	3.5 ± 0.9 b (4)	60.0 ± 5.0 a (4)	0.813 ± 0.051 b (4)
K2301	+	59.8 ± 7.4 a (5)	3.8 ± 0.3 b (5)	3.2 ± 0.2 b (5)	49.0 ± 8.0 a (5)	0.574 ± 0.044 a (5)
R13202	+	68.7 ± 5.7 a (8)	2.1 ± 0.3 a (9)	2.3 ± 0.1 a (8)	29.7 ± 4.2 c (9)	0.563 ± 0.59 a (9)

Table 9

Activities associated with nitrate reductase in the progeny of NR minus selections

Leaves of 9 day old nitrate induced plants were assayed as described in Chapter 2, Section 7.

The number of individual plants assayed (in duplicate) for each activity is given in brackets below the mean and standard error.

The numbers i, ii, iii, iv, v and vi indicate

(i) xanthine dehydrogenase presence detected by gel assay

(ii) nitrate content (µmol/gfw)

(iii) nitrate reductase (µmol nitrate reduced/h/gfw)

(iv) nitrate reductase (µmol nitrate reduced/h/gfw)

(v) nitrite reductase (µmol nitrite reduced/h/gfw)

(vi) cytochrome c reductase (µmol cytochrome c reduced/h/gfw)

Values were tested for significance at the 95% confidence limit by Students t-test (Appendix 1). The letters a, b, c signify:

a - not significantly different from the wild type

b - significantly greater than the wild type

c - significantly lower than the wild type

d - not significantly different from the NR^+ phenotype

nd - not determined

Table 9

Genotype	XDH (i)	Nitrate content (ii) (µmol/gfw)	Enzyme Activity (µmol/h/gfw) NADH-NR (iii)	FMNH2-NR (iv)	NiR (v)	NADH-CR (vi)
R9201 NR+	+	70.0 ± 9.0 a (4)	2.3 ± 0.4 a (6)	2.4 ± 0.2 a (5)	44.0 ± 5.7 a (6)	0.510 ± 0.065 a (6)
R11301 NR+	+	65.3 ± 1.8 a (6)	3.9 ± 0.5 b (7)	3.5 ± 0.3 a (7)	64.9 ± 15.8 b (7)	0.388 ± 0.039 a (6)
R9201 NR minus	-	153.5 ± 8.2 b (4)	0.0 c (6)	0.0 c (6)	65.3 ± 9.7 a (6)	0.528 ± 0.065 a (6)
R11301 NR minus	-	65.1 ± 6.0 da (5)	0.0 c (8)	0.0 c (8)	67.7 ± 10.7 c (8)	0.507 ± 0.076 a (6)
R12202 NR minus	-	29.4 ± 11.9 c (4)	0.0 c (5)	0.0 c (5)	44.2 ± 8.9 a (5)	1.048 ± 0.127 b (5)
R12801 NR minus	+	31.2 ± 2.8 c (5)	0.0 c (9)	0.0 c (10)	23.0 ± 1.7 c (8)	0.337 ± 0.051 a (10)

Mink and Golden Promise were significantly different. It is therefore important to use the correct wild type when comparing the selections to these values.

4.1.3 In vitro FMNH2-NR activity

From a total of 28 extractions, Maris Mink had a mean FMNH2-NR activity of 2.4 µmol NO2- produced/h/gfw, with a standard error of 0.2 µmol/h/gfw (table 8 and figure 14). Golden Promise possessed an average FMNH2-NR activity of 1.9 ± 0.2 µmol/h/gfw from a total of 24 independent assays (table 8 and figure 14). There was no significant difference, at the 5% level, between Maris Mink and Golden Promise. So the two wild type samples were combined to give a value of 2.2 ± 0.1 µmol/h/gfw (n=54) (table 8). This value was used to compare the FMNH2-NR activities from the selections.

4.1.4 In vitro nitrite reductase activity

The nitrite reductase activity found in Maris Mink was determined from 28 extractions to give a mean and standard error of 44.7 ± 3.8 µmol NO2- reduced/h/gfw (table 8 and figure 15). In Golden Promise the value of 46.0 ± 3.0 µmol/h/gfw from a sample size of 33 (table 8 and figure 15) was not significantly different from the Maris Mink sample. The combined wild type mean value of 45.4 ± 2.4 µmol/h/gfw (table 8 and figure 15) was used to compare all the nitrite reductase activities from the selections.

Figure 13

Bar chart of in vitro NADH-NR activities found in extracts from the progeny of selected plants

The mean (and standard error) of **in vitro** NADH-NR activity found in extracts from 9 day old seedlings is given for the M3 or M4 progeny of the selections K1803, K1901, K2301, K2401, R13202, R9201, R11301, R12202 and R12801. The experimental details are given in Chapter 2, Section 7.

A and B indicate the mean **in vitro** NADH-NR activities found in extracts from 9 day old Maris Mink and Golden Promise seedlings respectively.

Figure 14

Bar chart of in vitro FMNH2-NR activities found in extracts from the progeny of the selected plants

The mean (and standard error) of **in vitro** FMNH2-NR activity found in extracts from 9 day old seedlings is given for the M3 or M4 progeny of the selections K1803, K1901, K2301, K2401, R13202, R9201, R11301, R12202 and R12801. The experimental details are given in Chapter 2, Section 7.

A and B indicate the mean **in vitro** FMNH2-NR activities found in extracts from 9 day old Maris Mink and Golden Promise seedlings respectively.

nd = not determined

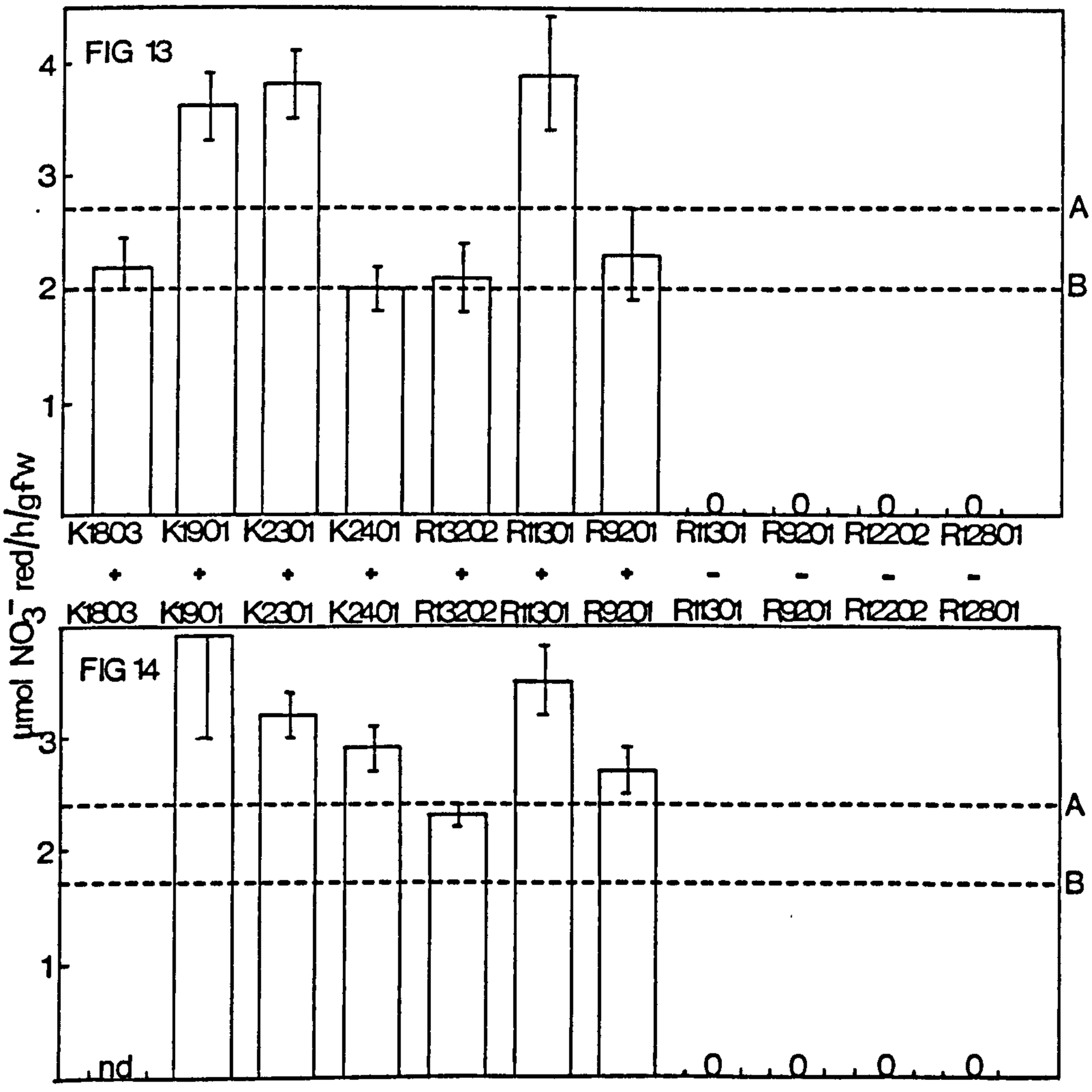


FIG 13
4
3
2
1
A
B
0
0
0
0
K1803
K1901
K2301
K2401
R13202
R11301
R9201
R11301
R9201
R12202
R12801
+
+
+
+
+
+
+
-
-
-
-
red/h/gfw

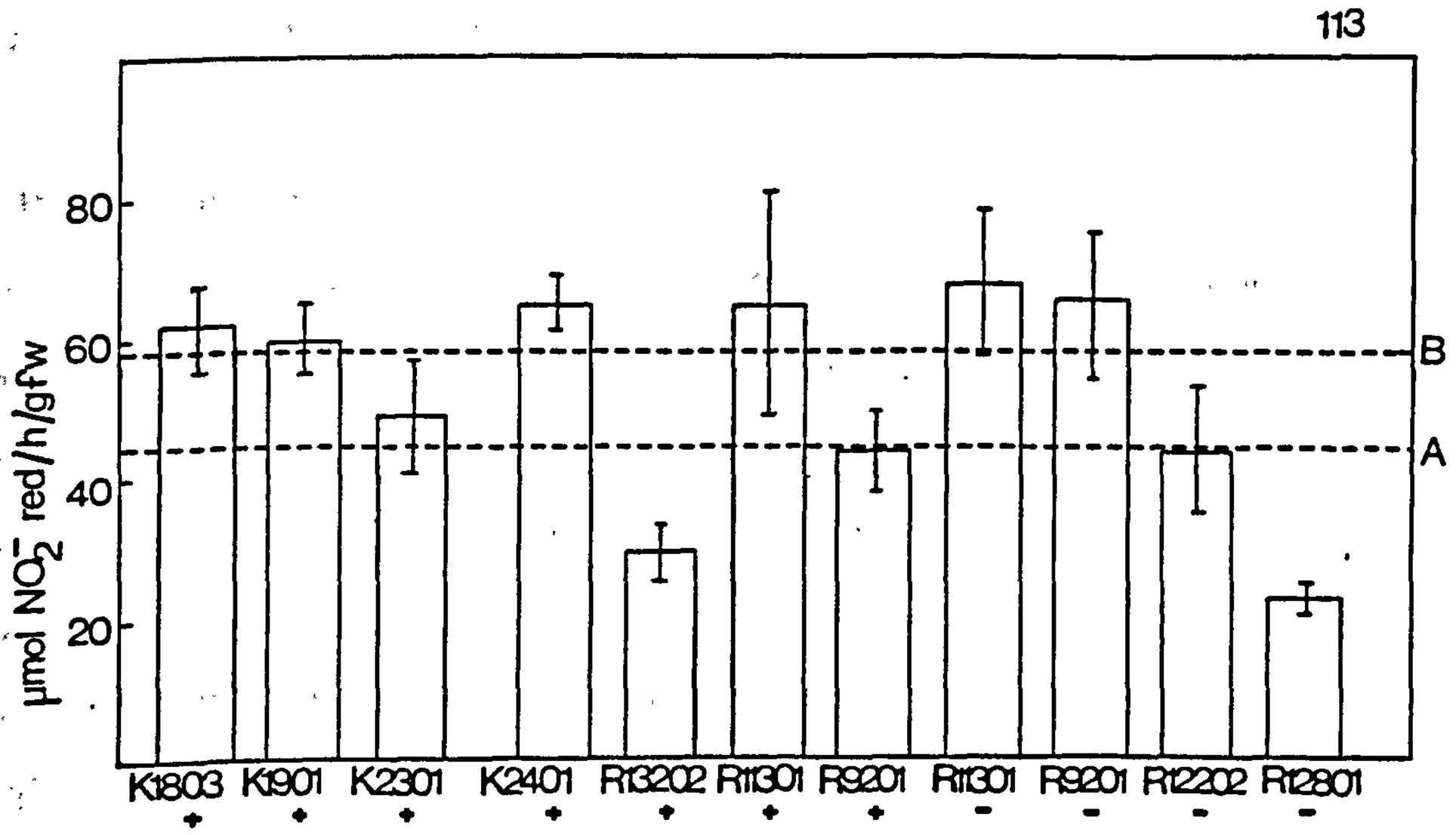
K1803
K1901
K2301
K2401
R13202
R11301
R9201
R11301
R9201
R12202
R12801
FIG 14
µmol NO3-
3
2
1
A
B
nd
0
0
0
0

Figure 15

Bar chart of in vitro nitrite reductase activities found in extracts from the progeny of selected plants

The mean (and standard error) of **in vitro** nitrite reductase activity found in extracts from 9 day old seedlings is given for the M3 or M4 progeny of the selections K1803, K1901, K2301, K2401, R13202, R9201, R11301, R12202 and R12801. The experimental details are given in Chapter 2, Section 7.

A and B indicate the mean **in vitro** nitrite reductase activities found in extracts from 9 day old Maris Mink and Golden Promise seedlings respectively.


113
80
60
40
20
µmol NO_2^- red/h/gfw
B
A
K1803
K1901
K2301
K2401
R13202
R11301
R9201
R11301
R9201
R12202
R12801
+
+
+
+
+
+
+
-
-
-
-

4.1.5 In vitro NADH-CR activity

The average level of NADH-CR activity found in Maris Mink calculated from 24 assays was 0.489 ± 0.029 µmol cytochrome c reduced/h/gfw (table 8 and figure 16). The same activity in Golden Promise was measured in 32 extracts. The average value for Golden Promise was 0.451 ± 0.044 µmol/h/gfw, once again not significantly different from the value determined for Maris Mink. The combined wild type mean was 0.467 ± 0.028 µmol/h/gfw (table 8).

4.1.6 Nitrate content

The nitrate content of 9 day old Maris Mink seedlings was determined as 62.7 ± 2.2 µmol nitrate/gfw after an 18 hour induction period, from a sample size of 24 (table 8 and figure 17). The Golden Promise nitrate content of 54.5 ± 5.8 µmol/gfw from a total of 30 extractions (table 8 and figure 17) was not significantly different from Maris Mink values. The two samples were combined to give a new mean of 58.0 ± 3.4 µmol/gfw (table 8).

4.2 Characterisation of K2401, K1803, K1901, K2301 and R13202

4.2.1 Xanthine dehydrogenase activity

Seven day old primary leaves of nitrate grown M3 seedlings of K2401, K1803, K1901, K2301 and R13202 possessed a hypoxanthine-dependent dehydrogenase band in the same position as the hypoxanthine-dependent band found in wild type extracts.

Figure 16

Bar chart of in vitro NADH-cytochrome c reductase activities found in extracts from the progeny of selected plants

The mean (and standard error) of **in vitro** NADH-cytochrome c reductase activity found in extracts from 9 day old seedlings is given for the M3 or M4 progeny of the selections K1803, K1901, K2301, K2401, R13202, R9201, R11301, R12202 and R12801. The experimental details are given in Chapter 2, Section 7.

A and B indicate the mean **in vitro** NADH-cytochrome c reductase activities found in extracts from 9 day old Maris Mink and Golden Promise seedlings respectively.

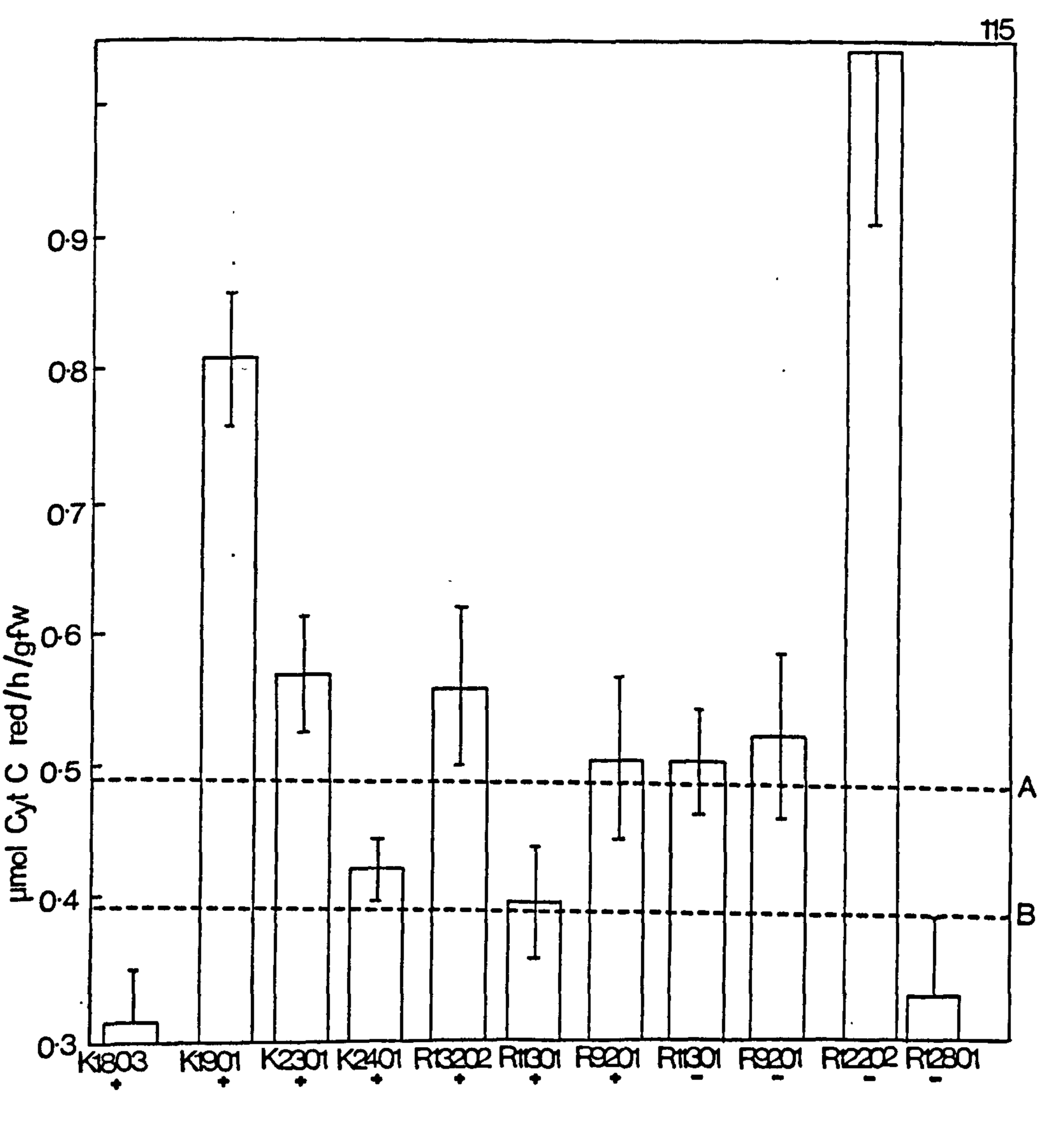
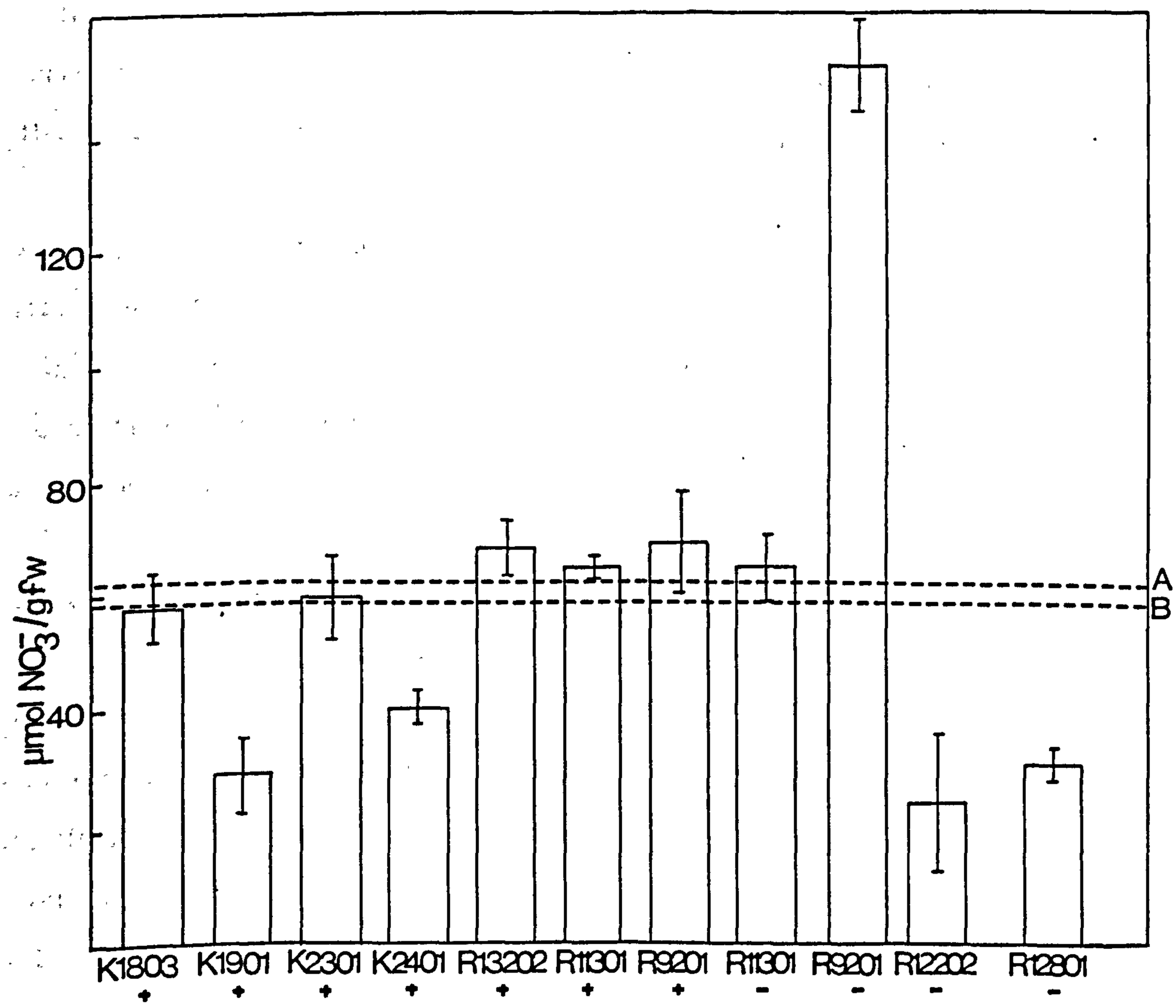

115
0·9
0·8
0·7
0·6
0·5
0·4
0·3
µmol Cyt C red/h/gfw
A
B
K1803
K1901
K2301
K2401
R13202
R11301
R9201
R11301
R9201
R12202
R12801
+
+
+
+
+
+
+
−
−
−
−

Figure 17

Bar chart of nitrate content found in extracts from the progeny of selected plants

The mean (and standard error) of nitrate content activity found in extracts from 9 day old seedlings is given for the M2 or M4 progeny of the selections K1803, K1901, K2301, K2401, R13202, R9201, R11301, R12202 and R12801. The experimental details are given in Chapter 2, Section 7.

A and B indicate the mean **in vitro** nitrate content from 9 day old Maris Mink and Golden Promise seedlings respectively.


120
80
40
μmol NO3-/gfw
A
B
K1803
K1901
K2301
K2401
R13202
R11301
R9201
R11301
R9201
R12202
R12801
+
+
+
+
+
+
+
-
-
-
-

4.2.2 In vitro NADH-NR activity

The levels of NADH-NR activity found in K2401, K1803 and R13202 (table 8 and figure 13) were not significantly different from the NADH-NR activity found in Golden Promise. K1901 and K2301 seedlings possessed more NADH-NR activity (table 8 and figure 13) than Golden Promise; these were calculated to be significantly higher than Golden Promise.

4.2.3 In vitro FMNH2-NR activity

K2401 and R13202 possessed FMNH2-NR activities (table 8 and figure 14) which were not significantly different from the wild type levels. The mean levels of FMNH2-NR activity found in K1901 and K2301 were significantly greater than that found in either wild types.

4.2.4 In vitro nitrite reductase activity

The mean levels of NiR found in K2401 and K1803 were significantly greater than either of the wild types (table 8 and figure 15). K1901 and K2301 possess levels of NiR (table 8 and figure 15) which were not significantly different from either of the wild types. The average level of NiR found in R13202 was less than that in the wild types (table 8 and figure 15).

4.2.5 In vitro NADH-CR activity

K2401, K1803, K2301 and R13202 possessed levels of NADH-CR activity which were not significantly different from those found in the wild types (table 8 and figure 16). K1901 had an average level of NADH-CR activity which was significantly greater than that of the combined wild types (table 8 and figure 16).

4.2.6 Nitrate content

The levels of nitrate found in K1803, K2401, K2301 and R13202 were found to be not significantly different from that in the wild types (table 8 and figure 17). K1901 contained significantly less nitrate than the wild types (table 8 and figure 17).

4.3 Characterisation of R9201, R11301, R12202 and R12801

Preliminary **in vivo** NR assays were used to identify NR+ and NR minus phenotype plants in the M4 segregating populations of R9201 and R11301.

4.3.1 Xanthine dehydrogenase activity

The NR+ plant extracts of R9201 and R11301 gave hypoxanthine-dependent dehydrogenase activity bands after electrophoresis on polyacrylamide disc gels (eg figure 18). Hypoxanthine-dependent dehydrogenase activity was not detected in extracts of the R9201 and R11301 NR minus plants (eg figure 18) nor in extracts from R12202. However, this activity was found in extracts of R12801.

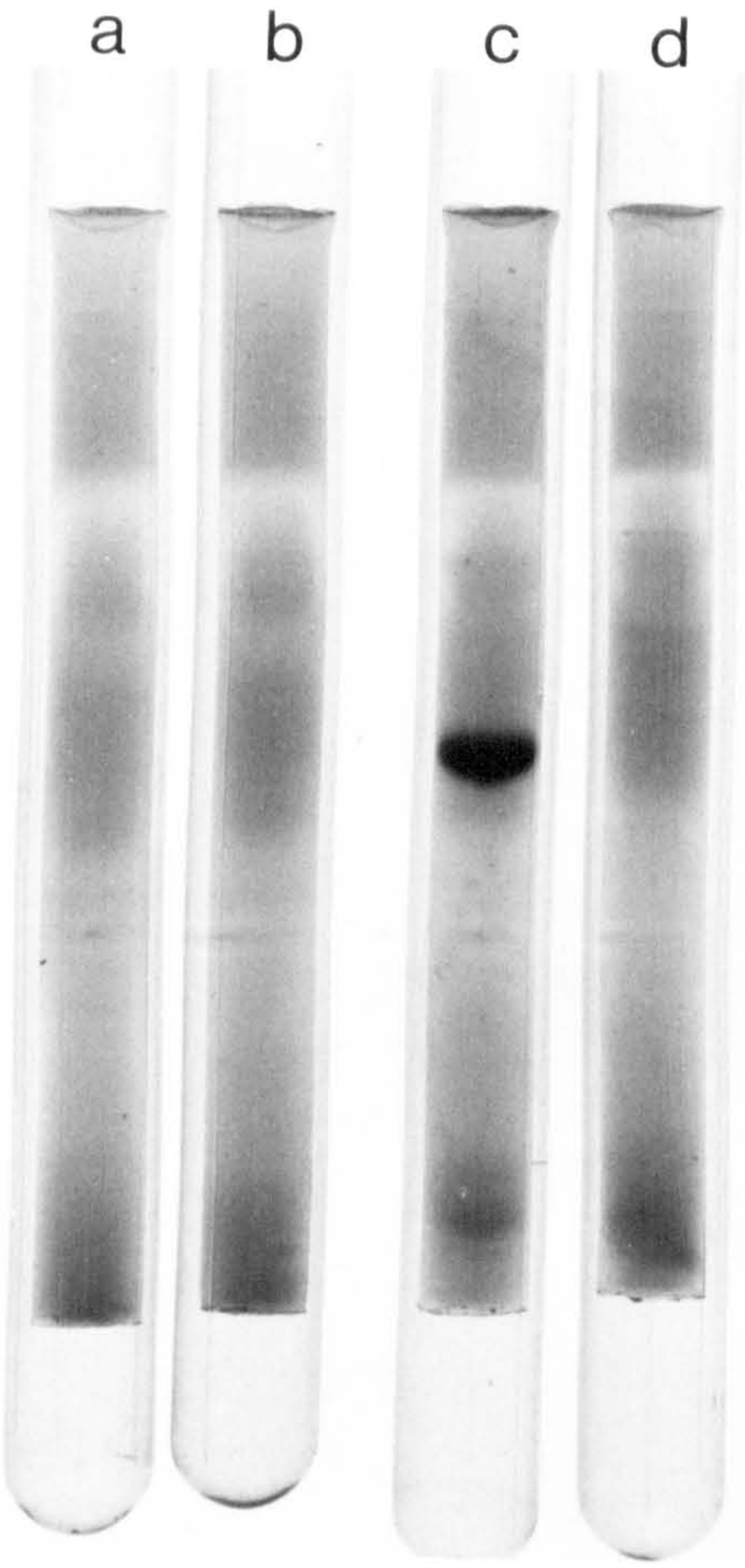
4.3.2 In vitro NADH-NR activity

The level of NADH-NR activity found in the R9201 NR+ plants was not significantly different from that detected in Maris Mink (table 9 and figure 13). However, the NR+ R11301 plants on average gave a significantly greater NADH-NR activity than was found in Maris Mink (table 9 and figure 13). The NR minus plants of R9201, R11301 , R12202 and R12801 possessed no detectable NADH-NR activity.

Figure 18

Xanthine dehydrogenase activity detected in extracts of R9201 NR+ and NR minus seedlings

The experimental conditions are given in Chapter 2, Section 5. Extracts from NR minus seedlings were electrophoresed in 5% PAGE gels a and b, whilst extract from NR+ seedlings were treated in an identical fashion in similar gels, c and d. The gels were stained for xanthine dehydrogenase activity in the presence (gels a and c) or absence (gels b and d) of hypoxanthine.


a
b
c
d

4.3.3 In vitro FMNH2-NR activity

The activities of FMNH2-NR in NR+ plant extracts of R9201 and R11301 were not significantly different from the wild type levels (table 9 and figure 14). The NR minus plants of R9201, R11301, R12202 and R12801 contained no detectable FMNH2-NR activity (table 9 and figure 14).

4.3.4 In vitro nitrite reductase activity

The NiR activity in the R9201 NR+ and NR minus plants were not significantly different from the wild types (table 9 and figure 15). The R11301 NR+ plants possessed significantly higher levels than those found in the wild types (table 9 and figure 15). The NiR activity found in R11301 NR minus plant extracts was not significantly different from R11301 NR+ plants (table 9 and figure 15). Similarly R12202 extracts gave a mean level of NiR activity which was not significantly different from the wild types (table 9 and figure 15). The NiR activity found in R12801 was, on the other hand, half that of the wild type levels; a significant difference from the other wild types (table 9 and figure 15).

4.3.5 In vitro NADH-CR activity

The average NADH-CR activities found in R9201 NR+ and NR minus, R11301 NR+ and NR minus, and R12801 plants were not significantly different from the wild type levels of NADH-CR activity (table 9 and figure 16). R12202 possessed approximately double these levels of NADH-CR activity (table 9 and figure 16).

4.3.6 Nitrate content

The nitrate content of leaves of 7 day old seedlings was measured after the pretreatment for 18 hours with 15 mM KNO3. The nitrate contents of the wild type plants, R9201 NR+ and R11301 NR+ and NR minus plants were all similar (table 9 and figure 17). The nitrate content of R9201 NR minus plants was significantly higher than the wild type values and for R12202 and R12801 the content was lower than this value (table 9).

5. CHLOROPHYLL CONTENT OF R12801 AND R13202

R13202 seedlings were pale green and grew slowly to maturity in soil. R12801 seedlings when grown on compost were initially pale green, however, as each individual leaf aged, it became greener until it was similar to the wild type leaves. Therefore, there were many shades of green leaves on the same R12801 plant (figure 19).

Individual plants of both R13202 and R12801 occasionally showed damaged (but not withering) primary leaves when grown on compost.

6. CONCLUSIONS

6.1 Mutagenesis with gamma rays and recovery of viable plants

In comparable experiments using 2.6×10^5 M2 barley (cv. Maris Mink) seeds after azide mutagenesis, Bright **et al** (1983) selected 33 plants, of which survived; two had wild type levels of NR activity

Figure 19

A comparison of the shades of green found in the leaves of an R12801 plant

R12801 was grown in compost under the conditions described in Chapter 2, Section 2.1.

12801
67 DAY OLD

and three were shown to be NR minus. One of these NR minus plants, R9401, was further characterised. Therefore, the success rate for chlorate selection (defined as the rate for chlorate selection divided by the number of plants screened) was 1.3×10^{-4} for Bright **et al** (1983). This was similar to my selection rate of 2.1×10^{-4}. These numbers suggest that I might have been slightly less stringent in selecting plants, however, all the apoprotein mutants characterised so far are leaky and might well not be totally chlorate resistant. This less stringent selection would be one way of possibly isolating apoprotein mutants from chlorate screening. Since mutagenesis rates (based on the number of chlorophyll mutants identified) were similar for azide and gamma radiation it was reasonable to suggest that the number of NR minus plants isolated from gamma irradiated M2 seeds would be approximately that found for azide treatment. Bright **et al** (1983) isolated 1 plant which survived and was characterised as having the NR minus phenotype. This gives a success rate of NR minus mutant selection of 0.107, derived by dividing the number of NR minus plants obtained by the number of selected plants. Of the 10 plants I selected one might have expected one surviving NR minus plant. It was disappointing that the only selected plant which survived was a wild type plant with respect to nitrate assimilation. This also suggests that the system for maintaining NR minus plants was not optimal.

The selection of plants with chlorate after pretreatment with ammonium ions is rather interesting as nitrate flux and not ammonium is thought to induce nitrate reductase. Since R9201, at least, was found to be NR minus, this suggests the following possibilities; some nitrate was

present in the pretreatment allowing partial induction of NR (this could have come from nitrification during the ammonium treatment as these experiments were not carried out in sterile conditions) or, ammonium ions can also induce NR in these plants. Ammonium ions did not alter the level of NR activity in barley under nitrate induced conditions (Deane-Drummond, 1982).

6.2 K2401, K1803, K1901, K2301 and R13202 are wild type plants

All the progeny of these selections possessed NADH-NR and FMNH2-NR activity (K1901 and K2301 possessed NADH-NR and FMNH2-NR which were greater than the wild type activities). This infomation indicates that K2401, K1803, K1901, K2301 and R13202 have no defects in the overall electron transport chain of NR. Defects in the distal end would be detected by loss of FMNH2-NR activity and the proximal end by loss of NADH-CR activity.

The proximal parts of the NR electron transport chains for K2401, K1803, K1901, K2301 and R12302 were found to be unaffected by mutagenic treatment, since the level of total NADH-CR (ie both constitutive, non-NR related; and nitrate inducible, NR-related) in all these selections were similar to that of the wild type or greater.

K2401, K1803, K1901, K2301 and R13202 all possessed xanthine dehydrogenase activity. When this last fact is coupled with the data presented above, it becomes clear that K2401, K1803, K1901, K2301 and R13202 were wild type plants with respect to nitrate assimilation. The NiR levels in K2401, K1803, K1901, K2301 and R13202 were found to be

either the same as the wild type or greater.

The nitrate contents of K2401, K1803, K2301 and R13202 were not significantly different from that found in the wild type. Although K1901 had only 50% of the wild type nitrate content, it had the same levels of **in vitro** NR and NiR activity as the wild type, suggesting that it had the potential to reduce as much nitrate and nitrite as the wild types (when supplied with an excess of nitrate or nitrite and reducing power).

K2401, K1803, K1901, K2301 and R13202 all grew to maturity and set plenty of seeds, similar to the wild type plants which escaped through the chlorate screens described by Bright **et al** (1983). If selected plants were actually wild type plants, they would not die during the rescue operation. I selected 10 plants using chlorate, 9 of these died. Bright **et al** (1983) selected 33 plants using chlorate, of which 28 died. From a total of 50 barley (cv. Winer) plants selected by partial resistance to chlorate, 45 possessed high levels (10% of the wild type) of NR activity (Tokarev and Shumny, 1977). Müller and Grafe (1978) selected 64 **N. tabacum** (cv. Gatersleben) cell lines using chlorate, 22 of these exhibited wild type levels of NR activity. Using chlorate to select **N plumbaginifolia** cell lines, Marton **et al** (1982a) isolated 136 chlorate resistant lines, of which 107 lines possessed wild type or decreased levels of NR activity. Evola (1983a) found that the four **N. tabacum** chlorate resistant lines also possessed NR activity. Nichols and Syrett (1978) isolated a total of 212 of chlorate resistant **Chlamydomonas reinhardii** mutants, of which 113 were

able to grow well on nitrate (indicative of the NR+ phenotype). All these selections which possess NR activity would also appear to be 'escapees' and are examples of the fact that any primary selection system will allow some wild type plants through. Those plants which were selected with chlorate and grew to maturity, producing many seeds, usually had the NR+ phenotype. This suggests that those selections which died on rescue from the chlorate screens, were likely to be mutants which would have been different from those previously selected and therefore valuable in extending the understanding of the nitrate assimilation pathway.

The survival of the NR+ selection R13202 through the screen could have been due to the lack of chlorophyll under the growth conditions used. R13202 might have had problems with maintaining energy requiring systems such as nitrate assimilation due to this lack of chlorophyll. This fact coupled with slow growth could be a reason for the survival of R13202 when grown in the presence of chlorate. Some chlorophyll mutants are known to lack NR activity (Warner and Kleinhofs, 1974).

6.3 R9201, R11301 and R12202 are MoCo mutants

The NR minus progeny of all these selections lacked both NADH-NR and FMNH2-NR activities. The lack of NADH-NR activites in these mutants was similar to the previously selected and characterised mutant R9401 at Rothamsted (Bright **et al**, 1983). All the other barley mutants so far characterised are leaky, possessing 0.6-8% of the wild type (cv. Steptoe and Winer) NR activity (Kleinhofs **et al**, 1980; Somers **et al**, 1983a). Wild type levels of NADH-CR activity were found in the NR

minus plants of R9201 and R11301, suggesting that, in these two mutants, the proximal end of the nitrate reductase electron transport chain was not affected. The level of NADH-CR activity found in R12202 was greater than that found in the wild type, which again suggests that there is no defect in the proximal end of the nitrate reductase electron transport chain in this line.

The NR minus progeny of R9201, R11301 and R12202 also lacked xanthine dehydrogenase activity, giving strong evidence that they each have a defective MoCo. These plants had wild type levels of nitrite reductase, indicating that the defects are purely associated with nitrate reductase and are not involved in the possible joint regulation of nitrate reductase and NiR as described for fungi (Cove, 1979).

The nitrate content of the NR minus plants of R9201, R11301 and R12202 were similar to wild type, suggesting that there was no defect in the nitrate uptake mechanism in these mutants. R12202 possessed only 50% of the wild type nitrate content, this is a sufficient level to induce NR so the lack of NR cannot be due to a primary lesion in nitrate uptake. There have been suggestions that NR may be involved in the nitrate uptake mechanism (Butz and Jackson, 1977), but the evidence from these mutants implies that there is no correlation between the nitrate content and NR activity. I have managed to characterise three barley lines (R9201, R11301 and R12202) as MoCo mutants. Bright et al (1983) identified a further barley line (R9401) as a MoCo mutant. Kleinhofs **et al** (1985), on the other hand, has characterised 3 MoCo lines (**nar2a** **nar3,** **nar4**) and 18 apoprotein lines in barley

cultivars, Steptoe and Winer. **nar3a** and **nar3b** were isolated by Tokarev and Shumny (1977). They isolated a third NR deficient line, Xno 29, in barly cv. Winer which was an apoprotein mutant. The difference between the selection procedures was that Kleinhofs et al (1978) selected plants on the basis of lowered **in vivo** NR activity compared to the wild type, whilst Tokarev and Shumny (1977) and Bright's group at Rothamsted used chlorate.

Using chlorate to select for NR mutants in **Nicotiana** species the following ratios (MoCo:apoprotein) were found: **N. tabacum** cv. Gatersleben, 4:6 (Müller and Grafe, 1978); **N. tabacum** cv. Xanthi, 4:0 (Buchanan and Wray, 1982); **N. tabacum** cv. Xanthi, 1:1 (Evola, 1983a and b); **N. plumbaginifolia**, 4:7 (Marton **et al**, 1982a and b); **N. plumbaginifolia** 6:26 (Negrutiu **et al**, 1983; Dirks **et al**, 1985). Braaksma and Feenstra (1982b) isolated a ratio (MoCo:apoprotein) of 2:8 in **A. thaliana**. The same group had previously isolated one MoCo mutant and no apoprotein mutants (using chlorate) in **Pisum sativum** cv. Rondo (Feenstra and Jacobsen, 1980). Three NR mutants were isolated in **P. sativum** cv. Juneau, giving a ratio (MoCo:apoprotein) of 1:2 using lowered levels of **in vivo** NR activity to identify them (Kleinhofs **et al**, 1978; Warner **et al**, 1982). Five MoCo and no apoprotein mutants were isolated from **Hyoscyamus muticus**, have been identified using chlorate as a selection method (Fankhauser pers. comm. in Kleinhofs **et al**, 1985). Another four MoCo mutants and no apoprotein mutants of **H. muticus** were isolated using amino acid auxotrophy and nitrate non-utilisation (Strauss **et al**, 1981; Lazar **et al**, 1983).

It would seem that three main factors contribute to the type of NR mutants selected; i) the mutagenic agent used, ii) bias caused by the use of chlorate or other selection methods and iii) the viability of the plants which have been mutagenised in the genes controlling NR activity. This is discussed in detail in Chapter 8.

The **N. tabacum** cv. Gatersleben **nia** and **cnx** mutants all lacked both NADH-NR and reduced benzyl viologen-NR activity, with the one exception of **nia95** (Mendel and Müller, 1979). The NR minus cell lines isolated in **N. tabacum** cv. Xanthi (Buchanan and Wray, 1982; Evola, 1983a), **N. plumbaginifolia** with one exception (Marton **et al**, 1982a) also lacked NADH-NR activity and a distal-NR activity (either FMNH2-NR or reduced benzyl viologen-NR activity).

6.4 R12801 is a possible NR apoprotein mutant

R12801 lacked NADH-NR and FMNH2-NR activity but possessed xanthine dehydrogenase activity. This suggests that R12801 could be a candidate for a nitrate reductase apoprotein mutation. The level of NADH-CR activity was the same as that found in the wild type. The presence of xanthine dehydrogenase and lack of FMNH2-NR activity was also found in the barley (apoprotein) **narl** mutants, with the exception of **narlh** (Kleinhofs **et al**, 1980). The levels of NADH-CR activity varied widely between the different **narl** mutants as did the levels of nitrite reductase activity (Kleinhofs **et al**, 1980). This is analogous to the situation found in the **N. tabacum nia** mutants (Mendel and Müller, 1979).

Another factor to be considered is the paleness of this mutant. Warner and Kleinhofs (1974) studied the nitrate and nitrite reductase activities of chlorophyll deficient mutants in barley and found that there was a significant correlation between the lack of nitrate reductase and either photosynthesis or the plastid structure; the two traits seem to be linked. The additional problems found in R12801, over and above the lack of nitrate reductase, could be caused by this. It is conceivable that the effect of the mutation was within the NR enzyme at the site which was not involved in the NADH-CR electron transport chain, so preventing NR activity but allowing NADH-CR activity.

All NR minus plants possessed wild type levels of nitrite reductase and their NR+ siblings also exhibited wild type levels of NADH-NR activity. This suggests that R9201, R11301, R12202 and R12801 have not got defective nitrate induction systems (which induce both NR and nitrite reductase activities), as is found in the **nirA** mutants of **A. nidulans** (Cove, 1979).

R9401 (Bright **et al**, 1983), **nar2a** and all the **nar1** mutants also have fully induced wild type levels of nitrite reductase in the presence of nitrate (Kleinhofs **et al**, 1980). The **N. tabacum** **cnxB** mutants also have an unaffected nitrite reductase activity (Buchanan and Wray, 1982).

To study the NR minus mutants further it would be interesting to see whether the MoCo mutants are allelic, or whether the mutants

characterised here are representatives of mutations occurring in different genes. Genetic crosses of these new mutants with R9401, the previously isolated MoCo mutant, would also reveal more information about these mutants. This is studied in detail in the following chapter.

The analysis of the progeny of a cross, between R12801 and a known apoprotein mutant for allelism would also be very useful in further understanding the nature of the defect associated with R12801.

CHAPTER 4

GENETICS OF R9201, R11301 AND R9401

1. INTRODUCTION

Genetic analysis of mutants can be used to ascertain the genetic behaviour of particular genes and the relationships between different genes. The mutants R9201 and R11301 were tested for 1) Mendelian inheritance of the mutant phenotypes and 2) allelism by crossing with each other and previously characterised NR mutants. These lines were as follows:-

1) Az 34 (Warner et al, 1977), an NR deficient mutant **(nar2a)**, characterised as a single recessive gene mutation affecting the production of an active MoCo (Kleinhofs **et al**, 1980).

2) Az 28 (Kleinhofs et al, 1980), an NR deficient mutant **(narld)**, in a single recessive nuclear gene. The **narl** locus is thought to code for the NR apoprotein (Kuo **et al**, 1981).

3) R9401 (Bright et al, 1983), characterised as containing a single recessive gene mutation affecting the formation of active cofactor.

2. METHODS

2.1 Selecting the parents for the crosses

The NR minus plants of the F2 populations from the crosses (R9201 x Maris Mink), (R11301 x Maris Mink), and (R9401 x Golden Promise) were grown in the glasshouse in vermiculite. The nutrient solution contained (mM); $(NH_4)_2SO_4$ (3.6 or 1.7), $CaCl_2.6H_2O$ (3.5 or 1.7), KCl (0.091), H_3BO_3 (0.121), $MnSO_4.5H_2O$ (0.018), $ZnSO_4.7H_2O$ (0.002), $NH_4Mo_7O_{24}.4H_2O$ (0.57 µM), $CuSO_4$ (0.002) and H_2SO_4 (0.055 µM). These plants reached maturity, but grew very slowly making

synchronisation with crossing partners difficult, they also set very low numbers of seeds. They could therefore, not be used directly in the crossing programme. Instead it was necessary to use plants heterozygous for the NR mutation (phenotypically NR+) from the F2 populations. The NR+ plants from the F2 generation could be either homozygous or heterozygous with respect to the affected gene under study. Only the heterozygous plants would carry the mutant allele and these plants would be present as two thirds of the NR+ plants available for crossing. However, it was not possible to discriminate between them prior to crossing. The parents of all crosses were also allowed to self fertilise, and analysis of the seeds allowed the determination of the genotype of the parent plants to be carried out. Those crosses which involved heterozygous R9201, R11301 and R9401 parents were studied further, the other crosses were discarded. The initial crosses were therefore carried out with several different NR+ individuals.

Since the homozygous NR defective lines, Az 34 and Az 28, had 50% and 15-20% wild type NR activity respectively (figure 20) and were also able to grow to maturity and set seed in Eff compost containing nitrate, they were used in the homozygous NR condition.

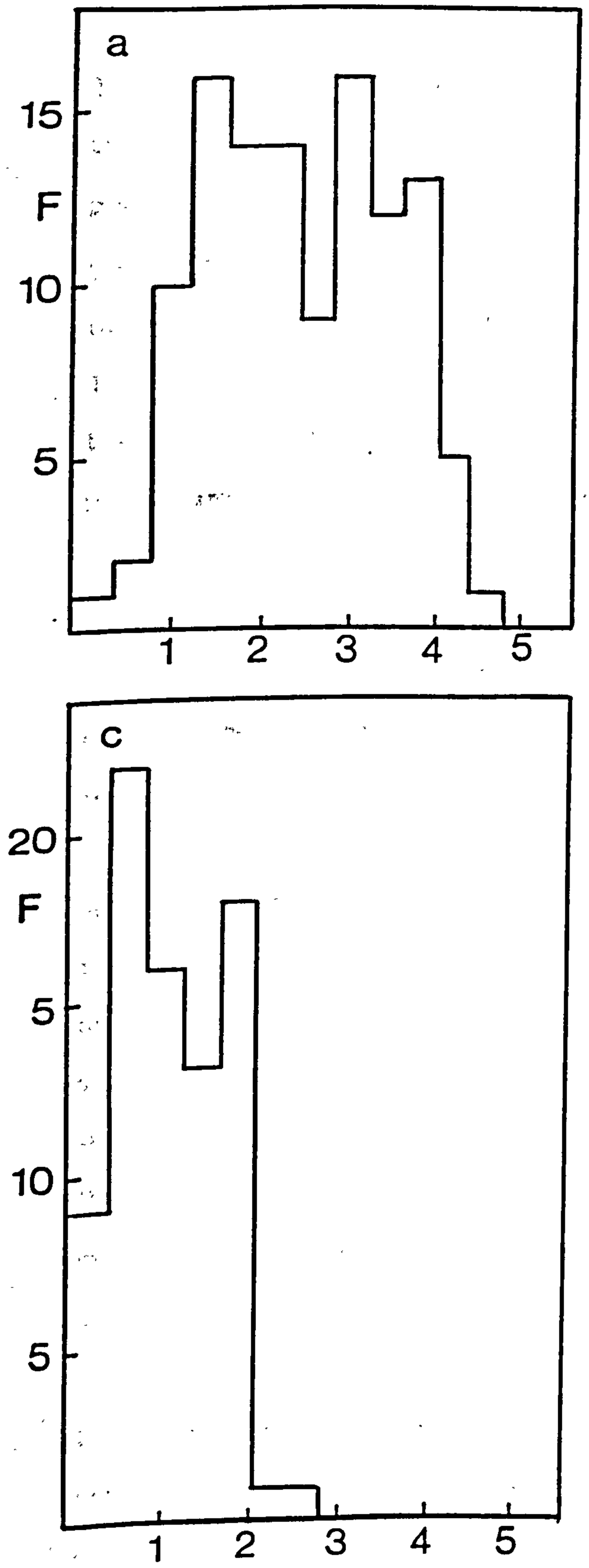
2.2 Setting up the crosses

The NR+ seedlings obtained from the F2 generations of the following crosses, (R9201 x Maris Mink), (R11301 x Maris Mink) and (R9401 x Golden Promise) were selected by their continued growth after two weeks in Eff compost. Homozygous NR minus plants died under these conditions (see figure 11).

Figure 20

Distribution of in vivo NR activity found in extracts of nitrate induced seedlings of (a) Maris Mink (b) Steptoe (c) Az 34 and (d) Az 28

A total of 280 plants were grown and assayed for in vivo NR activity under the conditions given in Chapter 4, Section 2.


a
b
c
d
F
15
10
5
20
1
2
3
4
5
μmol NO_3^- red/h/gfw

The NR+ seedlings were grown in a controlled environment cabinet under the conditions given in Chapter 2, Section 1. After two weeks the plants were repotted into 7" pots in fresh compost and placed in the glasshouse (temperature maintained at 16-18° C with no artificial lighting) and watered daily with the nutrient solution given above. The lines, Az 34, Az 28 and wild types were grown in Eff compost under the same conditions. In order to carry out reciprocal crosses, the plants were left to grow until the anthers developed.

2.3 Crossing

All three anthers were removed with fine forceps from each flower on single ears of individual plants. The ear was labelled and covered with a cellophane bag to prevent accidental cross-pollination.

After three to four days, when the ovaries had matured, selected pollen from a different line was used to fertilize the ear by brushing a mature anther over the style. Each flower on an ear was treated in the same fashion, the ear was then covered until the grains were mature. An attempt was made to cross every NR+ plant with each of the lines R9401, Az 34 and Az 28 and its parent cultivar Maris Mink. When mature, the F1 seeds were harvested, planted in Eff compost and grown up for analysis and to obtain F2 seeds.

2.4 Genotyping of parents

Self fertilised seeds from the lines, R9201, R11301 and R9401 were germinated in Eff compost in a controlled environmental cabinet (Chapter 2 Section 1) and scored for segregation of the NR minus

phenotype to determine which of the parent plants were heterozygous for the mutant trait. The NR activity of the progeny of self fertilized plants was estimated by the **in vivo** assay to facilitate screening of the large numbers necessary for genetic analysis. The NR minus plants of these lines withered after two weeks and finally died under these experimental conditions (figure 11).

2.5 Allelism testing

To study whether the mutations were allelic to each other and to the marker genes, the F2 seedlings of the relevant crosses were assayed for **in vivo** NR activity after seven days. Thirty F2 seedlings were grown on Eff compost under the following conditions: 150 $\mu Em^{-2}s^{-1}$; 80% relative humidity; 12 hour photoperiod; 18° C during the photoperiod and 15° C in the dark. The light was supplied by both tungsten bulbs and fluorescent lights. The plants were watered daily with demineralised water and watered with 15 mM KNO_3 16-18 hours prior to assaying. The plants were grown on for a further two weeks and then screened for withering. Withering under these conditions was shown to be a property of the plants with an NR minus phenotype (figure 11).

3. RESULTS

When studying the distribution pattern of **in vivo** NR activity of the crosses it was necessary to first examine the distribution of NR activities in the parents, so that the F2 progeny of the crosses could

be divided into the categories, wild type and non-wild type levels of NR activity.

3.1 In vivo NR activity of the parental cultivars

The **in vivo** NR activity values of individual seedlings of the wild type plants of cv. Maris Mink and Steptoe ranged from 0.1-5 µmol NO_2^- produced/h/gfw (data pooled from 14 experiments) (figure 20a and b). The values were approximately normally distributed with means and standard errors of 2.5 ± 0.1 (n = 110) and 2.2 ± 0.1 (n = 63) µmol/h/gfw for Maris Mink and Steptoe respectively. These values are not significantly different at the 5% confidence level when compared by Students t-test. Combining the data for these two cultivars gave a mean wild type value of 2.4 ± 0.1(n = 173) µmol/h/gfw. This wild type **in vivo** NR activity distribution overlapped considerably with the activity distributions of the NR deficient lines Az 34 and Az 28. Az 34 had a broad distribution of **in vivo** NR activity (figure 20c), with a mean and standard error of 1.1 ± 0.1 (n = 80) µmol/h/gfw (50% of the Steptoe mean activity). Az 28 had a tighter band of **in vivo** NR activity, (see figure 20d) with a mean value of 0.5 ± 0.1 (n = 27). However, this distribution did not appear to be uniform. The **in vivo** NR activities of the wild types, Maris Mink and Steptoe, and the NR deficient lines, Az 34 and Az 28 were determined as controls in each experiment in which their crossed progeny were being examined.

Figure 21

The three phenotypes (a) wild type (b) NR minus (withered) and (c) shrivelled in the F2 population of a cross between Az 28 and R11301

Plants were grown for three weeks in Eff compost under the conditions given in Chapter 4, Section 2; watered daily with demineralised water.

wt
withered

Az 34 and Az 28, although NR deficient, were able to grow on Eff compost but they both showed, in a number of cases, a distinct shrivelling of the primary leaf. Further development was unaffected in these plants, indicating that 'shrivelling' is distinct from the 'withering' and subsequent death found in the NR minus plants of the F2 populations of the backcrosses of R9201, R11301 and R9401 (see figure 21).

The R9201, R11301 and R9401 NR minus plants possessed less than 10% of the wild type mean **in vivo** NR activity, in future this level will be described as 'null' or 'zero' to aid clarity. Az 34 and Az 28 plants posssessing 50% and 15-20% of the mean wild type **in vivo** NR activity respectively, will be described as possessing 'low' **in vivo** NR activity.

3.1 Inheritance of the mutant genes in R9201, R11301 and R9401

The NR minus trait was inherited in the selfed progeny of heterozygous plants as a single recessive nuclear gene in the progeny of R9201 and R11301 (table 10). The observed proportion of NR minus plants (NR minus by assay) in R9201 and R11301 was not significantly different from the 3:1 ratio found in R9401 (table 10).

It was found that all the 185 plants (table 10) with a null phenotype developed the characteristic withering. The withering phenotype was inherited in a 3:1 (non-withering:withering) ratio in various generations of segregating R9201, R11301 and R9401 (table 10).

Table 10

Segregation patterns (NR+ : NR minus) in the F2 population arising from the self fertilisation of plants derived from backcrosses of R9201, R11301 and R9401

A total of 786 4-6 day old seedlings were assayed for **in vivo** NR activity.

Table 10

	Number of F2 seedlings				
	Observed		Expected		
Genotype	high	null	high	null	χ^2 (3:1)
R9201	163	52	161.25	53.75	0.08
R11301	200	58	193.5	64.5	0.87
R9401	238	75	234.75	78.25	0.18

This indicates that in all these cases a single mutant gene has been inherited in a Mendelian fashion.

3.3 Genotyping of the parent plants used in the crossing programme

For R9201, 11 of the 18 parent plants proved to be heterozygous (some of their progeny showed withering). From those 11, 41 of the 173 progeny studied showed the withering phenotype. This gave a χ^2 value of 0.156; not significantly different from the expected 3:1 ratio.

Similarly for R11301, 14 of the 20 parent plants proved to be heterozygous. The progeny of these 14 plants gave 60 withering plants out of a total of 207, which was also not significantly different from a 3:1 ratio ($\chi^2 = 1.8$).

For R9401, only one of the seven parents used was heterozygous. Four of the 20 progeny studied of this plant showed withering.

This again gives evidence of the inheritance of single recessive nuclear genes in R9201, R11301 and R9401 lines.

3.4 Genetic relationships amongst R9201, R11301 and R9401

The affected genes in the different mutants could be non-allelic or allelic. This was investigated by examining the inheritance of withering and levels of **in vivo** NR activities in the progeny of crosses.

3.4.1 Cross between R9201 and R9401

Heterozygous R9201 and R9401 plants were crossed to produce eight F1 seeds. Two F1 plants withered and died, one from each of two ears. This suggests that R9201 and R9401 are allelic. If the same gene is affected in both lines the double recessive genotype (one allele from each parent) is expected in one quarter of the F1 seeds. It is expected that two thirds of the remaining F1 would give withering plants in the F2 progeny and in fact, five out of the six remaining F1 plants did so, compared to the four expected. In each case, as expected, withering was observed in a 3:1 (non-withering:withering) ratio.

3.4.2 Cross between R9201 and R11301

Crosses between R9201 (heterozygous) and R11301 (heterozygous) plants were carried out in both directions. All twelve F1 seedlings showed no signs of withering and reached maturity. This immediately suggests that different gene loci were affected in R9201 and R11301. In this case there should be three distinguishable phenotypes present identified by the proportions of the withering seedlings and their F2 progeny (0, 4/16, 7/16).

When the F2 populations from individual F1 plants were studied, seven of the twelve F1 plants give no withering plants in the F2 where nine were expected.

Table 11

Segregating patterns (NR+ : NR minus) in the F2 population arising from the self fertilisation of F1 plants from crosses between R9201 and R11301

A total of 140 F2 seedlings were assayed for **in vivo** NR activity.

Table 11

Cross	in vivo NR activity		Suggested ratios
	high	low	
F1 [R11301/18 x R9201/16] 2	18	10	9:7/3:1
F1 [R11301/18 x R9201/16] 1	18	12	9:7/3:1
F1 [R9201/19 x R11301/18] 3	20	9	3:1
F1 [R11301/18 x R9201/16] 3	21	7	3:1
F1 [R9201/19 x R11301/18] 1	14	11	9:7

The F2 populations from the self fertilisation of the five individual F1 plants known to have withered plants in their progeny, were studied to see whether they were 9:7 (wild type levels of NR:nulls) or 3:1 (wild type:null levels of NR) ratios present. As can be seen from table 11 two of the five F1 plants gave a 3:1 ratio. Progeny from another two F1 plants gave ratios which were not significantly different from either a 3:1 or a 9:7 ratio. However, there was one F1 plant whose progeny gave a ratio which was not significantly different from a 9:7 ratio, but was significantly different from a 3:1 ratio (figure 22). This provides further evidence for segregation of two unlinked genes (if tightly linked an apparent 3:1 ratio would be found).

3.4.3 Cross between R11301 and R9401

The cross between heterozygous R11301 and R9401 was attempted, but no success was had in obtaining viable F1 seeds. However, it is possible to suggest that R11301 is not allelic to R9401, since it has been found that R9401 is allelic to R9201 and R9201 is not allelic to R11301.

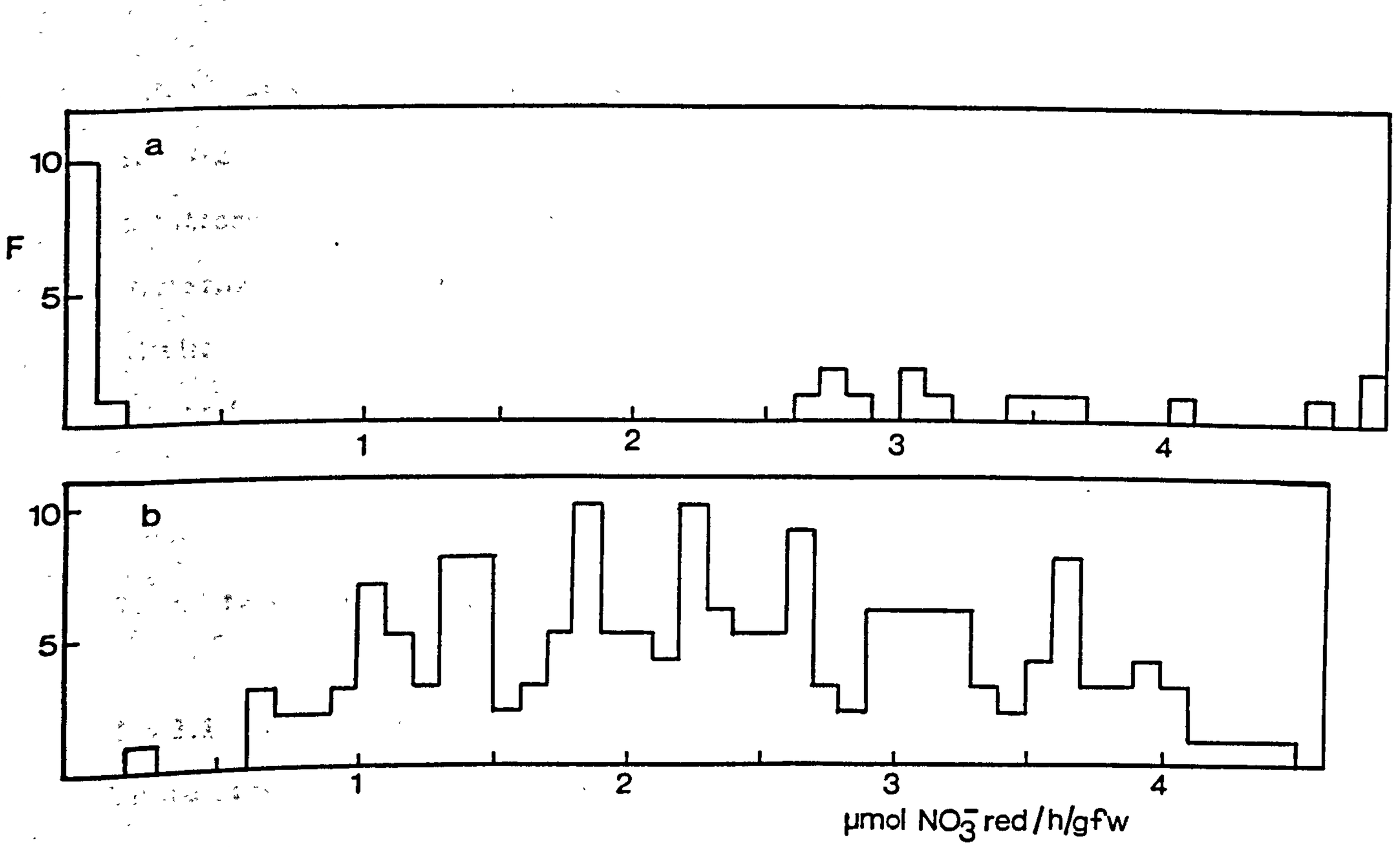
3.5 Genetic relationships of R9201 and R11301 with marker genes **narl** and **nar2**

The rationale behind this series of experiments was to see whether R9201 and R11301 were allelic to **narld** (apoprotein mutant gene) or **nar2a** (MoCo mutant gene) by analysing the progeny from these crosses.

Figure 22

Distribution of in vivo NR activity found in extracts of nitrate induced (a) F2 seedlings from the cross F1 [R9201/19 x R11301/18] 1 (n=25) and (b) wild type (n=137) seedlings

Seedlings were grown and assayed as detailed in Chapter 4, Section 2.


a
10
F
5
1
2
3
4
b
10
5
1
2
3
4
µmol NO_3^- red / h / gfw

Using the example of a cross between Az 28 **(nar1d)** and R9201 there are two possible relationships, allelism and non-allelism. The same is true for the relationship between Az 34 **(nar2a)** and R9201 or R11301.

3.5.1 Allelism of two mutant lines

If Az 28 and R9201 were non-allelic, they could be assigned the arbitrary genotypes of eeBB (homozygous for gene e) for Az 28 and EEBb (heterozygous for gene b) for R9201. However, if Az 28 and R9201 were allelic then the genotypes would be assigned the symbols, e'e' for Az 28 and Ee for R9201 (it is not specified whether e' is dominant with respect to e).

These two hypotheses are outlined in figure 23.

3.5.1.1 Non-allelism

There are two distinguishable outcomes in the F2 if the parent plants were non-allelic.

1. A quarter of the F2 population arising from the self fertilisation of the F1 genotype BbEe would have the R9201 NR minus withering phenotype. Within the same F2 population there would also be plants with wild type levels of NR activity and plants possessing low (Az 34) levels of NR activity which do not wither.

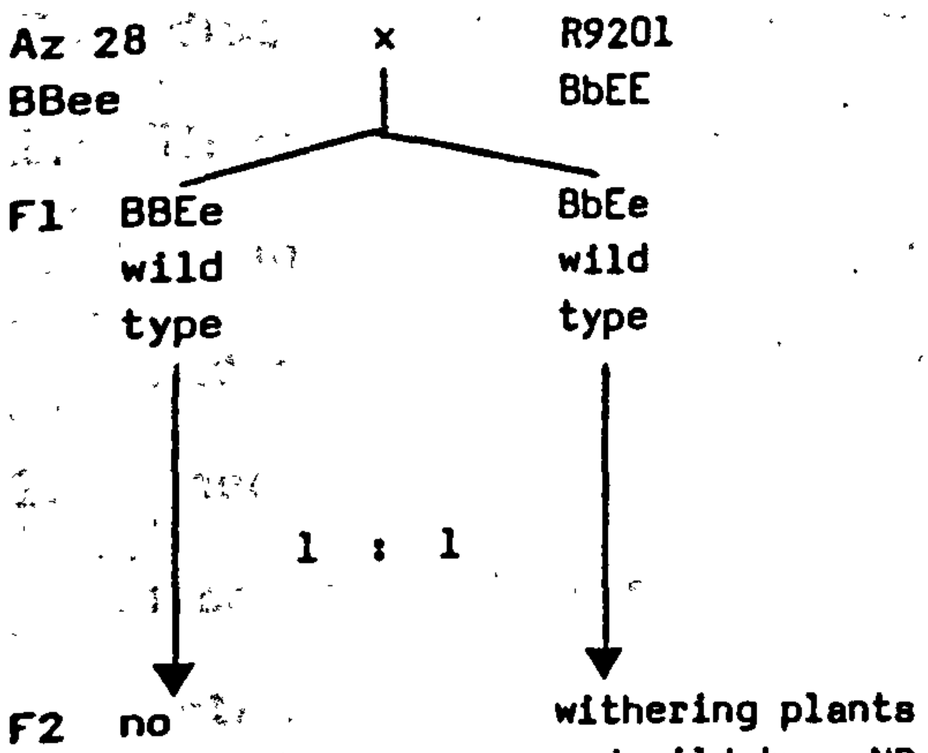
2. The F2 population formed from the self fertilisation of the other possible F1 genotype, BBEe, (figure 23a) would not contain any withering NR minus plants. F1 plants from this cross can therefore be discarded if they do not show withering in the following (F2)

Figure 23

Two hypotheses for the relationship between R9201 and Az 28

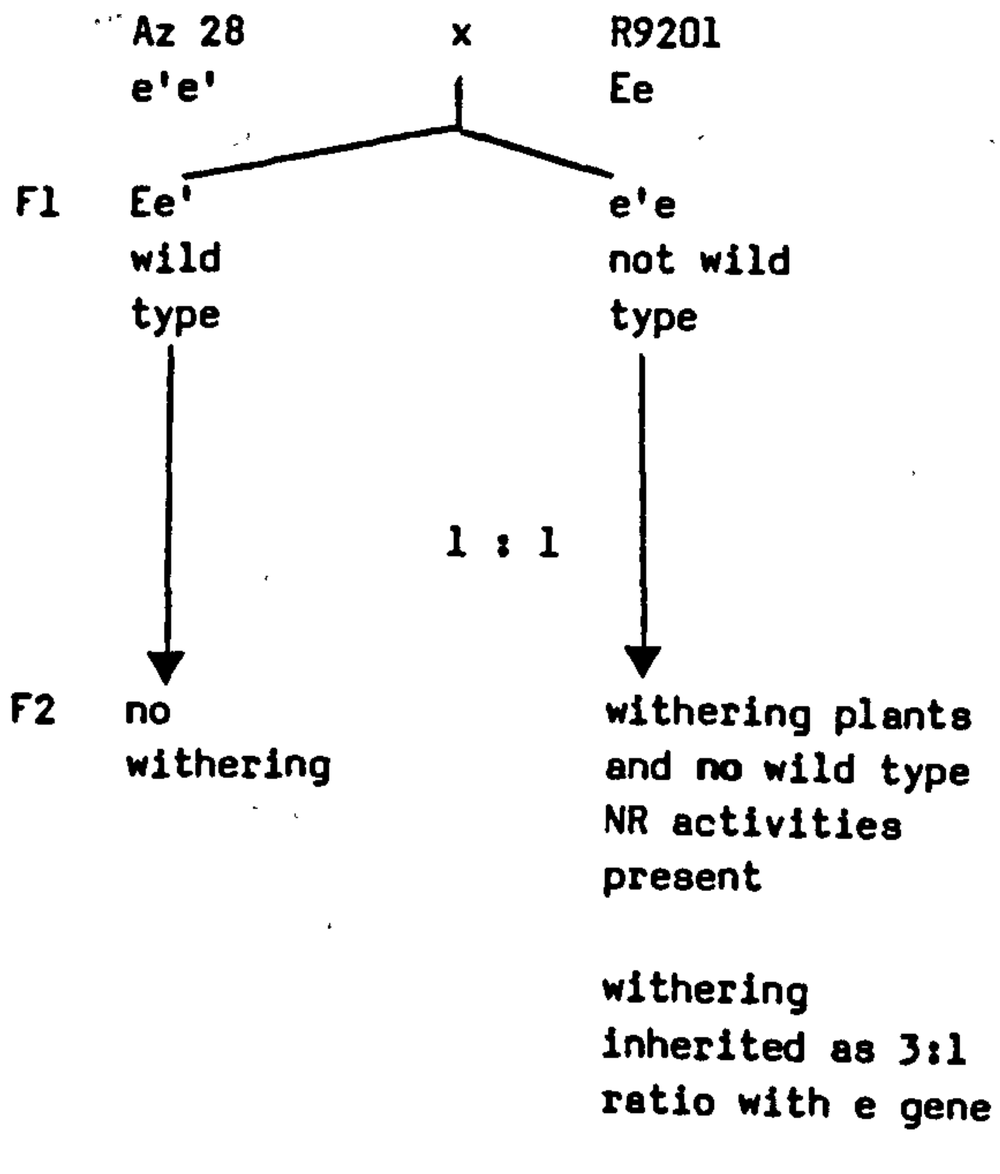
a) Non-allelic

Where Az 28 has the genotype BBee and R9201 has the genotype BbEE



b) Allelic

Where Az 28 has the genotype e'e' and R9201 has the genotype Ee



generation. Individual F2 plants arising from the F1 genotypes which possess no recessive R9201 mutant genes (b) would fall into two groups, one with wild type levels of NR activity and the other with Az 28 levels.

3.5.1.2 Allelism

Two diagnostic outcomes were envisaged for allelism (figure 23b).

1. There should be segregation of wild type and non-wild type levels of **in vivo** NR activities (expected to be intermediate between Az 28 and null levels) in the F1 generation.
2. There would be no withering plants in the F2 generation arising from the F1 plants which possessed wild type levels of **in vivo** NR activity. The F2 population from the self fertilised F1 plants which posssessed intermediate levels (ie between Az 28 and null) of **in vivo** NR activity would contain withering plants and no plants with wild type activities would be present.

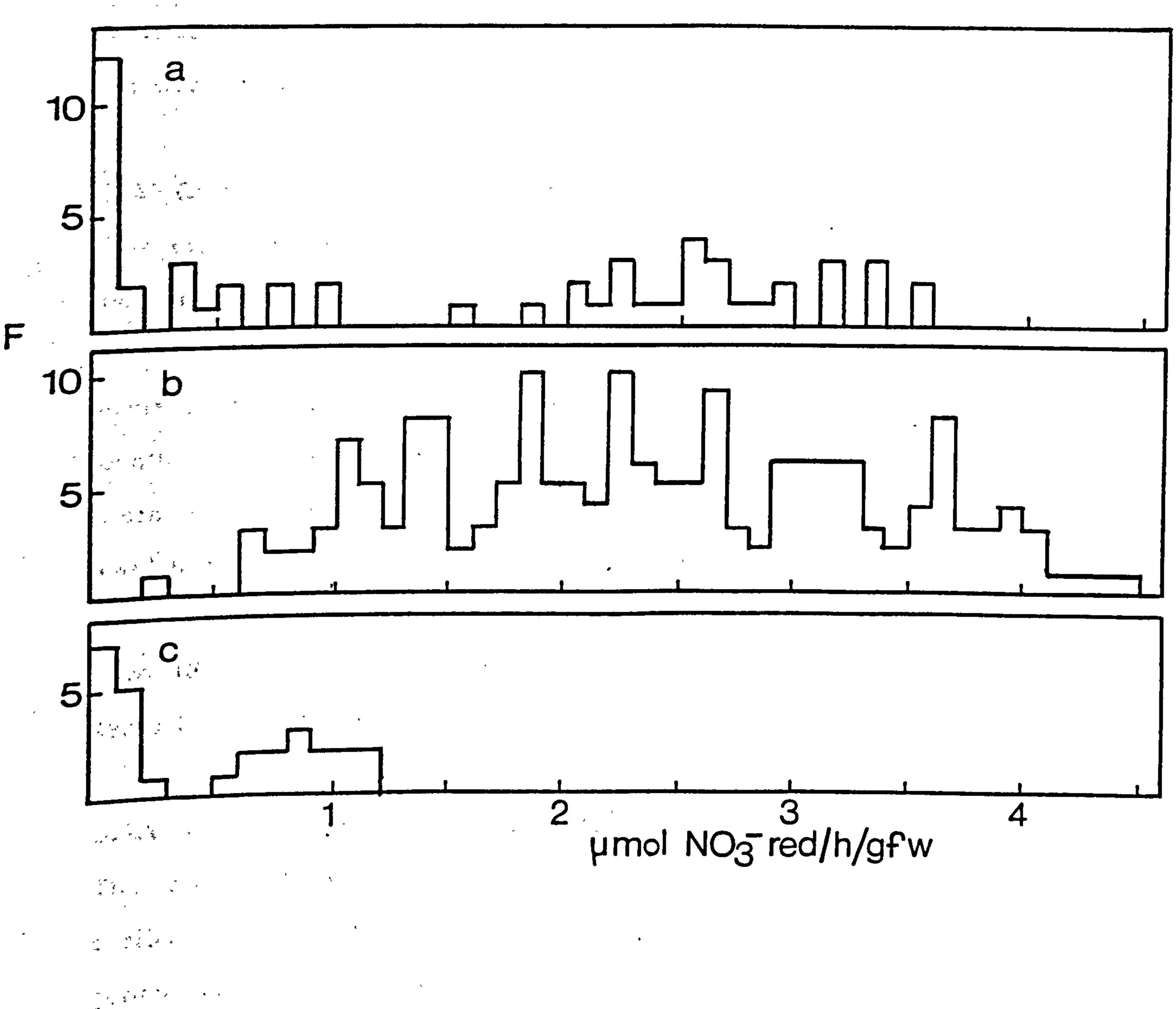
3.5.2 Cross between R9201 and Az 28

All of the fifteen F1 seeds from the reciprocal cross of R9201 x Az 28 which germinated, grew to maturity. The F2 population from each of the fifteen F1 plants were scored for withering and zero NR activity. The progeny from five of the seven F1 plants in which Az 28 was the male partner in the original cross, showed no withering plants. When Az 28 was the female partner in the original cross, four out of eight F1 plants contained withering plants, giving a total of six out of fifteen F1 plants that showed withering and had therefore received a mutant gene from R9201.

Figure 24

Distribution of in vivo NR activity found in extracts of nitrate induced (a) F2 seedlings from the cross R9201 x Az 28 (n=54) (b) wild type (n=137) and (c) Az 28 (n=27) seedlings

Seedlings were grown and assayed as described in Chapter 4, Section 2.


a
10
5
F
b
10
5
c
5
1
2
3
4
µmol NO3- red/h/gfw

The distribution of activities of the F2 population arising from a single F1 plant showed a broad spectrum containing both wild type levels of **in vivo** NR and withering plants (figure 24). The division between wild type activity and the low NR activity associated with Az 28 was unclear.

3.4 Cross between R11301 and Az 28

The cross between heterozygous R11301 and homozygous Az 28 was carried out in both directions.

None of the twelve F1 seeds which germinated, showed the withering and death phenotype. The progeny of individual self fertilised F1 plants were scored for the presence or absence of withering plants. Of the twelve F1 plants, four had withering plants in the F2. The progeny of one of these four F1 plants was examined for the distribution of **in vivo** NR activity amongst seedlings (figure 25). Plants with high (wild type) levels of NR and withering plants were present.

3.5.4 Cross between R11301 and Az 34

The cross between R11301 (heterozygous) and Az 34 (homozygous) was carried out in both directions. All of the seventeen F1 seeds which germinated, grew to maturity showing no signs of withering. Eight out of a total of seventeen F1 plants showed withering in the F2, demonstrating the expected 1:1 segregation of the gene from R11301 in this cross.

Figure 25

Distribution of in vivo NR activity in extracts of nitrate induced F2 seedlings from the cross R11301 x Az 28 (n=57)

The seedlings were grown and assayed as described in Chapter 4, Section 2.

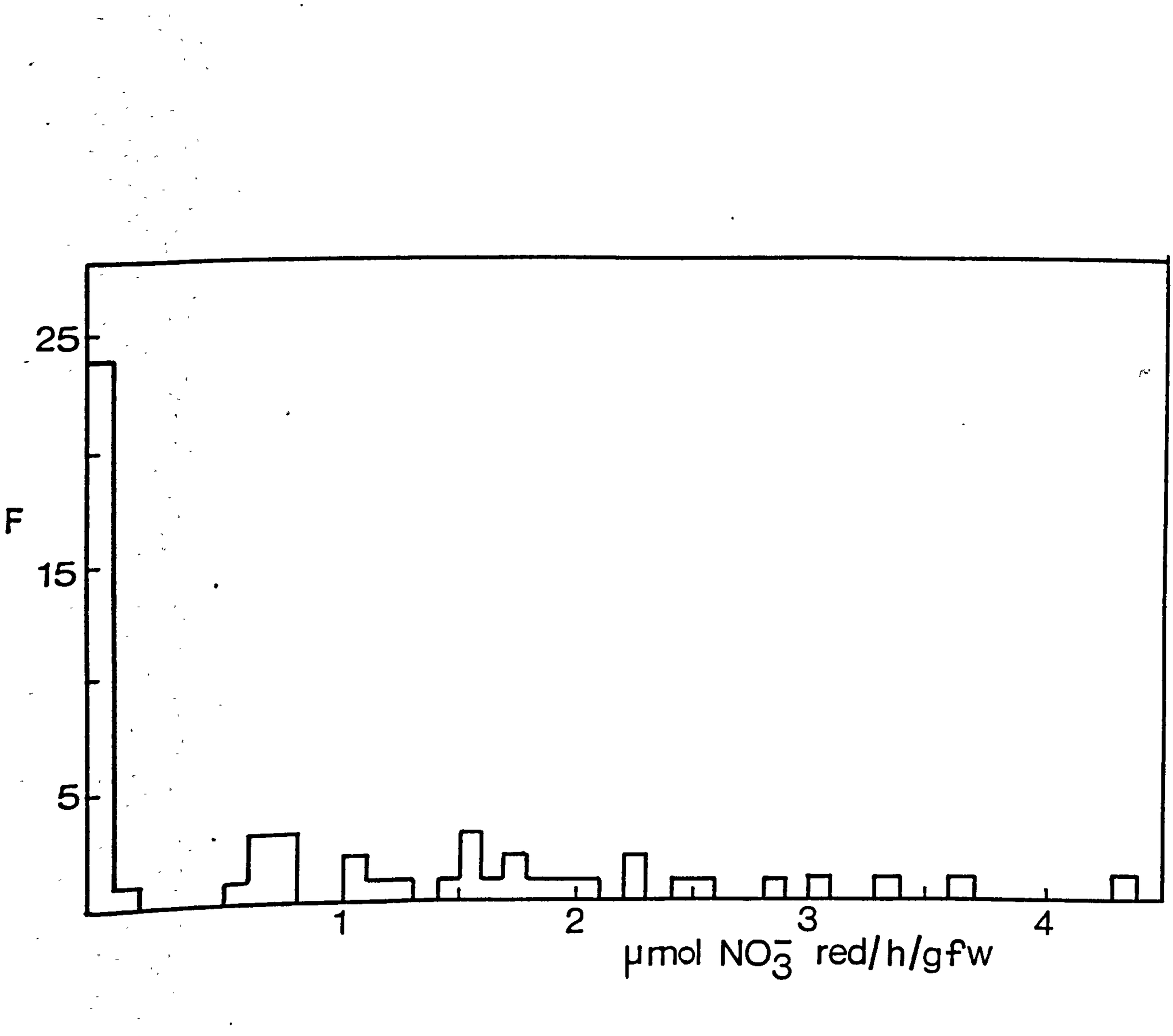
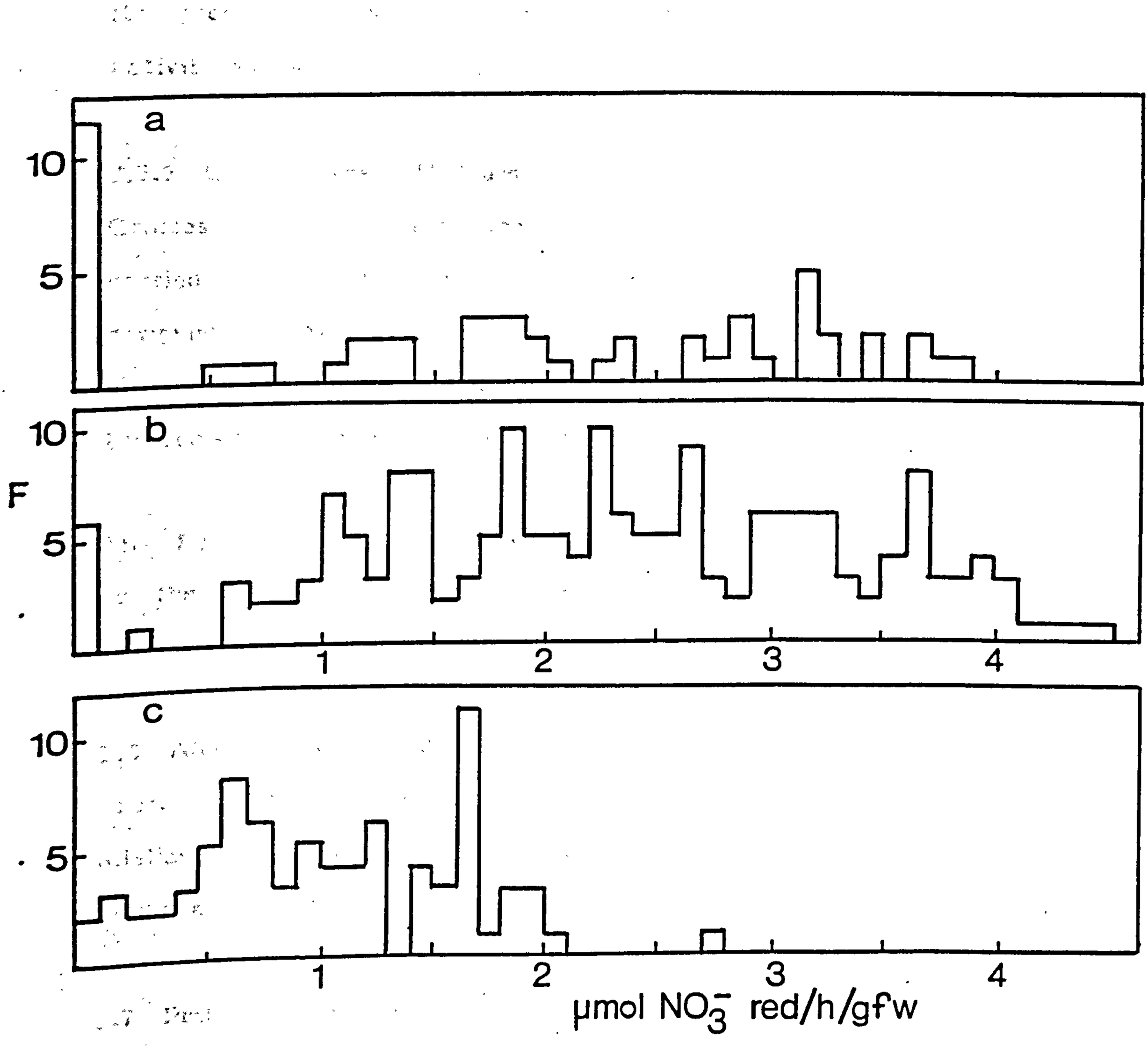

25
15
5
F
1
2
3
4
µmol NO_3^- red/h/gfw

Figure 26

Distribution of in vivo NR activity in extracts of nitrate induced (a) F2 seedlings from the cross R11301 x Az 34 (n=59) (b) wild type (n=137) and (c) Az 34 (n=30) seedlings

The seedlings were grown and assayed as described in Chapter 4, Section 2.


a
10
5
b
10
F
5
1
2
3
4
c
10
5
1
2
3
4
µmol NO3- red/h/gfw

As can be seen in the distribution pattern of NR activity in the F2 population arising from an F1 plant known to have withering plants in its progeny (figure 26), there are both plants with wild type NR activity and withering plants.

3.5.5 Cross between R9201 and Az 34

Crosses between the heterozygous NR+ R9201 and homozygous Az 34 were carried out in both directions. All the F1 seeds of this cross which germinated, showed no signs of withering and grew to maturity. The F1 plants whose progeny possessed no withering plants were discarded and the remaining F2 progeny were scored for **in vivo** NR activity.

The F2 populations from crosses in both directions gave similar results so the data from particular crosses were combined (figure 27). Plants with wild type levels of NR activity and withering plants were present.

3.6 Allelism from the data

From the data presented it would appear that: R9201 and Az 28 are non-allelic; R11301 and Az28 are non-allelic; R11301 and Az 34 are non-allelic and R9201 and Az 34 are non-allelic.

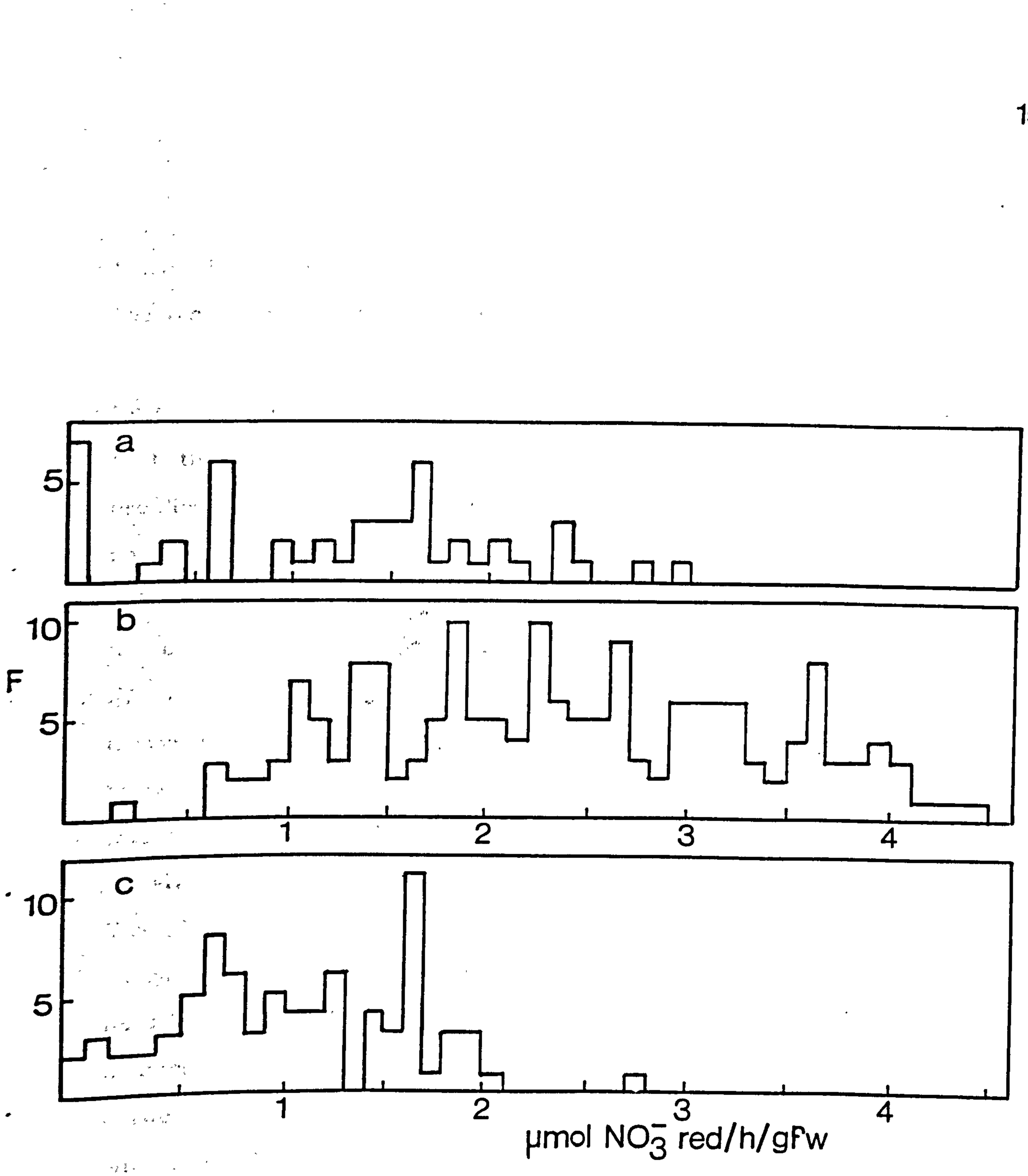
3.7 Problems of interpretation

The results from the **in vivo** assays had suggested that there were plants with wild type values in the same population as nulls. Owing to the wide spread of values from the **in vivo** NR assays (figures 20-27) it is difficult to distinguish values intermediate between those of the wild type and Az 34 plants and the tests were never designed to do

Figure 27

Distribution of in vivo NR activity in extracts of nitrate induced (a) F2 seedlings from the cross R9201 x Az 34 (n=60) (b) wild type (n=137) and (c) Az 34 (n=30) seedlings

The seedlings were grown and assayed as described in Chapter 4, Section 2.


a
5
b
10
F
5
1
2
3
4
c
10
5
1
2
3
4
μmol NO3- red/h/gFw

this. Using my results it cannot be said whether the upper values of the range are genuinely wild type or of this intermediate class.

New evidence has been found recently by Professor Kleinhofs to suggest that these interpretations may not be correct and that Az 34 and R9401 are indeed allelic (Kleinhofs pers. comm.). Since we know that R9401 and R9201 are allelic, this information would suggest that the gene affected in R9201 and Az 34 **(nar2a)** are themselves allelic. He and his coworkers used the **in vitro** NR assay on single leaf samples of the F1 and F2 plants arising from a cross between heterozygous R9401 and homozygous Az 34. In the F1 population he saw two classes of plants; those with wild type levels; and those with lower NR activities, intermediate between the wild type and Az 34. This was also seen using **in vivo** assays on the progeny of the same cross (Bright pers. comm.). The F2 seedlings from the F1 plants with intermediate levels were assayed by the same method and showed, as expected, some seedlings with no NR activity. No seedlings were found with wild type activities, but a large proportion of the plants had NR activity which was intermediate between the wild type and Az 34 values. The lack of seedlings with wild type NR activity provided clear evidence for the allelism of Az 34 and R9401.

Accepting then, that R9201, R9401 and Az 34 are allelic, this allows the further conclusion to be drawn; the crosses of R9201 x Az 34 and R9401 x Az 34 both show considerable complementation of NR activity in plants containing one allele from **nar2a** and one from R9401 or R9201. The allelism of **nar2a** with R9401 and R9201 allows these two alleles to

be designated **nar2b** and **nar2c** respectively, conforming to Kleinhofs nomenclature. The fact that the plants which contain one allele from Az 34 and one from R9201 or R9401 express a level of NR activity greater than the parent with highest activity, suggests that there is some level of intragenic complementation. The analogous F1 plants from the cross R9401 x R9201 did not show complementation as they withered and died in the same manner as their homozygous parents. Possible intragenic complementation of genes involved in MoCo formation has also been suggested for **A. nidulans** (Cove, 1979), **N. crassa** (Dunn-Coleman **et al,** 1984) and **P. chrysogenum** (Birkett and Rowlands, 1981). However Cove (1979) states that if there is intragenic complementation, it is rather weak. It suggests that different portions of the **nar2** gene product are non-functional in the **nar2a** and **nar2b** or **nar2c** mutants and that at least two steps are involved in the formation of the product of the enzyme encoded for by the **nar2** gene. Such complementation was not seen among various **narl** alleles (Kleinhofs **et al,** 1980).

4. DISCUSSION

1. The distributions of **in vivo** NR activities of the wild type controls, Maris Mink and Steptoe, were not significantly different from each other and could be treated as a single population. This evidence allows the interference caused by genes modifying NR activity to be discounted. These genes could have been derived from the varietal differences between Maris Mink and Steptoe. The modification of NR activity caused by such differences have been reported for wheat (Sherrard **et al,** 1976).

The wild type **in vivo** NR activity distribution pattern was very broad and overlapped the distributions of NR activity from both Az 34 and Az 28 populations. The broadness of the **in vivo** NR distributions was disappointing, preventing stronger conclusions being drawn about the relationships of R9201 and R11301 with the marker lines. The variation measured could have been caused by the small tissue samples. These are more variable because 1) the differences found between values would be less critical if the activities themselves were larger and 2) there is large variability of NR activity within tissues, this effect can be exaggerated by variations in the slicing of leaf material for the **in vivo** assay. It would also appear that the **in vivo** NR assay is not a sufficiently accurate method of determining NR activity (Lillo, 1983). This strongly suggests that further studies of the genetics of these mutants should use the far more labour intensive **in vitro** method to assay NR activity.

2. R9201, R11301 and R9401 all contain single recessive nuclear mutations. Crosses in both directions gave very similar results, this suggests that the NR minus pollen grains are not at a competitive disadvantage and that the pollen does not need to use nitrate. Cultured barley caryopses do not use nitrate for growth and protein synthesis (Bottacin **et al**, 1985). It is not possible to distinguish NR minus from normal seeds from ears known to be segregating. It seems therefore that nitrate is not necessary and may not be used at all as a nitrogen source during pollen formation or seed development.

3. It is clear that R11301 is not allelic to R9201, but R9201 is allelic to R9401. From this it follows that R9401 is not allelic to R11301.

4. Linkage patterns could not be simply studied due mainly to the overlaps of the various distributions. However, it can be tentatively concluded that the mutant genes in R11301 and R9201 are not closely linked.

5. In barley before these new lines, R9201 and R11301, were isolated, three different NR genes had been identified by the use of mutants. Two of these were MoCo genes, referred to as **nar2** (Kleinhofs **et al,** 1980) and **nar3** (Tokarev and Shumny, 1977). A futher MoCo mutant, referred to as **nar4**, has recently been identified (Kleinhofs **et al,** 1985). The MoCo mutant which were affected at the **nar3** or **nar4** genes were unavailable for this crossing programme so it is unclear whether it is allelic to R11301; **nar3** and **nar4** cannot be allelic to R9201 or R9401 since **nar3a** was shown to be non-allelic to Az 34 **(nar2a)** (Somers **et al,** 1983a). The **nar4** gene is known to be non-allelic to **nar2** and **nar3** (Kleinhofs **et al,** 1985).

These MoCo mutants are the only ones reported which are fully null and conditional lethel. In future it will be necessary to analyse the genetic relationships of all the available NR mutants. The availability of allelic series of mutants at the various loci (with varying levels of NR activity) will be useful in physiological and

biochemical studies of the regulation of nitrate assimilation. This intragenic complementation of **nar2a**, **b** and **c** lends further interest to the comparison of these MoCo mutants ability to associate the NR monomers into dimers. This is discussed in the following chapter.

CHAPTER 5

CYTOCHROME C REDUCTASE (NADH-CR) PROFILES OF NR MINUS MUTANTS

1. INTRODUCTION

NADH-CR activity is associated with the haemoflavoprotein subunits of the nitrate reductase enzyme (Kuo **et al**, 1980; Mendel and Müller, 1979; Nason **et al**, 1970). There are two other major sources of NADH-CR activity; mitochondrial and microsomal (Wallace and Johnson, 1978). Sucrose density gradients can be used to separate these different species on the basis of size and shape (Hames, 1984). Using 4-20% sucrose density gradients, over a 50% sucrose layer, three major peaks of NADH-CR activity were found in nitrate induced extracts of **H. vulgare** and **N. tabacum** (Wray and Filner, 1970; Mendel and Müller, 1979). These bands had sedimentation coefficients of approximately 13, 8 and 4 S. The 8 and 4 S bands were nitrate inducible. The 13 S NADH-CR activity was assumed to be associated with the endoplasmic reticulum because of its large S value (Mendel and Müller, 1979). The 8 S peak was only present under nitrate induced conditions and represents the active dimeric form of NR (Wray and Filner, 1970; Mendel and Müller, 1979).

In fungi it has been shown that during the reconstitution of the NR activity in an 8 S-deficient mutant (**nit1**) there is an apparent conversion of the 4 S to 8 S species, causing a decrease in the 4 S and increase in the 8 S NADH-CR activity (Ketchum **et al**, 1970; Nason **et al**, 1971). This indicates that the 8 S and 4 S NADPH-CR activities are related to each other and to NADPH-NR in fungi.

The 4 S peak was present under both induced and non-induced conditions, but was slightly more abundant in nitrate treated plants (Wray and Filner, 1970). It was suggested that the 4 S NADH-CR activity was either a precursor or dissociation product of NR (Wray and Filner, 1970; Small and Wray, 1980; Somers **et al**, 1983a).

MoCo is known to have two functions. One of these is catalytic and the other is dimerisation of the monomeric subunits which is also necessary for NADH-NR activity (Nason **et al**, 1971; Mendel **et al**, 1981).

The MoCo defect in some mutants prevents dimerisation of the haemoflavoprotein subunits (and therefore they lack an 8 S NADH-CR activity) for example, **nit1** from **N. crassa** (Nason **et al**, 1970) and **cnxC** from **N. plumbaginifolia** (Mendel **et al**, 1986). Other MoCo mutants possess both monomers and dimers, eg **cnxE** from **A. nidulans** (MacDonald **et al** 1974), B73 from **A. thaliana** (Braaksma and Feenstra, 1982b), **cnxA** from **N. plumbaginifolia** (Mendel **et al**, 1986). The dimers from these MoCo mutants (**cnxE**, B73 and **cnxA**) possess NADH-CR activity but no NADH-NR activity, suggesting that although the catalytic function of MoCo is defective, they still possess the ability to dimerise apoprotein monomeric subunits (Mendel and Müller, 1980). The mutant line B25 from **A. thaliana** possesses very little dimer whilst the **cnxE** (**A. nidulans**) and **cnxA** (**N. tabacum** and **N. plumbaginifolia**) exhibit close to wild type levels of dimer (MacDonald **et al**, 1974; Mendel and Müller, 1980; Mendel **et al**, 1986).

Table 12

Distribution of NADH-CR activity between dimer and monomer peaks from extracts of the mutants and wild types

Source	Percentage of NADH-CR activity in	
	Peak A (dimer)	Peak B (monomer)
Maris Mink	70 (7.7)	30 (3.5)
Golden Promise	69 (7.8)	31 (3.7)
R9201 NR+	64 (7.7)	36 (4.3-2.0)
R9201 NR minus	36 (7.3-6.1)	64 (4.0)
R11301 NR+	64 (7.7)	36 (2.1)
R11301 NR minus	74 (7.7)	26 (3.4)
R9401 NR minus	29 (7.7-6.1)	71 (4.7)
R12202 NR minus	0	100 (3.8)
R12801 NR minus	0	100 (4.7)

The areas under peaks A and B in figures 28 to 36 were calculated as a percentage of the total NADH-CR activity.

Number in brackets indicates the S value of the peaks.

Figure 28

A typical sucrose density gradient centrifugation analysis of an extract from 6 day old primary leaves of cv. Maris Mink prepared in the presence of 10 µM leupeptin

6 day old primary leaves were extracted with NR Extraction Buffer with 10 µM leupeptin. After centrifugation of the brei at 38,000 g for 20 minutes the NR activity of the extract was 8.91 µmol nitrite produced/h/gfw.

This figure shows the distribution of NADH-NR (▲) and NADH-CR (●) activities after analysis of an 0.4 ml aliquot of the extract prepared in the presence of 10 µM leupeptin on a 4-20% sucrose gradient .

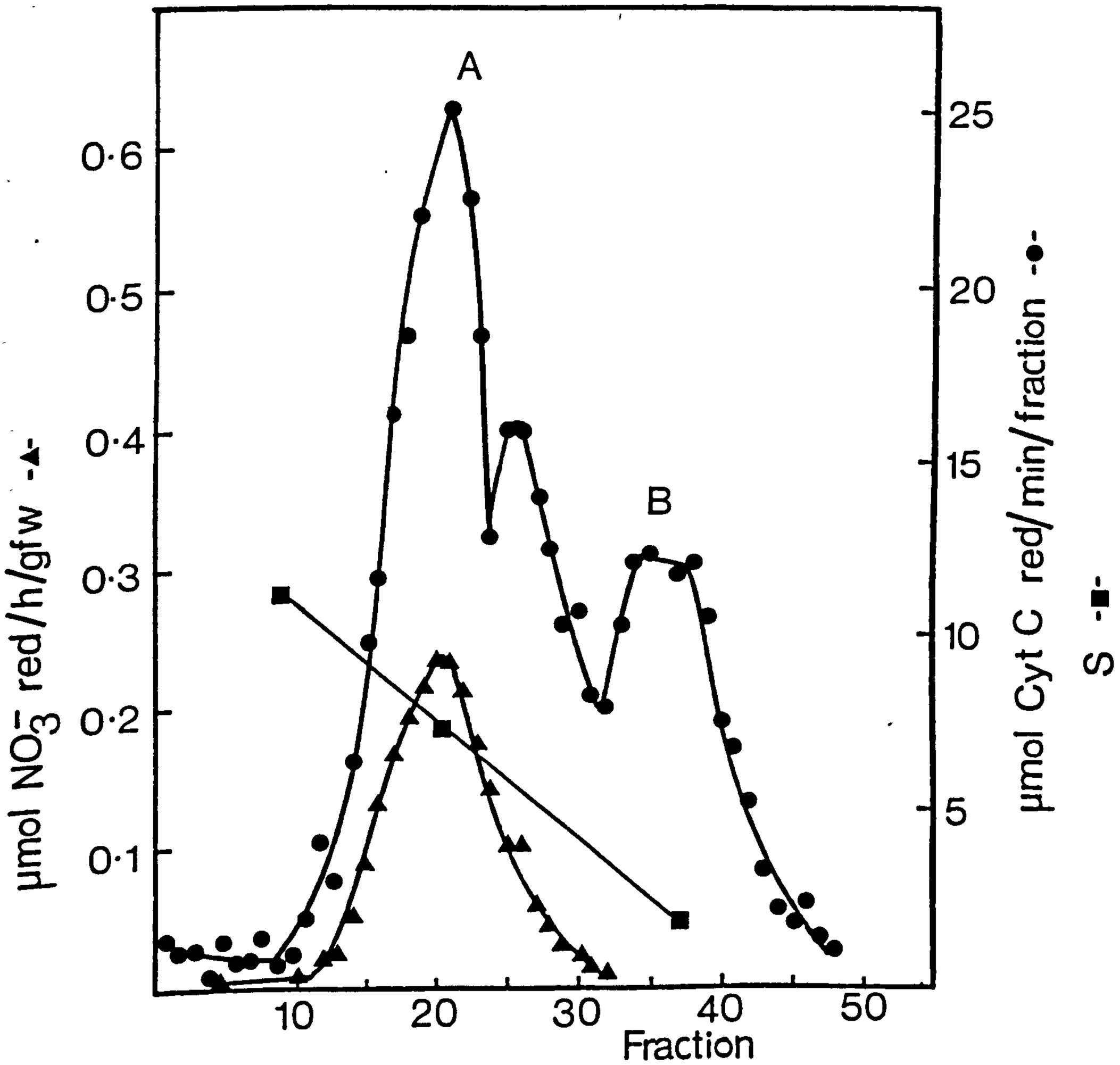
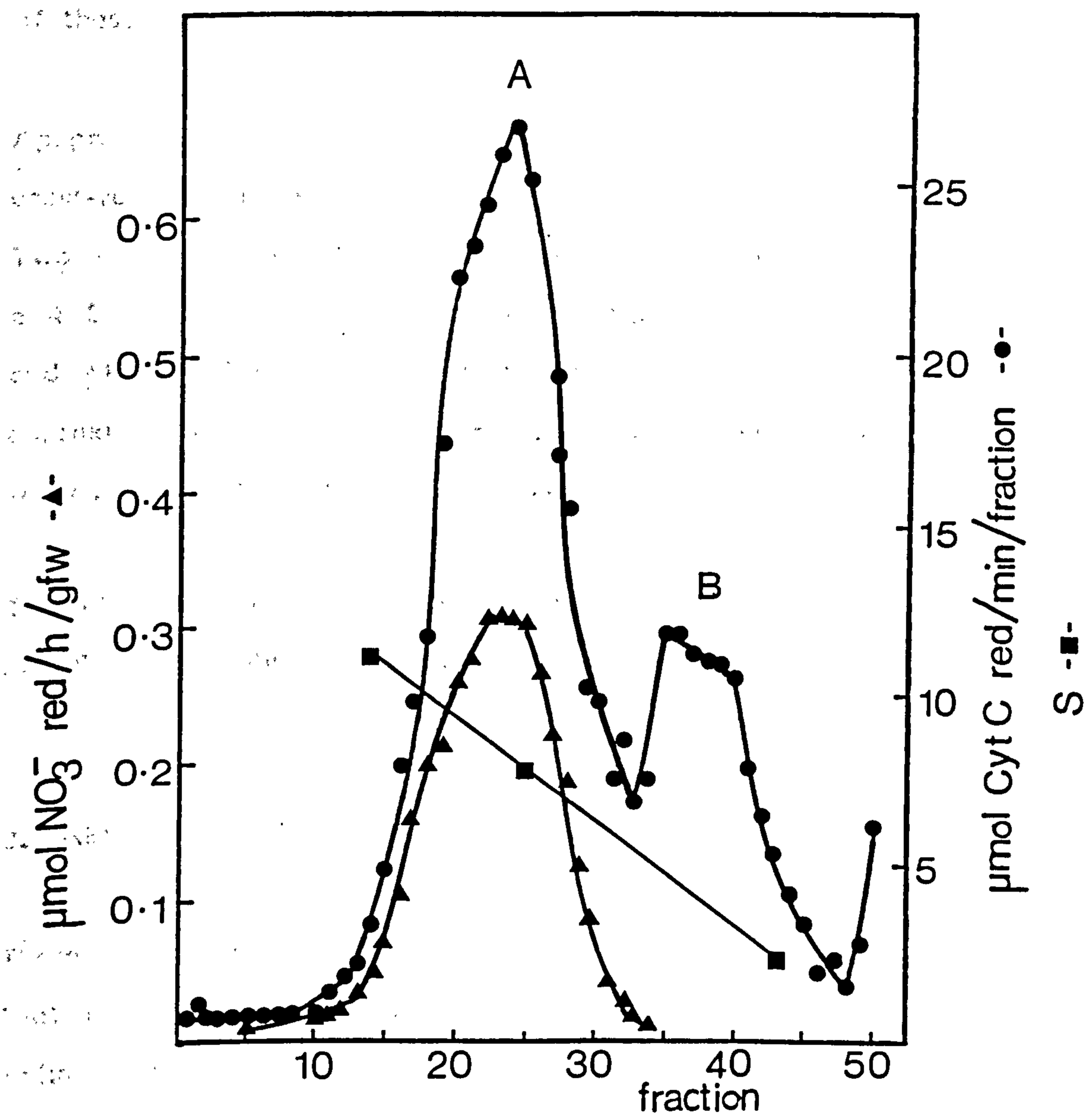

A
B
0·6
0·5
0·4
0·3
0·2
0·1
μmol NO_3^- red/h/gfw -▲-
25
20
15
10
5
μmol Cyt C red/min/fraction -●-
S -■-
10
20
30
40
50
Fraction

Figure 29

A typical sucrose density gradient centrifugation analysis of an extract from 6 day old primary leaves of cv. Golden Promise prepared in the presence of 10 µM leupeptin

6 day old primary leaves were extracted with NR Extraction Buffer with 10 µM leupeptin. After centrifugation of the brei at 38,000 g for 20 minutes the NR activity of the extract was 7.82 µmol nitrite produced/h/gfw.

This figure shows the distribution of NADH-NR (▲) and NADH-CR (●) activities after analysis of an 0.4 ml aliquot of the extract prepared in the presence of 10 µM leupeptin on a 4-20% sucrose gradient .


A
B
0·6
0·5
0·4
0·3
0·2
0·1
μmol NO_3^- red/h/gfw -▲-
25
20
15
10
5
μmol Cyt C red/min/fraction -●-
S -■-
10
20
30
40
50
fraction

One aim of this work was to see whether MoCo mutant barley lines R9201, R11301, R9401 and R12202 were able to dimerise the 4 S subunits of NR. This would give information about the nature of the MoCo defects in all of these lines.

Apoprotein mutants of barley showed wide variations in levels of NR cross-reacting material and NADH-CR profiles (Narayanan **et al**, 1983). Two classes of apoprotein mutants were found; those that possessed only a 4 S NADH-CR, equivalent to the **N. tabacum** apoprotein mutants (Mendel and Müller, 1979), and those that also have a dimer NADH-CR peak at approximately 8 S (Narayanan **et al**, 1983). **narlb**, **narlg** and **narli** were placed in the first class with **narld** in the second.

The other aim of this work was to examine the NADH-CR profile of the postulated apoprotein mutant, R12801.

2. RESULTS

These results were obtained using the method described in Chapter 2, Section 8. In addition to the homozygous NR minus plants, their NR+ siblings were examined in the M4 segregating populations from backcrosses of (R9201 x Maris Mink), (R11301 x Maris Mink) and (R9401 x Golden Promise). The parental types, Maris Mink and Golden Promise were also used as controls.

R12202 and R12801 (in the background of cv. Golden Promise) self-fertilised M3 seedlings were grown and assayed as homozygotes using Golden Promise seedlings as controls.

The results discussed below are based on at least two separate experiments for each mutant line and included appropriate controls for each centrifugation.

2.1 Cytochrome c reductase activity profiles of Maris Mink and Golden Promise

The sucrose density gradient sedimentation analyses of Maris Mink (figure 28) showed two major peaks (A and B) of NADH-CR activity at 7.7 S and 3.5 S respectively. The 7.7 S peak had a shoulder at 6.0 S in three of four individual Maris Mink NADH-CR profiles. These major peaks of NADH-CR had the relative distribution of 70% and 30% for peaks A and B respectively (table 12). NADH-NR activity cosedimented with the 7.7 S NADH-CR peak and therefore the CR associated with this peak is due to the presence of the active dimer NR enzyme. Three sucrose gradient sedimentation analyses of cv. Golden Promise gave two major NADH-CR activity peaks, A and B, at 7.8 S and 3.7 S (figure 29) which contained 69% and 31% NADH-CR activity respectively (table 12). The NADH-NR activity cosedimented with the 7.8 S NADH-CR (peak A). The secondary NADH-CR peak (peak B) sedimented at 3.7 S and like that of Maris Mink, was broad. There was considerable similarity between the profiles of these two parental types as can be seen when figure 28 is compared with figure 29.

Figure 30

A typical sucrose density gradient centrifugation analysis of an extract from 6 day old primary leaves of R9201 NR+ plants prepared in the presence of 10 µM leupeptin

6 day old primary leaves were extracted with NR Extraction Buffer with 10 µM leupeptin. After centrifugation of the brei at 38,000 g for 20 minutes the NR activity of the extract was 3.62 µmol nitrite produced/h/gfw.

This figure shows the distribution of NADH-NR (▲) and NADH-CR (●) activities after analysis of an 0.4 ml aliquot of the extract prepared in the presence of 10 µM leupeptin on a 4-20% sucrose gradient.

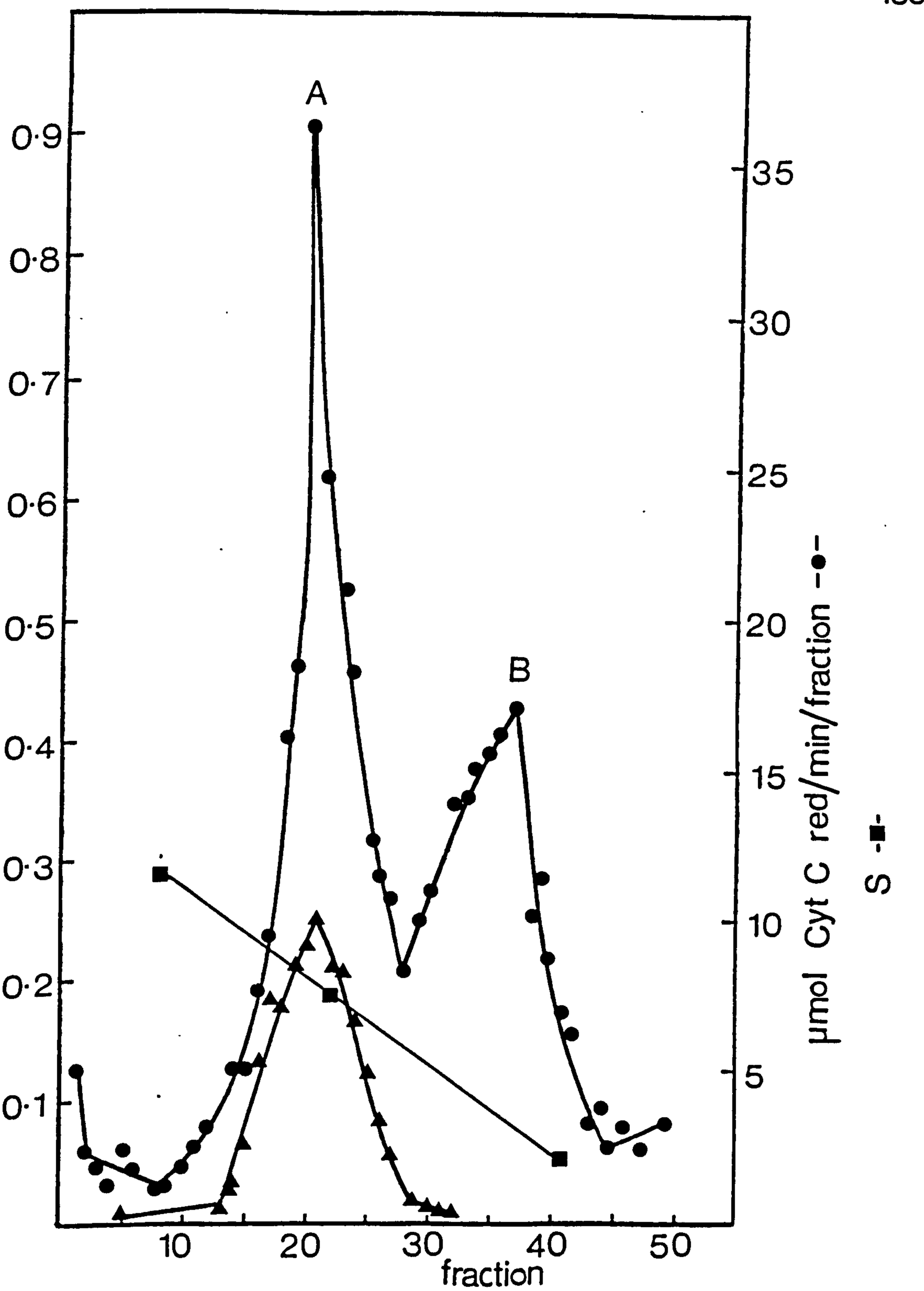

A
B
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
35
30
25
20
15
10
5
µmol NO_3^- red/h/gfw -▲-
µmol Cyt C red/min/fraction -●-
S -■-
10
20
30
40
50
fraction

Figure 31

A typical sucrose density gradient centrifugation analysis of an extract from 6 day old primary leaves of R9201 NR minus plants prepared in the presence of 10 µM leupeptin

6 day old primary leaves were extracted with NR Extraction Buffer with 10 µM leupeptin. After centrifugation of the brei at 38,000 g for 20 minutes the NR activity of the extract was 0.0 µmol nitrite produced/h/gfw.

This figure shows the distribution of NADH-NR (▲) and NADH-CR (●) activities after analysis of an 0.4 ml aliquot of the extract prepared in the presence of 10 µM leupeptin on a 4-20% sucrose gradient.

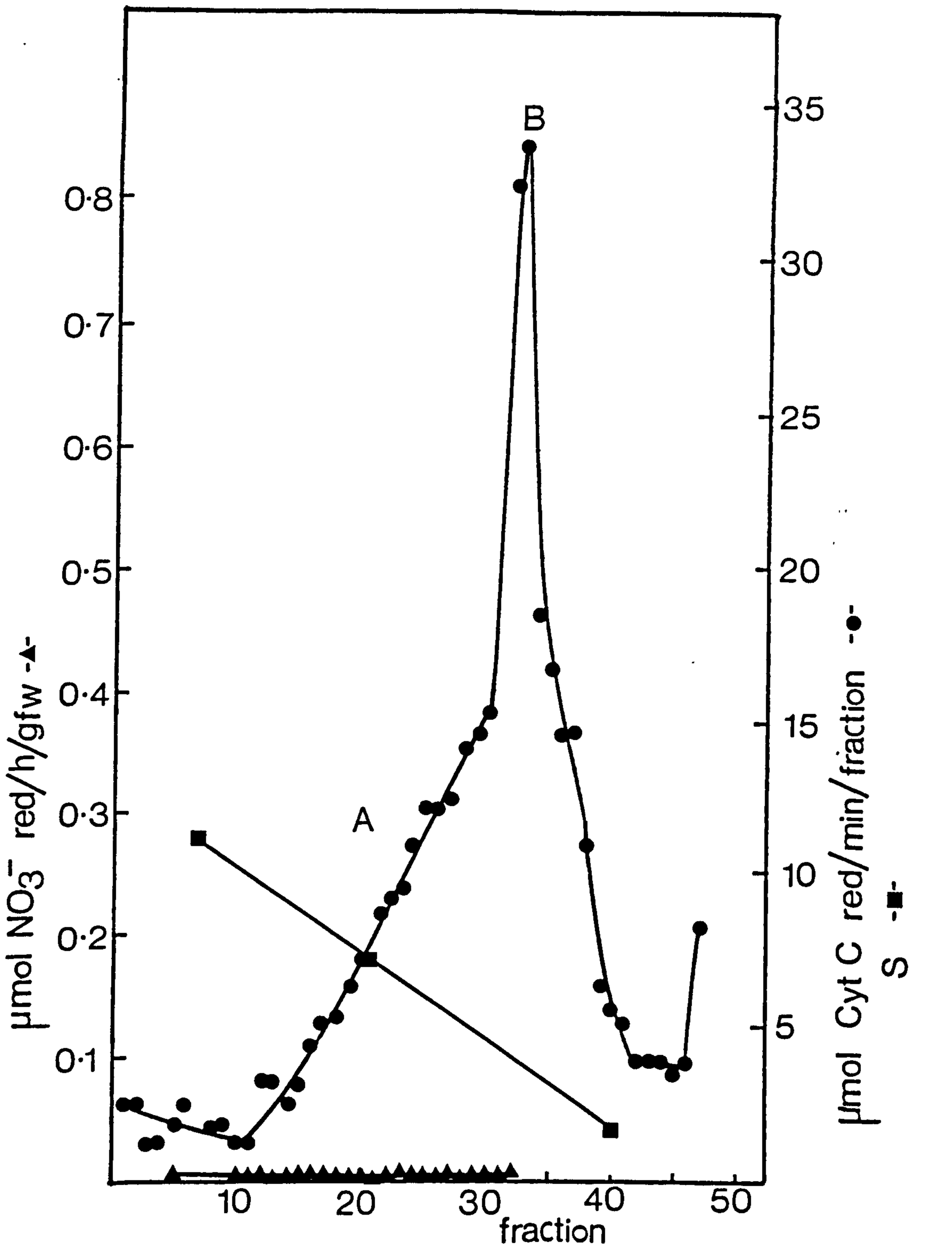
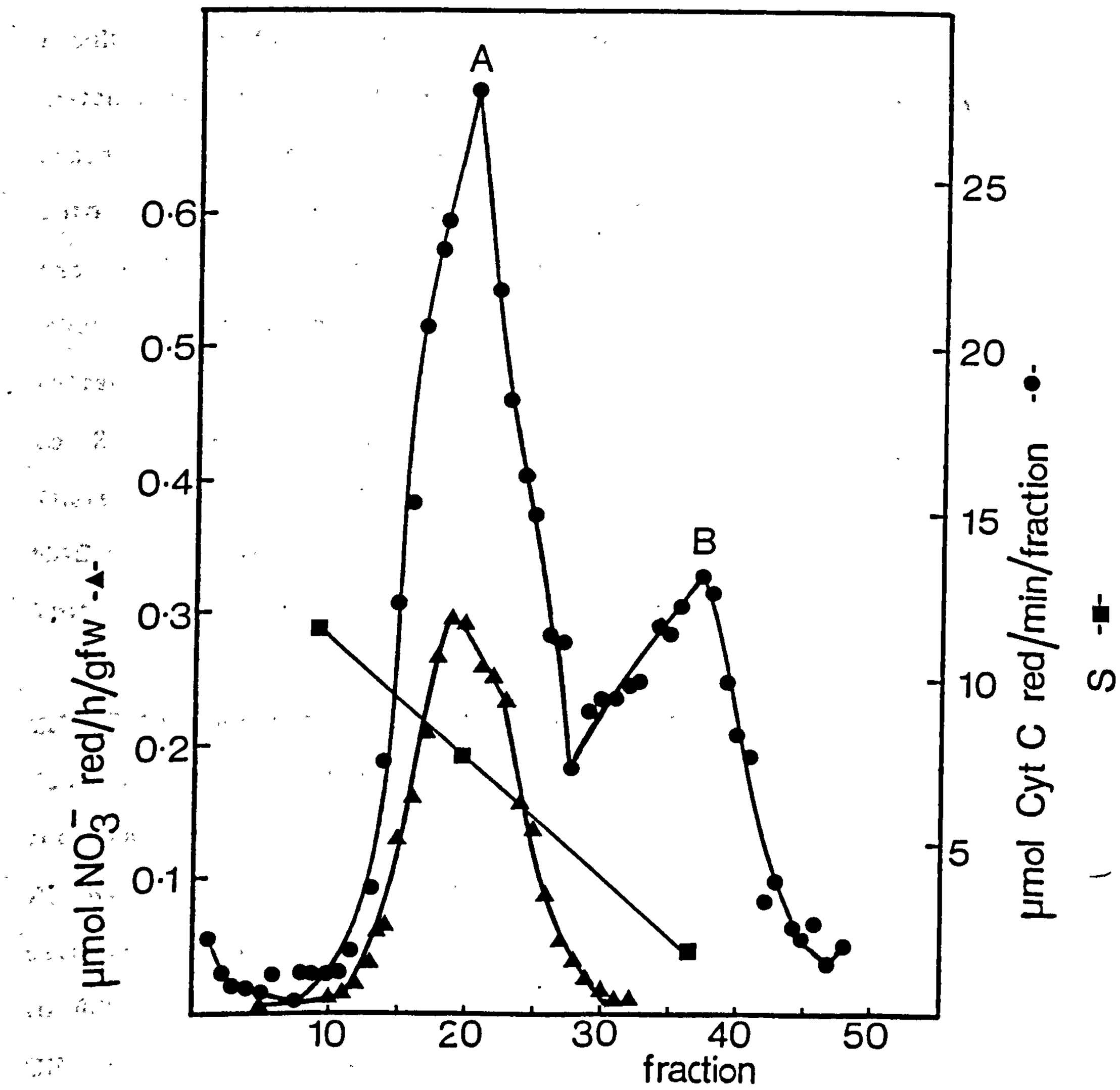

B
A
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
35
30
25
20
15
10
5
10
20
30
40
50
µmol NO3- red/h/gfw -▲-
µmol Cyt C red/min/fraction
S -■-
fraction

Figure 32

A typical sucrose density gradient centrifugation analysis of an extract from 6 day old primary leaves of R11301 NR+ plants prepared in the presence of 10 µM leupeptin

6 day old primary leaves were extracted with NR Extraction Buffer with 10 µM leupeptin. After centrifugation of the brei at 38,000 g for 20 minutes the NR activity of the extract was 2.18 µmol nitrite produced/h/gfw.

This figure shows the distribution of NADH-NR (▲) and NADH-CR (●) activities after analysis of an 0.4 ml aliquot of the extract prepared in the presence of 10 µM leupeptin on a 4-20% sucrose gradient.


A
B
0·6
0·5
0·4
0·3
0·2
0·1
25
20
15
10
5
10
20
30
40
50
µmol NO_3^- red/h/gfw -▲-
µmol Cyt C red/min/fraction
S -■-
fraction

2.2 Cytochrome c reductase activity profiles of R9201

The NADH-CR profile of R9201 NR+ plants showed a 7.7 S peak (A) and a broad peak (B) ranging from 2.8 S to 4.3 S (figure 30). Similar results were found when this experiment was repeated. The relative distribution of NADH-CR activity was 64% and 36% in peaks A and B respectively (table 12). Once again, the NADH-NR activity cosedimented with the 7.7 S NADH-CR activity (peak A), showing marked similarity to the wild type profiles. The situation was clearly different in the R9201 NR minus plants (figure 31). All three profiles from separate extractions gave a broad spread of NADH-CR activity ranging from 7.3 S to 2.7 S, with a clear peak of activity sedimenting at 4 S (peak B). There was, as expected, no NADH-NR activity. However 36% of the total NADH-CR activity was recovered in the 7.3 S to 6.1 S region (table 12). This suggests the presence of some inactive dimer.

2.3 Cytochrome c reductase activity profiles of R11301

Sucrose gradient sedimentation analyses of R11301 NR+ plants reproducibly gave a major NADH-CR activity sedimenting at 7.2 S (peak A) with two shoulders at 7.7 S and 5.9 S (figure 32). There was a secondary peak, B, of NADH-CR activity ranging from approximately 2 S to 4.8 S (figure 32). These peaks represented 64% and 36% of the NADH-CR activity (table 12). The NADH-NR activity cosedimented with the 7.7 S NADH-CR activity peak, giving the same sedimentation coefficient as the wild type NR activity. The shape and distribution of the NADH-CR activity for R11301 NR+ plants was very similar to those of the wild types. Two NADH-CR profiles of R11301 NR minus plants gave a 7.7 S peak of NADH-CR activity, peak A, with a shoulder at 6.9 S, broadening

Figure 33

A typical sucrose density gradient centrifugation analysis of extracts from 6 day old primary leaves of R11301 NR minus plants in the presence of 10 µM leupeptin

6 day old primary leaves were extracted with NR Extraction Buffer with 10 µM leupeptin. After centrifugation of the brei at 38,000 g for 20 minutes the NR activity of the extract was 0.0 µmol nitrite produced/h/gfw.

This figure shows the distribution of NADH-NR (▲) and NADH-CR (●) activities after analysis of an 0.4 ml aliquot of the extract prepared in the presence of 10 µM leupeptin on a 4-20% sucrose gradient.

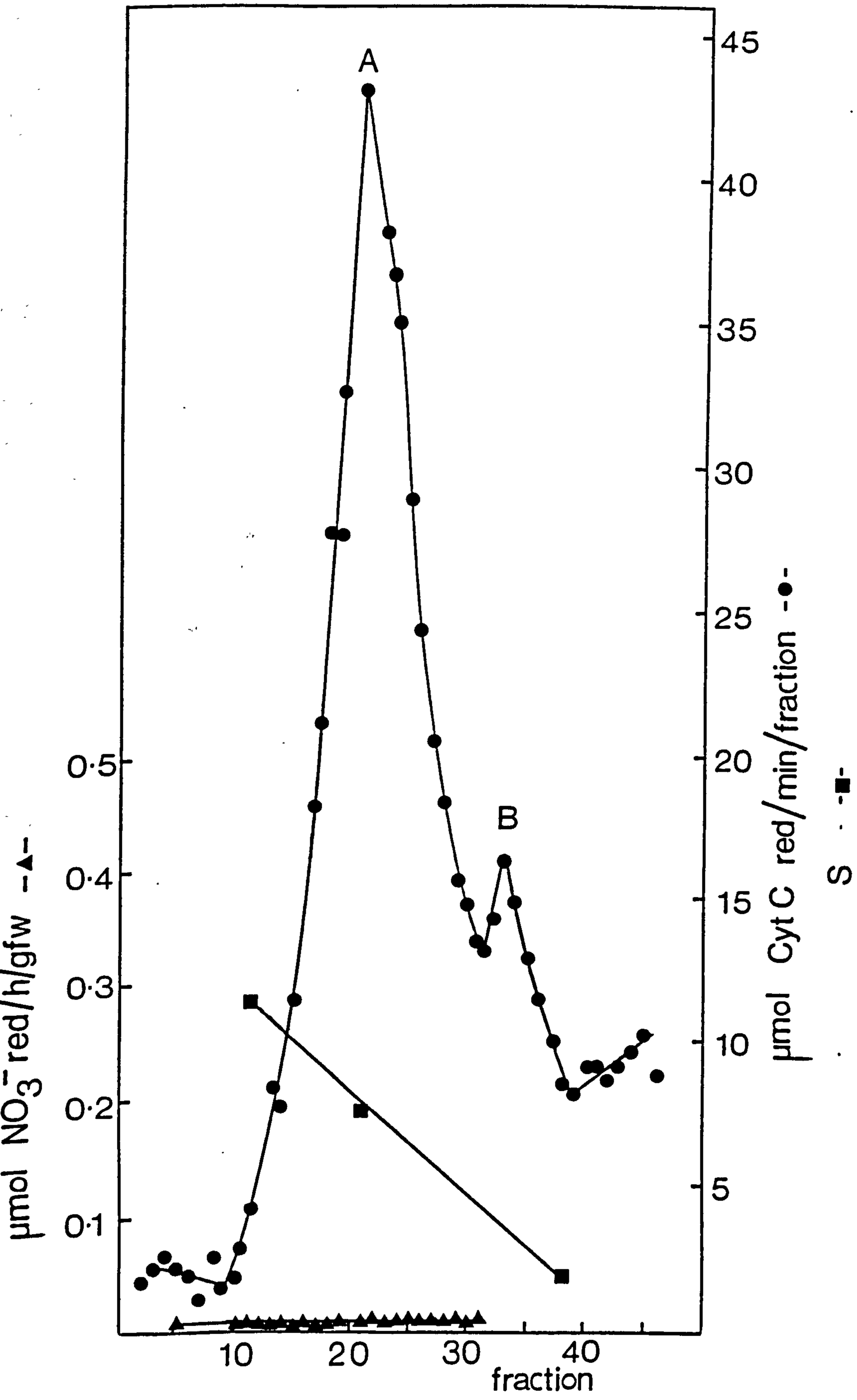
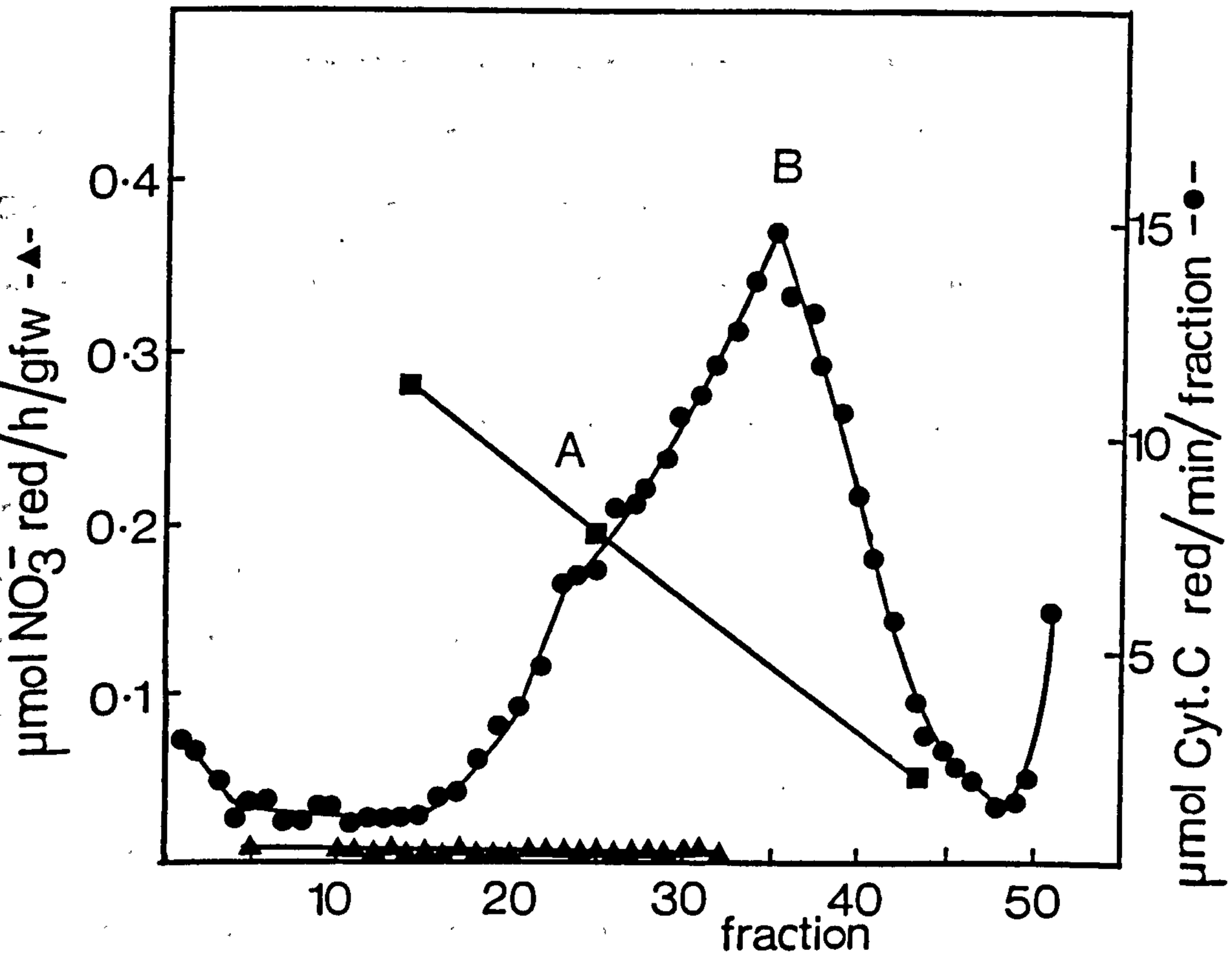

A
B
45
40
35
30
25
20
15
10
5
0·5
0·4
0·3
0·2
0·1
µmol NO3- red/h/gfw -▲-
µmol Cyt C red/min/fraction -●-
S -■-
10
20
30
40
fraction

Figure 34

A typical sucrose density gradient centrifugation analysis of an extract from 6 day old primary leaves of R9401 NR minus plants prepared in the presence of 10 µM leupeptin

6 day old primary leaves were extracted with NR Extraction Buffer with 10 µM leupeptin. After centrifugation of the brei at 38,000 g for 20 minutes the NR activity of the extract was 0.0 µmol nitrite produced/h/gfw.

This figure shows the distribution of NADH-NR (▲) and NADH-CR (●) activities after analysis of an 0.4 ml aliquot of the extract prepared in the presence of 10 µM leupeptin on a 4-20% sucrose gradient.


0·4
0·3
0·2
0·1
µmol NO3− red/h/gfw -▲-
A
B
15
10
5
µmol Cyt.C red/min/ fraction -●-
S -■-
10
20
30
40
50
fraction

out to a small peak, B, at 3.4 S (figure 33). The relative distribution of NADH-CR activity between peaks A and B was 74% and 26% (table 12). As expected, there was no NADH-NR activity present.

2.4 Cytochrome c reductase activity profiles of R9401

The NR+ plants from the backcross (R9401 x Golden Promise) produced an NADH-CR activity profile similar to that of the wild types with a major peak (A) at 7.7 S and a secondary peak (B) at 3 S (Wray **et al**, 1985).

The NR minus plants from the same F2 population gave a single broad peak ranging from 2.5 S to 7.6 S (figure 34). The NADH-CR activity sedimenting above 6.1 S was 29% (table 12). The distribution between peaks A and B was similar in all three profiles of R9401 NR minus plant extracts.

2.5 Cytochrome c reductase activity profiles of R12202 and R12801

The sucrose gradient sedimentation profiles of R12202 and R12801 gave only a single NADH-CR peak sedimenting at 3.8 S (figure 35) and 4.7 S (figure 36) respectively, suggesting no dimer was present in either of the mutant lines.

Figure 35

A typical sucrose density gradient centrifugation analysis of an extract from 6 day old primary leaves of R12202 NR minus plants prepared in the presence of 10 µM leupeptin

6 day old primary leaves were extracted with NR Extraction Buffer with 10 µM leupeptin. After centrifugation of the brei at 38,000 g for 20 minutes the NR activity of the extract was 0.0 µmol nitrite produced/h/gfw.

This figure shows the distribution of NADH-NR (▲) and NADH-CR (●) activities after analysis of an 0.4 ml aliquot of the extract prepared in the presence of 10 µM leupeptin on a 4-20% sucrose gradient.

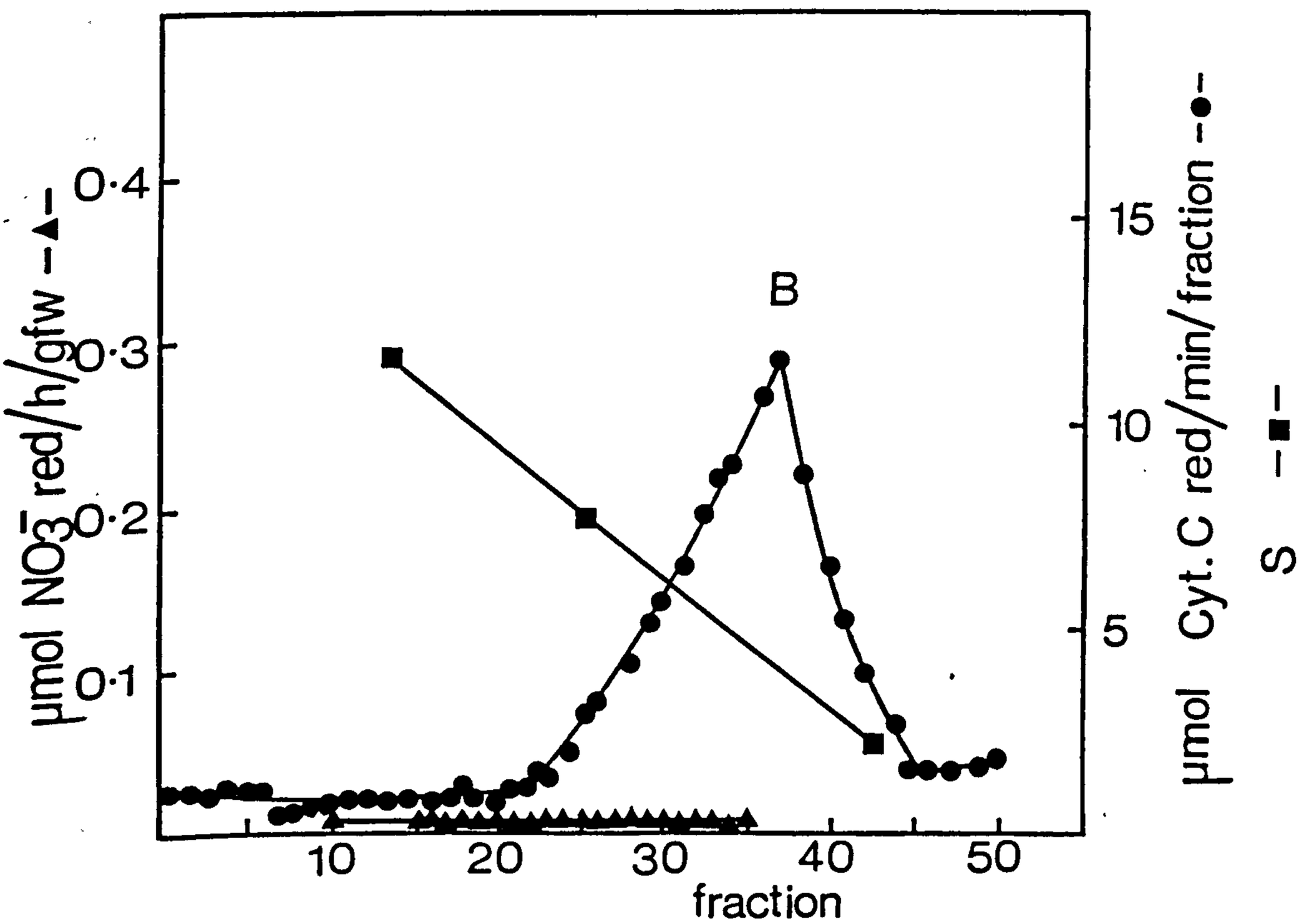
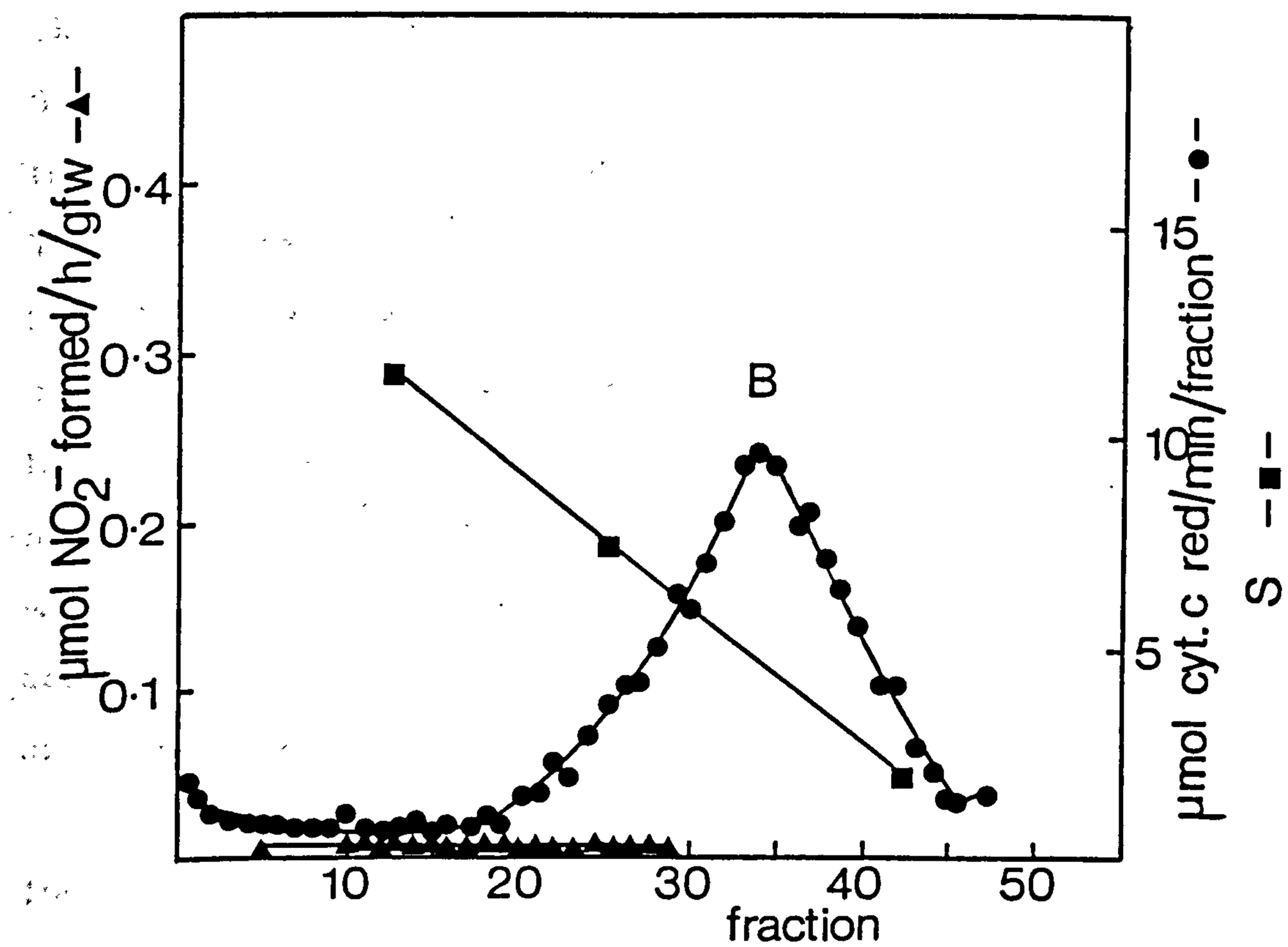

B
0·4
0·3
0·2
0·1
15
10
5
10
20
30
40
50
fraction
µmol NO3 red/h/gfw -▲-
µmol Cyt.C red/min/fraction -●-
-■-
S

Figure 36

A typical sucrose density gradient centrifugation analysis of an extract from 6 day old primary leaves of R12801 NR minus plants prepared in the presence of 10 µM leupeptin

6 day old primary leaves were extracted with NR Extraction Buffer with 10 µM leupeptin. After centrifugation of the brei at 38,000 g for 20 minutes the NR activity of the extract was 0.0 µmol nitrite produced/h/gfw.

This figure shows the distribution of NADH-NR (▲) and NADH-CR (●) activities after analysis of an 0.4 ml aliquot of the extract prepared in the presence of 10 µM leupeptin on a 4-20% sucrose gradient.


μmol NO_2^- formed/h/gfw -▲-
0·4
0·3
0·2
0·1
B
μmol cyt.c red/min/fraction -●-
15
10
5
S -■-
10
20
30
40
50
fraction

3. DISCUSSION

The Maris Mink and Golden Promise NADH-CR activity profiles were very similar to that reported previously for **H. vulgare** cv. Golden Promise (Small and Wray, 1980; Wray **et al**, 1985). Two peaks (7.9-7.6 S and 4 S) were also present in extracts of nitrate induced **N. tabacum** callus tissue (Mendel and Müller, 1979) and **N. crassa** (Nason **et al**, 1970). The NR+ plants from the F2 populations from (R9201 x Maris Mink), (R11301 x Maris Mink) and (R9401 x Golden Promise) gave NADH-CR activity profiles which were similar to the wild types. This suggests that alterations in the distributions between the NADH-CR activity peaks A and B of the NR minus plants taken from the same populations, were caused by recessive gene mutations and not by the mixed genetic background, nor due to other segregating mutations which might also have been present in the selected plants.

The NR minus gene mutations decreased the NR activity to less than 1% in these mutant lines (R9201, R11301, R9401, R12202 and R12801) and also changed distribution of the CR species. The distribution of NADH-CR activity of R9201 and R9401 NR minus plants were similar, with about 30% of the very broad 4 S peak covering the 6.1 S to 7.6 S range. This is similar to the situation found in **nar2a** (Narayanan **et al**, 1983). However, Wray **et al** (1985) in the same laboratory and using the same source of R9401 seeds, find that there is no 7.7 S NADH-CR activity in R9401. As Narayanan **et al** (1983) suggest, it is possible that the amount of dimer present in these mutants is very dependent on the exact **in vitro** conditions used, perhaps that is because the inactive dimeric

form of NR found in R9401 is more unstable than the wild type dimer.

R11301 NR minus seedlings gave an NADH-CR profile that included a 7.7 S peak of wild type proportions even though the mutant possessed no NR activity. This suggests that the MoCo of R11301 is not defective in its ability to dimerise apoprotein subunits and indicates that the mutation in R11301 is biochemically different from R9201 and R9401.

The R12202 NADH-CR profile gave a single broad band (3.8 S) of NADH-CR activity, distinguishing it from the other two classes of MoCo mutant lines (R9201/R9401/Az 34 and R11301).

The NADH-CR profiles from extracts of R9201, R9401 and Az 34 are clearly different from those given by R11301 NR minus plants lending further support to the genetic evidence that R11301 is not allelic with either R9201, R9401 or Az 34.

R12801, the putative apoprotein mutant, showed a very similar profile to the apoprotein mutants **narlb**, **narlg** and **narli** of barley (Narayanan **et al**, 1983) and the tobacco mutants **nia63** and **nia95** (Mendel and Müller, 1979) in having no dimer. This suggests that in all these mutants (R12801, **narlb**, **narlg**, **narli**, **nia63** and **nia95**), the defect in the apoprotein has affected regions required for dimer formation.

The 6.9 S shoulder and general broadening towards a 3.4 S peak on many of the profiles may be due to proteolytic cleavage. This would support

the hinge-domain theory presented by Small and Wray (1980).

I seem to have three classes of MoCo mutants which possess, a) wild type proportions of inactive NR dimer, b) less than wild type proportions of the inactive dimer and c) no detectable dimer. It is possible that classes b) and c) are a single group since it has been reported that the **nar2a** (Az 34) mutant possessed 30% of the wild type levels of 8 S NADH-CR activity (Narayanan **et al**, 1983), whilst **nar2b** (R9401) possessed no dimeric NADH-CR activity (Wray **et al**, 1985). Narayanan **et al** (1983) suggest that the experimental conditions are critical in determining the levels of inactive dimer present in extracts from **nar2a** mutants.

Five MoCo genes have been identified, of which mutations in the genes **chlE** (A. **nidulans**), **chlD** and **chlG** (E. **coli**), **cnxA** and **cnxB** (N. **tabacum**) and **cnxA** (N. **plumbaginifolia**) gave rise to lines which possessed the inactive dimeric NR macromolecules (Cove, 1979; Sperl and DeMoss, 1975; Mendel and Müller, 1985; Mendel **et al**, 1986; Miller and Amy, 1983; Buchanan and Wray, 1982). R11301 seems to be similar to this group.

R9401, R9201 and R12202 mutants may be similar to the **chlA (chlM** and **chlN**) and **cnxE** mutant from E. **coli** (Stewart and MacGregor, 1982), the **cnxABC, cnxG** and **cnxH** mutants from A. **nidulans** (MacDonald **et al**, 1974), the **nar2a** mutant from H. **vulgare** (Narayanan **et al**, 1983) and the N. **plumbaginifolia cnxC** (Mendel **et al**, 1986) mutants, since they all possessed some or no NADH-CR dimer.

To get a clearer understanding of the relationship between the barley MoCo genes a further investigation of the MoCo moiety from the mutants (R9201, R11301 and R12202) was undertaken (Chapter 6).

CHAPTER 6

THE MOLYBDENUM-CONTAINING COFACTOR OF NITRATE REDUCTASE

1. INTRODUCTION

Molybdenum is taken up by the plant, transported, modified and inserted into a preformed pterin structure (demolybdo-MoCo). This process of accumulation and insertion involves a series of steps as shown by the six distinct 99Mo-labelled species detected by Hinton and Mortenson (1985a) during pulse-chase experiments.

The amount of MoCo has been measured by the ability of extracts to reconstitute NR activity in the presence of inactive NR monomers from the **nit1** mutant of **N. crassa** and excess molybdate. This quantity of MoCo can be determined for all molybdoenzymes studied with the exception of nitrogenase (Pienkos **et al**, 1977a). Nason **et al** (1970) first demonstrated this using two NR minus strains, **nit1** and **nit3** of **N. crassa.** Extracts of **nit1** and **nit3** provided active 4.5 S NR monomers and active cofactor respectively (Nason **et al**, 1970). MoCo from diverse phylogenetic sources have been shown to function in this assay (Nason **et al**, 1970; Mendel, 1983; Narayanan **et al**, 1984).

There are three general methods for extracting MoCo:

a) Dialysis of a crude extract (Ketchum **et al**, 1970).

b) 1-5 minutes acidification of cell free extracts at pH 2-2.5 followed by neutralisation (Nason **et al**, 1971; Mendel **et al**, 1981).

c) Heat treatment of cell free extracts at 70-80° C for 90 seconds followed by rapid cooling (Mendel, 1983; Narayanan **et al**, 1984).

Dialysis releases a low amount of MoCo from nitrate induced and non-

induced tissue, indicating that some MoCo is not bound to molybdoenzymes and is 'free'.

Heat treatment has proved to be a most effective way of releasing MoCo from molybdoenzymes (Mendel, 1983; Narayanan **et al**, 1984) and, possibly, Mo-carrier proteins (Johnson, 1980). This 'bound' MoCo released by heating accounts for 95% of MoCo in nitrate induced **N. tabacum** cells (Mendel **et al**, 1982b). It is nitrate inducible, implying that the great majority of MoCo in induced tissue is associated with nitrate reductase (Mendel **et al**, 1982b).

The aims of work presented in this chapter were:

1. To measure the molybdenum content of the mutants R9201, R11301 and R9401 in order to see if uptake of molybdenum was the primary defect preventing the formation of an active MoCo.
2. To test whether repair of NR and xanthine dehydrogenase activity could be restored by growth of the mutants on high levels of molybdate. If this occurred, it would suggest that the mutants R9201, R11301 and R9401 were equivalent to **cnxA** in **N. tabacum** (Mendel **et al**, 1984) and **cnxE** in **A. nidulans** (Arst **et al**, 1970).
3. To assay the amount of MoCo in extracts of R9201, R11301, R9401 and R12202.
4. To discover whether extracts of the putative apoprotein mutant R12801 could form an active nitrate reductase in the presence of molybdate and MoCo from xanthine oxidase.

Table 13

Molybdenum content of whole wild type and mutant seedlings

Source	Molybdenum (ng/gfw) NR+	NR minus
Maris Mink	445 ± 67 (17)	0
Golden Promise	355 ± 153 (7)	0
R9401	380 ± 116 (5)	800 ± 175 (6)
R9201	607 ± 147 (8)	871 ± 123 (4)
R11301	405 ± 99 (5)	542 ± 145 (4)
R12801	372 ± 194 (5)	

7 day old seedlings were grown and extracted to determine the molybdenum content as described in Chapter 2 Section 6. The seedlings were supplied with half strength Hoaglands Solution which contained 25 μM sodium molybdate.

A total of 61 duplicated assays were performed in at least two independent experiments for each plant category. The number is, in each case, given in brackets after the mean and standard error.

2. RESULTS

2.1 Molybdenum content of seedlings

Molybdenum determinations were carried out as described in Chapter 2, Section 6. Plants were watered daily with half strength Hoagland Solution containing 25 µM sodium molybdate. The average molybdenum content of entire 7 day old induced seedlings of Maris Mink and Golden Promise were 445 (n=17) and 355 (n=7) ng molybdenum/gfw respectively (table 13). There was no significant difference between these two wild types when tested by Students t-test (Appendix 1). The two samples were therefore combined to give a value of 419 ± 64 ng molybdenum/gfw (n=24) for the mean and standard error. The molybdenum contents of NR+ plants segregating in the F2 populations from backcrosses of R9401, R9201 and R11301 were not significantly different from the wild type values (table 13). This was also true for the R9201, R11301 and R12801 NR minus plants (table 13). However, R9401 NR minus plants contained significantly greater levels of molybdenum from both their NR+ siblings and the wild type level (table 13).

2.2 Plant growth and NR activity in the presence of high levels of molybdate

Plants were grown in the presence of 25 µM to 10 mM sodium molybdate as described in Chapter 2, Section 10.

The NR activity of induced 7 day old wild type plants was not greatly affected by four days growth on molybdate (figure 37). Essentially the same effect was shown in extracts from 4, 5 and 6 day old plants except

Figure 37

In vitro NADH-NR activity of 7 day old nitrate induced wild type seedlings grown in the presence of high concentrations of molybdate

Seedlings were supplied with half strength Hoagland Solution, to which was added 15 mM nitrate and varying concentrations of soidum molybdate. This treatment was continued on a daily basis until the seedlings were 7 days old. Leaves were extracted and assayed for **in vitro** nitrate reductase activity as described in Chapter 2, Sections 3 and 5.

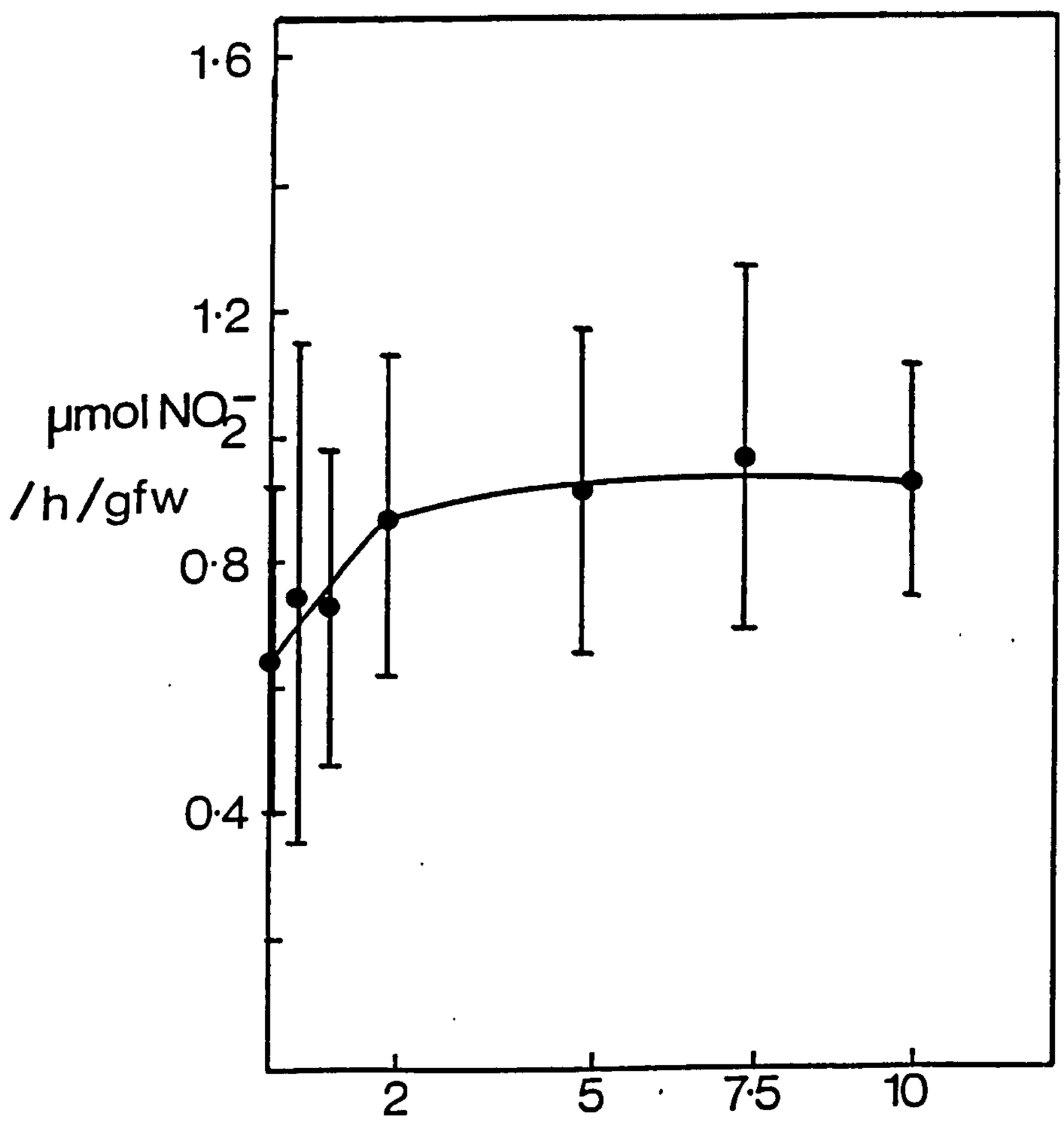

1·6
1·2
µmol NO_2^-
/h/gfw
0·8
0·4
2
5
7·5
10
mM Mo

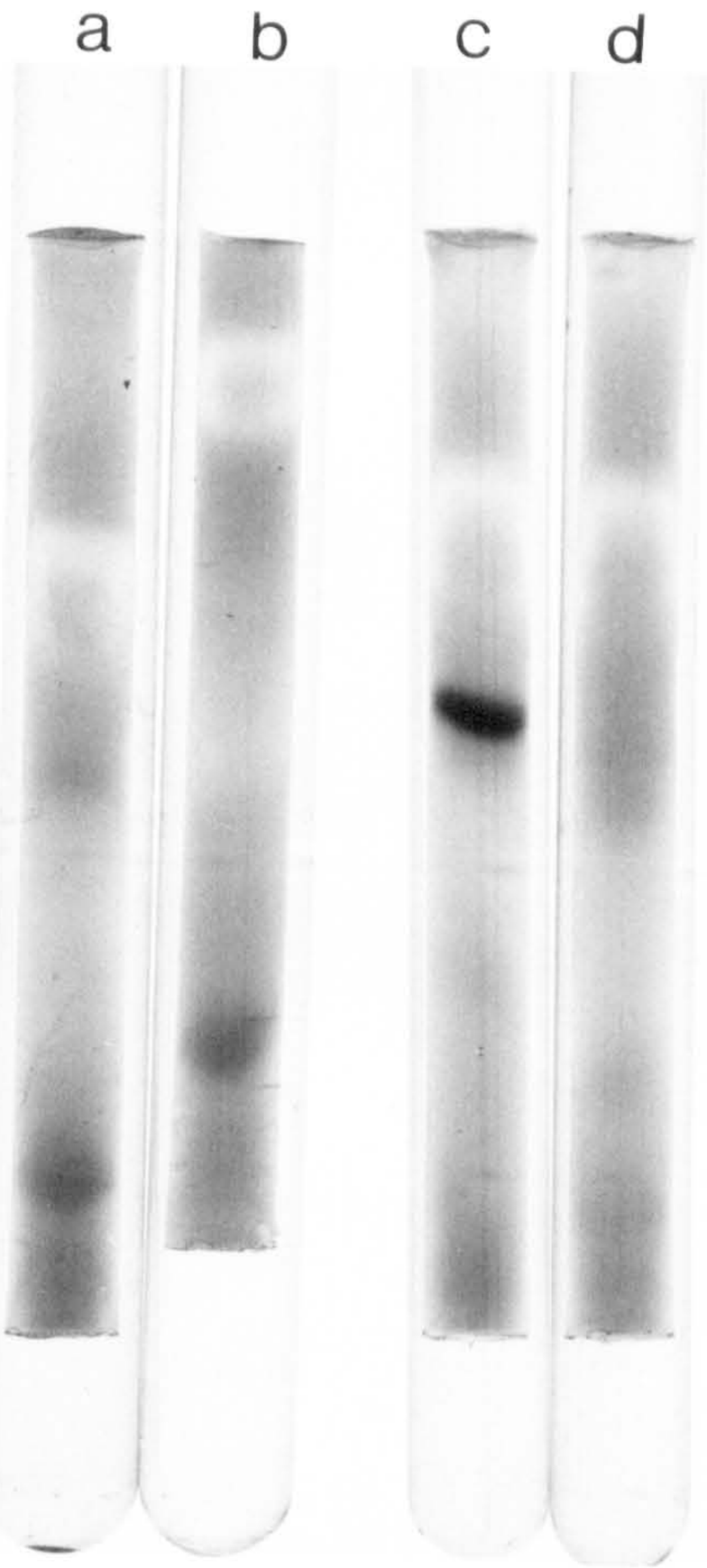
Figure 38

Xanthine dehydrogenase activity found in extracts from NR+ and NR minus R9201 shoots after growth on (a) normal or (b) high levels of molybdate for 48 hours

Nitrate induced 7 day old R9201 NR+ and NR minus seedlings were grown in the presence of 25 µM (a), or 2 mM (b) sodium molybdate. The experimental conditions are given in Chapter 2, Section 5.

5% and 7% PAGE were used in (a) and (b) respectively.

Extracts from NR+ seedlings were electrophoresed in gels c,d,e,f,g,h, whilst extracts from NR minus seedlings were electrophoresed in gels a,b,i,j,k,l. All gels were stained for xanthine dehydrogenase activity in the presence (a,c,e,g,i,k) and absence (b,d,f,h,j,l) of hypoxanthine.


a
b
c
d

e
f
g
h
i
j
k
l

that the NR activity was increased 1.5 to 2-fold. 10 mM molybdate in the growth medium is known to be toxic to tobacco callus (Mendel **et al**, 1981; Buchanan and Wray, 1982). On the basis of my preliminary experiments (figure 37) 2mM molybdate was used with the mutant plants as a compromise between toxicity and the high levels of molybdate required to ensure maximum response.

The R9201, R11301 and R9401 NR+ plants contained both NR (table 14) and xanthine dehydrogenase activity in the presence of normal and high levels of molybdate (eg figure 38), as did both wild types. None of the shoots from the NR minus plants of the same F2 populations showed any repair of NR (table 14) or xanthine dehydrogenase activities (eg figure 38).

Xanthine dehydrogenase and NR activities were not detected in the roots of R9401 NR minus plants after growth in the presence of high concentrations of molybdate (figure 39) suggesting that the MoCo is also lacking in the roots of these plants.

The other MoCo mutant, R12202, was not assessed for **'in vivo'** repair of NR and xanthine dehydrogenase due to a shortage of seeds.

2.3 MoCo content of seedlings

Molybdenum cofactor was measured by assaying the restoration of NR activity in **N. crassa** mutant **nit1** extract which depends on the dimerisation of NADPH-NR subunits in the presence of a second extract containing free MoCo and exogenous molybdate (Mendel, 1983). Extracts

Table 14

The effect of excess molybdate on NR activity of the wild type and mutant leaves induced for 48 hours in the presence of 25 µM of 2 mM sodium molybdate

	NR activity (µmol/h/gfw)	
Source	**+ 2 mM**	**+ 25 µM**
Maris Mink	5.2 ± 0.5 (5)	4.3 ± 0.3 (4)
Golden Promise	3.6 ± 0.5 (2)	1.6 ± 0.0 (2)
R9401 NR+	3.9 ± 0.8 (9)	3.1 ± 0.3 (7)
R9201 NR+	6.1 ± 1.1 (5)	5.9 ± 1.0 (5)
R11301 NR+	3.8 ± 0.2 (4)	4.4 ± 0.1 (3)
R9401 NR minus	0.0 (8)	0.0 (7)
R9201 NR minus	0.2 ± 0.0 (2)	0.1 ± 0.0 (4)
R11301 NR minus	0.1 ± 0.0 (4)	0.1 ± 0.0 (3)

A total of 74 duplicated assays were performed in at least two independent experiments for each plant category. The number is in each case given in brackets after the mean and standard error.

Figure 39

Xanthine dehydrogenase activity in extracts from R9401 NR+ and NR minus roots after growth on high levels of molybdate for 48 hours

Nitrate induced 7 day old NR+ and NR minus seedlings were grown in the presence of 2 mM sodium molybdate. The experimental details are given in Chapter 2, Section 5.

7% PAGE was used for this experiment.

Extracts from NR+ seedlings were electrophoresed in gels a,b,c,d, whilst extracts from NR minus seedlings were electrophoresed in gels e,f,g,h. The gels were stained for xanthine dehydrogenase activity in the presence (a,c,e,g) and absence (b,d,f,h) of hypoxanthine.

a
b
c
d
e
f
g
h

of 7 day old mutant leaves were made, the MoCo was released by heat treatment and preincubated with a **nit1** extract in the presence of molybdate to allow reconstitution of NR activity. A more detailed description of the method is given in Chapter 2 (Section 11).

2.3.1 The MoCo assay

The conditions for reconstitution of NR activity from **nit1** extract, MoCo and excess molybdate had already been optimised in the same laboratory (Mendel **et al,** 1985). The **nit1** monomers were shown to be in excess and a 40 minute preincubation gave maximum reconstitution (Mendel **et al,** 1985).

Molybdenum is known to be rapidly lost from the released cofactor (Lee **et al,** 1974; Amy, 1981) and, therefore, the presence of 5-10 mM molybdate in the **nit1** complementation mixture is a characteristic common to assays of MoCo from diverse phylogenetic sources ranging from bacteria (Claassen **et al,** 1981; Amy, 1981), to fungi (Lee **et al,** 1974) and plants (Mendel, 1983; Mendel **et al,** 1981; Mendel **et al,** 1985; Narayanan **et al,** 1984). I used a final concentration of 8 mM molybdate to achieve maximum levels of NR activity as suggested by Mendel (1983).

Some 99% of the total amount of extracted MoCo detected required the presence of exogenous molybdate in order to form active NR (table 15).

Using 100 µl **nit1** extract, Mendel **et al** (1985) found that the formation of active NR was proportional to the amount of MoCo added in a 50 µl aliquot.

The assay under these optimised conditions was checked using MoCo from nitrate-induced wild type barley cv. Maris Mink. The NADPH-NR activity detected was due to the mixing of the two extracts and not due to NR activity residing in either extract (table 15). Neither the **nit1** extract nor the heat treated extract from Maris Mink possessed any NR activity (table 15).

In my experiments there was considerable variation in the levels of MoCo activity between experiments but differences of usually less than 10 nmol/h/mg protein between triplicated assays of the same extract of MoCo. This difference between experiments could have been caused by differences in the plant growth conditions, plant extraction or the evacuation stages of the assay, since all other steps in the assay procedure were strictly followed. It seems unlikely that the plant growth conditions altered as the same watering and light regime was followed in all cases. The extraction of the plant material also seems to have been very consistent since all of the extracts contained similar levels of protein (data not shown).

Another possibility is that the ability of the frozen **nit1** extracts to form active NR changed with time. However, the **nit1** extract was tested periodically for its ability to restore NADPH-NR activity using heat treated commercial xanthine oxidase (which also possess MoCo) in the presence of excess molybdate and was found to be stable. It should be possible to minimise the possible variation due to the evacuation steps by evacuating for a standard period of time after full vacuum has been reached. Despite these variations between experiments the broad

Table 15

Validation of MoCo assay

Source	NR Activity (nmol/h/mg protein)
nitl extract	0
Maris Mink MoCo extract	0
nitl extract + Maris Mink MoCo extract + Mo	766
nitl extract + Maris Mink MoCo extract - Mo	3
Boiled **nitl** + Maris Mink MoCo extract + Mo	0

analysis remains clear.

The residual MoCo found in extractions made without molybdate (table 15) could be due to the retention of molybdate by a small percentage of MoCo during heat treatment.

2.3.2 MoCo content of extracts from wild type, R9201, R11301, R9401, R12202 and R12801

Using a Students t-test (Appendix 1) there was no significant difference between the amount of MoCo in extracts from cultivars Maris Mink and Golden Promise (table 16). Therefore, these two wild type samples were combined to give a pooled mean of 700 ± 96 nmol/h/mg protein (n=8). The R9201, R11301 and R9401 NR+ plants possessed levels of MoCo which were not significantly different from the pooled wild type and the other appropriate controls (table 16). There was evidence of a slight decrease in the MoCo content of the NR+ plants compared to the wild type which might be due to the presence of the single mutant allele in some of the phenotypically NR+ plants.

The R9201, R9401, R12202 and R12801 NR minus plant extracts had lower amounts of MoCo than the wild types and NR+ siblings (table 16). R11301 NR minus plant extracts gave a mean MoCo content similar to that of the wild type and their NR+ siblings (table 16). R12202 and R12801 had only 6% and 13% respectively, of the MoCo content of Golden Promise, their parental type (table 16).

Table 16

MoCo content of the wild type and mutant seedlings in the presence of molybdate in the MoCo extraction buffer

Source of MoCo	Reconstituted NR activity (nmol/h/mg protein from MoCo extractions)	
Maris Mink	766 ± 114 (4)	a
Golden Promise	634 ± 164 (4)	a
R9401 NR+	548 ± 80 (6)	a
R9201 NR+	610 ± 9 (2)	c
R11301 NR+	575 ± 97 (3)	c
R9401 NR minus	71 ± 21 (3)	df
R9201 NR minus	19 ± 5 (4)	df
R11301 NR minus	677 ± 187 (7)	ce
R12202 NR minus	39 ± 36 (3)	d
R12801 NR minus	80 ± 38 (4)	d

A total of 40 assays were performed. These values are expressed as mean ± standard error of (n) extracts, each determined in triplicate from at least 2 independent experiments.

Values were tested for significance at the 95% confidence limit by Students t-test (Appendix 1).

The letters a,b,c,d,e,f signify:

a = not significantly different from the combined wild type value of 700 ± 96 nmol/h/mg protein

b = significantly different from the combined mean

c = not significantly different from the relevant wild type

d = significantly different from the relevant wild type

e = not significantly different from NR+ siblings

f = significantly different from NR+ siblings

2.4 Reconstitution of NADH-NR activity in extracts of R12801 using MoCo supplied by xanthine oxidase

Two methods of reconstitution (of NADH-NR activity) were tried. For the first method an extract of R12801, the putative apoprotein mutant, was tested for its ability to supply NR monomer in a reconstitution assay in the presence of molybdate and MoCo extracted from commercial xanthine oxidase. The second method was essentially the same except that the NR extraction buffer was used to make an extract of R12801 and the volumes involved in the reconstitutions were larger. Further details of the methods are given in Chapter 2, Section 12.

Under both conditions the levels of NR activity reconstituted by xanthine oxidase MoCo, molybdate and **nit1** extract were high (3,000 - 4,500 nmol/gfw/h). However, no activity was recovered using extracts of R12801 as subunit donors. This suggests that the R12801 might not be able to supply apoprotein subunits for reconstitution in the presence of free MoCo and excess molybdate. This could be taken to be an indication that R12801 was a mutation in the NR structural gene. However, these results must be viewed with caution as the correct positive control experiment was not carried out. This would consist of using an extract of a mutant which has a defective MoCo (and therefore no NR activity), but which would, when supplied with active MoCo from xanthine oxidase and molybdate, form active dimeric NR. A possible candidate for this might be R11301.

3. DISCUSSION

As a result of the work described here, a clearer understanding of the relationships between the different genes involved in the synthesis of MoCo has been achieved.

R9201, R9401 and R11301 NR minus plants had molybdenum contents which were at least as high as wild type plants. They are, therefore, unlikely to be mutants in the uptake or accumulation of molybdenum. The level of molybdate found in all barley lines tested (R9201, R11301, R9401 and R12801) were comparable to those levels described for mutant and wild type **N. tabacum** recorded by Mendel **et al** (1984), none of which were defective in molybdate uptake. Wray **et al** (1985) also found that both R9401 NR minus and NR+ plants possessed the same amount of molybdenum (10-11 µg/g dry weight). The NR+ plants had molybdenum contents which were comparable to those detected in solution cultured barley and are approximately 10-fold greater than the standard values for soil growth plants (McGrath pers. comm.). It is therefore unlikely that the molybdenum uptake is limiting NR production in the mutants.

Molybdenum-deficient plants have been shown to possess very low NR activities, but when presented with molybdenum and nitrate for a period of 8 hours, maximum levels of NR activity were achieved (this did not happen with nitrate alone) (Afridi and Hewitt, 1964). This again suggests that the 48 hour treatment with 2 mM molybdate would have allowed sufficient molybdate to be taken up by the plants.

The measurement of molybdenum content does not, however, define where the molybdenum is within the plant and cell. It is possible that the entry into the plant is satisfactory but the movement within the plant or cell is defective. In **C. pasteurianum** 99Mo has been followed into six separate proteins in pulse-chase experiments (Hinton and Mortenson, 1985a and b). These proteins could be affected without altering the overall molybdenum content of the seedlings.

In one class of MoCo mutants in bacteria (Stewart and MacGregor, 1982), fungal (MacDonald **et al**, 1974; Dunn-Coleman, 1984) and plant (Mendel and Müller, 1985; Mendel **et al**, 1986), systems the NR and xanthine dehydrogenase activities were restored by growth on high levels of molybdate or by extraction of the enzyme in the presence of molybdate. This was not the case for R9201, R9401 and R11301.

A low level of molybdenum is not toxic to Golden Promise over a long period of time, since the growth shown by R9401 NR minus embryos grown on sterile medium containing 1 mM sodium molybdate (and glutamine as the sole nitrogen source) for a month was similar to the wild type (Bright **et al**, 1983). This experiment also suggests that the requirement of the plant for molybdate is not blocked in those MoCo mutants.

The NR and xanthine dehydrogenase activity was not repaired in either the roots or shoots of R9401 NR minus plants. This suggests that the gene affected by the R9401 mutation is expressed in both roots and leaves. This appears to be different from the situation found in

spinach roots and leaves. Immunological studies indicate that the NR proteins found in spinach roots and shoots are different proteins, suggesting that they might be products of different genes (Ferrario **et al**, 1983)

Assays of MoCo content reveal two classes of MoCo mutants, one with low MoCo (R9201, R9401 and R12202) and the other with high (wild type) McCo content (R11301). This is another clear difference between R11301 and R9201, R9401 and R12202. This supports the genetic analysis, in Chapter 4, which suggests that R11301 was caused by a mutation at a different gene (non-allelic) to Az 34 **(nar2a)**, R9401 **(nar2b)**, and R9201 **(nar2c)**.

3.1 R11301

R11301 NR minus plants do not possess NR or xanthine dehydrogenase activity after a period of growth on high levels of molybdate. An extract of R11301 prepared in the presence of molybdate exhibited no NR activity. Extracts from R11301 NR minus plants do, however, show restoration of NR activity in the presence of **nit1** extract (subunit donor) and molybdate. These three facts taken together with the presence of wild type levels of dimer in crude extracts of R11301 NR minus plants (Chapter 5), indicate that the defect in the R11301 plants is in the final step of the molybdopterin synthesis prior to the insertion of molybdenum (figure 40). The missing enzyme which effects this change would seem to be present in the **nit1** extract since MoCo from R11301 in the presence of molybdenum and a **nit1** extract forms active NR, whilst an extraction of R11301 in the presence of molybdate

Figure 40

Proposed model for the functions of the MoCo genes identified in barley and other higher plants

GENE		PATHWAYS	PHENOTYPE & EXPRESSION
Nicotiana	H.vulgare		
		Molybdopterin synthesis	
cnxC and cnxD		R12202 ?	
			1. No NR activity 2. Inefficient dimerisation 3. Easily dissociated to give NR monomers
		R12202 ? nar 2	
			1. No NR activity 2. Efficient dimerisation
cnxB		R11301	
			1. No NR activity 2. Efficient dimerisation 3. Growth on high Mo (or extraction in the presence of Mo) restored NR activity
cnxA			
			1. NR activity
			ACTIVE MoCo
			"Mo"
			Molybdenum uptake, transport and modification

does not. This enzyme would allow the formation of a MoCo which would be able to mediate effective dimerisation.

The mutated gene found in R11301 is not analogous to those found in the **cnxA** and **cnxE** mutants found in **N. tabacum** (Mendel and Müller, 1985) and **A. nidulans** (MacDonald **et al**, 1974) respectively.

3.2 R9401 and R9201

R9401 and R9201 NR minus plants did not show restored NR and xanthine dehydrogenase activity when grown in the presence of high levels of molybdate. Neither was any NR activity detected if R9201 or R9401 NR minus plants were extracted in the presence of molybdate. R9201 and R9401 NR minus plant extracts lacked or possessed only small amounts of the 7.7 S dimeric NADH-CR activity (Chapter 5).

I found that R9401 **(nar2b)** and R9201 **(nar2c)** possessed 10 and 3% of the wild type MoCo respectively. These are similar to the 6% of the wild type MoCo content found in Az 34 **(nar2a)** (Narayanan **et al**, 1984). This is interesting since Az 34 has 5% (Kleinhofs **et al**, 1980) of the wild type (cv. Steptoe) **in vitro** NADH-NR activity. Since R9201 and R9401 possess no **in vitro** NADH-NR (Chapter 3) and 3-10% of the wild type MoCo content, Az 34 might therefore be expected to possess more than 6% of the wild type MoCo content. The MoCo levels detected in cv. Steptoe by Narayanan **et al** (1984) were about 10-fold smaller than those I found for cv. Maris Mink and Golden Promise. It is perhaps possible that not all the MoCo was detected by Narayanan **et al** (1984) since the level of NR found in Steptoe was of the same order as found in both

Maris Mink and Golden Promise.

Wray et al (1985) reported that R9401 NR minus plants possessed 0.2% of the wild type MoCo content. The differences between these results could be due to slight variations in the extraction conditions. My results essentially confirm this finding but indicate a higher MoCo content, approximately 13% of the wild type value.

The defect in **nar2** barley mutants seems to be at an earlier stage of the MoCo synthesis than R11301 since there is inefficient dimerisation (as exhibited by R9201) and greater instability of the dimers that do form (as shown by the differences in profiles reported for R9401) (figure 40). The **nit1** extract is not able to convert inactive R9201 or R9401 MoCo into the structurally and catalytically active MoCo of the wild type, therefore the **nar2** gene could be analogous to the **cnxC** gene in **N. plumbaginifolia** (Mendel **et al**, 1986)

3.3 R12202

Since R12202 lacked MoCo activity, it is clear that the defect in R12202 is not same as that found in R11301. However, it is not possible to determine whether R12202 is biochemically distinct from **nar2a**, **b** and **c**. This again fits in with the CR profiles given in the previous chapter.

It is possible that either R12202 is allelic to the **nar2** mutants or that the R12202 gene product is involved in an earlier step in the MoCo

synthesis than the **nar2** gene product (figure 40) since no dimer was found and there was no restoration of NR activity when R12202 MoCo was incubated with **nit1** extract and excess molybdate.

However, it is possible that the ability of the defective MoCos to dimerise subunits to differing degrees is an allelic effect rather than a genetic phenotype. Cove (1979) points out that within a class of **A. nidulans** mutants some strains possessed dimers at wild type levels or greater, whilst others exhibited both monomers and dimers and a further group displayed only monomers. Cove (1979) also suggests that some of the **A. nidulans** mutant strains which exhibit some dimer might be slightly leaky and possess low levels of NR activity; Az 34 **(nar2a)** would fit into this group. It is clear that a larger number of allelic barley mutants should be isolated and characterised before R12202 can be classified as being either in or out of the **nar2** gene mutant group.

3.4 R12801

R12801, the putative apoprotein mutant, lacked the ability to reconstitute NADH-NR activity when supplied with MoCo from xanthine oxidase and also possessed very little MoCo activity.

R12801 with 13% of the wild type MoCo activity is very different from the **nar1** mutants which possessed 48-100% of the wild type MoCo content (Narayanan **et al**, 1984). R12801 was apparently unable to supply apoprotein subunits for reconstruction with excess free MoCo (supplied by xanthine oxidase) and molybdate.

3.5 The mutant gene in R9401 is not expressed in roots or shoots

Most of the work described so far has involved the measurement of enzyme activities in leaf extracts, therefore in this chapter one mutant, R9401, was examined in detail to see whether the shoot phenotype (NR minus, XDH minus) was also found in the roots. It was clear from my results that this MoCo gene is expressed in both shoots and roots of wild type plants.

It would be interesting to establish whether the effects of a mutation in one of the MoCo genes disturb nitrate uptake, this is examined with R9401 in the next chapter. Another area to explore would be, whether a second NR activity (using NAD(P)H as electron donor) is present in wild type and mutant plants, since this activity has been reported for the barley mutant **narla** and not the wild type (cv. Steptoe) (Dailey **et al**, 1982b). Further analyses of NR activity in the roots and shoots of the wild type and mutant plants are carried out in the following chapter.

CHAPTER 7

NITRATE UPTAKE IN R9401, LOCATION OF NADH-NR AND NADPH-NR IN R9401, R9201, R11301 AND R12801

1. INFLUX OF NITRATE INTO R9401

1.1 INTRODUCTION

A preliminary study of the nitrate uptake across the plasmalemma of roots in the segregating M4 population of R9401 was carried out in collaboration with Dr. M. Drew (LARS).

Nitrate uptake in barley roots is mediated by specific carriers or channels which follow Michaelis-Menten kinetics (Rao and Rains, 1976a). The transport of nitrate through the plasmalemma is known to be dependent on metabolic energy (Rao and Rains, 1976a).

Previous experiments showed that barley plants deprived of nitrate possessed, altered uptake characteristics; Imax increases (Drew pers. comm.) and Km decreases (Lee, 1982).

Ammonium ions have been implicated in the control of nitrate uptake (Drew **et al**, 1984; Pitman, 1967). When present in the external medium ammonium is a potent inhibitor of nitrate uptake in barley (Lewis **et al**, 1982; Rao and Rains, 1976a) and corn plants (MacKown **et al**, 1982a and b) and also affects nitrate reduction, this has been well documented (Minotti **et al**, 1969a and b; Clarkson and Warner, 1979; Lycklama, 1963; Frith and Nichols, 1975; Oaks **et al**, 1977). Most experiments studying the effect of ammonium on nitrate uptake use relatively long experimental periods (usually 2-24 hours) which makes it difficult to decide whether ammonium directly affects uptake per se or via inhibition of nitrate reductase (Deane-Drummond and Glass,

1983b). This phenomenon (of decreased uptake of nitrate in the presence of ammonium) could be studied directly using NR minus plants, since nitrate is not metabolised and there will be very low levels of ammonium produced from nitrate reduction. We asked whether NR minus plants have altered uptake parameters.

The measurement of a radioactive tracer would reflect the fate of nitrogen applied to the root and not of nitrogen previously stored in the plant. Obviously an ideal tracer for this experiment would be an isotope of nitrogen with a long half-life. Those tracers which are available for use are 15NO3-, 13NO3- and 36ClO3-.

15NO3- has the disadvantage that it is not radioactive and can only be measured by using mass spectroscopy, and the sensitivity of this method of detection is lower than the measurement of radioactivity (Talonizte **et al**, 1984).

Although 36-chlorine has a long half-life (3×10^5 y) and it is a $\beta+$ emitter, these particles have very low penetration power, and only single readings can be taken for each plant since they have to be prepared before sampling to enhance the signal for detection. 36-Chlorate has been used as a tracer for influx experiments since it is an analogue of nitrate. However, it is unclear whether the carriers, or channels in the plasmalemma, which are specific for nitrate have the same affinity for chlorate (Deane-Drummond, 1985).

13N has been used previously for measuring the processes of nitrogen

fixation (Nicholas **et al**, 1961; Thomas **et al**, 1977; Schubert **et al**, 1981) and nitrogen assimilation (Shoukit **et al**, 1978). As a tracer 13N has several advantages for studies in short term influx experiments as described below;

1) Nitrate itself can be used, ensuring that any difference in specificity (Km) or velocity (Imax) of uptake is not likely to be due to the substrate.

2) 13N emits positrons (β+), which on annihilation, produce two 0.511 MéV gamma rays. These gamma rays can be detected outside the system under study due to their great penetration capacity. This means that the system need not be destroyed after each reading.

3) The half-life of 13N is 9.96 minutes, so that the time for which the accumulation of transported ion can be measured accurately is limited to about 40 minutes.

4) To measure carrier mediated influx of nitrate across the plasmalemma it is necessary to limit the uptake period to less than one half-life for cytoplasmic exchange. For nitrate this is about 17 minutes (Deane-Drummond and Glass, 1982). This excludes the use of 15-N as a tracer and is ideal for the short lived but high specific activity of 13-N.

On the basis of these arguments 13NO3- was used in the experiments described below. One of the problems associated with these experiments is that because of the very short half-life combined with the expense of producing high levels of 13NO3-, the numbers of plants involved were necessarily small.

1.2 RESULTS

For details of the experimental conditions see Chapter 2, Section 13.

From a total of 270 M4 segregrating R9401 seeds, 215 germinated. After four days growth in the presence of 1 M nitrate 21 withering plants were identified as NR minus. The nutrient solution was then changed to contain 1 M NH4+ as the sole nitrogen source, since prolonged exposure of NR minus plants to nitrate is toxic. When 13 days old, both NR+ and NR minus plants were exposed to 1 M nitrate (12 hours prior to experiment) in order to fully induce NR activity and any nitrate transport mechanisms. After rinsing off the induction buffer, 10 NR minus and 10 NR+ plants were exposed to either 1, 4 or 20 or 100 mM nitrate containing high specific radioactivity of 13NO3-. A further 11 NR minus plants and 9 NR+ plants were subjected to the same treatment in a separate experimental run. The levels of radioactivity uptake and **in vivo** NR activities were measured.

It was necessary to calculate the rate of decay for 13N in order to allow for the amount lost during the experiment. This allows an estimation of the initial uptake rate of nitrate to be made. The 13N decay was found to be 10.3 ± 0.2 minutes (Drew pers. comm.) which was very close to the expected value of 9.96 minutes (McNaughton and Presland, 1983).

18-Fluorine, 13NO2- and 13NH4+ are all contaminants of the 13NO3- produced from pure water. 13NO2- and 13NH4+ were removed by boiling the solution under alkali and acid conditions (McNaughton and Presland,

Table 17

Rate of 13NO3- uptake in roots of R9401 NR+ and NR minus plants in the presence of increasing external nitrate concentrations

[NO3-] (mM)	Uptake Rate (µmol/gfw root/min) NR+	NR minus
1	0.161 ± 0.022 (5)	0.117 ± 0.033 (5)
4	0.501 ± 0.062 (4)	0.264 ± 0.025 (6)
20	3.172 ± 0.634 (5)	0.952 ± 0.215 (5)
100	6.768 ± 1.097 (5)	1.97 ± 0.09 (5)

For details of experimental conditions see Chapter 2, Section 13.

The number of individual plants assayed from two independent experiments is given in brackets after the mean and standard error.

1983). The remaining 18F is, like 13N, a positron emitter, but it has a much longer half-life (110 minutes). The samples were, therefore, recounted one hour later, thus contamination by 18F could be allowed for.

In this experiment the 18F contamination was 3-5% of the initial counts (Drew pers. comm.).

Since both experimental runs were in agreement, the data were pooled. The NR minus plants took up less nitrate in all treatments (table 17). The results were quite variable, this could well be due to the small sample size, leading to less uniformity of the plant size and vigour than would be found with a larger sample size. The NR minus plants were generally less vigorous than their NR+ siblings due to the pretreatment with nitrate. This might have accentuated the differences in nitrate uptake.

These results from the different treatments were then combined to obtain the kinetic parameters, Km and Imax for the NR+ and NR minus plants. Km is a measure of the affinity of the nitrate uptake system for nitrate and Imax a measure of the maximal initial influx rate. The smaller the Km, the greater the affinity for nitrate of the uptake system.

The Imax was estimated from a) the intercept on the 1/v axis of a Lineweaver-Burk plot (figure 41), b) the intercept on the vertical axis (Imax) of a Hofstee plot (figure 42) and c) the slope (1/Imax) of an

Figure 41

Lineweaver-Burk plot of 13NO3- uptake in R9401 NR+ and NR minus seedlings

The mean rate (and standard error) of 13NO3- uptake (I) in seedlings subjected to external nitrate concentration of 1, 4, 20 and 100 mM were determined as described in Chapter 2, Section 13.

The symbols (■) and (●) signify the NR+ and NR minus phenotypes.

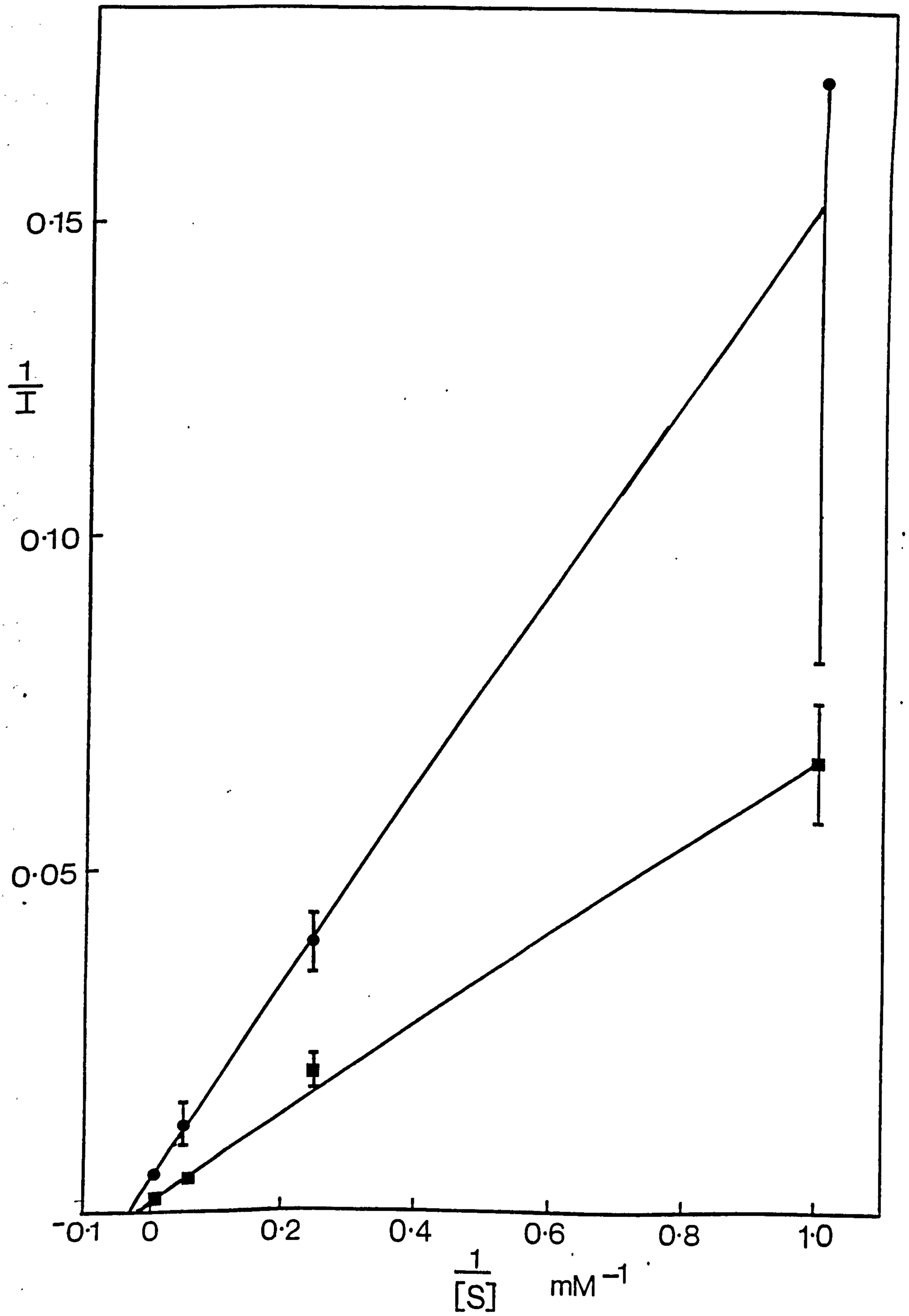

0·15
0·10
0·05
1/I
-0·1
0
0·2
0·4
0·6
0·8
1·0
1/[S] mM⁻¹

Figure 42

Hofstee plot of 13NO3- uptake in R9401 NR+ and NR minus seedlings

The mean rate (and standard error) of 13NO3- uptake (I) in seedlings subjected to external nitrate concentrations of 1, 4, 20, 100 mM were determined as given in Chapter 2, Section 13.

The symbols (■) and (●) signify the NR+ and NR minus phenotypes.

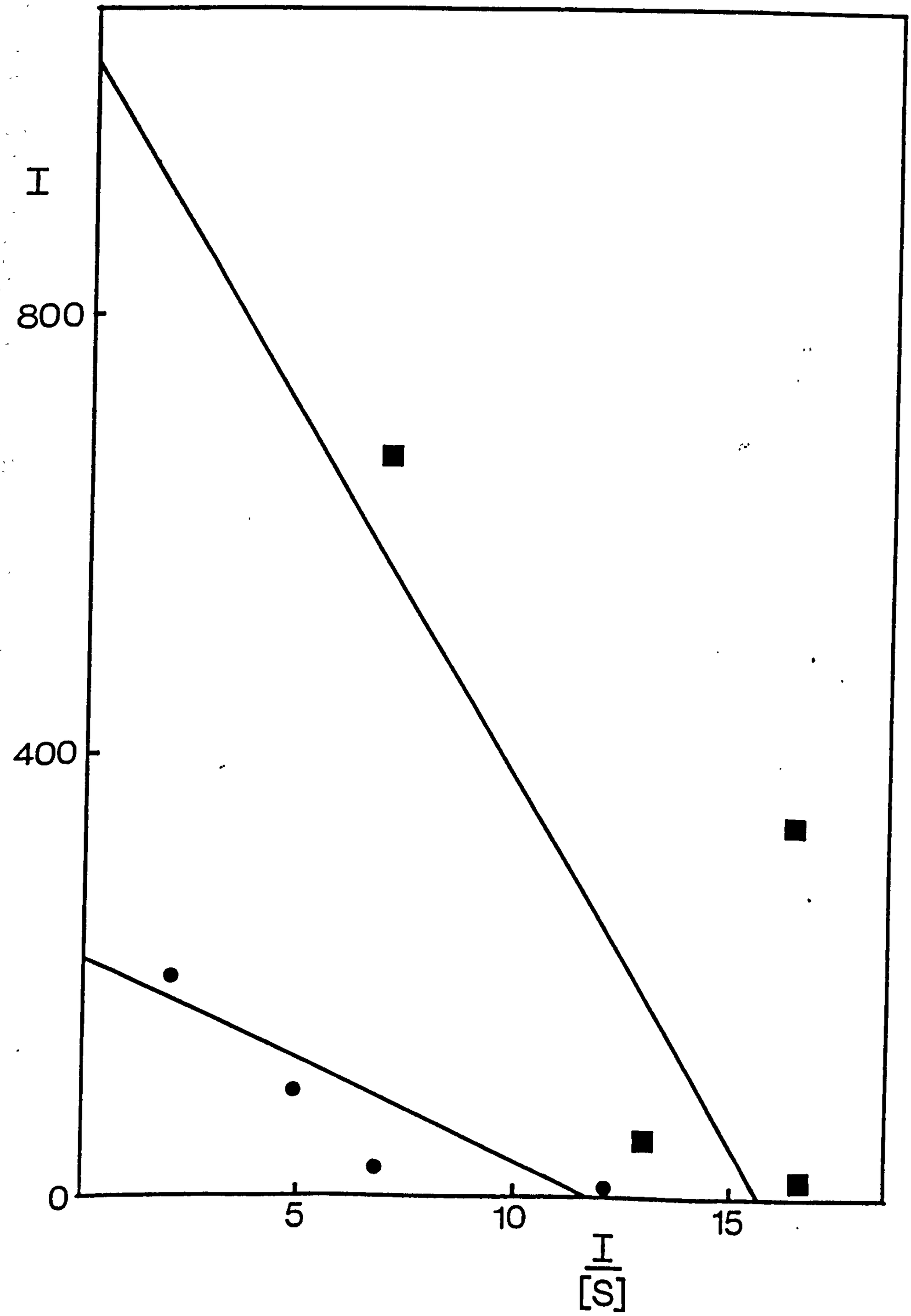
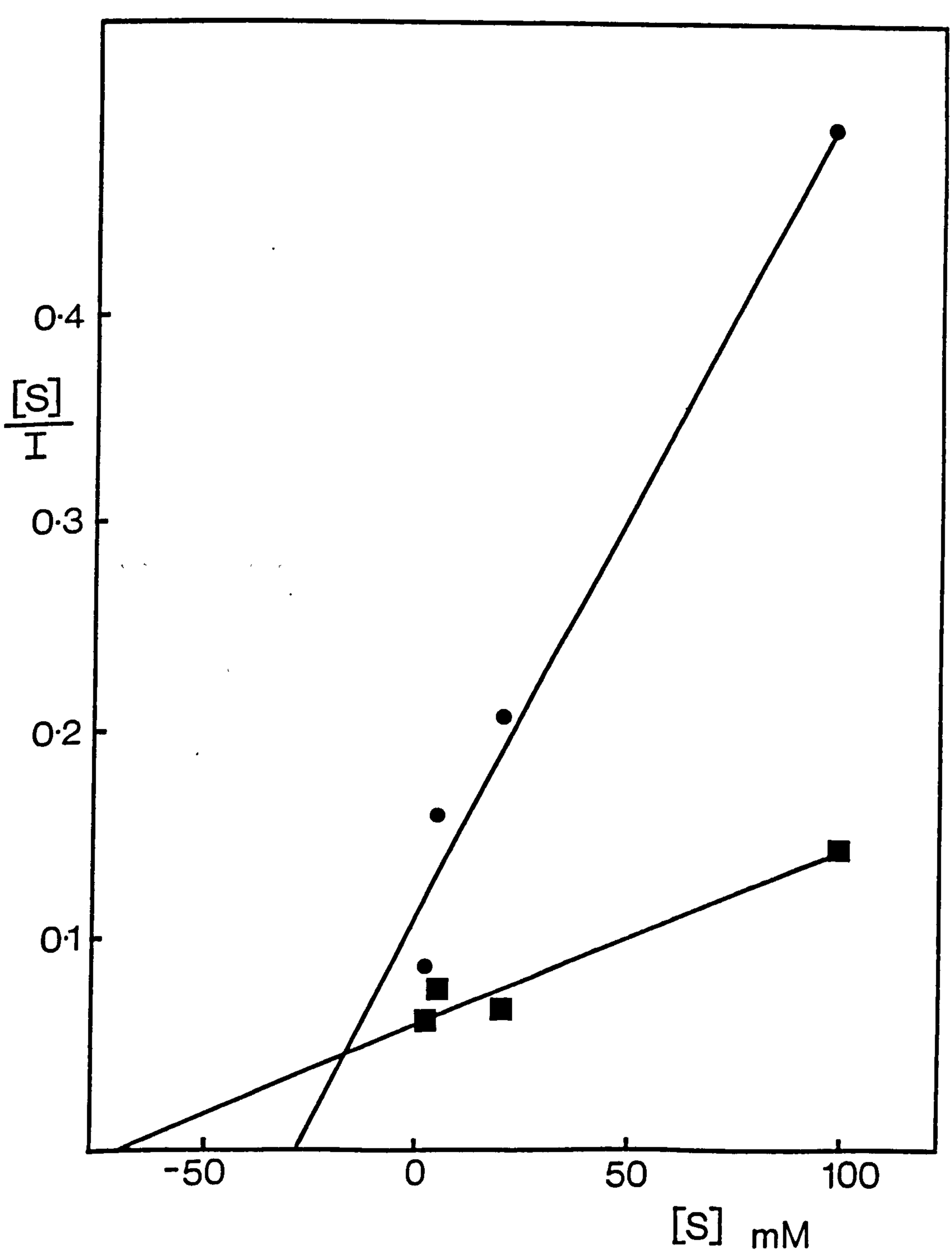

I
800
400
0
5
10
15
I
[S]

Figure 43

Eadie plot of 13NO3- uptake in R9401 NR+ and NR minus seedlings

The mean rate (and standard error) of 13NO3- uptake (I) in seedlings subjected to external nitrate concentrations of 1, 4, 20, 100 mM were determined as described in Chapter 2, Section 13.

The symbols (■) and (●) signify the NR+ and NR minus phenotypes.


0·4
0·3
0·2
0·1
[S]
I
-50
0
50
100
[S] mM

Eadie plot (figure 43). The Km was estimated in a similar fashion from a) the intercept on the horizontal axis (-1/Km) of a Lineweaver-Burk plot (figure 41), b) the slope (-Km) of a Hofstee plot (figure 42) and c) the intercept on the vertical axis (Km/Imax) of an Eadie plot (figure 43). These estimates for Km and Imax are given in table 18. The three methods of estimation gave broadly similar values for these kinetic parameters and were of the same order as those calculated by Dr. M. Drew using a log least squares computer programme to evaluate the results (table 18). There was no significant difference between any of the Km or Imax values obtained by the various methods of estimation.

The Km estimates obtained for both NR minus and NR+ plants were substantially higher than that found for barley cv. Midas (Drew pers. comm.) which was determined in a parallel set of experiments carried out on the same day. The Km values are generally greater than those found in the literature for barley (Deane-Drummond and Glass, 1982), **A. thaliana** (Doddema and Telkamp, 1979), **P. sativum** (Deane-Drummond, 1985) and **Chara corallina** (Deane-Drummond, 1984). Interestingly the Km of NR+ plants was greater than that of their NR minus siblings.

The average Imax value of the NR+ plants fits in with the previously published values for higher plants ranging from 2-30 umol/h/gfw (Glass **et al**, 1985; Deane-Drummond and Glass, 1983a; Deane-Drummond and Glass, 1982; Lee, 1982; Doddema and Telkamp, 1979; Deane-Drummond, 1985) and is very similar to the Imax (NO_3^-) value given by Lee (1982) for barley cv. Midas.

Table 18

Estimated Km and Imax values from graphs (figure 41-43) for 13NO3- influx experiments on NR+ and NR minus plants from an M4 segregating population of R9401

Type of plot		Km (µM)	Imax (µmol/gfw/10 min)
Lineweaver-Burk (figure 41)	NR+	71	1000
	NR minus	40	263
Eadie (figure 42)	NR+	72	1098
	NR minus	31	259
Hofstee (figure 43)	NR+	68	1192
	NR minus	19	214
Average	NR+	70 ± 1	1097 ± 55
	NR minus	30 ± 6	246 ± 16
log. least squares*	NR+	74	998
	NR minus	33	214

* Kindly supplied by Dr. M. Drew

However, the estimated average Imax for R9401 NR minus plants was significantly different from that of their NR+ siblings.

1.3 CONCLUSIONS

The estimates of Km suggest that the uptake system in the R9401 NR minus plant has different characteristics from the NR+ plants. This was supported by the Imax estimate which also gave a greater value for the NR+ plants. The Imax value estimated for the NR+ was approximately the same as Dr. M. Drew obtained, on the same day, using cv. Midas (Drew pers. comm.), and in the literature. Since Epstein and Hagen (1952) suggest that a two fold difference in the Km is the minimum experimental difference required to indicate a genuine difference, these conclusions must be tentative. Further work to show whether poor growth affected nitrate uptake should be done. This could be achieved if pretreatment of both NR minus and their NR+ siblings with glutamine or nitrate was carried out, followed by the same experiment described above, since the NR minus plants grow almost as well on glutamine as the wild type plants (Bright **et al**, 1983).

Another way of obtaining more uniform material would be to use cultured roots. Homozygous seedlings would also increase the vigour, as the exposure to nitrate would not be required for identification of NR minus plants.

1.4 DISCUSSION

Deane-Drummond and Glass (1982) did similar experiments using $^{36}ClO_3^-$ as a tracer and found that Km values were highly variable between

different experiments.

It would appear that there might be a difference in the Imax and Km of the NR minus plants when compared to their NR+ siblings. The differences in Imax could be due to the poorer growth of the NR minus plants. There would be less demand for high levels of nitrate influx for a slower growing plant. The difference in Km between the NR minus and their NR+ siblings could also be attributed to this. Alternatively; there might be a genuinely lower Km in the NR minus plants, because these plants would have a much lower internal level of ammonium ions and reduced N arising from the reduction of nitrate.

Additionally, the higher Km found (for the uptake of nitrate) in NR+ plants might be caused by feedback regulation using ammonium ions or a further metabolite of nitrate. Despite these changes in uptake parameters, the R9401 NR minus plants contained approximately the same amount of nitrate (on a fresh weight basis) as their NR+ siblings (Bright **et al**, 1983), suggesting that the overall influx of nitrate into NR minus plants is unimpaired.

2. TISSUE DISTRIBUTION OF NR ACTIVITY

2.1 INTRODUCTION

Whilst doing the experiment described above, the **in vivo** levels of NR were determined to identify the NR minus plants in all four treatments. When the distribution of total **in vivo** NR in roots and shoots were

Table 19

In vivo NR activity of shoots and roots of segregating R9401 plants involved in the 13NO3- uptake experiments

[NO3-] (mM)		In vivo NR activity (µmol/h/gfw) shoots	roots	total	% in roots
1.0	NR+	1.21 (3)	0.37 (3)	1.58	23
	NR minus	0.03 (4)	0.49 (4)	0.53	94
4.0	NR+	0.94 (2)	0.29 (2)	1.23	24
	NR minus	0.10 (6)	0.34 (6)	0.44	77
20	NR+	1.51 (3)	0.14 (3)	1.65	8
	NR minus	0.05 (4)	0.21 (4)	0.26	81
100	NR+	2.13 (2)	0.10 (2)	2.23	4
	NR minus	0.05 (5)	0.32 (5)	0.37	86

For details of experimental conditions see Chapter 2, Section 13.

The number of individual plants assayed from two independent experiments is given in brackets after the mean

studied, it seemed that a far greater proportion of the total NR activity resided in roots of the NR minus plants when compared to their NR+ siblings (table 19).

There could be several reasons for this relatively high proportion of NR residing in the roots of NR minus plants, including;

1) the expression of a different NADH-NR activity from that displayed by the shoots of NR+ plants,

2) the presence of a bispecific NAD(P)H-NR,

3) more nitrite contamination around the roots of the NR minus plants caused by the associated microflora and

4) greater storage of nitrite in the roots of NR minus plants.

This was investigated in 7 day old plants grown in vermiculite. **In vitro** NADH-NR and NADPH-NR activities were determined in NR+ and NR minus roots and leaves (Chapter 2, Section 4). **In vitro** activities were assessed in preference to the **in vivo** NR activity since it was known that neither NADH (NADPH) nor KNO3 were limiting during the short incubation period.

For the experiments discussed in the following sections, the segregating M4 populations of R9201, R11301 and R9401 were used. The M3 population of R12801 was also studied. The results were obtained from at least three independent extractions, assayed in duplicate, from at least two different experiments.

2.2 RESULTS AND CONCLUSIONS

2.2.1 Nitrite on the surface and within roots

The presence of nitrite either on the root surfaces or within cells is not corrected for when measuring **in vivo** NR activity. Using the **in vitro** NR assay it is possible to include blanks lacking NADH to allow for this. It was found that these controls gave similar absorbances whether the plant had the NR+ or NR minus phenotype, indicating that nitrite contamination was not a significant contribution under these circumstances.

2.2.2 Tissue distribution of NADH-NR activity in the wild types and plants with the NR+ phenotype

The roots of cv. Maris Mink possessed, on average, 3% of the total measured NADH-NR activity (table 20). The distribution was very similar in cv. Golden Promise where 5% of the total NADH-NR activity was found in the roots (table 20). When these levels of NADH-NR activity were converted from µmol/h/gfw to µmol/h/mg protein the distribution of the activity between the roots and shoots was similar to that found by Miflin (1967), since the roots had a lower protein content.

The roots of NR+ plants from the segregating M4 populations of R9401 and R11301 possessed 8 and 20% of the total NADH-NR activity respectively (table 20). The shoots of the phenotypically NR+ R9201 plants possessed all the NADH-NR activity (table 20).

Table 20

The distribution of in vitro NADH-NR between shoots and roots of the mutants and wild type plants

	In vitro NADH-NR activity (µmol/h/gfw)			
	shoots	roots	total	% in roots
Maris Mink	2.97 (15)	0.09 (8)	3.06	3
Golden Promise	1.18 (19)	0.06 (7)	1.18	5
R9401 NR+	0.78 (10)	0.07 (4)	0.85	8
R9201 NR+	1.26 (8)	0.00 (5)	1.26	0
R11301 NR+	1.08 (8)	0.27 (4)	1.35	20
R9401 NR minus	0.03 (19)	0.03 (12)	0.06	48
R9201 NR minus	0.00 (11)	0.00 (3)	0	-
R11301 NR minus	0.02 (8)	0.05 (4)	0.07	69
R12801 NR minus	0.02 (12)	0.03 (4)	0.05	60

7 day old seedlings were grown as described in Chapter 2, Section 3. They were assayed for **in vitro** NADH-NR activity after a 48 hour incubation period of supplementing with 15 mM KNO3.

The number of individual plants assayed from at least two independent experiments is given in brackets after the mean

2.2.3 Tissue distribution of NADH-NR activity in R9401, R9201, R11301 and R12801 NR minus plants

The shoots and roots of R9201 NR minus plants possessed no detectable **in vitro** NR activity which requires NADH as the source of reductant (table 20). Alternatively, R11301, R9401 and R12801 NR minus plants exhibited 50-100 fold less NADH-NR activity in their shoots than their NR+ siblings and relevant wild type plants (table 20).

The absorbance measurements obtained for root NR activity are low and close to the limits of detection.

2.2.4 The distribution of NADH-NR activity

The amount of nitrate normally reduced in the roots of higher plants is still a matter of debate. The uptake and reduction of nitrate varies with nitrogen nutrition, species and even between varieties (Reed and Hageman, 1980; Epstein and Jefferies, 1964; Epstein, 1972; Clarkson and Hanson, 1980; Perby and Jensen, 1983). According to Miflin (1967) the specific activity of nitrate reductase in barley is slightly higher in roots than leaves. However, if the activity is calculated in terms of fresh weight, the shoots (leaves) have much greater NR activity (Beevers and Hageman, 1969). In cotton the bulk of nitrate taken up by the root is transported to the leaves, the amount reduced in the root is minimal (Radin, 1977).

The leaves of the wild type barley plants possessed more than 90% of the total **in vitro** NADH-NR activity. This fits in well with the data of Campbell and Remmler (1986), who used ELISA tests to show that 5% of the **Z. mays** NR protein was present in the roots compared to the leaves.

Table 21

The distribution of NADPH-NR between extracts of shoots and roots from the mutants and wild type plants

	NADPH-NR activity (nmol/h/gfw)			
	shoots	roots	total	% in roots
Maris Mink	10.0 (15)	4.1 (8)	14.1	29
Golden Promise	15.2 (18)	1.7 (7)	16.9	10
R9201 NR+	5.1 (10)	16.5 (4)	21.6	76
R11301 NR+	13.6 (8)	23.9 (4)	37.5	64
R9401 NR+	5.1 (10)	16.5 (4)	21.6	76
R9201 NR minus	0.0 (11)	0.0 (3)	0	-
R11301 NR minus	1.9 (8)	4.2 (4)	6.1	69
R9401 NR minus	2.4 (19)	3.4 (12)	5.8	59
R12801 NR minus	0.1 (11)	1.4 (4)	1.5	93

7 day old seedlings were grown as described in Chapter 2, Section 2. They were assayed for **in vitro** NADPH-NR activity after a 48 hour incubation period of supplementing with 15 mM KNO3.

The number of individual plants assayed from at least two independent experiments is given in brackets after the mean

From this investigation it is clear that the vast majority of the NADH-NR activity (90-100%) in NR+ plants, as in the wild types, resides in the leaves.

The reduced level of NR activity in these NR minus plants has been brought about by at least a 90 fold decrease in activity in the leaves of NR minus plants compared to their NR+ siblings.

If this minimal residual activity is genuine, and since R9401, R9201 and R11301 are MoCo mutants, there are several possible explanations for the presence of this activity; a) the formation of active MoCo limits the formation of active NR in the leaves, b) more MoCo is produced in the roots in these mutants then in the shoots, allowing a greater proportion of total NR activity to be found in the roots, c) the shoot MoCo was originally present but, being defective, it is turned over faster compared to the root MoCo or d) the possible expression of either a second root-specific NADH-NR or a bispecific NAD(P)H-NR gene product requires less MoCo.

2.3 NADPH-NR ACTIVITY AND TISSUE DISTRIBUTION IN THE WILD TYPES AND NR+ PLANTS

In order to check whether a bispecific NAD(P)H-NR gave rise to these minimal levels of NR activity, the activity and distribution of in vitro NADPH-NR was determined for the mutants and wild types.

The controls cv. Maris Mink and Golden Promise possessed small but measurable amounts (1-3% of the NADH-NR activity) of NADPH-NR activity (table 20 and 21). Approximately the same levels of NADPH-NR activity were found in the R9201, R11301 and R9401 NR+ plants (table 21). The roots of the wild type plants exhibited 29 and 10% of the total NADPH-NR activity detected (table 21). The NR+ phenotype plants of R9201, R11301 and R9401 displayed 60-80% of their NADPH-NR activity in the roots (table 21).

2.3.1 NADPH-NR activity and tissue distribution in the R9401, R9201, R11301 and R12801 NR minus plants

Only 3 to 10% of the NADH-NR activity found in the NR minus plants could be detected using NADPH as the source of reductant (tables 20 and 21). Between 55 and 95% of this activity was located in the roots (table 21).

2.3.1 The distribution of NADPH-NR activity

It would appear that there were minimal levels of NADPH-NR activity present in the roots and shoots of all plants tested here. No method of inhibiting phosphatase activity was used, therefore it was difficult to decide whether these low levels of apparent NADPH-NR activity are due to the proposed bispecific NAD(P)H-NR as in **narla** or the normal NADH-NR activity. However, they do give an upper limit for the level of NADPH-NR activity in the mutants. This value falls to 1-3% for the NR+ and wild type plants.

Dailey **et al** (1982b) using a similarly prepared extract from the shoots of their barley wild type, Steptoe, found 1% of the total NR activity (ie the addition of NR activities when using NADH and NADPH as independent reductants) when supplying NADPH as reductant. However, the **narla** (structural gene defect) mutant line (possessing about 25% of the wild type activity) when supplied with NADPH, gave 82% of the total NR activity (Dailey **et al**, 1982b). Using **nar2**, their MoCo mutant line, which still possesses some NR activity (figure 20), under the same conditions gave 14% of the total NR activity when NADPH was the reductant supplied. The NADPH-NR activity for **nar2a** was 100 µmol/g/fgw (Dailey **et al**, 1982b). This is 10-fold greater than the levels found in R9201, R11301 and R9401. Essentially the NADH-NR activities are lost in both roots and shoots of NR minus plants. The residual levels of apparent activity are very low and close to the limits of detection.

This minimal NR activity was found in the roots and shoots of the NR minus plants tested (when NADH was used as the reductant), suggesting that there is basically no NADH-NR or NAD(P)H-NR as both of these enzymes can use NADH. It was therefore unneccessary to include systems to prevent phosphatases from converting NADPH to NADH. On this basis it seems sensible to suggest that none of the mutants studied here (R9201, R11301, R9401 and R12801), nor Maris Mink or Golden Promise possess anything more than a minimal level of NADPH-NR activity when compared to Steptoe and its mutants (Dailey **et al**, 1982b).

From this study of the NADH and NADHPH-NR activities it is clear that the MoCo required for all possible NR activities is inactive in both roots and shoots. It also indicates that the high levels of **in vivo** NR activities described in the previous section must be caused by artifacts. Two obvious sources for this would be root surface contamination or high levels of nitrite found within the tissue.

R12801, the most likely apoprotein mutant candidate, does not have this high residual level of NADH-NR or high level of NADPH-NR suggesting that R12801 is not identical to the **narla** mutant

CHAPTER 8

GENERAL DISCUSSION

Four chlorate resistant barley selections (R9201, R11301, R12202 and R12801) were identified as NR minus mutants. None of these selections were nitrate or molybdate uptake mutants. Three lines (R9201, R11301 and R12202) were classified as cofactor (MoCo) mutants, similar to the previously characterised R9401 mutant (Bright **et al**, 1983), since as well as being NR minus they lacked xanthine dehydrogenase activity. R12801, although lacking NR activity, exhibited xanthine dehydrogenase activity suggesting that a possible NR apoprotein gene mutation was present.

None of the MoCo mutants isolated at Rothamsted, including R9401, could grow with nitrate supplied as the nitrogen source. The profiles of **in vitro** nitrate reductase, nitrite reductase and cytochrome c reductase activities for the newly characterised MoCo mutants (R9201, R11301 and R12202) were similar to those described for the barley MoCo mutants, R9401 (Bright **et al**, 1983) and Az 34 (designated **nar2a**) (Kleinhofs **et al**, 1980) and also the tobacco MoCo mutants, **cnxA**, **cnxB** and **cnxC** (Mendel and Müller, 1985; Mendel **et al**, 1984; Mendel **et al**, 1986) (table 22). It is more difficult to suggest that R12801 (which is not a MoCo mutant) is caused by an apoprotein gene mutation, because the levels of nitrite reductase and cytochrome c reductase activities of the nine allelic barley apoprotein **(nar1)** gene mutant plants varied considerably from plant to plant (Kleinhofs **et al**, 1980). This was also true for the 36 allelic NR apoprotein mutant callus cell lines in **N. tabacum** described by Mendel and Müller (1979).

Genetic analysis has shown that:-

1. The NR minus trait in R9201, R11301 and R9401 is inherited in a Mendelian fashion as single recessive nuclear mutations.
2. R9201 is allelic to R9401.
3. R9201 and R11301 are non-allelic.

The implications from more detailed studies of the relationship between R9401 and Az 34 (**nar2a**) suggest that R9401 is allelic with the **nar2a** mutant (Kleinhofs pers. comm.). Since the mutations in R9401 and R9201 are allelic and R9401 and **nar2a** are allelic, it follows that all three are allelic with each other, therefore R9401 and R9201 have been designated **nar2b** and **nar2c** respectively.

1. SPECTRUM OF MUTANTS ISOLATED

Using nitrate non-utilisation as a selection method three classes of NR mutants, apoprotein, MoCo, and regulatory, were isolated in **A. nidulans** (Cove, 1979) and **N. crassa** (Dunn-Coleman **et al**, 1984). The ability to grow and remain healthy in the presence of chlorate has provided only two of these classes of mutants (apoprotein and MoCo) which have been clearly identified in higher plants (table 23).

The use of chlorate to positively select for plants or cell lines lacking in NR activity is more stringent than either nitrate non-utilisation (Cove, 1979) or reduced **in vivo** NR activity as used by Kleinhofs et al (1980) to select barley mutant plants (table 23). As a consequence of this greater stringency, there is little possibility of using chlorate to select quantitative mutants (ie. those with low

Table 22

Enzyme activities associated with higher plant NR mutants

The following symbols +,- and nd indicate the following:

\+ = presence of activity

\- = absence of activity

nd = not determined

* = not designated

References

1. Bright **et al** (1983)
2. Present work
3. Kleinhofs **et al** (1980)
4. Mendel and Müller (1985)
5. Mendel **et al** (1984)
6. Mendel **et al** (1986)
7. Müller and Grafe (1978)
8. Somers **et al** (1983a)
9. Kleinhofs **et al** (1985)

Table 22 Enzyme activities associated with higher plant NR mutants

	mutant	gene	XDH	[NO3-]	NADH-NR	FMNH2-NR	NiR	NADH-CR	Ref
MoCo Mutants									
H. vulgare	R9401	nar2b	-	+	-	-	+	+	1
	R9201	nar2c	-	+	-	-	+	+	2
	R11301	*	-	+	-	-	+	+	2
	R12202	*	-	+	-	-	+	+	2
	Az 34	nar2a	-	nd	8%	-	+	+	3
	Xno18/19	nar3	-	nd	3%	-	nd	varies	8
	Az 72	nar4	-	not fully characterised					
N. tabacum		cnxA	-	nd	-	nd	+	+	4
		cnxB	-	+	-	nd	+	+	5
N. plumbaginifolia		cnxA	-	nd	-	nd	nd	+	6
		cnxB	-	nd	-	nd	nd	+	6
		cnxC	-	nd	-	nd	nd	+	6
Apoprotein Mutants									
H. vulgare	R12801	*	+	+	-	-	+	+	2
		narla-li	+	nd	-	-	varies	varies	3
H. tabacum		nia	+	nd	-	nd	varies	varies	7

residual NR levels). There are also indications that there are mutants which are difficult to maintain after isolation, suggesting that the rescue and growth conditions used are not optimal for these mutants. In my case nine barley chlorate resistant plants were isolated which failed to reach maturity on transfer from the selection system (Chapter 3). Kleinhofs **et al** (1978) also describe the isolation of chlorate resistant barley plants which they were unable to propogate. It is possible that some of these selections were representatives of the 'missing' regulatory mutants. These regulatory mutants are likely to lack NR activity (in order to survive the chlorate screen) and possibly, other nitrate assimilation enzyme activities which are commonly regulated with nitrate reductase. This might well explain the inability of the selections to grow with either ammonium or glutamine as the nitrogen source (Chapter 3). It is clear that the recovery and growth conditions are not completely successful and prevent, at present, the characterisation of these potentially interesting selections.

2. DIFFERENCES IN THE RATIO OF APOPROTEIN: MoCo MUTANTS SELECTED IN BARLEY

The number of apoprotein mutant plants isolated in barley by Kleinhofs **et al** (1985) was larger than that reported in this thesis. On the other hand, far more MoCo mutant plants were characterised during the present study than by Kleinhofs **et al** (1985). Factors which may influence the proportions of apoprotein and MoCo NR mutant plants are; 1) plant species, including the level of ploidy, 2) different mutagenic treatment, 3) selection method and stringency of selection and 4) the

Table 23

Selection of higher plant NR mutants

* = unconfirmed

References

1. Bright et al (1983)
2. Present work
3. Kleinhofs et al (1985)
4. Tokarev and Shumny (1973)
5. Müller and Grafe (1978)
6. Buchanan and Wray (1982)
7. Evola (1983a)
8. Evola (1983b)
9. Marton et al (1982a)
10. Marton et al (1982b)
11. Negrutiu et al (1983)
12. Dirks et al (1985)
13. Braaksma and Feenstra (1982)
14. Fankhauser (pers. comm. in Kleinhofs et al, 1985)
15. Strauss et al (1981)
16. Lazar et al (1983)
17. Feenstra and Jacobsen (1980)
18. Kleinhofs et al (1978)
19. Warner et al (1982)
20. Steffen and Schieder (1984)

Table 23 Selection of higher plant NR mutants

	Species	Screen	MoCo : Apoprotein	Ref
H. vulgare	cv. Maris Mink	chlorate	4 : 0	1,2
	cv. Golden Promise	chlorate	0 : 1*	2
	cv. Steptoe	lowered in vivo NR	2 : 19	3
	cv. Winer	chlorate	2 : 1	3,4
N. tabacum	cv. Gatersleben	chlorate	4 : 36	5
	cv. Xanthi	chlorate	4 : 0	6
	cv. Xanthi	chlorate	1 : 1	7,8
N. plumbaginifolia		chlorate	4 : 7	9,10
		chlorate	6 : 26	11,12
A. thaliana		chlorate	2 : 8	13
H. muticus		chlorate	5 : 0	14
		amino acid auxotrophy and nitrate non-utilisation	4 : 0	15,16
Pisum sativum	cv. Rondo	chlorate	1 : 0	17
	cv. Juneau	lowered in vivo NR	1 : 2	18,19
Petunia hybrida		chlorate	3 : 1	20

presence of multiple genes or isoenzyme genes and the possible differential expression of these in different tissues.

2.1 Plant species, including the level of ploidy

The use of different species and cultivars can contribute to the difference in apoprotein:MoCo gene mutation ratio. As can be seen in table 23, **N. tabacum** was used to select 36 apoprotein and 4 MoCo mutant cell lines (Müller and Grafe, 1978; Müller, 1983), whilst Marton **et al** (1982a and 1982b) selected 7 apoprotein and 4 MoCo mutant cell lines from **N. plumbaginifolia.** Cultivars can also affect the spectrum of mutants obtained. As described above, Müller and Grafe (1978) used **N. tabacum** cv. Gatersleben to isolate a 36:4 (apoprotein:MoCo) ratio, whilst Evola (1983a) isolated 1 apoprotein and 1 MoCo mutant cell line from **N. tabacum** cv. Xanthi. In both cases the mutagenic agent was N-ethyl-N-nitrosourea and both groups used chlorate as the selection system.

The ploidy level of the species mutagenised can affect both the total number of selections (table 24) and the ratio of apoprotoein:MoCo gene mutations (table 23). This can be seen when comparing the number of NR minus lines occurring spontaneously in **N. plumbaginifolia** and **N. tabacum.** Marton **et al** (1982a) suggest, this major discrepancy can be attributed to the fact that **N. tabacum** is allodihaploid (and therefore two independent mutations at analogous loci are required to obtain an NR minus line). Whilst **N. plumbaginifolia** is a haploid species (and therefore requires a single gene mutation to cause the NR minus phenotype).

Table 24

The effect of increasing the mutagenic treatment on the number of chlorate resistant lines isolated

1. Marton **et al**, 1982a
2. Müller and Grafe, 1978
3. Murphy and Imbrie, 1981

Table 24 The effect of increasing the mutagenic treatment on the number of chlorate resistant lines isolated

Species	Mutagenic treatment	Frequency of chlorate resistant selections	Ref
N. plumbaginifolia	None	6×10^{-5}	1
	60Co 15 Jkg^{-1}	33	
	60Co 20 Jkg^{-1}	150	
	None	6×10^{-5}	1
	ENU* 0.15 mM	50	
	ENU* 0.30 mM	110	
N. tabacum cv. Gatersleben	None	2×10^{-9}	2
	ENU* 0.25 mM	50	
R. damascena	None	9 x 10-4	3
	Ultraviolet 14 Jm^{-2}	16	
	(254 nm) 42 Jm^{-2}	28	

*ENU is N-ethyl-N-nitrosourea

2.2 Mutagenic treatment

The usual level of spontaneous mutations which can be selected with chlorate is in the region of 1 in 100,000 to 1 in 10,000 (Marton **et al,** 1982a; Murphy and Imbrie, 1981). Mutagenic agents can increase the number of selections obtained above this level. The frequency of chlorate resistant cell lines can be enhanced 3-25 fold (table 24) depending on the mechanism of mutagenesis of the mutagen used and the level of exposure that the plant material is subjected to.

The mutants which were studied in the present work were produced by treatment of the M1 with either azide or gamma radiation (table 6).

Azide is thought to cause point mutations by base substitution (Kleinhofs and Smith, 1976) via the metabolic intermediate azidoalanine (Owais **et al,** 1983). Gamma radiation is thought to cause deletions and chromosome rearrangements (Auerbach, 1976). Since the mechanisms for azide and gamma ray mutagenesis are different, it seemed likely that different gene mutations might arise allowing the identification of new classes of NR mutant plants. However, no NR mutants were obtained from a total of 4.8×10^4 M2 seedlings (from gamma irradiated M1) screened with chlorate. Similar numbers of chlorophyll mutants were identified in the M2 seedlings from both gamma irradiated and azide treated M1 seeds, indicating that the amount of gamma radiation used was as effective a 1 mM sodium azide treatment. This suggests that the gamma ray dose given to the M2 seeds was large enough to cause parallel numbers of single gene mutations (as assessed by ratios of chlorophyll mutants), but not large enough to cause death or sterilisation of the

M1 plants which would have reduced the number of mutations available for study in the M2. Azide, having less direct effect on the survival of the M1 plants, can be used at higher doses to create mutations.

Another reason for the lack of NR mutants from the gamma irradiated M2 seedlings could have been the use of the cultivar Golden Promise rather than Maris Mink (as used for the azide treatment). However, this is unlikely to be a major reason for the lack of mutants since azide treated Golden Promise provided R12202 (an NR minus mutant).

2.3 Selection method

It is possible that using chlorate to select plants or cell lines restricts the classes of mutants selected. The chlorate selected barley plants, isolated at Rothamsted, which had a defective nitrate assimilation system possessed no **in vitro** NR activity and were all MoCo mutants, none of these mutants could grow on nitrate. This suggests that the MoCo genes identified in these NR minus plants are essential for NR activity. All the barley apoprotein gene mutants so far characterised are leaky, possessing 0.5-2.4% of the wild type levels of **in vitro** NADH-NR activity (Kleinhofs **et al**, 1980). These barley apoprotein mutants were all allelic and were selected (with the exception of **Xno29**) on the basis of low **in vivo** NR activity. **Xno29** was isolated using 8 mM chlorate for a period of two days, as were **Xno18** and **Xno19** (both MoCo gene mutants) (Tokarev and Shumny, 1977). All these were leaky mutants exhibiting 11%, 3% and 2% respectively of the wild type level of **in vitro** NADH-NR activity (Somers **et al**, 1983a). A more stringent regime of 10 mM chlorate for a period of nine days was

utilised at Rothamsted to isolate the non-leaky MoCo mutants.

A possible conclusion that can be drawn from the leaky nature of the barley apoprotein gene mutants, is that no mutation caused by treatment with azide in this gene in barley is responsible for total loss of NR activity. If this were true, it would ensure that no apoprotein gene mutant would be selected by using a stringent chlorate screen.

The results from different laboratories using similar techniques for mutant plant isolation (eg. the work in this thesis and Tokarev and Shumny, 1977) also emphasizes the point that individual workers will select to different criteria.

2.4 Multiple genes and isoforms

A possible reason for the leaky nature of apoprotein mutant plants could be that a second NR apoprotein gene or multiple genes are present and expressed in the barley genome. A mutation in the NR apoprotein gene which codes for the major NR subunit species would not prevent the formation of other species of NR subunits, which would produce low levels of NR activity. Dailey **et al** (1982b) suggest that the residual 1.5% of the wild type **in vitro** NADH-NR activity exhibited by **narla** mutant plants could be attributed to a second (NAD(P)H) bispecific NR species. This would indicate the presence of a second NR apoprotein gene. From my results (Chapter 7) there is little evidence of an NADPH-NR enzyme in either the mutants characterised or in the wild type cultivars, Maris Mink and Golden Promise.

Since the MoCo gene mutants plants isolated at Rothamsted all died when supplied with nitrate as the nitrogen source, it is clear that these MoCo genes are essential for NR activity. This would argue against the presence of multiple forms of products of the MoCo genes and could indicate the presence of singly expressed MoCo genes in the barley genome.

The mutant MoCo gene isolated in R9401 affected NR and xanthine dehydrogenase activities in both shoots and roots, indicating that in the wild type plants this gene is expressed in both tissues. This lack of differential gene expression suggests that the R9401 MoCo gene product is also essential for NR activity in the roots and again implies that multiple gene products are not responsible for this step in the formation of MoCo.

At present one apoprotein structural gene **narl** has been clearly identified in barley (table 25). The same is true for **A. nidulans** (MacDonald and Cove, 1974), **N. crassa** (Dunn-Coleman **et al**, 1984) and **P. chrysogenum** (Birkett and Rowlands, 1981). The situation in **N. tabacum** is more complex, there are two NR apoprotein structural genes (Müller, 1983) (table 25). These two genes are thought to be identical (Müller, 1983), since **N. tabacum** is an allodihaploid species which arose from the hybridisation of two early species of Nicotiana (**N. sylvestris** (2n=24) and **N. tomentosiformis** (2n=24)) (Gray **et al**, 1974).

Table 25

NR co-factor MoCo References

1. Müller and Grafe, 1983
2. Mendel **et al**, 1984
3. Mendel **et al**, 1986
4. Kleinhofs **et al**, 1985
5. Braahome and Feenstra, 1982b
6. Warner **et al**, 1982
7. Mendel, 1983
8. Marton **et al**, 1982a

Table 25 NR co-factor (MoCo) and apoprotein genes identified in higher plants

Genes	N. tabacum	N. plumbaginifolia	H. vulgare	A. thaliana	P. sativum
MoCo	cnxA1 cnx A2(ref1)	cnxA (ref3)	nar2(ref4)	chl-2(ref5)	nar2(ref6)
	cnxB(ref2)	cnxB "	nar3 "	chl-3 "	
		cnxC "	nar4 "		
		cnxD "			
Apoprotein	nial nia2(ref7)	NA(ref8)	narl(ref4)		narl(ref6)

There have been at least three MoCo genes identified in barley (**nar2**, **nar3** and **nar4**) (table 25). It is not clear whether R11301 is allelic to **nar3**, **nar4** or whether it is a representative mutant from another MoCo genetic locus in barley. R12202 could be allelic with **nar2**, **nar3**, **nar4** or non-allelic to them all. It would therefore seem that there are a minimum of three and maximum of five MoCo genes identified in barley so far. As can be seen in table 25, three MoCo genes have been identified in **Nicotiana** species (Mendel and Müller, 1985; Mendel **et al**, 1984; Mendel **et al**, 1986), whilst only two have been identified in A. **thaliana** (Braaksma and Feenstra, 1982b). Five (A. **nidulans**) and four (**N. crassa**) genes concerned with the formation of MoCo have been identified in fungi (Cove, 1979; Dunn-Coleman, 1984). Since the MoCo component from diverse species can substitute for each other and form active NR species, this suggests that there are likely to be five genes involved in the synthesis of MoCo in all organisms, including barley.

3. THE CONDITIONAL LETHAL NATURE OF SOME MoCo MUTANTS

Glaser and DeMoss (1972) suggest that the use of chlorate to select for NR minus lines in E. **coli** causes a bias in the distribution of mutants selected. They propose, since MoCo was common to anaerobic nitrate respiration and aerobic formate dehydrogenase (in E. **coli**), that chlorate resistance (which is associated with non-leaky MoCo mutants) would preferentially select for these MoCo mutants. However, they then go on to suggest that if MoCo is common to important pathways, many mutations affecting MoCo might well be lethal. This suggestion of a lethal phenotype is supported by work reported on higher plant mutants.

Bright **et al** (1983) described the difficulties encountered in maintaining MoCo mutants, whilst both Evola (1983b) and Müller (1983) had problems with regeneration of fertile plants from MoCo defective tobacco callus cell lines.

It is also possible to grow MoCo NR minus mutant plants which are fertile and set seed when ammonium or glutamine is supplied as the nitrogen source. This implies that the MoCo gene products, whilst essential for growth on nitrate, are not essential for plant metabolism and growth under ammonium or glutamine conditions. Xanthine dehydrogenase might well have a more important role in the legumes which transport nitrogen in the form of ureides (eg soybean) since it is required for purine oxidation (figure 44). In soybean plants xanthine dehydrogenase is found in far greater concentration in the nodules than in any other plant organ, whilst amide transporting legumes (eg, pea, alfalfa and white lupin) exhibit low or no xanthine dehydrogenase activity (Triplett, 1985). Nitrogen assimilation in legumes can effectively bypass nitrate reductase by using the nitrogen fixing capacity of the symbiotic bacteria (eg Rhizobium species) found in the nodules. In ureide transporting legumes the further metabolism of this nitrogen would require xanthine dehydrogenase (figure 44). This requirement for either NR activity or xanthine dehydrogenase activity for the assimilation of nitrogen could be the reason why no MoCo mutant plants have been reported for **Glycine max.** There are, however, reports of possible MoCo mutants in the legume **P. sativum** (Kleinhofs **et al**, 1978; Feenstra and Jacobsen, 1980; Warner **et al**, 1982). **P. sativum** transports amides and therefore mutants in the MoCo genes would presumably not be lethal.

Figure 44

Nodule metabolism in amide- and ureide-transporting plants

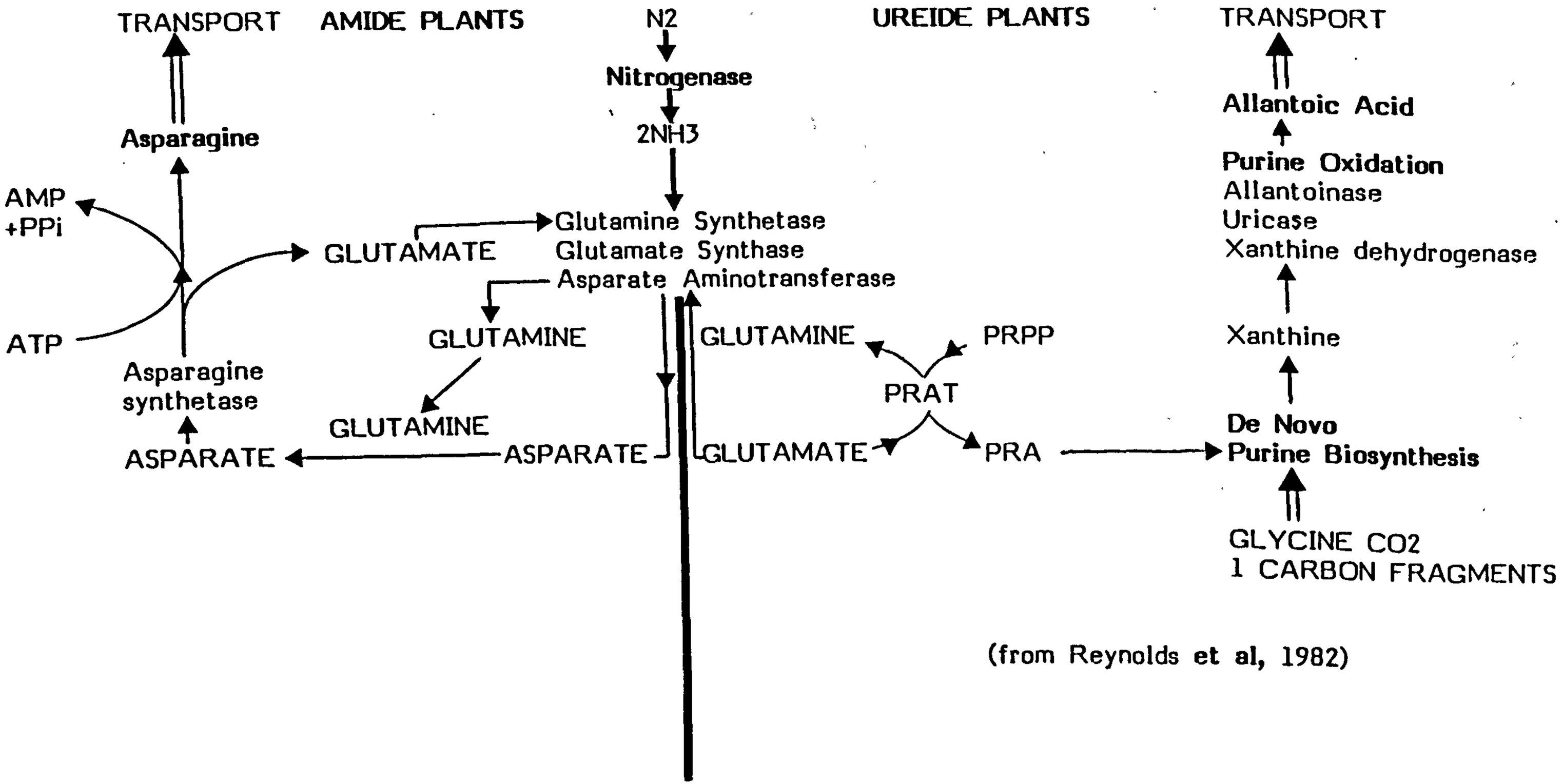
The abbreviations PRA, PRAT and PRPP indicate:

PRA = phosphoribosyl amine

PRAT = 5-phosphoribosyl pyrophosphate amidotransferase

PRPP = Syn,5-phosphoribosyl pyrophosphate

Figure 44
Nodule metabolism in amide- and ureide-transporting plants



(from Reynolds **et al**, 1982)

4. IS R12801 AN NR APOPROTEIN STRUCTURAL GENE MUTANT?

If R12081 is an apoprotein gene mutant it is rather unusual since more than one gene is affected.

The pieces of evidence which indicate that the mutation in R12801 is at an NR apoprotein gene locus are; R12801 lacks **in vitro** NR (NADH-NR and FMNH2-NR) activity but exhibits xanthine dehydrogenase activity (Chapter 3); R12801 displays low levels of nitrate induced monomeric subunits and no dimeric NR (table 12). The lack of dimeric NR was also found in extracts of **nia95** and **nia63** from **N. tabacum** (Mendel and Müller, 1979) and **narlb, narlg** and **narli** mutant plants from **H. vulgare** (Narayanan **et al**, 1983). However, other evidence suggests that R12801 is not a simple NR apoprotein structural gene mutant. These facts include; R12801 seedlings, unlike all other NR mutants isolated at Rothamsted, grow slowly to maturity when nitrate is supplied as the nitrogen source; levels of nitrite reductase activity and nitrate content are low; R12801 seedlings contain very little MoCo (whilst the **narl** mutants isolated by Kleinhofs **et al** (1980) were shown to posses 45-100% of the wild type MoCo levels (Narayanan **et al**, 1984); R12801 seedlings were initially very pale green with no NR activity, but the leaves darken with age and the NR activity is increased, this phenotype is inherited in selfed progeny.

The following suggestions are possible reasons for the R12801 phenotype:

1) A development or chlorophyll mutation which affects the

expression of nitrate and nitrite reductase activities.

2) The R12801 phenotype could have arisen from multiple gene mutations.

3) R12801 could be caused by an 'induction' mutation, ie the defect in the apoprotein gene prevented the positive regulation of the MoCo and nitrite reductase structural genes in the presence of nitrate. Nitrite reductase and MoCo are known to be induced in concert with nitrate reductase by the presence of nitrate in **N. tabacum** (Mendel **et al**, 1982b). Narayanan **et al** (1984) however, found that **H. vulgare** cv. Steptoe MoCo was not induced by nitrate.

If R12801 is an NR apoprotein structural gene mutant it, follows that it is not equivalent to the barley **narl** gene mutant plants. All the anomalies found in R12801 suggest that other genes (as well as **narl**) have also been directly or indirectly affected.

In order to identify whether R12801 is an apoprotein gene mutant it is necessary to cross R12801 with a **narl** gene mutant. It would also be interesting to try to segregate out the pale green phenotype from the NR minus plants in order to discover whether multiple mutations caused the R12801 phenotype. If R12801 was found to be non-allelic to **narl**, it would be valuable to get a clearer picture of the developmental aspects of nitrate assimilation using this mutant. This could be achieved by measuring the levels of, for example, glutamine synthetase (GS), chlorophyll content, NR and nitrite reductase activities of R12801 and the wild type. It could then be seen whether there was any correlation between these activities in the wild type and R12801 since

GS activity and chlorophyll content are known to be developmentally regulated (Lara **et al**, 1983).

5. FURTHER NOVEL BARLEY MoCo GENE MUTANTS CHARACTERISED

5.1 R11301

R11301 has been shown to be genetically and biochemically distinct from **nar2** mutant barley plants. R11301 is also biochemically different from plants with mutations at the **nar3** locus in barley (table 26). The **nar4** gene mutant has not been sufficiently characterised in terms of its biochemistry to allow comparison. To prove that R11301 is affected by a mutation at different genetic locus from either **nar3** or **nar4**, crosses should be carried out. An alternative technique to apply if crossing proved difficult would be somatic hybridisation of cell lines derived from R12801 and **nar3** and **nar4** mutant plants.

The biochemical features which distinguish R11301 from the other MoCo mutants (including R9201, R9401 and R12202) are:

1) Wild type levels of (inactive) NR (figure 33).
2) NR and xanthine dehydrogenase activities are not repaired by growth on high levels of molybdate in the presence of nitrate (Chapter 6).
3) Lack of NR activity when extracted in the presence of molybdate, but the exhibition of wild type levels of MoCo when reconstitution experiments are carried out with **nit1** extracts in the presence of molybdate (table 16).

Table 26

Comparison of higher plant and fungal MoCo mutants

The symbols +, -, +/- and nd indicate the following:

+ = wild type levels of activity

- = no detectable activity

+/- = some strains possessed dimeric NR, whilst other allelic strains did not.

nd = not determined

a **In vivo** repair of NR and xanthine dehydrogenase activity when grown in the presence of high concentrations of molybdate as described in Chapter 2, Section 10.

b Presence of wild type levels of dimeric NR in extracts subjected to centrifugation on sucrose density gradients as described in Chapter 2, Section 8.

c MoCo content as measured by the ability of a MoCo extraction of the mutant to reconstitute active NR in the presence of a **nit1** extract and excess molybdate as described in Chapter 2, Section 11.

Table 26 Comparison of higher plant and fungal MoCo mutants

Species			in vivo repair[a] of NR and XDH	dimer[b]	MoCo content[c]
H. vulgare	R9401	nar 2b	-	29%	-
	R9201	nar 2c	-	36%	-
	Az 34	nar 2a	nd	30%	-
	R11301		-	+	+
	R12202		nd	-	-
		nar 5	nd	+	20%
N. tabacum		cnx A	+	+	+
		cnx B	-	+	+
N. plumbaginifolia		cnx A	+	+	+
		cnx B	-	-	+
		cnx C	-	-	-
A. thaliana	B73		+	+	nd
	B25		-	-	nd
A. nidulans		cnx E	+	+	nd
		cnx A	-	+/-	nd
		cnx B	-	+/-	nd
		cnx F	-	+/-	nd
		cnx G	-	+/-	nd
		cnx H	-	+/-	nd

All these facts suggest that the defect in R11301 NR minus plants is in the gene which codes for the enzyme required for the last stage in the synthesis of active MoCo (before molybdenum is inserted) (figure 40). This enzyme can be provided by a **nit1** extract and allows the formation of active NR. The presence of wild type levels of dimeric, but inactive, NR in extracts from R11301 NR minus plants also indicates that this enzyme is likely to be the final enzyme involved in the molybdopterin synthesis.

R11301 has a novel phenotype when compared with other higher plant MoCo mutants (table 26).

5.2 R9201, a new **nar2** allele

R9201 was shown to be allelic to **nar2b** (R9401) and was designated **nar2c.** **2c.** It would seem likely that the **nar2** gene product is required earlier in the molybdopterin synthesis pathway since there is either non or up to 30% dimeric (but inactive) NR (table 26) and no detectable MoCo (table 16) found in **nar2** mutant extracts. However, it is important to bear in mind that very few allelic mutant plants are available and that intermediate levels of dimer might be an allelic rather than a genetic effect (ie phenotypic).

5.3 R12202

R12202 cannot be distinguished from R9401 or R9201 on the basis of the NADH-NR or FMNH2-NR activities, or the levels of nitrate or nitrite reductase activity present. On the other hand, there does seem to be a difference in the NADH-CR profiles; R12202 has no inactive NR dimer and

no MoCo present. This could imply that the R12202 defective gene product is required in the molybdopterin synthetic pathway prior to the **nar2** gene product, since some of the **nar2** mutants appear to possess some dimer whilst R12202 does not. Again it is possible that this lack of dimer is merely an allelic effect of a different mutation in the **nar2** gene. In order to clarify the situation it would be necessary to cross R12202 with a **nar2** mutant plant.

6. FUTURE WORK

6.1 Isolation of more mutants

In future it is clear that more mutants (both non-allelic and allelic to those previously identified) of nitrate assimilation in barley need to be isolated and characterised. Identification of regulatory mutants would broaden our understanding of the control of nitrogen flux and would lead to greater perception of the relationships between nitrate and other areas of metabolism. The characterisation of more allelic mutants would allow allelic variation in activities to be distinguished from variation caused by mutations in different genes. A spectrum of mutant alleles would also be helpful for physiological studies of nitrate assimilation, including for example, nitrate and molybdenum uptake.

6.2 Genetic analyses

Genetic crosses of R12202 and R12801 with plants of known mutant classes are needed to determine if whether R12202 is allelic with **nar2**,

nar3, or **nar4**. These genetic crosses are also needed to decide if R12801 possesses a defective apoprotein gene and to define whether this is **nar1**. It would also be very interesting to see whether the mutation in R11301 is allelic to **nar3** or **nar4** (previously unavailable for crossing).

6.3 NR antibody work

Monoclonal NR antibody raised against purified wild type NR subunits would be useful for assessing the amounts of NR protein (as determined by the levels of cross-reacting material (CRM)) produced in the mutants and wild types. It would be instructive to compare these level with the levels of NR activity detected. If the level of CRM found in extracts of R12801 was greatly reduced, it would suggest that R12801 was an apoprotein mutant.

6.4 Molecular characterisation of nitrate assimilation

It would be valuable to clone the apoprotein, the MoCo and the regulatory genes since it is clear that learning about the molecular details of a system provides greater understanding of its control (eg the glutamine synthetase enzyme of **P. vulgaris**). It might well be possible to use the cloned sequences of **E. coli** MoCo genes (eg the **chlA** gene) or the **A. nidulans** apoprotein gene **niaD** to probe for analogous genes in barley. It would be instructive to compare sequences of the wild type and the defective genes from the NR minus mutant plants. It might be possible to identify which areas of these gene sequences are essential for the functioning of the proteins associated with NR activity. It should also be possible to create new and interesting

mutants using site-directed mutagenesis. The recognition sites and mechanisms involved in the nitrate induction of nitrate reductase (and other enzymes) could also be studied at the molecular level. It would also be worthwhile to see whether the apoprotein gene or any particular MoCo gene normally limits NR activity; this could be done by increasing the copy numbers. Once the MoCo genes were isolated it would be exciting to translate the message **in vitro** and use this to raise antibodies against the products in order to identify the proteins involved in the formation of an active MoCo.

In conclusion, the more that is known about nitrate reductase and nitrate assimilation the more they can be manipulated for the benefit of Mankind.

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Appendix 1

Test of significance using Students t-test

Null hypothesis: The mean of the population from which the sample NADH-NR activities of Golden Promise was taken is not significantly different from the mean in *vitro* NADH-NR activities of the population from which the Maris Mink sample was taken.

Using the formulae.

$$t_{df} = \frac{\bar{Y}_1 - \bar{Y}_2}{S\bar{Y}_1 - \bar{Y}_2} \qquad df = n_1 + n_2 - 2$$

$$\text{where} \quad S^2 \text{ pooled} = \frac{\sum_{i=1}^{n1} (y_{1i} - \bar{y}_1)^2 + \sum_{i=1}^{n2} (y_{2i} - \bar{y}_2)^2}{n_1 + n_2 - 2}$$

$$S\,\bar{y}_1 - \bar{y}_2 = \sqrt{S^2 \text{ pooled} \left(\frac{1}{n_1} + \frac{1}{n_2}\right)}$$

where $\bar{y}_1$ = mean of Golden Promise values

$\bar{y}_2$ = mean of Maris Mink values

$S\bar{y}_1 - \bar{y}_2$ standard error of the difference of the means

Using for example the *in vitro* NADH-NR activities of Golden Promise and Maris Mink

Golden Promise	Maris Mink
$\bar{y}_1 = 2.0$	$\bar{y}_2 = 2.7$
$n_1 = 30$	$n_2 = 27$
$\Sigma y_1 = 69.35$	$\Sigma y_2 = 74.1$
$\Sigma y_1^2 = 171.3$	$\Sigma y_2^2 = 226.47$
$\sum_{i=1}^{n_1}(y_{1i} - \bar{y}_1)^2 = 171.3 - \frac{(69.35)^2}{30}$	$\sum_{i=1}^{n_2}(y_{2i} - \bar{y}_2)^2 = 226.47 - \frac{(74.1)^2}{27}$
$= 29.85$	$= 23.11$

$$s^2 \text{ pooled} = \frac{29.85 + 23.11}{30 + 27 - 2} = 0.96$$

$$s_{\bar{y}_1 - \bar{y}_2} = \sqrt{0.96 \times \left(\frac{1}{30} + \frac{1}{27}\right)} = 0.26$$

$$t_{55} = \frac{2.7 - 2.0}{0.26} = 2.70$$

$$t_{55}\ (5\%) = 2.008$$

t_{55} is larger than t_{55} (5%) and therefore the Null hypothesis is rejected.

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A conditional-lethal molybdopterin-defective mutant of barley

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Summary. The molybdenum cofactor of the barley mutant R9401 is not able to reconstitute NADPH nitrate reductase activity from extracts of the *N. crassa nit-1* mutant nor is it able to effect dimerisation of the nitrate reductase subunits present in the R9401 mutant. Unphysiologically high levels of molybdate cannot restore nitrate reductase and xanthine dehydrogenase activity to mutant R9401 in vivo nor reactivate the Mo-co factor in vitro. The results indicate that the defect in mutant R9401 lies in the pathway leading to the formation of a functional molybdopterin moiety and that the same nuclear gene is involved in the synthesis of both shoot and root molybdenum cofactor.

Introduction

The molybdenum of higher plant assimilatory nitrate reductase and, with the exception of nitrogenase, of all other molybdo-enzymes so far examined is carried on a dissociable, dialysable, oxygen-sensitive structure, the molybdenum cofactor (Mo-co) (Johnson 1980). The Mo-co is a complex between a novel pterin, molybdopterin, and molybdenum (Johnson et al. 1980; Johnson and Rajagopalan 1982; Wahl et al. 1984). The Mo-co is involved at the catalytic centre of nitrate reductase and it is also responsible for dimerisation (Nason et al. 1971) of the two ca. 100,000 mol. wt. flavohaemoprotein subunits which, together with probably two molybdenum cofactors, comprise the 7.7S holoenzyme (Kuo et al. 1980; Campbell and Wray 1983; Redinbaugh and Campbell 1985).

Mutants altered in the synthesis of a functional Mo-co have been isolated in a variety of organisms including filamentous fungi and higher plants (reviewed by Garrett and Amy 1978; Cove 1979; Dunn-Coleman et al. 1984). Barley Mo-co mutants have been reported in the barley cultivars Steptoe (Warner et al. 1977; Somers et al. 1983) and Winer (Tokarev and Shumny 1977). Genetic analysis shows that they represent two Mo-co loci designated *nar2* and *nar3* (Kleinhofs et al. 1983). The nature of the defects in these mutants is unknown. We previously reported on the isolation of a barley Mo-co mutant, R9401, (Bright et al. 1983) in the cultivar Maris Mink. We show here that the defect in mutant R9401 lies in the molybdopterin biosynthetic pathway and that the same nuclear gene is probably involved in the synthesis of both shoot and root molybdopterin.

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Abbreviations. BSA: bovine serum albumen; GSH: glutathione (reduced); *NEM:* N-ethylmaleimide

Methods

Selection and growth. Mutant R9401, of barley, *Hordeum vulgare* cv. Maris Mink, was selected and grown to maturity as described previously (Bright et al. 1983). The experiments reported below were conducted on the F_2 progeny of two F_1 plants derived from the cross R9401 × cv. Golden Promise. Growth of plants was carried out essentially as previously described (Small and Wray 1980).

Identification of nitrate reductase-minus and nitrate reductase-plus plants amongst the F_2 progeny. Tips (5–10 mg in weight) of four-day-old nitrate-treated plants were ground in a mortar with 1 ml of 50 mM potassium phosphate buffer, pH 7.5, containing 0.1 mM EDTA 10 µM FAD, 1 mM cysteine and 3% (w/v) bovine serum albumin (fraction V, Sigma, St. Louis, MO, USA). The brei was transferred to a test-tube, 0.1 ml of 0.1 M KNO_3 and 0.1 ml of 1 mM NADH were added and after incubation at 25° C for 30 min nitrite production was detected by the addition of 1 ml of 1% (w/v) sulphanilamide in 3 M HCl and 1 ml of 0.02% (w/v) naphthylethylenediamine dihydrochloride. Absence of nitrite production by a shoot-tip homogenate (as shown by the absence of the characteristic red-pink colour) showed that the shoot-tip was derived from a plant lacking nitrate reductase activity.

Sucrose density gradient centrifugation was carried out as described previously (Small and Wray 1980).

Assay of molybdenum cofactor was performed by the method of Mendel et al. (1985). One unit of Mo-co activity will reconstitute the *nit-1* NADPH nitrate reductase to 1 nmole nitrite produced per min. Specific activity is expressed as units per mg protein of Mo-co extract.

In vitro reconstitution of nitrate reductase from barley extracts by xanthine oxidase molybdenum cofactor was performed essentially by the method of Mendel and Muller (1985). Seven day old nitrate grown shoots were extracted in deaerated 0.1 M potassium/sodium phosphate buffer, pH 7.5, containing 3 mM GSH, 1 mM EDTA, 20 mM sodium molybdate, 0.01 M FAD and 3% BSA (5 ml buffer/g tis-

sue). After centrifugation at 32,000 *g* for 15 min, 0.5 ml of the supernatant was incubated anaerobically for 30 min at 25° C with 0.5 ml of Mo-co released from bovine xanthine oxidase (Sigma). An aliquot was then assayed for nitrate reductase activity. The Mo-co source was prepared as follows: 10 µl (300 µg) xanthine oxidase was transferred to 5 ml of 50 mM potassium/sodium phosphate pH 7.5 containing 0.5 mM EDTA, 20 mM GSH, 25 mM sodium molybdate, deaerated, heated at 70° C for 90 s and transferred to ice.

Assay of nitrate reductase, cytochrome c reductase and xanthine dehydrogenase was performed as described previously (Wray and Filner 1970; Bright et al. 1983). *Molybdenum content* was assayed in dry leaves of plants grown in liquid culture on ammonium using an automated flame ionisation system.

Results

Molybdenum cofactor activity in wild-type barley and in F_2 progeny plants segregating for nitrate reductase activity

Molybdenum-cofactor activity was assayed by measuring the extent of the ability of Mo-co, released by heat-treatment of tissue extracts, to reconstitute NADPH nitrate reductase activity from extracts of the *Neurospora crassa nit*-1 mutant. The Mo-co activity of wild-type plants and of F_2 progeny plants containing nitrate reductase activity was similar (Table 1) and comparable to the values reported previously (Mendel et al. 1985). However, Mo-co activity was almost completely absent from extracts of both shoots and roots of F_2 progeny lacking nitrate reductase activity (Table 1).

Nitrate reductase and xanthine dehydrogenase activity cannot be repaired in vivo by high levels of molybdate

Neither nitrate reductase activity (data not shown) nor xanthine dehydrogenase activity (Fig. 1 a, b) could be detected in either shoot or root extracts when F_2 progeny plants lacking nitrate reductase activity were grown in the presence of 2 mM sodium molybdate, a concentration some 80-fold higher than that present in the usual nutrient solution. This level of molybdate slightly increased extractable nitrate reductase activity in wild-type plants.

Table 1. Shoot and root molybdenum-cofactor activities in wild-type barley cv. Golden Promise and F_2 progeny of the cross between cv. Golden Promise and the Nitrate Reductase-minus cv. Maris Mink R9401

Plant and organ type	Mo-co activity (units/mg protein)
Shoots	
Wild-type cv. Golden Promise	36.60 ± 2.70 (3)
Nitrate reductase-plus F_2 progeny	34.80 ± 2.77 (3)
Nitrate reductase-minus F_2 progeny	0.07 ± 0.02 (6)
Roots	
Wild-type cv. Golden Promise	28.6 ± 4.9 (3)
Nitrate-reductase-plus F_2 progeny	35.2 ± 7.0 (3)
Nitrate-reductase-minus F_2 progeny	0 ± 0.0 (6)

Assays were carried out as described in Methods on shoots and roots derived from 5-day-old plants which had been grown in the presence of 10 mM KNO_3. Figure in bracket indicates number of independent determinations

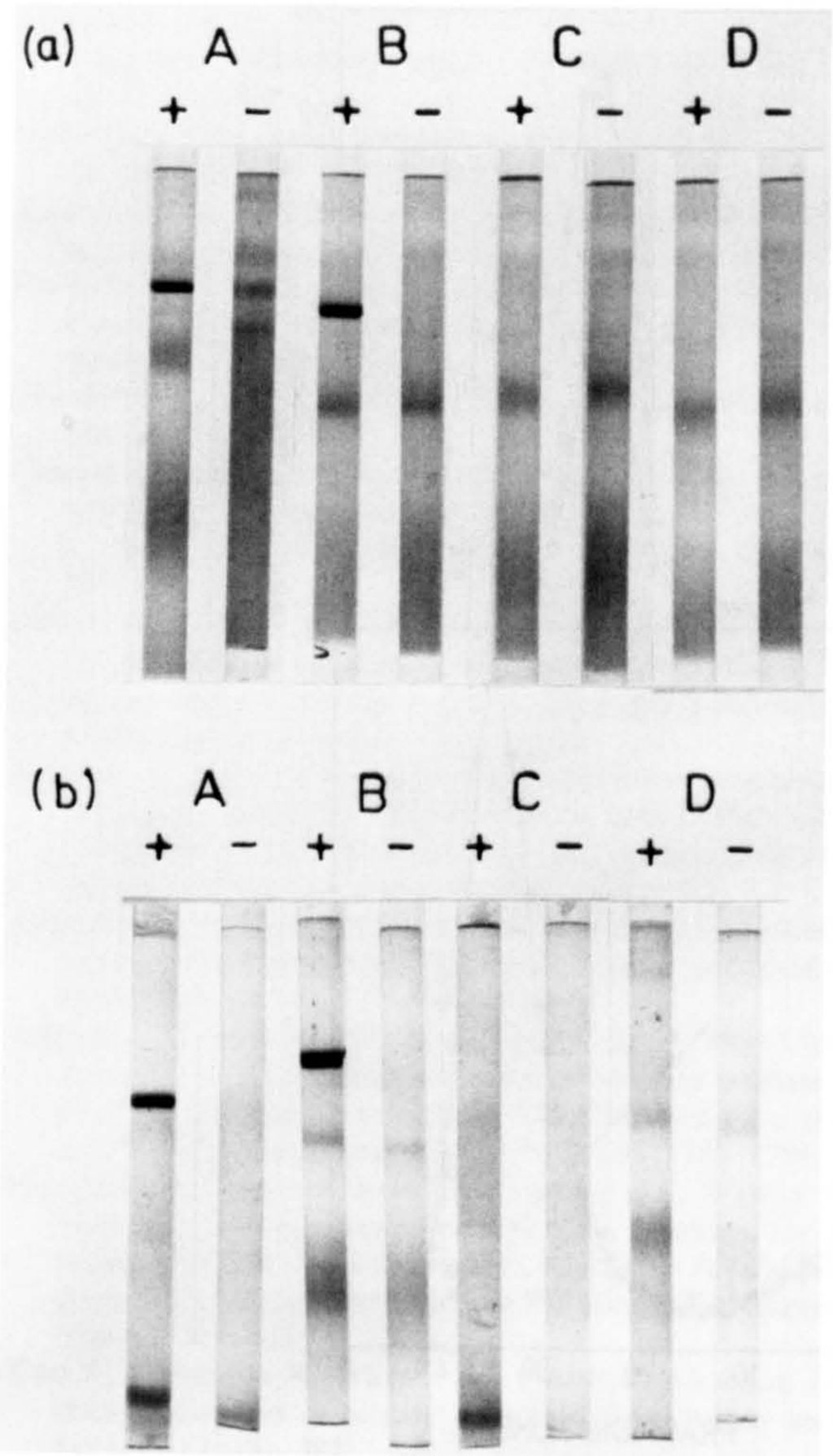

Fig. 1 a, b. Specific staining for xanthine dehydrogenase activity after gel electrophoresis of extracts from: **a** shoot of an F_2-progeny plant possessing nitrate reductase activity grown in the absence (A) and presence (B) of 2 mM sodium molybdate or of an F_2 progeny plant lacking nitrate reductase activity grown in the absence (C) or presence (D) of 2 mM sodium molybdate; **b** root of an F_2 progeny plant possessing nitrate reductase activity grown in the absence (A) and presence (B) of 2 mM sodium molybdate or of an F_2 progeny plant lacking nitrate reductase activity grown in the absence (C) or presence (D) of 2 mM sodium molybdate. +, indicates the presence of hypoxanthine in the xanthine dehydrogenase activity stain; −, is a control in which hypoxanthine was omitted

Sucrose density gradient analysis of cytochrome c reductase species present in shoot extracts of wild-type and F_2 progeny plants

Sucrose density gradient analysis of extracts of wild-type barley shoots showed the presence of a major peak of NADH-cytochrome c reductase activity which co-sedimented with NADH nitrate reductase activity at 7.7 S (Fig. 2 a). This 7.7 S NADH-cytochrome c reductase species was missing from extracts of F_2 progeny plants lacking

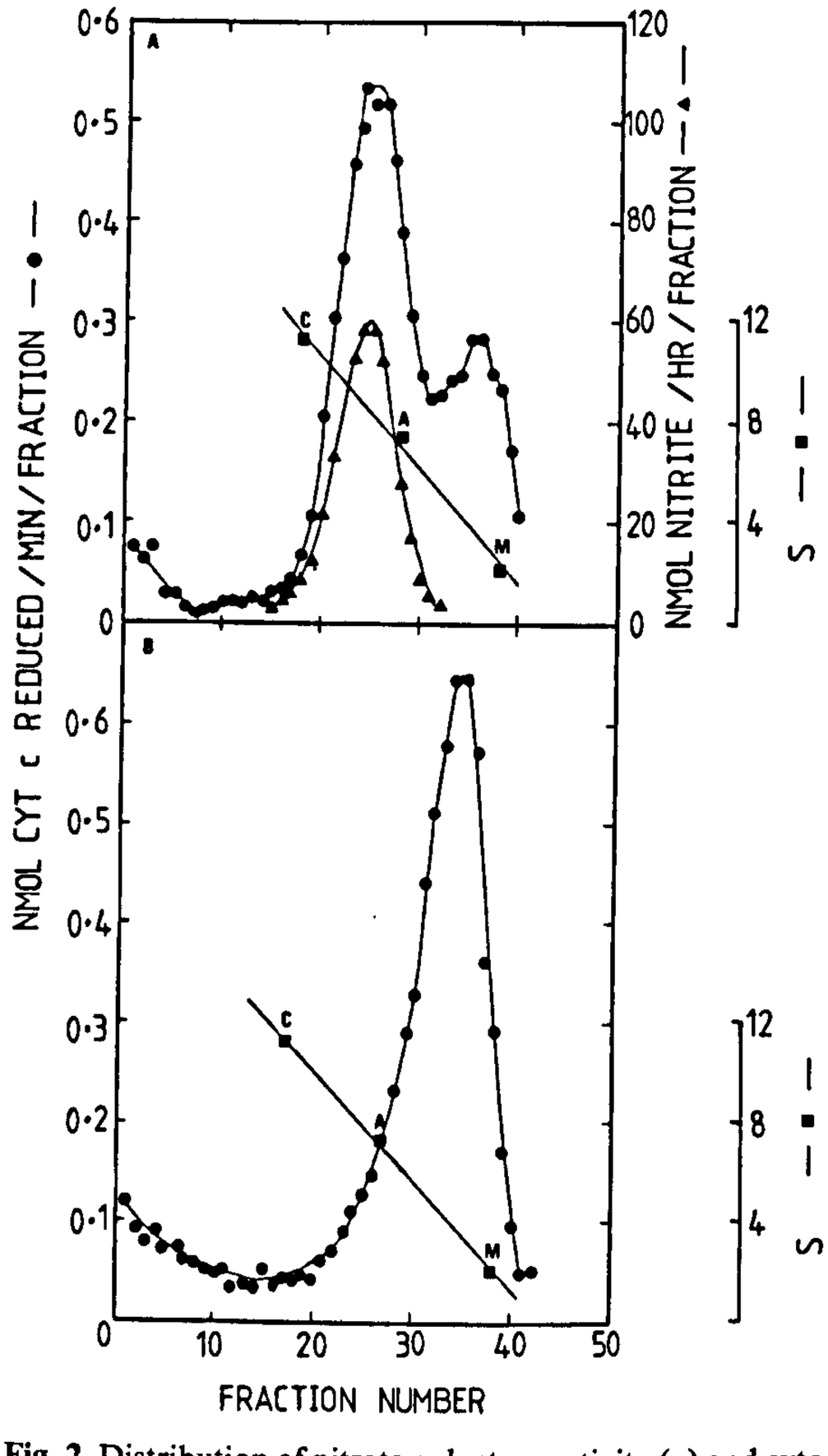

Fig. 2. Distribution of nitrate reductase activity (▲) and cytochrome c reductase activity (●) after sucrose density gradient centrifugation of cell-free extracts of (*A*) wild-type barley cv. Golden Promise grown on nitrate and (*B*) F_2 progeny plant lacking nitrate reductase activity grown on nitrate. Conditions of centrifugation are described in Small and Wray (1980). *C*, *A* and *M* denote the positions of the reference proteins catalase (11.3S), alcohol dehydrogenase (7.4S) and myoglobin (2.04S) after centrifugation

Table 2. Reconstitution of nitrate reductase activity in shoot extracts of F_2 progeny plants lacking nitrate reductase by molybdate and by molybdate plus bovine xanthine oxidase molybdenum-cofactor

Plant Type	Nitrate reductase activity repaired by	
	Molybdate	Molybdate + Mo-co
	(nmoles/h/g fresh wt)	
Wild-type cv. Golden Promise	2.28 ± 0.05 (4)	1.26 ± 0.03 (4)
Nitrate reductase-minus F_2 progeny	0 ± 0.00 (6)	0.4 ± 0.07 (6)

Assays were carried out as described in the Methods section with plants grown in the presence of 10 mM KNO_3

Figure in bracket indicates number of independent determinations

nitrate reductase activity whilst the peak of NADH-cytochrome c reductase activity in the 3–4S region was greatly elevated (Fig. 2b).

In vitro repair of nitrate reductase activity by bovine xanthine oxidase molybdenum cofactor in the presence of molybdate

Extraction of shoots of F_2 progeny plants lacking nitrate reductase activity in the presence of EDTA, 20 mM sodium molybdate and 3 mM reduced glutathione did not repair nitrate reductase activity (Table 2). However if these shoot extracts were subsequently incubated with Mo-co released from bovine xanthine oxidase by heat-treatment then nitrate reductase activity was repaired to approximately 30% of the wild-type level (Table 2). The reason for the decrease in nitrate reductase activity in wild-type cell free extract in the presence of exogenous Mo-co is not clear.

Discussion

The barley mutant R9401 was isolated by virtue of its resistance to chlorate. It was unable to grow on nitrate as sole nitrogen source and since it lacked both shoot nitrate reductase and xanthine dehydrogenase activity we suggested that it carried a defect in shoot Mo-co synthesis (Bright et al. 1983).

The inability of the shoot Mo-co of mutant R9401 to reconstitute NADPH-NR activity from the monomer flavohaemoprotein subunits present in extracts of the *N. crassa* Mo-co mutant *nit-1* (Nason et al. 1970; 1971) supports this suggestion (Table 1). It is, however, not clear from this data whether the defect is in catalytic function, dimerisation ability or both. Attempts to demonstrate dimerisation by analysis of reconstitution mixes on sucrose gradients were unsuccessful, even in the wild-type, due to the presence of extremely large amounts of monomer cytochrome c reductase activity.

Dimerisation of the apoprotein flavohaemoprotein subunits, which carry NADH-cytochrome c reductase activity (Wray and Filner 1970; Small and Wray 1980), is a function of the molybdopterin moiety of the Mo-co since demolybdo-molybdenum cofactor, generated either as a consequence of molybdenum starvation (Hewitt et al. 1977; Gewitz et al. 1981; Ramadoss et al. 1981) or mutation (Mendel et al. 1981) is able to effect dimerisation. Dimerisation appears to involve interaction of the molybdopterin with a thiol ligand on the apoprotein since NEM- treated flavohaemoprotein subunits cannot be dimerised by molybdenum cofactor Wahl et al. 1984).

Sucrose density gradient analysis of the cytochrome c reductase species present in shoot extracts of mutant R9401 showed the 7.7 S cytochrome c reductase species, characteristic of the ca. 200,000 mol wt nitrate reductase holoenzyme (Wray and Filner 1970; Small and Wray 1980), was missing and that there was a greatly elevated peak of activity at 3.1 S (Fig. 2b). This 3.1 S cytochrome c reductase species is most likely a degradation product of the flavohaemoprotein subunit rather than the subunit itself (Brown et al. 1981; Campbell and Wray 1983). We conclude from this that the molybdenum-cofactor is defective in both dimerisation ability and catalytic function and that the mutation carried by mutant R9401 is within the molybdopterin biosynthetic pathway. A mutation, *cnxC*, (equivalent to NX21) of *Nicotiana plumbaginifolia*, which is phenotypically simi-

lar to that of mutant R9401, has been described (Marton et al. 1982; Mendel et al. 1982; Xuan et al. 1983).

Two loci, *cnxA* (Müller and Grafe 1978; Mendel et al. 1981; Mendel and Müller 1985) and *cnxB* (Buchanan and Wray 1982) have been identified in *N. tabacum* (Xuan et al. 1983) which are probably involved in the insertion of molybdenum into molybdopterin (Mendel and Müller 1985) and in molybdenum processing (Mendel et al. 1984), respectively. Unlike the situation in *N. tabacum cnxA* mutants neither nitrate reductase nor xanthine dehydrogenase activity can be restored in vivo to mutant R9401 by either long term (Bright et al. 1983) or short term (Fig. 1) growth in the presence of unphysiologically high levels of molybdate and nitrate reductase activity could not be restored in vitro in the presence of exogenous molybdate, reduced glutathione and EDTA (Table 2). Mendel and Müller (1985) have speculated that reduced glutathione may supply thiol groups required for molybdenum liganding and that EDTA may be involved in molybdenum chelation. The molybdenum content of wild type and mutant R9401 seedlings was similar (10–11 μg/g fresh weight). Our observations are consistent with the conclusion that mutant R9401 is defective in molybdopterin biosynthesis.

The defect in mutant R9401 does not lie in the gene specifying the apoprotein since shoot NADH-cytochrome c reductase activity (a measure of apoprotein) is nitrate-inducible (Bright et al. 1983) and Mo-co released from bovine xanthine oxidase by heat-treatment is able to reconstitute NADH-nitrate reductase activity from shoot extracts of mutant R9401 (Table 2).

Immunochemical evidence indicates that the nitrate reductase of spinach roots and leaves are different proteins (Ferrario et al. 1983) suggesting that they might be the products of different genes. We looked to see whether the root Mo-co of mutant R9401 was also defective and found that, for those properties examined, it behaved the same as the shoot Mo-co (Table 1 and Fig. 1). This provides some support for the suggestion that at least one of the steps in molybdopterin synthesis is governed by the same gene in roots and shoots and also an explanation of why mutant R9401 is unable to grow on nitrate as sole nitrogen source (Bright et al. 1983).

When analysed by sucrose density gradient centrifugation extracts of both the barley *nar2* and *nar3* mutant show the presence of a ca. 8S cytochrome c reductase species (Narayanan et al. 1983) unlike R9401 (Fig. 2b). Whether mutant R9401 carries a mutation at a locus different from either *nar2* or *nar3* awaits allelism testing.

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