THE BIOCHEMICAL GENETICS OF SULPHATE ASSIMILATION IN BARLEY (*HORDEUM VULGARE*)

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A thesis submitted to the University of St. Andrews for the degree of Master of Science

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This thesis is dedicated to my Dad, David Bates.

I know he would have been so proud.

I wish I'd written faster.

DECLARATION

I, Amanda Frances Gilkes, hereby certify that this thesis, which is approximately 31,000 words in length, has been written by me, that it is a record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

I hereby certify that the candidate has fulfilled the conditions of the resolution and regulations appropriate for the degree of M.Sc. in the University of St Andrews and that the candidate is qualified to submit this thesis in application for that degree.

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ABSTRACT

The sulphate assimilation pathway was investigated in barley (*Hordeum vulgare*). The enzymes ATP sulphurylase, thiosulphate reductase, sulphite reductase, serine acetyltransferase and cysteine synthase proposed as steps in the pathway of higher plants, have been detected in barley seedling leaves.

Cysteine synthase was purified ca 400 fold from extract of 7 day old barley seedling leaves to give an activity of 52µmols cysteine produced/min/mg protein. Differential centrifugation of mechanically ruptured protoplasts showed cysteine synthase was likely to be predominantly or exclusively located within the chloroplast. Specific staining for cysteine synthase activity of non-denaturing polyacrylamide gels suggest the presence of a single form of the enzyme in barley. Development of cysteine synthase activity in barley seedlings was shown not to be dependent on light but was influenced by nutritional status.

Selenate, which was shown to be toxic to barley seedlings, was used as the positive selection agent in a screen designed to isolate whole plant mutants defective in sulphate assimilation. Over 70,000 M_2 seedlings (mutagenised in the M_1 with 2mM sodium azide) were screened for resistance to selenate. Nine M_2 seedlings were selected as most likely to be defective in sulphate assimilation. M_3 progeny were obtained from four of the M_2 selections. The M_3 progeny showed no resistance to selenate and specific activity staining of non-denaturing polyacrylamide gels showed the presence of functional ATP sulphurylase, thiosulphate reductase, sulphite reductase and cysteine synthase. These results suggest it is unlikely that these M_2 selections carry a heritable mutation in sulphate assimilation.

TABLE OF CONTENTS

CHAPTER 2 - MATERIALS AND METHODS

CHAPTER 3 - ENZYMOLOGICAL STUDIES ON THE SULPHATE ASSIMILATION PATHWAY IN WILD TYPE BARLEY

CHAPTER 4 - DEVELOPMENT OF A POSITIVE SELECTION SCREEN FOR IDENTIFYING SELENATE-RESISTANT BARLEY PLANTS

CHAPTER 1

INTRODUCTION

1.1 Sulphur metabolism

Sulphur (S) is an essential nutrient for all plants and is taken up in the form of the inorganic anion sulphate. It is the second most abundant element, after nitrogen, to be taken up and metabolised. Sulphur is required by plants for the synthesis of the sulphur containing amino acids cysteine and methionine, for other S compounds of primary metabolism and for a wide range of secondary metabolites. Whilst inorganic sulphur is assimilated to cysteine in plants and bacteria, animals do not have the necessary assimilation pathway and require a dietary source of these sulphur containing amino acids.

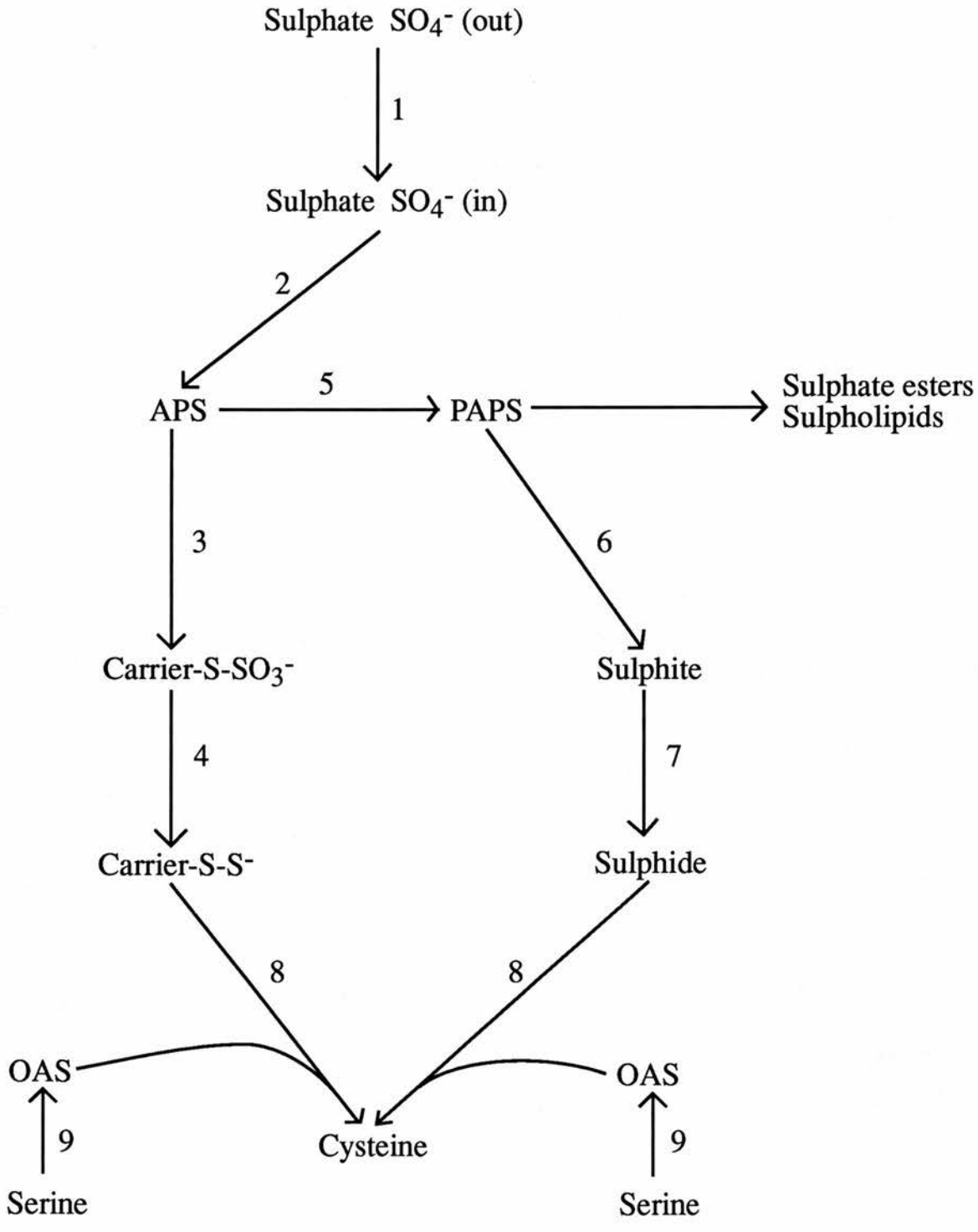
Inorganic sulphate (SO_4^-) must be reduced to the level of sulphite (SO_3^-) for the formation of sulpholipids. These occur exclusively as a component of the chloroplast membrane and are required for the correct functioning of intact chloroplasts. Sulpholipid formation is a major biosynthetic pathway, accounting for about 50% of the plants total sulphur requirement (Harwood, 1980).

For the synthesis of the amino acids cysteine and methionine, and a variety of coenzymes, the inorganic sulphate needs to be reduced to the level of sulphide (S^-). The reduction of sulphate and the incorporation of the reduced sulphur into cellular components is termed assimilatory sulphate reduction or sulphate assimilation.

1.1.1 Sulphate assimilation

There are numerous reviews of sulphate assimilation in higher plants including Anderson, 1980,1990; Giovanelli *et al.*, 1980; Schmidt, 1986; Schmidt and Jager, 1992, and De Kok *et al.*, 1993. The proposed pathways by which higher plants assimilate sulphate are

Fig 1.1 Possible pathways of sulphate assimilation in higher plants



1. Sulphate uptake; 2. ATP-sulphurylase; 3. APS sulphotransferase; 4. Thiosulphate reductase; 5. APS kinase; 6. PAPS reductase; 7. Sulphite reductase; 8. Cysteine synthase; 9. Serine acetyltransferase.

summarised in Fig 1.1. The first step in the pathway is the uptake of inorganic sulphate from the soil. Sulphate (SO_4^{2-}) uptake is an energy dependant process (reviewed by Cram, 1990). The enzyme ATP sulphurylase catalyses the formation of the activated sulphate, adenosine-5'-phosphosulphate or adenylylsulphate (APS) (Anderson, 1980; Schiff and Fankhauser, 1981). Two pathways are possible from this point:

a) Bound intermediate pathway

Tracer studies using ^{35}S and enzymatic studies reviewed by Anderson (1980) and Schiff (1983) suggested that sulphate assimilation then followed a carrier bound or bound intermediate pathway. The carrier may be a low molecular weight thiol, possibly glutathione (Tsang and Schiff, 1978) or a somewhat larger molecule (Schmidt and Schwenn, 1971). The activated sulphate from APS is transferred onto a carrier (car-SH) in a reaction catalysed by the enzyme APS sulphotransferase (Schmidt 1972,1976a,b; Schiff and Fankhauser 1981 and Abrams and Schiff 1983). The bound sulphite ($car\text{-}S\text{-}SO_3^-$) is then reduced to bound sulphide ($car\text{-}S\text{-}S^-$) by thiosulphate reductase (Schmidt *et al.*, 1984) formerly called thiosulphonate reductase (Schmidt 1973 and Schmidt *et al.*, 1974). Cysteine synthase then catalyses the formation of L-cysteine from the bound sulphide and O-acetyl-L-serine (OAS) (Smith, 1972; Ngo and Shargool, 1974; Ng and Anderson, 1978a; and Schmidt, 1990). OAS is produced from serine by serine acetyltransferase (Smith, 1972; Nakamura *et al.*, 1987; Brunold and Suter,1982).

b) Free intermediate pathway

During the course of this project Schwenn (1989) proposed PAPS reductase as the "missing link" in an alternative free intermediate sulphate assimilation pathway. In this pathway APS is

converted to adenosine 3'-phosphate 5'-phosphosulphate (3'-phospho-adenylylsulphate) (PAPS) by APS kinase then PAPS reductase catalyses the reduction of PAPS to free sulphite and adenosine-3',5'-bisphosphate (3',5'-PAP). Free sulphite is reduced by sulphite reductase to free sulphide which is then incorporated into L-cysteine by cysteine synthase. PAPS is believed to be the sulphate source for sulpholipid synthesis in plants (Harwood, 1975; Kleppinger-Sparace and Mudd, 1990).

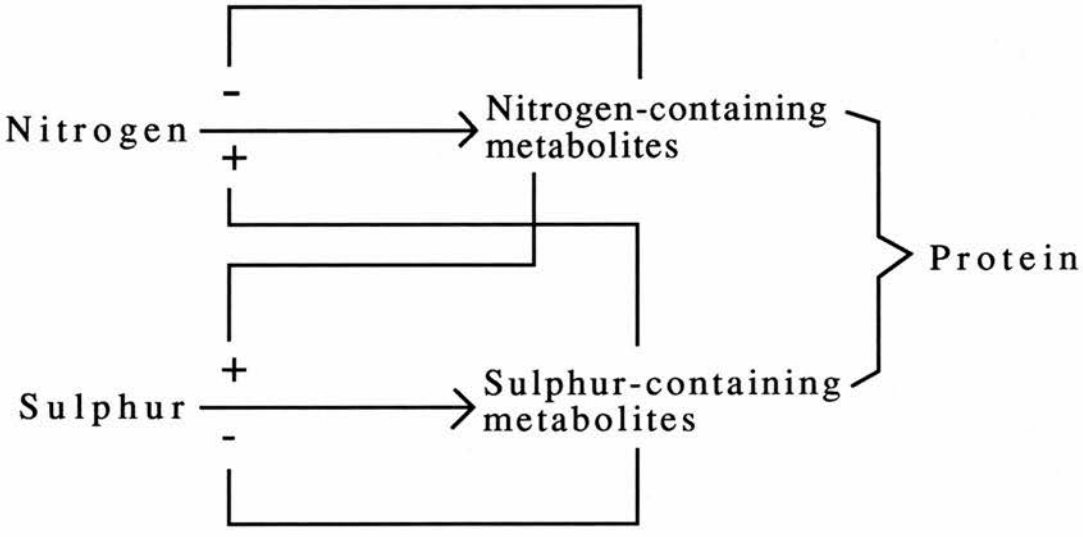
The suggestion of PAPS reductase as the missing link in a free intermediate pathway has called into question the previously held theory of the bound intermediate assimilatory pathway. Which pathway operates in plants is still a matter of controversy.

Once cysteine has been produced methionine can be synthesised from it via the trans-sulphurication pathway involving cystathionine and homocysteine as intermediates (Giovanelli *et al.*, 1980 and Thompson *et al.*, 1982).

1.1.2 Regulation of the pathway

Regulation of the sulphate assimilation pathway in higher plants has been reviewed by Schmidt (1986), Brunold (1993) and Stulen and De Kok (1993). Plants assimilate nitrogen and sulphur primarily into protein, where they occur in the molar ratio of about 25:1 (Reuveny and Filner, 1977). A mechanism to co-ordinate and balance the flow of these two essential components is necessary to meet the needs of net protein synthesis. A system of reciprocal regulatory coupling of the sulphate assimilation pathway with the convergent nitrate assimilation pathway has been suggested (Fig 1.2) (Reuveny and Filner, 1977; Reuveny *et al.*, 1980 and Brunold and Suter, 1984). This system

Fig 1.2 Diagram of the possible reciprocal regulatory coupling between nitrogen and sulphate assimilation pathways



- and + represent the negative and positive control mechanisms (Reuveny *et al*, 1980).

controls the two pathways by "(i) negative feedback of both pathways by their own end products when the reciprocal pathway is not rate limiting and (ii) positive regulation of each pathway by signals originating in the other pathway when the former is not limiting for growth" (Reuveny *et al.*, 1980). The initial step, catalysed by nitrate reductase, is believed to be the rate limiting step in the nitrate assimilation pathway (Beevers, 1981). The reaction steps of sulphate assimilation catalysed by the enzymes ATP-sulphurylase and APS sulphotransferase appear to be the most susceptible to regulatory signals (Smith, 1980; Brunold and Schmidt, 1978; Brunold and Suter, 1984; Neuenschwander *et al.*, 1991). In higher plants APS the product of ATP sulphurylase is not only the substrate for sulphate reduction, but also for sulpholipid biosynthesis (Ellis, 1969). Brunold (1990) suggests that APS sulphotransferase is more susceptible to regulatory signals than ATP-sulphurylase and the key control enzyme of sulphate assimilation. Sulphate reduction is repressed at normal levels of the substrate sulphate and derepressed at low concentrations (Reuveny and Filner,1977; Reuveny *et al.*, 1980). Sulphur deficiency results in an expanded free amino acid pool, with high levels of arginine and asparagine, and decreased levels of sulphur amino acids (Smith, 1980). The decrease in nitrate reductase activity under conditions of sulphur deficiency may be due to this increase in free amino acids (Freidrich and Schrader, 1978; Neuenschwander *et al.*, 1991). In cells starved for nitrogen, derepression of ATP-sulphurylase by sulphur limitation does not occur (Reuveny *et al.*, 1980). This evidence adds to the control mechanisms already mentioned; (iii) the negative regulation of nitrate assimilation and the positive regulation of sulphate assimilation by sulphur starvation, (iv)

the negative regulation of sulphate assimilation by nitrogen starvation.

Light has been shown to play a regulatory part in sulphate assimilation (Brunold, 1990; Neuenschwander *et al.*, 1991). APS sulphotransferase activity falls to 10% of that of a light control 24 hours after transfer to the dark, ATP-sulphurylase and cysteine synthase activity were unchanged (Neuenschwander *et al.*, 1991).

Regulation of sulphate uptake (reviewed by Clarkson *et al.*, 1993) will also play a role in the regulation of sulphate assimilation.

1.2 The enzymes of sulphate assimilation

1.2.1 ATP-Sulphurylase

ATP-sulphurylase (adenosine triphosphate-sulphate adenylyl transferase) (EC 2.7.7.4) (reviewed by Schmutz, 1990) catalyses the first step in the sulphate assimilation pathway; the formation of adenosine 5'-phosphosulphate and pyrophosphate (PPi) from adenosine triphosphate and sulphate.

$$SO_4^{2-} + ATP \longrightarrow APS^{2-} + PPi$$

The pyrophosphate formed is subsequently hydrolysed by a pyrophosphatase to give orthophosphate. This second reaction serves to reduce the extremely unfavourable free energy change for the formation of APS.

ATP-sulphurylase has been detected in cultured tobacco cells (Reuveny and Filner, 1976 and 1977); the leaves of pea (Brunold and Suter; 1989; von Arb and Brunold, 1985), beans (von Arb and Brunold, 1985), maize (Burnell,1984; Ghisi *et al.*, 1987; Schmutz and Brunold, 1985), cabbage (Osslund *et al.*, 1982), soya (Adams and Johnson, 1968) and spinach (Shaw and Anderson, 1972). ATP-sulphurylase activity has been at least partially purified from the shoots of cabbage (Osslund *et al.*, 1982) and spinach (Shaw and Anderson, 1972) and also the roots of maize (Onajobi *et al.*, 1973). Brunold and Suter (1989) showed that pea roots contained 50% less ATP-sulphurylase activity than shoots and also showed that the enzyme is exclusively or almost exclusively located within the proplastids. The enzyme has been found to be associated with the chloroplasts of spinach (Balharry and Nicholas, 1970) and maize

(Burnell, 1984). Two forms of ATP-sulphurylase were found in spinach leaf; the more abundant form (83 to 84% of the total activity) is present in the chloroplasts, the second (16 to 17% of the total activity) was found to be cytosolic (Lunn *et al.*, 1990). Schmutz and Brunold (1985) found that in maize leaves ATP-sulphurylase activity was present in the bundle sheath cells but not the mesophyll cells although Ghisi *et al* (1987) suggest that the enzyme is present in both cell types.

A light dependant increase in enzyme activity in maize leaves was demonstrated along with observations that the enzyme activity was greatest as the structural development of the plastids neared completion (Ghisi *et al.*,1987). Von Arb and Brunold (1985) found that ATP-sulphurylase activity was no longer measurable when pea and bean leaves were fully expanded.

Osslund *et al* (1982) suggest that in cabbage leaves the enzyme exists as an non-spherical dimer with a subunit molecular weight of 57,000. Gel filtration studies of ATP-sulphurylase from corn roots indicate a molecular weight of 42,000 (Onajobi *et al* , 1973).

Regulation of ATP-sulphurylase in cultured tobacco cells has been studied (Reuveny and Filner, 1977). These studies showed that the enzyme is repressed when cells are grown on readily assimilated sulphur sources, such as sulphate, L-cysteine or L-methionine, but it is derepressed during growth on slowly assimilated sulphur sources, such as L-djenkolate (L-S,S'-methylenebis cysteine) or glutathione, or during sulphur starvation. Derepression of cells starved of sulphur does not occur if the cells are also starved of nitrogen. It is suggested that regulation of ATP-sulphurylase in cultured tobacco cells is by both a negative feedback mechanism and a positive mechanism. Those

compounds which affect the development of the enzyme *in-vivo* had no effect on the *in-vitro* enzyme activity.

The assay of ATP-sulphurylase is made difficult by the extremely unfavourable equilibrium constant, which is given as about 10^{-8} by Robbins and Lipmann (1958), resulting in a lack of accumulation of the product. The forward and reverse reaction have both been used for the determination of the enzyme activity. Methods where the formation of $AP^{35}S$ from $^{35}SO_4^{2-}$ and ATP is measured are time consuming because of the necessary separation of $AP^{35}S$ from $^{35}SO_4^{2-}$ by high voltage paper electrophoresis (Ellis ,1969; Reuveny and Filner, 1976) or centrifugation (Reuveny and Filner, 1977). Also high amounts of radioactive SO_4^{2-} are required. The molybdate method (Wilson and Bandurski, 1958), where molybdate is substituted for sulphate, results in the formation of the unstable adenosylphosphomolybdate and pyrophosphate, with the pyrophosphate being hydrolysed by endogenous or added pyrophosphatase and the amount of phosphate produced taken as a measure of the ATP-sulphurylase activity. This method has the disadvantages that the wrong substrate is used, the hydrolysis of the pyrophosphate may be incomplete and any ATPases present in an extract will give a high background. The reverse reaction has been used in the spectrophotometric assay method of Burnell (1984). ATP produced in the reverse reaction can be measured using the luciferin-luciferase system (Balharry and Nicholas, 1970; Schmutz and Brunold, 1982a).

A technique for visualising ATP-sulphurylase after polyacrylamide gel electrophoresis has been described (Skyring *et al.*, 1972).

1.2.2 APS sulphotransferase

Adenosine 5'-phosphosulphate sulphotransferase (APS sulphotransferase, adenylyl-sulphate: thiol sulphotransferase) (EC number not assigned) (reviewed by Brunold and Suter, 1990) is a key enzyme in the proposed carrier-bound pathway of sulphate assimilation. It catalyses the transfer of the sulphogroup of APS, formed by ATP-sulphurylase, to a low molecular weight carrier thiol (Car-SH), forming "carrier-bound sulphite" ($Car\text{-}S\text{-}SO_3^-$):

$$APS^{2-} + Car\text{-}SH \longrightarrow Car\text{-}S\text{-}SO_3^- + AMP^{2-} + PPi$$

In *Chlorella* the carrier seems to be reduced glutathione (Tsang and Schiff, 1978), but remains unidentified in higher plants. Thiols, such as dithiothreitol, dithioerythritol or glutathione may be used as acceptors of the sulphonyl group of APS (Tsang and Schiff,1976).

Schmidt has detected APS sulphotransferase activity in more than 50 plant families (1975a,b;1976a,b). Other workers have also detected the enzyme in pea (Brunold and Suter, 1989), spinach (Fankhauser and Brunold, 1978), *Nicotiana sylvestris* (Jenni *et al.*, 1980), Norway spruce (Suter *et al.*, 1992; Schupp and Rennenberg, 1992; Bosma *et al.*, 1991) and maize and wheat (Schmutz and Brunold, 1982a). Enzyme activity has been demonstrated in both roots and leaves of pea, with roots containing 30% of the activity of shoots. Most of the activity in the root was found in the first centimetre, located within the proplastids (Brunold and Suter, 1989). Within the leaves of spinach activity is localised exclusively in the chloroplasts (Schwenn *et al.*, 1976; Fankhauser and Brunold, 1978).

The APS sulphotransferase from spinach has a molecular weight of 110,000 and a K_m for APS of 13μM (Schmidt, 1976b). This

compares with a molecular weight of 115,000 and K_m for APS of 29μM in Norway spruce (Suter *et al.*, 1992).

Germinating sunflower seeds contain low levels of activity which increase up to the 35th day of seedling growth (Schmidt 1976a). Similarly Norway spruce buds show low activity which increases during shoot development, parallel to the chlorophyll content (Suter *et al.*, 1992). Norway spruce shoots also show seasonal changes with APS sulphotransferase activity increasing in February and March. Enzyme activity in pea and bean leaves was no longer measurable in fully expanded leaves (Von Arb and Brunold, 1985). 5'AMP is a competitive inhibitor of the enzyme (Schmidt, 1976a,b). Changes in enzyme level appear to be more important in regulating the APS sulphotransferase step than variation in enzyme activity (Brunold and Suter, 1990). A 50-110% increase in extractable activity on a fresh weight basis in cultures of *Lemna* when grown with 2mM arginine, asparagine or glutamine was shown by ^{15}N-density labelling experiments to be due to increased enzyme synthesis, *de novo* (Suter *et al.*, 1986). Growth on reduced sulphur compounds decreases the extractable enzyme activity but has no effect *in-vitro*. (Schmidt 1976a; Suter *et al.*, 1992). These findings would explain higher levels of activity in developing leaves as cysteine and methionine would be used up in protein synthesis.

APS sulphotransferase activity has been determined by a method based on the formation of ^{35}S-sulphite from ^{35}S-APS in the presence of the artificial carrier, DTE. The ^{35}S-sulphite produced is liberated as $^{35}SO_2$ by the addition of acid and then trapped by a base. The trapped radioactivity is used a measure of APS sulphotransferase activity (Schiff and Levinthal,1968; Brunold and Suter 1990).

1.2.3 Thiosulphate reductase

Organic thiosulphate reductase (formerly called thiosulphonate reductase) catalyses the reduction of carrier bound sulphite to carrier bound sulphide using ferredoxin as electron donor, according to the following reaction

$$Car\text{-}S\text{-}SO_3^- + 6Fd_{red} + 6H^+ \longrightarrow Car\text{-}S\text{-}S^- + 6Fd_{ox} + 3H_2O$$

Thiosulphate reductase activity has been detected in *Chlorella* (Schmidt *et al.,* 1984), spinach (Schwenn and Hennies, 1974), and cultured tobacco cells (Hart and Filner, 1969). Tobacco cells can utilise thiosulphate as their only sulphur source (Hart and Filner, 1969).

Activity has been purified from *Chlorella fusca* and shown to consist of four distinct proteins of molecular weights 28, 26.5, 55 and 24kDa. All activities could use DTE (dithioerythritol) as electron donor (Schmidt *et al.,* 1984).

Thiosulphate reductase activity has been determined by measuring sulphide production from thiosulphate using DTE as electron donor (Schmidt *et al.,* 1984). Skyring and Trudinger (1972) suggest a modification of their native gel staining method for sulphite reductase which allows the detection of thiosulphate reductase.

1.2.4 APS kinase

APS kinase (ATP-adenylsulphate 3'-phosphotransferase, EC. 2.7.1.25) catalyses the formation of PAPS (adenosine 3'-phosphate 5'-phosphosulphate) in an ATP-dependent reaction:

$$APS + ATP \longrightarrow PAPS + ADP$$

PAPS is then used for sulphate reduction in "normal" bacteria, some photosynthetic bacteria, and some cyanobacteria, but not dissimilatory sulphate reducers (Roy and Trudinger, 1970). Some early studies showed no evidence for the formation of PAPS in plants (Asahi, 1964; Balharry and Nicholas, 1970; Ellis, 1969). However, APS kinase has now been identified in spinach (Burnell and Anderson, 1973), french beans and maize (Mercer and Thomas, 1969). In both these studies the enzyme was demonstrated to be present in the chloroplasts. PAPS is believed to serve in plants as the sulphate source for synthesis of sulphate esters and sulpholipids. It may also function as the activated sulphate source for sulphate assimilation if the free intermediate pathway exists.

APS kinase has not been purified from a plant source. Interference from 3'-nucleotidases in crude extracts has often resulted in inaccurate and misleading results when assaying for APS kinase activity. The method described by Burnell and Whatley (1975), which partially overcomes these problems involves the spectrophotometric determination of sulphate or APS-dependent production of ADP in the presence of pyruvate kinase and lactate dehydrogenase.

1.2.5 PAPS reductase

The demonstration of the existence of a thioredoxin-dependent PAPS reductase in spinach (Schwenn, 1989), cabbage (*Brassica pekinenis*) and tobacco cell cultures (J. Schwenn, unpublished) threw open the question of which pathway of sulphate assimilation operates in plants. PAPS reductase (PAPS sulphotransferase) (EC number not assigned) converts PAPS into sulphite and adenosine-3',5'-bisphosphate (3',5'-PAP):

$$PAPS + Thio(SH)_2 \longrightarrow SO_3H^- + 3',5'\text{-}PAP + H^+ + Thio(S)_2$$

If PAPS reductase is active in plants then it is possible that they reduce sulphate by a mechanism completely homologous to yeast and bacteria. Schwenn estimated the molecular weight of the enzyme to be 72,000. The sulphite produced by PAPS reductase would be metabolised by the ferredoxin dependent sulphite reductase.

PAPS reductase activity was determined by Schwenn (1989) as the thioredoxin dependent formation of [^{35}S] sulphite from [^{35}S]PAPS.

1.2.6 Sulphite reductase

In higher plants sulphite reductase (SiR) (EC 1.8.7.1), reviewed by von Arb (1990) catalyses the six electron reduction of free sulphide to free sulphite with reduced ferredoxin as physiological electron donor (Hennies, 1975; Aketagawa and Tamura, 1980; Krueger and Siegel 1982b; Hirasawa *et al.*, 1987):

$$SO_3^{2-} + 6Fd_{red} \longrightarrow S^{2-} + 6Fd_{ox} + 3H_2O$$

Hirasawa *et al.* (1987) suggest that SiR forms an electrostatic complex with ferredoxin similar to those formed by other ferredoxin dependant plant enzymes such as nitrite reductase and glutamate synthase. Schiff and Fankhauser (1981) suggested that SiR was not involved in the main sulphate reduction pathway but acts on SO_3^{2-} liberated from car-S-SO_3^- by thiols or produced from SO_2 taken up from a polluted atmosphere. However, if PAPS reductase is in fact involved in primary sulphate assimilation then SiR becomes part of the free intermediate pathway.

SiR has been detected in the leaves of spinach (Asada, 1967; Asada *et al.*, 1969; Schmidt and Trebst, 1969; Hennies, 1975; Krueger and Siegel, 1982a,b), pea (Mayer, 1967; von Arb and Brunold, 1983 and 1985), rape (Koguchi *et al.*, 1988), wheat (Sawhney and Nicholas, 1975; Schmutz and Brunold, 1984) and maize (Schmutz and Brunold, 1984 and 1985). It has been detected in the roots of barley (Mayer, 1967) and pea (Mayer, 1967; Brunold and Suter, 1989). Schmutz and Brunold (1985) showed SiR to be present in both the mesophyll and bundle sheath cells of maize leaves. Cell fractionation studies, suggest that most if not all of the leaf enzyme activity is located in the chloroplasts (Schmidt and Trebst, 1969; Mayer, 1967; Sawhney and Nicholas, 1975) and that at least 50% is tightly bound to the thylakoid membrane in peas (von Arb and Brunold, 1983). In roots the plastids are the suggested location (Brunold and Suter, 1989; Mayer, 1967).

SiR has been purified from a number of plant species, and purified to homogeneity from spinach. Aketagawa and Tamura (1980) reported the specific activity of the purified spinach enzyme to be 44.6 µmol sulphide formed per min per mg of protein with reduced ferredoxin as the electron donor. Aketagawa and Tamura (1980) and Krueger and Siegel (1982a) reported a subunit molecular

weight of between 69-71,000 with the enzyme capable of existing as a dimer of M_r 136,000 or a tetramer of M_r 270,000 depending on the ionic environment. Spinach SiR contains 1 mol of siroheme and one Fe_4S_4 centre per subunit (Krueger and Siegel, 1982a,b). Spinach SiR catalyses both the reduced ferredoxin and reduced methyl viologen-dependent six-electron reduction of sulphite and nitrite. However, the K_m of SiR for sulphite is at least 2 orders of magnitude less than for nitrite. Despite marked similarities between SiR and NiR they have been shown to be two distinct enzymes (Sawhney and Nicholas, 1975; Krueger and Siegel, 1982a; Schmutz and Brunold, 1985; von Arb and Brunold 1985). Rates of reduction with reduced ferredoxin as electron donor are greater than with methyl viologen.

A number of possible assay systems to measure SiR activity involve: i) formation of methylene blue from the sulphide produced directly in the reaction mixture (Siegel, 1965) or from H_2S after acidification (Tamura *et al.*, 1978); ii) estimation of the radioactivity of $H_2{}^{35}S$ formed from $^{35}SO_3{}^{2-}$ using Cd^{2+} solution as a distillation trap; iii) measurement of sulphide using a S^{2-} electrode (Ng and Anderson, 1979); iv) determination of the absorbance change of ferredoxin at 422nm or methyl viologen at 600nm (Krueger and Siegel, 1982a); and v) measurement of cysteine production in a coupled assay linked to cysteine synthase (von Arb and Brunold, 1983). The disadvantages of each of these methods has been summarised by von Arb and Brunold (1983). Skyring and Trudinger (1972) have discribed a method of specific activity staining in non denaturing polyacrylamide gels where the sulphide produced is precipitated as black FeS.

1.2.7 Cysteine synthase

Cysteine synthase (O-acetyl-L-serine sulphydrylase or O-acetyl-L-serine (thiol)-lyase, EC.4.2.99.8) catalyses the last step in both possible schemes for assimilatory sulphate reduction, the formation of L-cysteine from O-acetyl-L-serine (OAS):

Kinetic experiments have shown that this is the only way sulphur is incorporated into organic compounds in plants (Anderson, 1980; Giovanelli *et al.*, 1980).

In higher plants and cyanobacteria the name cysteine synthase usually, as throughout this thesis, refers only to O-acetyl-L-serine (thiol)-lyase and not the O-acetyl-L-serine (thiol)-lyase/serine acetyltransferase complex seen in *S. typhimurium*. It is generally believed that in higher plants no such complex formation exists, although Droux *et al.* (1992) report 3-5% of the total cysteine synthase activity in spinach leaf chloroplasts to be associated with serine acetyltransferase in a complex of molecular weight 310,000 kDa. The existence of such a complex would allow high levels of OAS to be maintained at the active site of cysteine synthase and could also prevent the release of pyridoxal phosphate from the enzyme. However, the matter is still open to question.

Cysteine synthases have been detected in, and at least partially purified from, a wide variety of plants. Two isoforms of the enzyme have been detected in spinach (Fankhauser and Brunold, 1979; Droux *et al.*, 1992; Saito *et al.*, 1992; 1993), *Quisqualis indica* var. *villosa* (Murakoshi *et al.*, 1986) and watermelon (*Citrullus vulgaris*) (Ikegami *et al.*, 1988a). Three forms of cysteine synthase were

reported in pea (Ikegami *et al.*,1987) and spinach (Lunn *et al.*, 1990). Only a single form of the enzyme was found in barley (Rosichan *et al.*, 1983) and in wheat (Ascano and Nicholas, 1977) leading to the suggestion that grasses contain only a single isoenzyme.

Localisation studies have shown 68-86% of the total cysteine synthase activity in peas and white clover (*Trifolium repens*) to be associated with the chloroplast stroma (Ng and Anderson, 1978a) and at least 20% to be within the chloroplasts and proplastids of spinach shoots and roots respectively (Fankhauser and Brunold, 1979). The enzyme was found mainly in the chloroplast and cytosolic fractions from spinach protoplasts but some activity was found in the mitochondrial fraction of spinach and pea leaves (Lunn *et al.*,1990). Droux *et al.* (1992) found that greater than 98% of the activity associated with the chloroplasts of spinach was in the soluble protein fraction. In cauliflower buds cysteine synthase was reported in the cytosol, proplastids and mitochondria (Rolland *et al.*, 1992), although Fankhauser *et al.*, (1976) found no appreciable levels of activity associated with the mitochondria or peroxisomes. Cysteine synthase was purified to homogeneity from the fruit chromoplasts (pigmented plastids) of bell pepper (*Capsicum annus*) (Romer *et al.*, 1992).

Cysteine synthase has two identical subunits each containing one molecule of pyridoxal phosphate. The values given for the molecular weight of the subunits varies between 26,000 in pea and *Brassica juncea* (Ikegami *et al.*, 1987 and 1988b) to 35,000 in spinach (Droux *et al.*, 1992) and bell pepper (Romer *et al.*, 1992). In *Quisqualis indica* and *Citrullus vulgaris* the subunit molecular weight of both isoforms was reported as 29,000kDa (Murakoshi *et al.*, 1986 and Ikegami *et al.*, 1988a). In recent studies, full length cDNA clones for cysteine synthase have been isolated. The deduced amino-acid

sequences of clones from spinach (Saito *et al.*, 1992) and *Capsicum annum* (Romer *et al.*, 1992) showed 53% and 56% homology respectively with the cysteine synthases from both *E. coli* and *S. typhimurium*.. Hell *et al.* (1993) reported 70 to 77% amino acid homology between cysteine synthase from spinach and from *Capsicum annum* chromoplasts. Sequence comparison of a cDNA from spinach leaf chloroplasts with several pyridoxal phosphate containing proteins revealed the presence of a lysine residue assumed to be involved in co-factor binding (Rolland *et al.*, 1993).

Cysteine synthase is specific for O-acetyl-L-serine; other derivatives including L-serine, L-homoserine and N-acetyl-serine are not substrates (Schmidt, 1977a,b; Droux *et al.*, 1992). Cysteine synthases from selenate accumulator and non-accumulator species catalyse the synthesis of selenocysteine when sulphide is replaced by selenide (Ng and Anderson, 1978b). Some isoforms of cysteine synthase have been shown to catalyse the formation of particular heterocyclic β-substituted alanines e.g. mimosine, quisqualic acid (Ikegami *et al.*, 1987, 1988a, 1990; Murakoshi *et al.*, 1986). Cyanide can be used by some isoforms of the enzyme to form β-cyano-L-alanine (Ikegami *et al.*, 1987, 1988a,b; Schmidt, 1990). Rosichan *et al.* (1983) reported cysteine synthase from barley accepts sodium azide to form the mutagenic metabolite β-azido-alanine.

Cysteine synthase activity in the plastids of bell pepper increased during fruit development with the highest levels of activity in the chromoplasts of red fruits (Romer *et al.*, 1992). It has been suggested that cysteine synthase is up-regulated during chromoplast development as part of an active program of antioxidant production during fruit ripening. Sulphur nutrition did not affect cysteine synthase levels in tobacco cells but specific activity declines in cell

depleted of nitrogen, increasing when nitrogen is added to nitrogen-depleted cells (Smith, 1980). These results support those of Reuveny and Filner (1977) who suggested that regulation of sulphate assimilation was coupled to nitrate assimilation.

In *in-vitro* enzyme studies the addition of pyridoxal 5'-phosphate seemed to protect the enzyme from inactivation (Rolland *et al.*, 1992) possibly by preventing co-factor loss as suggested by Bertagnolli and Wedding (1977). Pretreatment with cysteine inactivated the purified clover enzyme and although the normal activity was not enhanced by the addition of 0.1-1mM pyridoxal 5'-phosphate, preincubation of the inactivated enzyme with 0.1mM pyridoxal 5'-phosphate restored activity to 23% of the original (Ng and Anderson, 1978a).

Cysteine synthase activity is most usually measured by the spectrophotometric determination of cysteine as a red ninhydrin complex (Gaitonde, 1967). At non physiological pH's, above 9, the back reaction of the enzyme is favoured where sulphide is produced from cysteine and acetate. This back reaction can be used as a method of specific staining for cysteine synthase on native gels (Schmidt. 1990).

1.2.8 Serine acetyltransferase

O-acetyl-L-serine is the product of the acetylation of serine, a reaction catalysed by the enzyme serine acetyltransferase or serine transacetylase (EC.2.3.1.30)

O-acetyl-L-serine is the substrate for cysteine synthase as described previously.

The enzyme has been demonstrated in a number of plant species; *Phaseolus vulgaris* (Smith, 1972; Smith and Thompson, 1971), wheat (Ascano and Nicholas, 1977), cauliflower inflorescence (Rolland *et al.*, 1992), rapeseed (Ngo and Shargool, 1974; Nakamura *et al.*, 1987) and spinach leaves (Brunold and Suter, 1982; Lunn *et al.*, 1990 and Droux *et al.*, 1992).

Recent studies in *Brassica oleracea* (Rolland *et al.*, 1992) and spinach leaves (Lunn *et al.*, 1990) suggest that serine acetyltransferase is present in all cell compartments, chloroplasts, plastids, cytosol and mitochondria. Droux *et al.* (1992) found greater than 98% of the serine acetyltransferase located within the chloroplast was in the soluble protein fraction. Between one and two thirds of total activity in *Phaseolus vulgaris* was reported to be present in the mitochondria (Smith, 1972).

Serine acetyltransferase has been shown to exist in a complex with cysteine synthase in the bacterium *Salmonella typhimurium* (Kredich *et al.*, 1969). This bifunctional protein complex of molecular weight 309,000 consists of 1 molecule of serine acetyltransferase (molecular weight 160,000) and 2 molecules of cysteine synthase (molecular weight 68,000). The evidence for and against the existence of a serine acetyltransferase/cysteine synthase complex in higher plants is conflicting. In gel filtration studies on rape leaves (Nakamura *et al.*, 1988) and spinach (Droux *et al.*, 1992) the enzyme eluted at positions corresponding to molecular weights of 350,00 and 310,000 respectively suggesting a complex as seen in *S. typhimurium*. The cysteine synthase activity of the proposed complex in rape was very low and the ratio of cysteine synthase to serine

acetyltransferase activity was extremely low compared to that in *Salmonella*. Unlike Nakamura *et al.*, (1988), Bertagnolli and Wedding (1977) found no complex activity in *Phaseolus*. Smith and Thompson (1971) showed serine acetyltransferase purified 50 fold from bean seedlings was free from cysteine synthase activity; with the enzyme being specific for serine, not utilising either homoserine or threonine as substrates. L-cysteine and CoA inhibited the enzyme but pyridoxal phosphate had no effect on the activity. Cysteine synthase purified from wheat was shown to be devoid of serine acetyltransferase activity (Ascano and Nicholas, 1977).

Serine acetyltransferase activity can be measured using a cysteine synthase coupled assay method (Nakamura *et al.*, 1987); as the appearance of thionitrobenzoic acid, formed by a disulphide interchange between CoA liberated from acetyl-CoA and 5'5'-dithiobis(2-nitrobenzoic acid) (Ellmans reagent) (Kredich and Tomkins, 1966) or as ^{14}C labelled N-acetylserine (Smith and Thompson, 1969).

1.3 Selenium and it's metabolism by higher plants

Selenium is a naturally occurring analogue of sulphur. It is found widely distributed in the soils and rocks of the earth's crust. The anions of selenium, selenate (SeO_4^{2-}) and selenite (SeO_3^{2-}) are toxic to most higher plants. Only a limited number of species, the selenate accumulators or selenate indicators, many of which belong to the genus *Astragalus*, tolerate high endogenous levels of selenium compounds. Selenate accumulator plants may contain tissue levels of several thousand μg selenium/g (reviewed in Brown and Shrift, 1982). Selenium interactions in the sulphur metabolism of higher plants has recently been reviewed by Anderson (1993).

Selenate and selenite are readily absorbed from the soil by the roots and translocated to other parts of the plant. Selenate and sulphate are taken up and transported via the sulphate transport system in barley (Ferrari and Renosto, 1972). ATP sulphurylase has been shown to activate selenate to form APSe (Burnell, 1981; Wilson and Bandurski, 1958). From here the mechanism for the reduction of APSe to selenite is uncertain. Ng and Anderson (1978b) suggest that selenite is reduced to selenide (Se^{2-}) in the chloroplasts in a light-dependant reaction involving glutathione reductase. The findings of Bosma *et al.* (1991) that sulphite reductase cannot reduce selenite supports this non-enzymic theory. Cysteine synthases from selenate accumulator and non-accumulator species have been shown to catalyse the synthesis of selenocysteine when sulphide is replaced by selenide (Ng and Anderson, 1978b). Selenocysteine can then be converted to selenocystathionine and probably via the transsulphuration pathway to selenomethionine (Brown and Shrift, 1982; Dawson and Anderson, 1988).

The toxic effects of selenium have been attributed to the incorporation of Se-containing amino acids into proteins causing structural changes which affect protein function (Brown and Shrift, 1980; Burnell, 1981). Enzymic dysfunction and plant death follow.

Selenium tolerant accumulator plants synthesise methylselenocysteine and selenohomocysteine, two selenoamino acids rarely detected in non-accumulators. These cannot act as substrates for cysteinyl tRNA synthetase and are therefore excluded from incorporation into protein (Burnell, 1981).

Sensitivity to seleno-amino acids has been used to isolate mutant cultured plant cell lines altered in their cystine and sulphate uptake (Flashman and Filner, 1978; Furner and Sung, 1983).

1.4 Selection of barley plants deficient in sulphate assimilation

1.4.1 Mutant analysis

The isolation and characterisation of mutants has been used to study the genetic and biochemical regulation of metabolic pathways in micro-organisms, where large populations can be screened relatively easily for individuals lacking specific enzyme activities or products. The selection of mutants in higher plants (reviewed by Blonstein and King, 1986) is more difficult due to greater genetic and complexity and longer generation times. In order to overcome these problems it is necessary to induce high mutation frequencies within a plant population and to be able to identify the desired mutant phenotype amongst the wild type individuals.

High frequencies of mutations can be produced in populations of leaf protoplasts or cell suspensions using chemical mutagens (ethylmethane sulphonate (EMS), N-methyl-N'-nitro-nitrosoguanidine and N-ethyl-N-nitrosourea) or ionising radiation (X-rays and gamma-rays). One disadvantage of selecting mutants in cell culture is that it may not be possible to regenerate whole, fertile plants making it impossible to study the mutation by conventional genetic analysis or to study its effect on the physiology and biochemistry of the intact plant. Another disadvantage is the possibility that some mutations may not be expressed in the artificial environment of cell tissue culture.

The chemical mutagens, EMS and sodium azide, and ionising radiation can induce homozygous recessive mutations with the second (M_2) generations of self pollinating plants treated in the first (M_1) generation (Blonstein and King, 1986). Sodium azide has been used in

the study described here to induce homozygous mutations in populations of barley.

1.4.2 Mutagenic action of sodium azide

Inorganic azide (N_3^-) is converted to the mutagenic organic metabolite, β-azidoalanine by the enzyme cysteine synthase (O-acetyl serine(thiol)lyase) as described previously (section 1.2.7)(Rosichan *et al.*, 1983). Azide appears to act as a base substitution mutagen and is dependant on defective excision repair (Kleinhofs and Smith, 1976).

1.4.3 Whole plant mutant selection

Selection procedures need to be simple, quick and inexpensive to allow large numbers of individuals to be tested. The easiest way to screen a population for a mutant phenotype is to apply positive selection, that is a screen which kills wild type plants and allows only the desired mutant to survive.

For example the use of a toxic analogue where mutations confer resistance to the toxic effects of the analogue or a metabolic derivative of it, makes identification of the mutant phenotype simple. This approach has been used successfully to isolate nitrate reductase deficient mutants in barley using chlorate as a positive selection agent (Wray, 1986). In this example nitrate reductase reduces chlorate to the toxic compound chlorite which accumulates and kills the plant. Mutants lacking a functional nitrate reductase are chlorate resistant and can easily be identified.

1.4.4 Selection of barley plants deficient in sulphate assimilation

As selenate is known to be toxic it is suggested that it may be used as the positive selection agent in a screen to identify individual barley plants, which, for whatever reason, lack a complete, functional sulphate assimilation pathway. A wide range of mutations are likely to lead to selenate resistance including those altering sulphate uptake and the structural and/or regulatory *loci* for each enzyme. Even if both of the possible pathways are active in higher plants such a screen should identify individuals with mutations affecting steps common to both pathways; sulphate uptake, ATP sulphurylase, serine acetyltransferase and cysteine synthase. Mutations in steps unique to either pathway would still be selectable if the *in-vivo* activity of the pathway containing the mutation was much greater than the *in-vivo* activity of the pathway in which the mutation was not located. In the same way mutations at steps with multiple isoenzymes could be identified if one isoenzyme has a far greater activity *in-vivo*. An example of this would be selection of chlorate resistant mutants in *Arabidopsis thaliana* (Cheng *et al.*, 1988; Wilkinson and Crawford,1991). Arabidopsis has at least two functional nitrate reductase genes (*NIA1* and *NIA2*) which contribute different amounts to the total nitrate reductase activity. Mutation of the *NIA1* gene eliminates only a small fraction of the total activity and does not noticeably affect chlorate sensitivity. However, mutation of the *NIA2* gene removes most (90%) of the nitrate reductase activity, enough so that the plants are resistant to chlorate and can be selected from the positive screen. Anthranilate synthase, an enzyme involved in tryptophan biosynthesis, is another example of an enzyme in

Arabidopsis encoded by two genes where one is expressed at 10-fold higher levels than the other (Niyogi and Fink, 1991).

The toxicity of seleno-amino acids has been used to isolate mutant cell culture lines altered in cysteine uptake (Flashman and Filner, 1978). Selenate-resistant mutants of *Streptomyces* defective in sulphate uptake have been isolated (Lydiate *et al.*, 1988).

1.5 Aims

The aims of the work described here were:

i) To isolate mutant barley plants defective in sulphate assimilation, *i.e.* individuals altered in a gene which affects the normal functioning of the pathway.

ii) Genetical characterisation of the mutants to provide information on the genetic *loci* involved in determining the individual steps of the pathway and on the mode of inheritance of the mutation (whether it is nuclear or organelle encoded).

iii) Biochemical characterisation of the mutants to provide evidence for the pathways of sulphate and selenium metabolism, and information on the regulation of sulphate assimilation in barley and it's regulatory coupling to the nitrate assimilation pathway.

iv) To develop a fuller understanding of the biochemistry of sulphate assimilation in wild type barley, it's regulation and localisation of the pathway enzymes.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

All biochemicals and common chemicals used were of standard laboratory grade unless otherwise stated. Where appropriate, chemical formulae and common names used in the text are given.

Acetyl-coenzyme A, adenosine-3'-phosphate-5'-phosphosulphate (PAPS), adenosine-5'-phosphosulphate (APS), 1-amino-2-naphthol-4-sulphonic acid (8-anilino-1-naphthalenesulphonic acid) (ANSA), barium chloride ($BaCl_2.2H_2O$), bis[2-hydroxyethyl]iminotris-[hydroxymethyl]-methane (bis-tris), Coomassie brilliant blue G-250, L-cysteine HCl, cytochrome C type III (equine), DEAE sephadex A50, 1,1'-dimethyl-4-4'-bipyridium chloride (methyl viologen), 6,6' dithiodinicotinic acid, dithiothreitol (DTT), ferrous sulphate ($FeSO_4.7H_2O$), Fiske-SubbaRow reducer, glutamine, glycine, hydrogen peroxide 30% solution, lead acetate, L-malic acid, 2-(N-morpholino)ethanesulphonic acid (MES), N,N-dimethyl-p-phenylenediamine sulphate (DPD), O-acetyl-L-serine (OAS), Percoll, pyridoxal phosphate, Sephadex G100-120, sodium bisulphite ($NaHSO_3$), sodium sulphite (Na_2SO_3), sorbitol, tricine, zinc chloride ($ZnCl_2$) were obtained from Sigma Chemical Co. Ltd. Poole, Dorset, England.

Acetic acid, acrylamide, ammonium persulphate, calcium chloride ($CaCl_2.6H_2O$), copper chloride ($CuCl_2.2H_2O$), copper sulphate ($CuSO_4.5H_2O$), ethylenediaminetetra acetic acid, disodium salt (Na_2EDTA), ferric monosodium salt (NaFe EDTA), ethanol, ferric chloride ($FeCl_3$), glycerol, hydrochloric acid "AristaR" grade, magnesium chloride ($MgCl_2.6H_2O$), manganese sulphate ($MnSO_4.5H_2O$), methanol, N'-methylene bisacrylamide, β-

mercaptoethanol, ninhydrin, N-1-naphthylethylenediamine dihydrochloride (NED), N,N,N',N'-tetramethyl-ethylenediamineethane (TEMED), orthophosphoric acid "AristaR" grade, potassium chloride, potassium phosphate (KH_2PO_4/K_2HPO_4), potassium nitrate (KNO_3), potassium nitrite (KNO_2), potassium sulphate (K_2SO_4), sodium azide (NaN_3), sodium dithionite ($Na_2S_2O_4$), sodium dodecyl sulphate (SDS), Sodium selenate ($NaSeO_4$), sodium sulphide (Na_2S), sucrose "AnalaR" grade, sulphanilamide, sulphuric acid, trichloroacetic acid (TCA), zinc sulphate ($ZnSO_4$), were obtained from British Drug Houses Ltd., Poole, Dorset, England.

Ammonium molybdate ($(NH_4)_6Mo_7O_{24}.4H_2O$), bromophenol blue, sodium molybdate ($Na_2Mo_4.2H_2O$), were obtained from Fisons Ltd., Loughborough, Leicestershire, England.

Ammonium sulphate ($(NH4)_2SO_4$) (Pronalys AR grade), boric acid, magnesium sulphate ($MgSO_4.7H_2O$), sodium bicarbonate, sodium cyanide (NaCN),were obtained from May and Baker Ltd., Dagenham, England.

Adenosine-5'-triphosphate (ATP), bovine serum albumin (fraction V) (BSA), inorganic pyrophosphatase (PPase), tris(hydroxymethyl)methylamine, were obtained from Boehringer Mannheim, GmbH, West Germany.

Digitonin was obtained from Aldrich Chemical Company Ltd., Gillingham, Dorset and gelatin from Oxoid. Cellulase and macerozyme, both produced by Yakult, Honsha Company Ltd., Tokyo, Japan were obtained through a UK supplier.

2.1.2 Seed and plant growth materials

Barley (*Hordeum vulgare*) seed was obtained from William Watt, Seed Merchants, Cupar, Scotland and the Scottish Crop Research Institute, Invergowerie, courtesy of Dr. W. Thomas.

M_1 barley seed, cultivars Golden Promise, Klaxon, Tweed, Natasha, Vista, Doublet, Digger, Corniche, (azide treated as described in section 2.2.1.1) was grown in field plots at the Scottish Crop Research Institute and M_2 seed collected with a combine harvester courtesy of Dr.W. Thomas.

Vermiculite was obtained from Hoben Davis Ltd., Newcastle, Staffs, England. Potting compost (Fisons M2) was supplied by the local Botanic Gardens.

2.2 Methods

2.2.1 Mutant production, isolation and maintenance

2.2.1.1 Barley seed mutagenesis

Wild type seeds of barley were mutagenised by chemical mutagenesis using the procedure described by Kleinhofs *et al.* (1978). Seed of various cultivars were hydrated in trays of tap water at 0-4°C for 16 hours and then transferred to 10 litre flasks containing 3-4 litres of tap water which was aerated vigorously for a further 8 hours at room temperature. The flasks were placed in a fume cabinet and the water was replaced with 3 litres 0.1M potassium phosphate buffer pH 3 and sodium azide to give a final concentration of 2mM. After aeration of this solution for a further 2 hours, the seeds were washed in several changes of tap water for 30 minutes, blotted to remove excess liquid and air dried.

2.2.1.2 Growth of barley seedlings on different nitrate and sulphate regimes

Barley cultivar Golden Promise wild type seed were sown in vermiculite in trays (21.2 x 16.7cm). 100 seed were sown per tray. Trays of seed were treated with nutrient solution based on that of Hoagland and Arnon (1938). The basic medium used contained nitrogen as 20mM KNO_3 and sulphur as 1mM $MgSO_4.7H_2O$, 0.4µM $ZnSO_4.7H_2O$ and 0.2µM $MnSO_4.5H_2O$. It also contained 0.2mM NaFe EDTA, 0.5mM KH_2PO_4, 0.02mM H_3BO_3, 0.05µM $Na_2MoO_4.2H_2O$. For sulphate-free medium (-SO_4) 1mM $MgCl_2$, 0.4µM $ZnCl_2$ and 0.2µM MnCl replaced the sulphur salts whilst nitrate-free medium (-NO_3) did not have KNO_3 added.

Sulphate- and nitrate-free media (-SO_4 -NO_3) lacked 20mM KNO_3 and chloride salts replaced the sulphate salts as above.

Seeds were germinated in the light (115μE.m^2.s^{-1}) at 25°C and examined 14 days after sowing.

2.2.1.3 Selecting the conditions for the selenate screen

Golden Promise wild type seed (100) was sown in trays (21.2 x 16.7cm) of vermiculite and watered with distilled water during germination in the dark at 25°C. After 3 days the trays were transferred to the light and treated with one of the four modified half-Hoaglands nutrient solutions described above. On the 5th day after sowing sodium selenate was included in the nutrient solutions at a range of concentrations. Watering was carried out from above with a sprinkler or by immersing the trays in an outer tray containing the appropriate nutrient solution. Growth of the seedlings was monitored. Seedling height and weight were recorded on day 13.

2.2.1.4 Screening for selenate resistant barley seedlings

M_2 seed of a number of barley cultivars (mutagenised with azide in the M_1) was sown in trays of vermiculite to give a germination rate of about 200 seedlings per tray, and watered with modified half-Hoaglands nutrient solution lacking both sulphate and nitrate (-SO_4-NO_3) during germination in the light (115μE.m^{-2}.s^{-1}) up to day 5. From day 6 the seedlings were treated with the same -SO_4-NO_3 nutrient solution containing either 0.2, 0.5 or 1mM sodium selenate on alternate days. Seedlings were assessed on day 14 after sowing by comparison to a tray of control M_2 seedlings put through the same regime as above without the addition of selenate.

2.2.1.5 Maintenance of selected seedlings

After selection seedlings were transferred individually into pots of compost and grown on in the glasshouse. Seedlings were watered with tap water and monitored regularly.

Some selections were later transferred from compost into hydroponic culture. The roots of seedlings were washed free of compost. Seedlings were then supported in small pots of gravel with their roots protruding into a sulphate-free hydroponics solution containing (in 1 litre), K_2HPO_4 (230mg), NaFeEDTA (47mg), $MgCl_2.6H_2O$ (305mg), $CaCl_2.6H_2O$ (273mg), H_3BO_3 (7.5mg), $(NH_4)_6Mo_7O_{24}.4H_2O$ (0.67mg), $MnCl_2.4H_2O$ (3.5mg), $ZnCl_2$ (0.313mg), $CuCl_2.2H_2O$ (0.409mg) and KCl (6.75mg) with Bis Tris (1.046g), KNO_3 (0.1101g) and cysteine (0.1756g). In some cases cysteine was replaced with Na_2SO_4 (0.134g).

2.2.2 Growth of barley seedlings for tissue extraction

Wild type barley seedlings used in the biochemical analysis were grown in trays of vermiculite and treated with modified half Hoaglands nutrient solution lacking nitrate. The seeds were germinated in the dark at 25°C for four days then transferred to the light ($115\mu E.m^{-2}.s^{-1}$) for two days.

2.2.3 Enzyme assays

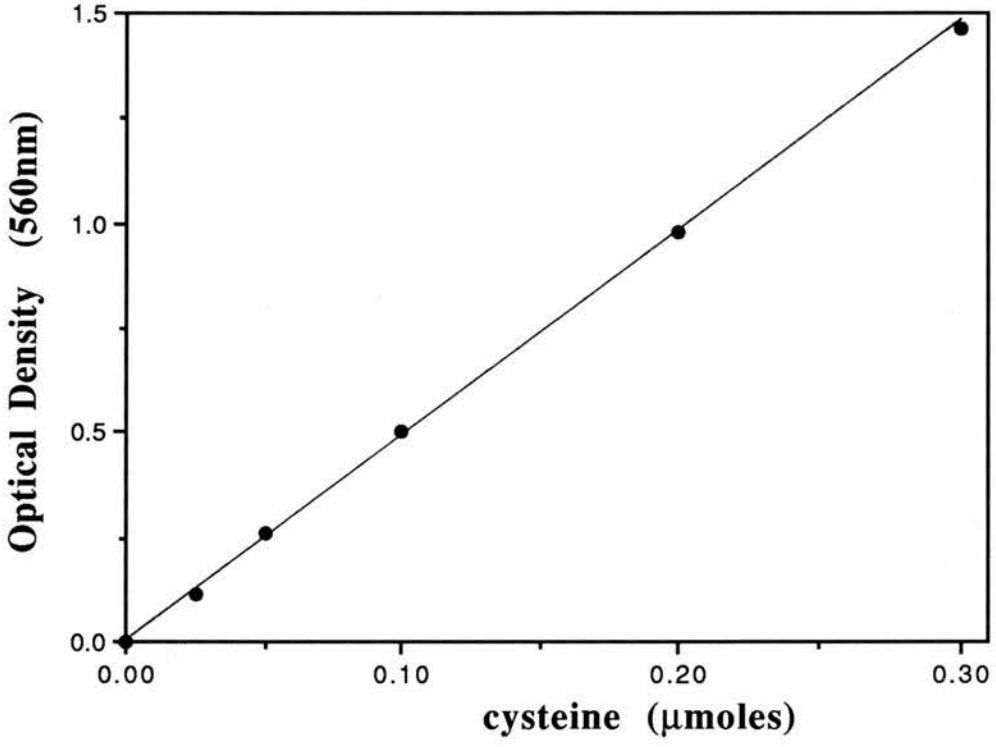
2.2.3.1 Cysteine synthase

Cysteine synthase activity was assayed using a modification of the method of Ng and Anderson (1978a). Barley tissue was ground in a mortar with 0.1M potassium phosphate buffer, pH 7, 1mM EDTA and 1mM DTT. The tissue to buffer ratio was 1:10 (wt:vol). The homogenate was centrifuged at 15,000 rpm in a SS34 rotor of a Sorval RC5 centrifuge for 15 minutes and the supernatant decanted and used as the enzyme source. All procedures were carried out at 4°C.

The assay mix contained 0.2M potassium phosphate pH 7.8, 2mM EDTA, 2mM DTT, 15mM O-acetyl-L-serine, 6mM Na_2S and enzyme extract (usually 30µl) in a final volume of 1ml. The assay, initiated by the addition of the Na_2S was performed at 25°C for 15 minutes, and terminated by the addition of 0.2ml 1.5M TCA. Controls were performed by withholding the enzyme extract until after the addition of the TCA.

Cysteine content was then determined using the method of Gaitonde (1967). 0.5ml of the assay solution was transferred to a test-tube and 0.5ml glacial acetic acid and 0.5ml acid ninhydrin reagent (made up fresh daily in the proportions 0.25g ninhydrin : 6ml acetic acid : 4ml conc. HCl, and mixed for at least 30 minutes to give a solution) were added. Tubes were vortexed, covered with glass marbles and placed into a boiling water-bath for 10 minutes. After rapid cooling of the tubes with tap water 3.5ml 96% ethanol was added to each and mixed by vortexing. The optical density was read at 560nm within 1 hour and the cysteine content determined from a calibration curve (0 - 0.3µmols cysteine, Fig 2.1).

Fig 2.1 Cysteine standard curve



The plot shows a typical standard curve demonstrating the direct relationship between standard amounts of cysteine and optical density at 560nm over the range 0 - 0.3μmols cysteine, after determination of cysteine by the method of Gaitonde (1967).

2.2.3.2 β-Cyanoalanine synthase

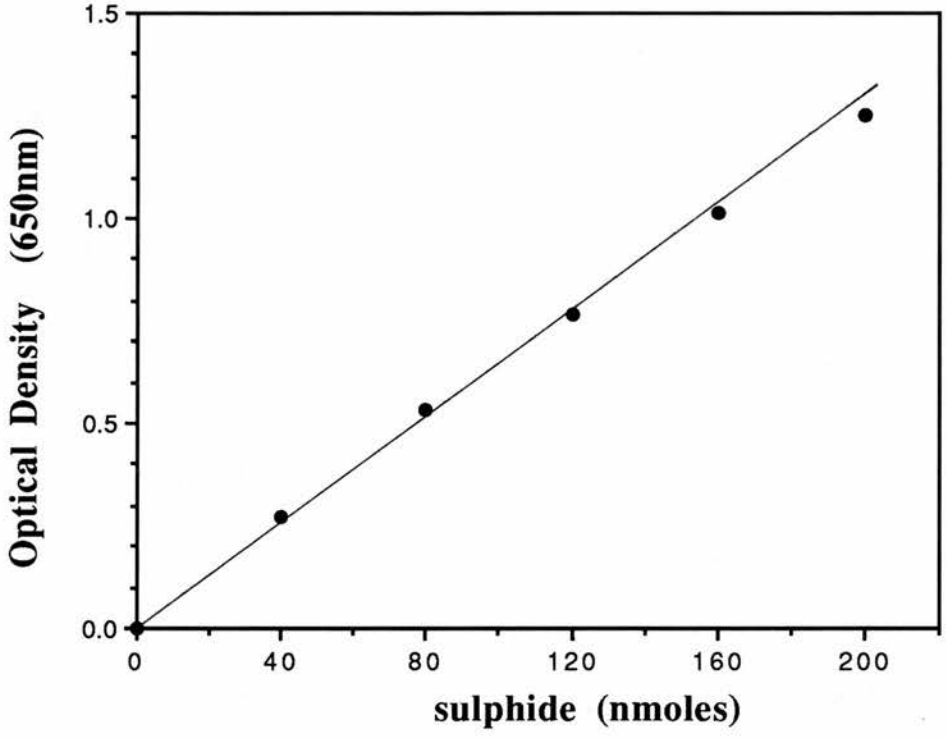
β-Cyanoalanine synthase (BCS) activity was assayed using a modification of the method of Miller and Conn (1980). The sulphide produced in the assay was determined by the method of Siegel (1965).

Tissue was extracted in Tris-HCl buffer pH 8.5 (1g/10ml) and centrifuged at 15,000 rpm in the SS34 rotor of a Sorval RC5 centrifuge at 4°C for 15 minutes to obtain the enzyme extract, as for cysteine synthase. 1ml enzyme extract (diluted with extraction buffer as necessary, usually 1 in 10) was pre-incubated at 30°C for 1 minute. 0.5ml 50mM NaCN in Tris-HCl buffer pH 8.5 was then added followed immediately by 0.2ml 10mM cysteine HCl in Tris-HCl buffer pH 8.5. Tubes were stoppered, shaken, and after incubation at 30°C for 20 minutes the reaction was terminated by the addition of 0.5ml DPD reagent (0.02M N,N-dimethyl-p-phenylene diamine sulphate in 7.2M HCl) and 0.5ml 0.3M $FeCl_3$ in 1.2M HCl. The tubes were restoppered, vortexed and left to stand at room temperature for 20 minutes for the colour to develop. The tubes were opened and left for at least 30 minutes in a closed fume hood (to remove any CN gas evolved after the addition of the acid reagents) before centrifuging in a microfuge to remove precipitated protein. The optical density was read at 650nm within 1 hour and the sulphide content determined from a standard curve (0 - 200nmols sulphide, Fig 2.2).

2.2.3.3 Thiosulphate reductase

Thiosulphate reductase activity was assayed using the method of Schmidt *et al.* (1984), by following sulphide production from

Fig 2.2 Sulphide standard curve



The plot shows a typical standard curve demonstrating the direct relationship between standard amounts of sulphide and optical density at 650nm after determination of sulphide by the method of Siegel (1965), over the range 0 - 200nmols sulphide.

thiosulphate using DTE as the electron donor. Sulphide was determined according to the method of Siegel (1965).

Tissue was extracted in Tris-HCl buffer, pH 8.5 (1g/10ml) and centrifuged at 15,000 rpm in the SS34 rotor of a Sorval RC5 centrifuge at 4°C for 15 minutes to obtain the enzyme extract. The reaction mixture consisted of 0.1M Tris-HCl buffer pH 9.0, 2mM DTE, 0.2mM $Na_2S_2O_3$ and enzyme extract in a total volume of 1ml. The reaction was started by the addition of $Na_2S_2O_3$ and incubated at 37°C for 30 minutes. The reaction was stopped by the addition of the reagents for sulphide determination (0.5ml DPD reagent (0.02M N,N-dimethyl-p-phenylene diamine sulphate in 7.2M HCl) and 0.5ml 0.3M $FeCl_3$ in 1.2M HCl). The reaction mixture and sulphide determination reagents were mixed well by vortexing and left to stand at room temperature in the dark for 20 minutes for the colour to develop. The optical density was read at 650nm within 1 hour and the sulphide content determined from a standard curve (0 - 200nmols sulphide, Fig 2.2). Tubes were kept stoppered throughout the sulphide determination to minimise any loss.

2.2.4 Non-denaturing polyacrylamide gel electrophoresis

Tube gels (85 x 5mm) contained 10% (w/v) acrylamide and 0.27% (w/v) methylene-bis-acrylamide in 0.1M Tris-glycine buffer pH 8.9, 240µl of 250mg/ml ammonium persulphate and 0.025% (v/v) TEMED. The reservoir buffer was 0.1M Tris-glycine buffer pH 8.9. The gels were pre-equilibrated for 30 minutes prior to sample loading at constant voltage with an initial current of 2-3mA/gel. Samples (up to 200µl tissue extract, 200µg protein) were mixed with 20µl loading buffer (0.002% bromophenol blue, 10% glycerol) before loading onto the gel. Electrophoresis was performed at 4°C overnight at a constant voltage with an initial current of 0.5mA/gel.

2.2.5 Gel staining for specific enzyme activity

2.2.5.1 Cysteine synthase

A method for staining native gels for cysteine synthase activity was developed based on the back reaction of the enzyme at pH's above 9, which forms O-acetyl-L-serine and free sulphide from cysteine. The position of the sulphide was visualised by it's precipitation as brown/black lead sulphide (A. Schmidt, pers. comm. to J.L. Wray and Schmidt, 1990).

Gels were loaded with extract from tissue homogenised as for the solution assay in 0.1M potassium phosphate buffer pH 7, 1mM EDTA and 1mM DTT (Ng and Anderson, 1978a) and electrophoresis carried out as described above. After electrophoresis gels were removed from the tubes by rimming with distilled water and placed immediately into 100 x 13mm screw-top tubes containing 5ml 0.1M Tris-glycine buffer, pH 10, 1mM lead

acetate and 15mM cysteine HCl (the lead acetate must be dissolved in the buffer before the cysteine is added and the pH adjusted). Gels were incubated at room temperature with continuous agitation for at least 2 hours.

Cysteine synthase activity appears as a brown/black band on a clear gel.

2.2.5.2 β-cyanoalanine synthase

The gel staining method used for cysteine synthase was modified to detect the enzyme β-cyanoalanine synthase which catalyses the formation of β-cyanoalanine from cysteine and cyanide (Blumenthal *et al.*, 1968; Hendrickson and Conn, 1969; and Miller and Conn, 1980).

Tissue was extracted and gels loaded, electrophoresed and removed as for cysteine synthase. The incubation mix was the same as for cysteine synthase with 0.5mM NaCN added. β-cyanoalanine synthase activity appeared as a heavy brown/black band on a clear gel. As the substrates for the cysteine synthase back reaction are present a band of cysteine synthase activity would also be expected. A second paler band also appeared at a position corresponding to cysteine synthase. Cysteine synthase activity bands appear more rapidly in the presence of NaCN (A. Schmidt, pers. comm. to J.L. Wray).

2.2.5.3 Sulphite reductase

Sulphite reductase (SiR) activity in gels was visualised using the method of Skyring and Trudinger (1972). Tissue was extracted 1g in 10ml 0.1M Tris HCl buffer pH 8, and the gels were loaded and electrophoresed as before.

The incubation mix, consisting of 0.1M Tris-HCl buffer pH 6.8, 2mM $FeSO_4.7H_2O$ and 0.12% methyl viologen, was degassed under vacuum in a desiccator for approx. 5 minutes then the desiccator chamber was flooded with oxygen-free nitrogen. The degassed solution was poured immediately into 100 x 13mm screw-top tubes, 9ml per tube (filled to overflowing), and 200mg reduced iron powder was added to each. Tubes were sealed, shaken well and placed onto a rocking/rolling table for 10-15 minutes until the methyl viologen was reduced to a deep blue colour. The tubes were then opened and the gel inserted followed by 13mg anhydrous Na_2SO_3 (resulting in a final concentration of 14mM Na_2SO_3 in the incubation mix). The tubes were resealed and returned to the rocking/rolling table to incubate at room temperature for 2.5 hours. The incubation mix remained deep blue throughout.

After incubation the gels were removed and rinsed under running distilled water to remove as much of the iron powder adhering to the gel as possible. Gels were transferred to distilled water for up to 3 hours to allow the blue reduced methyl viologen to be auto-oxidised.

SiR activity appears as a black band on a clear gel.

2.2.5.4 Thiosulphate reductase

The method of staining for thiosulphate reductase activity was as described for sulphite reductase except that 25.5mg $Na_2S_2O_3.5H_2O$ replaced Na_2SO_3 as the substrate. Gels were incubated for 5 hours and took 1 hour for the reduced methyl viologen to auto-oxidise in distilled water. Activity was again seen as a black band on a clear gel.

2.2.5.5 ATP-sulphurylase

A method based on that of Skyring *et al.* (1972) was followed for staining for ATP-sulphurylase activity in native gels.

Tissue was extracted in Tris-HCl buffer pH 8 (1g in 10ml), as for sulphite reductase and thiosulphate reductase, and gels loaded and electrophoresed as described previously. Gels were incubated in screw-top tubes containing 5mM ATP, 10mM $Na_2MoO_4.2H_2O$ and 5mM $MgCl_2$ in Tris-HCl buffer, pH 7 for between 30 minutes and one hour. The gels were then rinsed well and transferred to tubes containing 5ml 10mM lead acetate in 2% acetic acid and agitated for approximately 15 minutes. Activity was visualised as a white band on a clear gel. The gels were rinsed again in distilled water and placed into 1% acetic acid for storage.

2.2.6 Subcellular localisation of cysteine synthase

2.2.6.1 Preparation of barley protoplasts

Barley protoplasts were prepared using the method of R.M.Wallsgrove (pers. comm.), as described below.

Second leaves of 7 day old Golden Promise wild type seedlings grown in compost with a 16 hour day length were used. The lower epidermis was peeled from each leaf and the leaf was incubated, peeled side down on 60ml 1.5% cellulase and 0.5% macerozyme in digestion medium containing 0.4M sorbitol, 10mM MES pH 5.5, 1mM $CaCl_2$ and 1mM $MgSO_4$ in a rectangular container 10 x 15cm. After incubation at 25-28°C in the dark for 2 hours the container was gently agitated to release the protoplasts and the solution strained through two layers of muslin. The container was rinsed with digestion medium.

The resulting crude protoplast suspension was divided between 50ml centrifuge tubes. After centrifugation at 100g and 4°C for 5-8 minutes in the Sorval RC5B centrifuge (HB4 rotor), the supernatant was removed by aspiration and discarded. The protoplasts were washed twice to remove traces of the enzymes by resuspending the pellets in gradient medium; 400mM sorbitol, 25mM tricine pH 7.2, 1mM $MgSO_4$ and 1mM $CaCl_2$, and centrifuging as before. The supernatant was discarded and each pellet resuspended in 5ml gradient medium containing 25% Percoll and transferred to 15ml Corex centrifuge tubes. 2ml gradient medium with 15% Percoll was carefully layered on top of the protoplasts followed by 1ml gradient medium without percoll. Tubes were left on ice for up to 1 hour and then centrifuged at 500g 4°C for 5-6 minutes. Intact protoplasts were harvested from the 0-15% Percoll interface with a

wide-bore Pasteur pipette. These protoplasts were washed with excess Percoll free gradient medium and pelleted at 100g for 5 minutes. Protoplasts were resuspended in gradient medium for fractionation.

2.2.6.2 Fractionation of the protoplasts

The protoplasts were ruptured by a single pass through 20μm nylon mesh fixed to a syringe. A volume of lysed protoplast preparation was centrifuged at 15-1600g in the SS34 rotor of the Sorval RC5 for 1 minute at 4°C to give the P1 (chloroplast enriched) fraction, the supernatant was decanted and centrifuged at 10,000g for 10 minutes at 4°C to give the P2 (mitochondria and peroxisome enriched) fraction and the soluble fraction S. Both the P1 and P2 pellets were resuspended in gradient media. Assays for organelle marker enzymes and chlorophyll content were carried out on all fractions and the initial protoplast preparation to check the success of the fractionation.

2.2.6.3 Total chlorophyll content

The chlorophyll content of each fraction and the initial protoplast preparation were determined spectrophotometrically (Wintermans and DeMots, 1965). Up to 100μl of sample was added to ice-cold 96% ethanol to give a total volume of 1ml. The mixture was vortexed well and then centrifuged in a microcentrifuge. The absorbance of the supernatant was read at 654nm and the chlorophyll concentration in μg/ml calculated from:

$$[\text{chlorophyll}] = \frac{1000 \times A_{640}}{39.8}$$

2.2.6.4 Marker enzyme assays

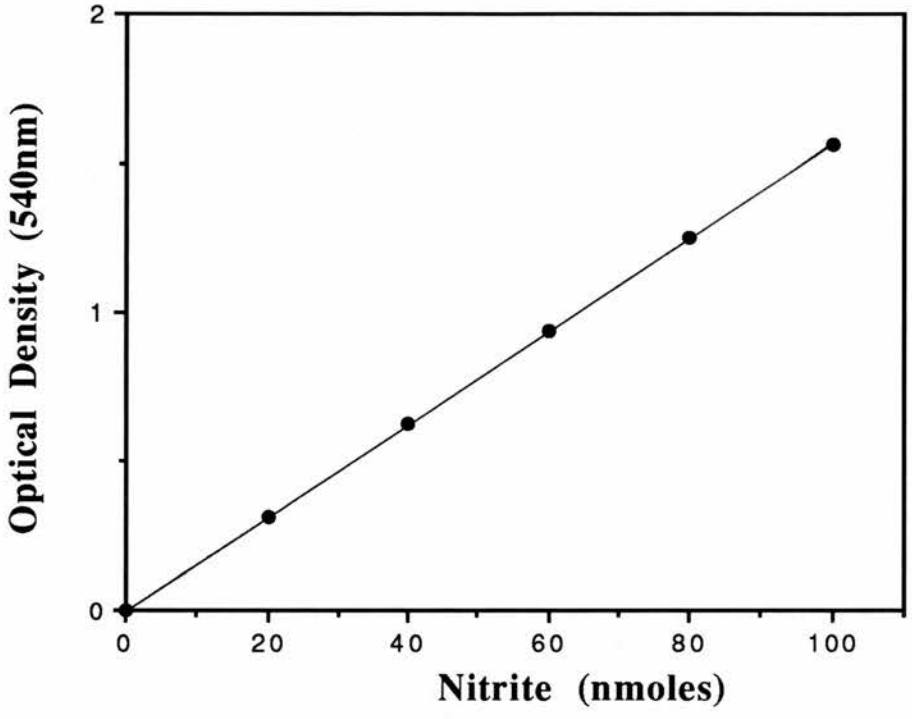
2.2.6.4.1 Nitrite reductase - chloroplasts

Nitrite reductase activity was assayed according to the method of Wray and Filner, (1970) except the tubes were not flushed with nitrogen. The assay mix contained 50mM potassium phosphate buffer pH 7.5, 2mM KNO_2, 1mM methyl viologen, and 0.1ml fraction in a final volume of 1ml. The reaction was started by the addition of 0.2ml 10mg/ml sodium dithionite in 95mM sodium bicarbonate. Controls consisted of the above but lacked sodium dithionite. The assays were carried out at 25°C for 20 minutes and were terminated by vigorous aeration of the assay mix which oxidised the blue reduced methyl viologen to the colourless leuco form. 30µl of the assay mix was added to 970µl H_2O followed by 1ml 1% sulphanilamide in 3M HCl and 1ml 0.02% NED. Full colour development occurred within 15 minutes after which the optical density was read at 540nm (Snell and Snell, 1949). The amount of nitrite reductase was calculated from a previously established standard curve (0-100nmoles KNO_2, (Fig 2.3)).

2.2.6.4.2 Cytochrome c oxidase - mitochondrial membrane bound

Cytochrome c oxidase was assayed by the method of Hackett (1964). 1ml of 5mg/ml horse heart cytochrome c was reduced by adding a small quantity of solid sodium dithionite (tip of small spatula) so that the ratio of A_{550} : A_{565} is greater than 10. Excess dithionite was removed by desalting on a Sephadex G-25 column with a 2ml bed volume, pre-equilibrated with 100mM Potassium

Fig 2.3 Nitrite standard curve



The plot shows a typical standard curve demonstrating the direct relationship between standard amounts of nitrite (0 - 100nmoles KNO_2) and optical density at 540nm after determination of nitrite by the method of Snell and Snell (1949).

Phosphate buffer pH 7.0. 0.5ml reduced cytochrome c was loaded onto the column, washed in with 0.5ml buffer then 0.75ml buffer. The orange coloured cytochrome c was collected.

After incubation of 100µl fraction with 100µl 0.1% digitonin for approximately 2 minutes at room temperature, 700µl of 100mM buffer and 100µl of the reduced horse heart cytochrome c were added and the absorbance at 550nm at 30°C followed. The cytochrome c oxidase activity was calculated from the decrease in absorbance due to the oxidation of reduced cytochrome c using the following formula:

$$\frac{dA_{550}}{dt} = \frac{Ered - Eox}{Ered} \times \frac{dA_{550(red)}}{dt}$$

Where $E_{red} = 28.4 \times 10^{-3}$ cm^2 mol^{-1}

and $E_{ox} = 8.1 \times 10^{-3}$ cm^2 mol^{-1}

2.2.6.4.3 Fumarase - soluble mitochondrial enzyme

Fumarase was assayed using the method of Racker (1950). The increase in absorbance at 240nm and 30°C was recorded for 100µl of sample in a 1ml reaction volume containing 50mM potassium phosphate buffer pH 7.4, and 50mM malate. Activity was calculated using $E_{240} = 2.6 \times 10^6$ cm^2 mol^{-1}.

2.2.6.4.4 Catalase - peroxisome marker

Catalase activity was determined using the method of Luck (1965) by measuring the loss of H_2O_2 spectrophotometrically. Hydrogen peroxide (30%) was diluted with 100mM potassium phosphate buffer pH 7.0 (usually about 100µl H_2O_2 in 10ml), to

give an A_{240} of about 1.0. 100µl sample was added to 0.9ml of diluted H_2O_2 solution in a 1ml cuvette and the change in absorbance at 240nm at 30°C was recorded. Catalase activity was calculated from $E_{240} = 3.6 \times 10^6\ cm^2\ mol^{-1}$.

2.2.7 Partial purification of cysteine synthase

100g of 7 day old barley seedling tissue was extracted by grinding in a mortar and pestle, in batches, with a total of 900ml extraction buffer consisting of 0.1M potassium phosphate pH 7.0, 1mM EDTA and 1mM DTT. After centrifugation for 15 minutes at 30,000g in the SS34 rotor of the Sorval RC5 centrifuge the supernatant was collected and used as the crude extract. Protein precipitating between 40 and 60% ammonium sulphate saturation was collected by centrifugation at 18,000g and dissolved in a minimum (about 200mls) of buffer B (30mM potassium phosphate pH 8.0, 1mM EDTA, 10mM mercaptoethanol). This solution was dialysed overnight against 2 changes of 2l of buffer B.

The dialysate was then centrifuged at 15,000g in the MSE 18 and the resulting supernatant applied to a DEAE Sephadex A50 column (4cm x 19cm) which had been pre-equilibriated with buffer B. The column was washed extensively with buffer B and eluted with a linear gradient of increasing potassium phosphate concentration, 50-500mM. Approximately 12.5ml fractions were collected and every fifth wash fraction and second gradient fraction assayed for cysteine synthase activity and protein (E_{280}). Fractions with significant cysteine synthase activity were pooled and concentrated by precipitation with 80% ammonium sulphate. After resuspension in buffer B and overnight dialysis, the solution was applied on to a Sephadex G100 column (2.5cm x 93cm) which had been equilibrated with buffer B. 9.5ml fractions were collected during elution with buffer B and each assayed for cysteine synthase activity and protein (A_{280}). Fractions with significant activity were pooled and applied to a second DEAE sephadex A50 column (1.5cm x 4.0cm). The column was washed with buffer B, then eluted with a

linear gradient of increasing potassium phosphate concentration (30 - 500mM). 5ml wash fractions and 3ml gradient fractions were collected and assayed. Fractions containing significant activity were pooled, and concentrated using an Amicon stirred cell followed by Amicon microconcentrators. All procedures were carried out at 4°C. Aliquots were taken at each stage of the purification for assay for cysteine synthase activity. Linearity of the phosphate gradients was checked using a conductivity meter and the assay method of Peterson (1978).

2.2.8 Protein determination

Protein was determined according to Bradford (1976). The reagent consisted of 100mg Coomassie blue G-250, 55ml 96% ethanol and 110ml orthophosphoric acid made up to a volume of 1 litre with distilled water, and filtered twice through Whatman No.1 filter paper.

5ml of reagent was added to 0.1ml of appropriately diluted sample and mixed. After full colour development, which occurred within 10 minutes, the optical density was read at 595nm. Calibration curves (0-100μg protein) were prepared with BSA as standards, (Fig 2.4).

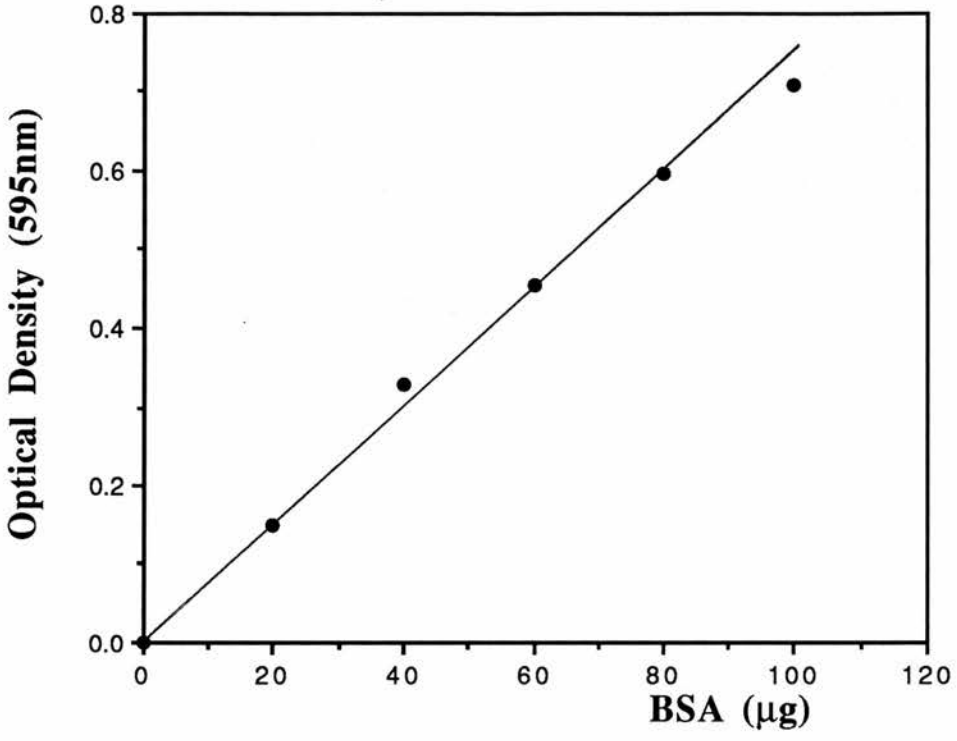
2.2.9 Phosphate determination

Phosphate was determined by two methods. When rapid estimates of phosphate concentration were required an ion selective conductivity meter was used.

For accurate determinations of phosphate concentration the method of Peterson (1978) was followed. The 0.25% stock ANSA (1-amino-2-naphthol-4-sulphonic acid) reagent solution was prepared by dissolving 0.125g ANSA in 48.75ml 15% sodium bisulphite then adding 20% sodium sulphite dropwise (approx. 2ml), until the solution clears. This stock was diluted 1 in 10 with distilled water prior to use.

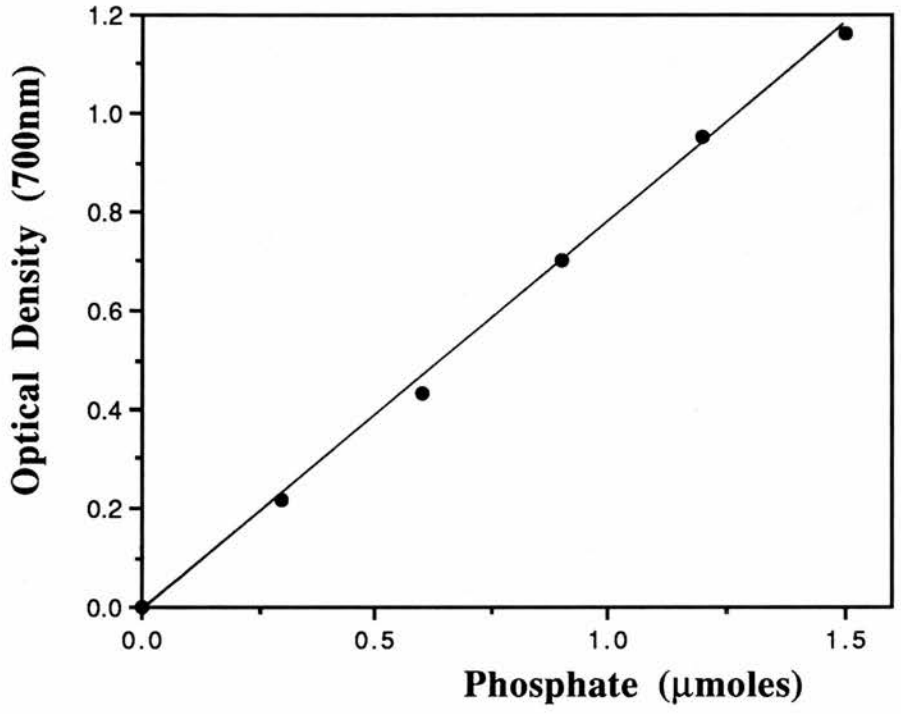
For the assay 1ml 2.5% ammonium molybdate in 4M HCl, 0.8ml 10% SDS (BDH specially pure) and 0.2ml 0.025% ANSA reagent were added to 3ml diluted sample or standard and mixed well. The tubes were left to stand at room temperature for 30 minutes. During this time a blue colour developed. The optical density was read at 700nm and the phosphate concentration

Fig 2.4 Protein calibration graph



The plot show a typical standard curve demonstrating the direct relationship between protein content and optical density at 595nm after incubation with Bradford's reagent.

Fig 2.5 Phosphate calibration graph



The plot a typical standard curve demonstrating the linear relationship between phosphate content and optical density at 700nm after determination of phosphate by the method of Peterson (1978).

determined from a calibration curve (0-1.5μmoles phosphate, Fig 2.5).

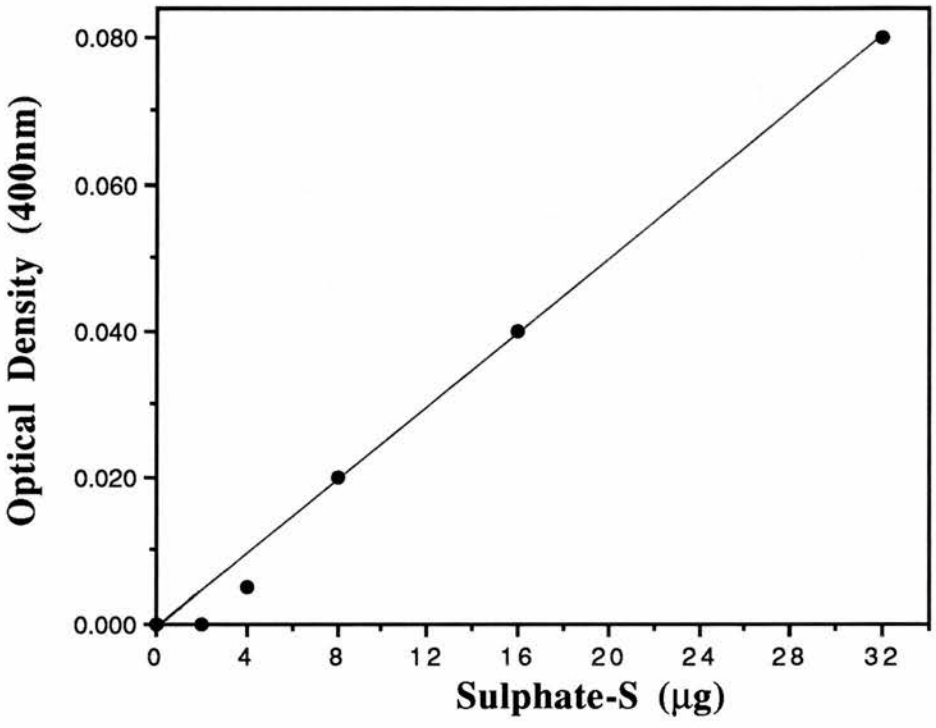
2.2.10 Sulphate determination

Sulphate was determined using a modification of the method of Tabatabai and Bremner (1970).

Barium chloride - gelatin reagent was prepared dissolving 0.6g gelatin in 200ml of hot (60-70°C) water. The solution was allowed to stand at 4°C for 16-18 hours, then the semigelatinous liquid was brought to room temperature and 2g $BaCl_2.2H_2O$ added and dissolved with swirling. The reagent was allowed to stand at room temperature for 2 hours before use.

1ml barium chloride - gelatin reagent was added to 10 mls of aqueous sample or K_2SO_4 standard (0-32μg sulphate-S) and mixed by swirling for a few seconds. After standing at room temperature for 40 minutes the absorbance of the resulting cloudy liquid was read at 400nm. Sulphate-S content of the samples were read from a calibration curve prepared with each assay (Fig 2.6).

Fig 2.6 Sulphate-S standard curve



The plot show a typical standard curve demonstrating the direct relationship between sulphate-S and optical density at 400nm obtained using the method of Tabatabai and Bremner (1970)

CHAPTER 3

ENZYMOLOGICAL STUDIES ON THE SULPHATE ASSIMILATION PATHWAY IN WILD TYPE BARLEY

3.1 Results

Two types of assay have been used in the investigations into the enzymes of sulphate assimilation in wild type barley; conventional solution enzyme assays and specific activity staining following non-denaturing polyacrylamide gel electrophoresis.

3.1.1 Solution assays

It was hoped to develop assays for each of the enzymes of the possible pathways of sulphate assimilation. These assays would then be developed as screening tests for use with putative selenate resistant mutants and also to look at the localisation and regulation of the enzymes in wild type barley.

To this end three of the enzymes have been examined; cysteine synthase, serine acetyltransferase and thiosulphate reductase. An assay was also developed for β-cyanoalanine synthase, an enzyme not associated with the pathway but which was visualised in the specific staining of non-denaturing polyacrylamide gels for cysteine synthase activity.

3.1.1.1 Cysteine synthase

Cysteine synthase was the first enzyme to be considered. Activity was determined using a modification of the method of Ng and Anderson (1978a) in which the cysteine produced from O-acetyl-L-serine and sulphide is measured by the method of Gaitonde (1967). The method for measurement of cysteine was intended to be utilised in coupled assay methods for sulphite reductase (Von Arb and Brunold, 1983) and serine acetyltransferase (Nakamura *et al.*, 1987).

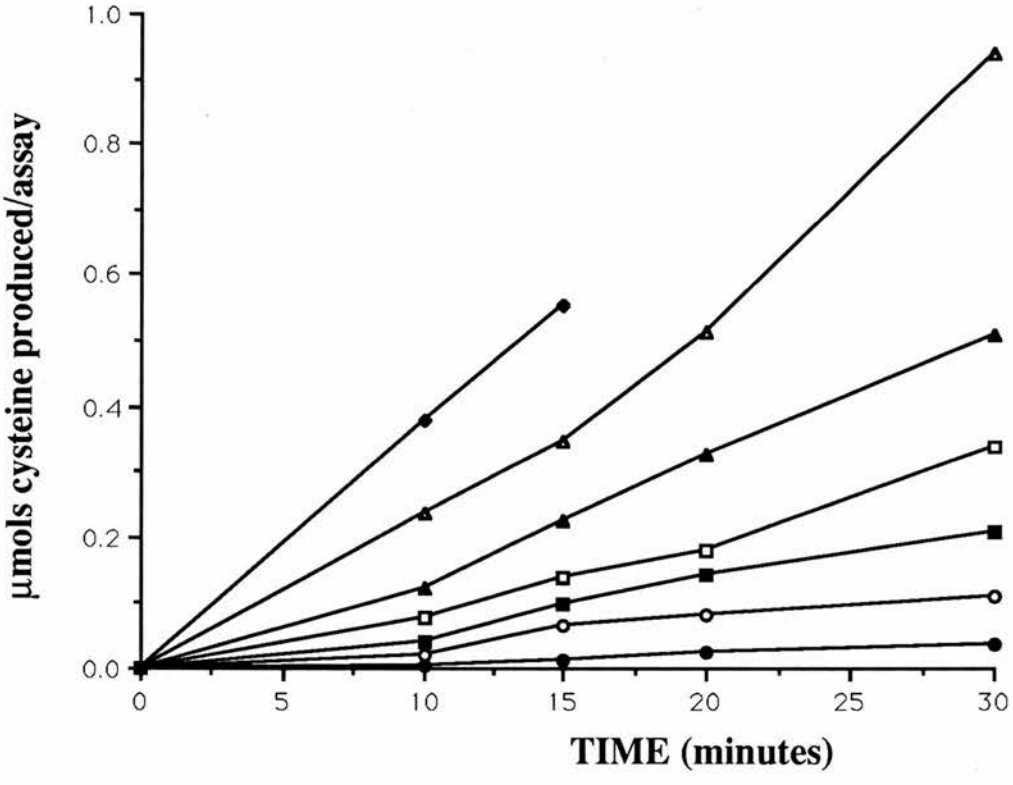
In the cysteine synthase assay extract is incubated with O-acetyl-L-serine and sulphide, the enzyme reaction is stopped by the addition

of TCA. Aliquots of the assay solution are transferred to other test-tubes for cysteine determination. Ninhydrin reagent and acetic acid are added and the tubes placed in a boiling water bath for ten minutes. After rapid cooling with tap water, ethanol is added to each tube and the contents mixed thoroughly. The optical density is read at 560nm and the cysteine content is determined from a standard curve. Investigations into the reproducibility of the cysteine synthase assay method showed that the time period between terminating the enzyme assay and commencing the cysteine determination did not affect the results. However, after the addition of the ethanol the absorbance needed to be recorded as soon as possible as the pink colour produced begins to fade slowly after one hour standing at room temperature.

Cysteine synthase activity, demonstrated as cysteine production was shown to be dependant on the presence of both substrates, O-acetyl-L-serine and sulphide, and extract in the assay mix. Cysteine production was shown to be proportional to the volume of extract in the assay mix and the incubation time (Fig 3.1 and 3.2). From these results, 15 minutes and 30µl crude extract (extracted 1g in 10mls 0.1M potassium phosphate buffer pH 7, containing 1mM EDTA and 1mM DTT) were chosen for routine use. Crude extract from 14 day old wild-type barley seedlings contained cysteine synthase activity of between 16 and 42 µmols cysteine produced/mg protein/hr.

Cysteine activity in the crude extract was shown to be stable for at least 4 hours at room temperature(21°C). The results of investigation into the stability of cysteine synthase at a range of higher temperatures is summarised in Fig 3.3. Incubation of the crude extract at 80°C resulted in total loss of activity after two minutes. After two minutes at 70°C, 50 percent of the activity was lost. Incubation at 50°C and 60 °C for 20 minutes reduced activity by 7

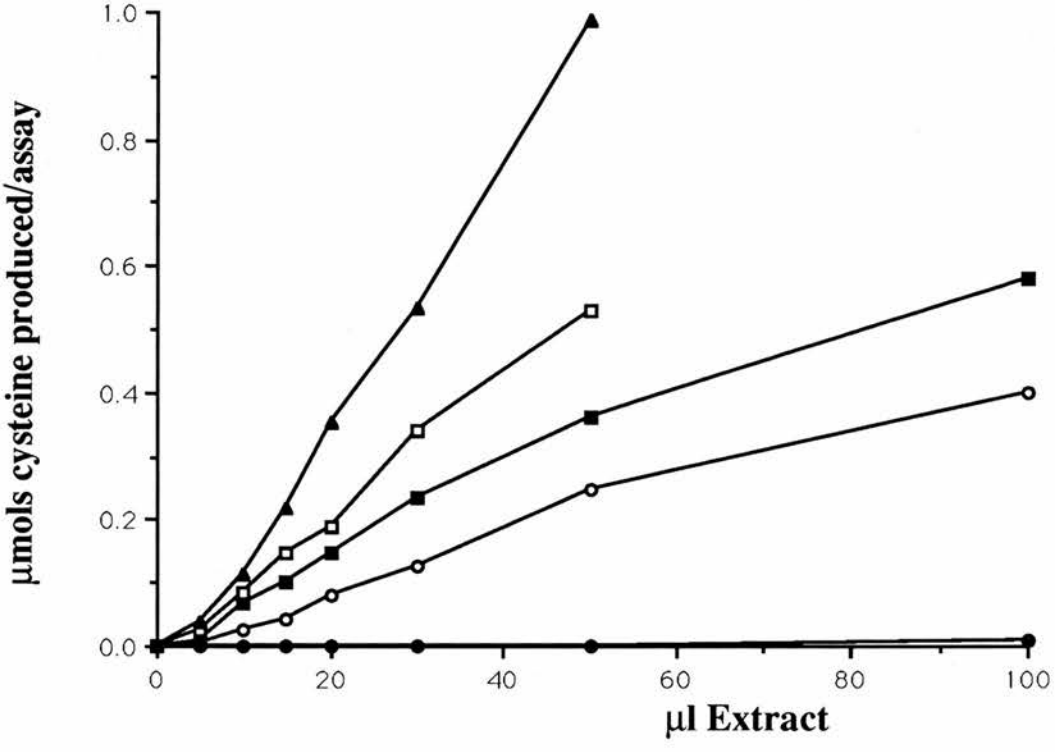
Fig 3.1 Plot showing the relationships between incubation time and cysteine production in the assay for cysteine synthase activity



Leaf tissue from 7 day old wild type barley cultivar Golden Promise seedlings was extracted in 0.1M potassium phosphate buffer pH 7, containing 1mM EDTA and 1mM DTT. Various volumes of extract were assayed for up to 30 minutes for cysteine synthase activity using a modification of the method of Ng and Anderson (1978a). Cysteine was determined by the method of Gaitonde (1967). The results are expressed as the mean activity (µmols cysteine produced/ assay) of duplicate assays from a single experiment.

The volumes of extract included in the assay mix were 5µl (●), 10µl (○), 15µl (■), 20µl (□), 30µl (▲), 50µl (Δ), and 100µl (◆).

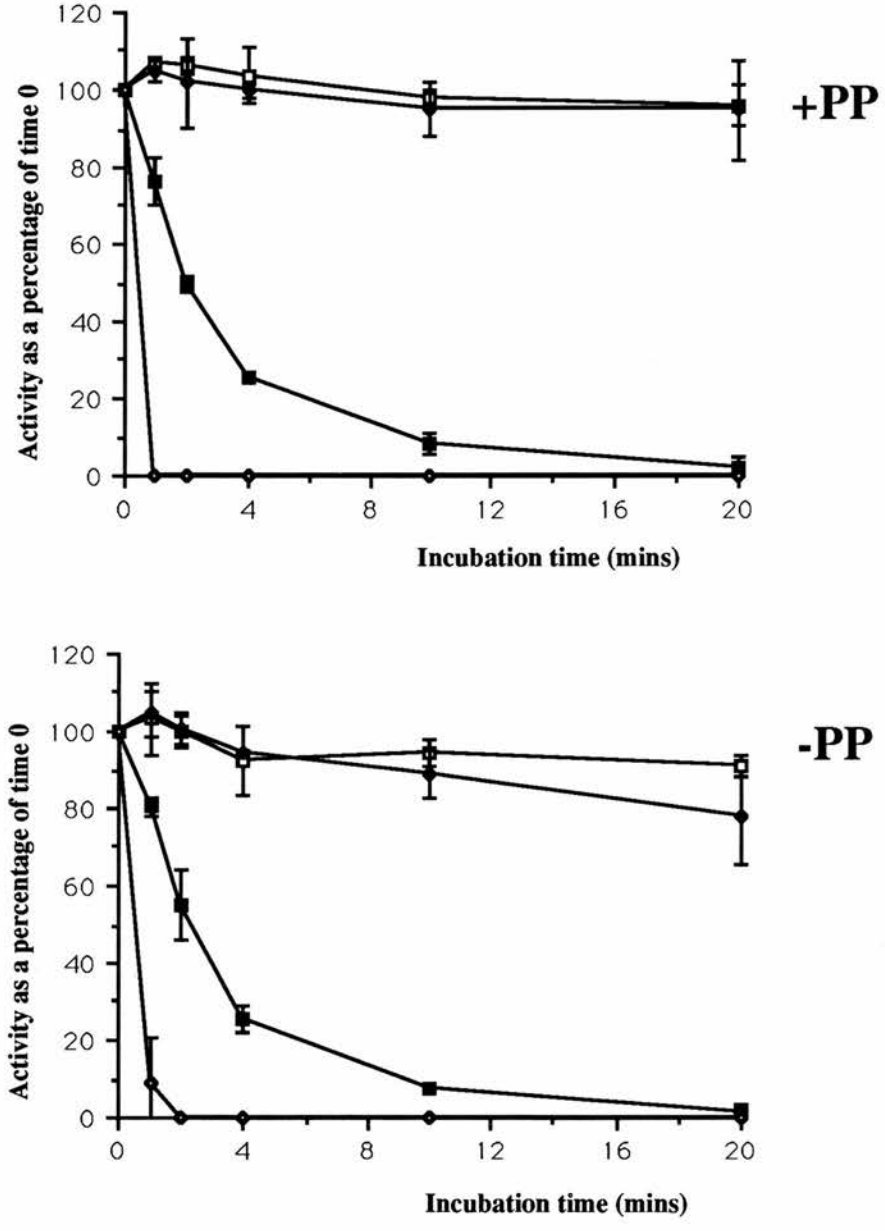
Fig 3.2 Plot showing the relationship between extract volume and cysteine production in the assay for cysteine synthase activity



Leaf tissue from 7 day old wild type barley cultivar Golden Promise seedlings was extracted in 0.1M potassium phosphate buffer pH 7, containing 1mM EDTA and 1mM DTT. Various volumes of extract were assayed for up to 30 minutes for cysteine synthase activity using a modification of the method of Ng and Anderson (1978a). Cysteine was determined by the method of Gaitonde (1967). The results are expressed as the mean activity (µmols cysteine produced/ assay) of duplicate assays from a single experiment.

The assay times used were 0 minutes (●), 10 minutes (O), 15 minutes (■), 20 minutes (□) and 30 minutes (▲).

Fig 3.3 Effect of temperature on the stability of cysteine synthase in the presence and absence of pyridoxal phosphate



Leaf tissue from 7 day old wild type barley cultivar Golden Promise seedlings was extracted in 0.1M potassium phosphate buffer pH 7, containing 1mM EDTA and 1mM DTT with or without 1mM pyridoxal phosphate (PP) added. Aliquots of each extract were incubated at 50 (□), 60 (◆), 70 (■) and 80°C (◇) for 1, 2, 4, 10 and 20 minutes before assay for cysteine synthase activity, with and without 1mM pyridoxal phosphate in the incubation mix. Results are shown as the mean of two separate experiments ± standard deviation.

The mean specific activities of unheated extracts (time 0) were 22.53 ± 2.38 µmols cysteine produced/mg protein/hr +PP and 23.85 ± 3.03 µmols cysteine produced/mg protein/hr -PP (n=8)

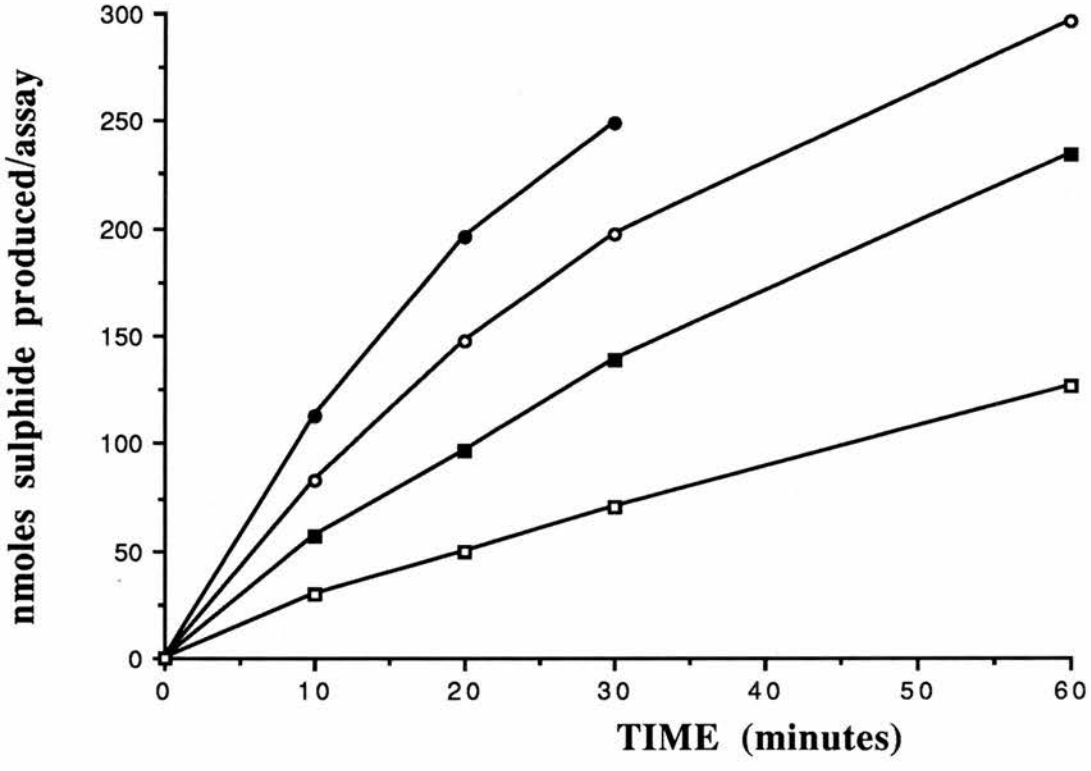
and 20 percent respectively. The inclusion of 1mM pyridoxal phosphate in the extraction and assay buffer did not affect the stability of cysteine synthase on heating over a 20 minute time period at these temperatures.

3.1.1.2 β-cyanoalanine synthase

β-cyanoalanine synthase, a mitochondrial enzyme found in higher plants is not an enzyme of the sulphate assimilation pathway. The enzyme catalyses the formation of β-cyanoalanine from L-cysteine and cyanide but has also been shown to form cysteine from O-acetyl-L-serine and sulphide, and exchange sulphide, rather than cyanide, into cysteine (Hendrickson and Conn, 1969). The enzyme can catalyses the cysteine synthase forward reaction although at only one twenty-fifth of the rate at which it forms β-cyanoalanine. It can also catalyse the back reaction of cysteine synthase, the reaction used in the specific activity stain for cysteine synthase (section 2.2.5.1). Since β-cyanoalanine synthase can exhibit some limited cysteine synthase activity in both the assay systems used it was felt necessary to develop a method to quantify β-cyanoalanine synthase activity.

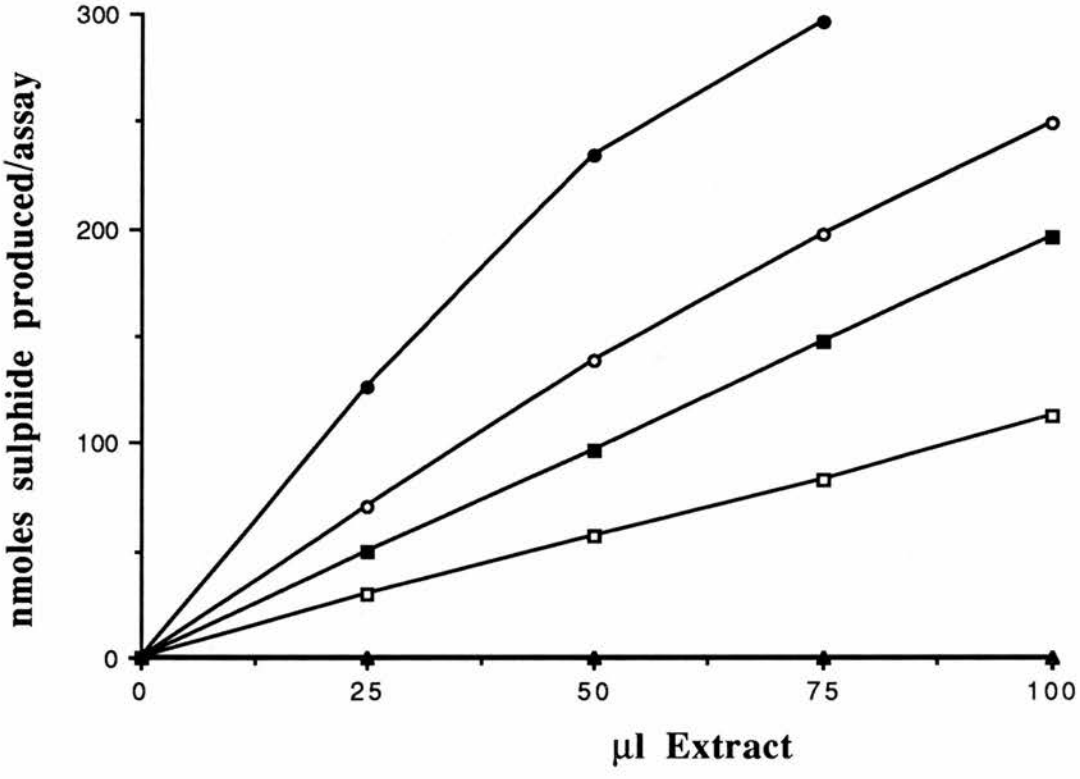
β-cyanoalanine synthase activity was assayed using a modification of the method of Miller and Conn (1980) in which the sulphide formed from cysteine and cyanide is measured by the method of Siegel (1965). An extra step was included to safely remove any cyanide gas evolved after the addition of the acid reagents. β-cyanoalanine synthase activity was dependent on the presence of both cysteine and cyanide in the incubation mixture and no sulphide was produced if the extract was boiled for 2 minutes before assay. Activity was shown to be proportional to the volume of extract added to the incubation mixture and the incubation time (Fig 3.4 and Fig

Fig 3.4 Plot showing the relationship between incubation time and sulphide production in the assay for β-cyanoalanine synthase activity



Leaf tissue from 7 day old wild type barley cultivar Golden Promise seedlings was extracted 1g/10ml Tris HCl pH 8.5. Various volumes of extract were assayed for up to 60 minutes for β-cyanoalanine synthase activity as described in section 2.2.3.2. The volumes of extract included in the assay mixes were 25µl (□), 50µl (■), 75µl (O), and 100µl (●). The results are expressed as the means of duplicate assays from a single experiment.

Fig 3.5 Plot showing the relationship between extract volume and sulphide production in the assay for β-cyanoalanine synthase activity



Leaf tissue from 7 day old wild type barley cultivar Golden Promise seedlings was extracted 1g/10ml Tris HCl pH 8.5. Various volumes of extract were assayed for up to 60 minutes for β-cyanoalanine synthase activity as described in section 2.2.3.2. The assay times used were 0 minutes (▲), 10 minutes (□), 20 minutes (■), 30 minutes (O) and 60 minutes (●). The results are expressed as the means of duplicate assays from a single experiment.

3.5). From these results, 20 minutes incubation time and 100µl crude extract (extracted 1g to 10mls Tris-HCl buffer pH 8.5) were chosen for routine use. Crude extract from 14 day old barley cultivar Golden Promise wild type leaves contained β-cyanoalanine synthase activity of 72-105nmoles sulphide produced/mg protein/min.

3.1.1.3 Serine acetyltransferase

Two methods for the assay of serine acetyltransferase were attempted. In the method of Ngo and Shargool (1974) cleavage of the thioester bond of acetyl-CoA, when CoA is liberated during the formation of O-acetyl-L-serine from L-serine and acetyl-CoA, is measured spectrophotometrically using the thiol reagent dithiodinicotinic acid. This method was unsuccessful since the addition of dithiodinicotinic acid to the reaction mix always caused turbidity. A problem that was not resolved.

The second method used was the coupled assay described by Nakamura *et al.* (1987, 1988) in which the O-acetyl-L-serine produced is converted by cysteine synthase to cysteine which is then measured by the method of Gaitonde (1967). 50 µl of extract was added to the reaction mixture containing 0.12M K-Pi pH 8, 10mM Na_2S, 40mM L-serine, 0.03% BSA and 2.5mM acetyl CoA (total reaction volume 200 µl). After 20 minutes at 30°C the reaction was stopped by the addition of 0.2 ml 4M HCl. The reaction mixture was centrifuged for 3 minutes at 15,000 rpm in a microcentrifuge. To a 200 µl aliquot of the supernatant was added 200 µl acetic acid and 200 µl acid ninhydrin reagent (made up as for cysteine synthase, section 2.2.5.1). The mixture was incubated in a boiling water bath for 10 minutes, then cooled rapidly. 1.4 ml 96% ethanol was added and mixed by vortexing. The absorbance of the resulting solution was

read at 560nm. Cysteine content was determined from a calibration curve established by adding known amounts of cysteine to the assay mixture and reading their absorbance without incubation at 30°C.

Initially the assay was tried using barley leaf tissue extracted 1g/10 ml in cysteine synthase extraction buffer (0.1M K-Pi pH 7, 1mM Na_2EDTA and 1mM DTT) and centrifuged as for cysteine synthase. No cysteine production was detected when 50 µl of this extract was assayed. The inclusion of 20% (v/v) glycerol in the extraction buffer did not result in detectable cysteine production. When 3% BSA and 5% PVP (Polyclar AT) were added to the extraction buffer an activity of 0.03 µmoles cysteine produced/g tissue/hr was obtained. Once partially purified cysteine synthase was available the assay was repeated using 40 µl extract (2 g/10 ml cysteine synthase extraction buffer) and 60 µl partially purified cysteine synthase (cysteine synthase activity 0.1836 µmoles cysteine produced/min) included in the reaction mixture. A serine acetyltransferase activity of 3.75 µmoles cysteine produced/g tissue/hr was obtained. This activity was calculated from a cysteine content that was only just detectable on the standard curve. The high error level inherent in taking readings so close to zero meant that replicate assay results varied too much for the assay method to be considered for routine quantitative use.

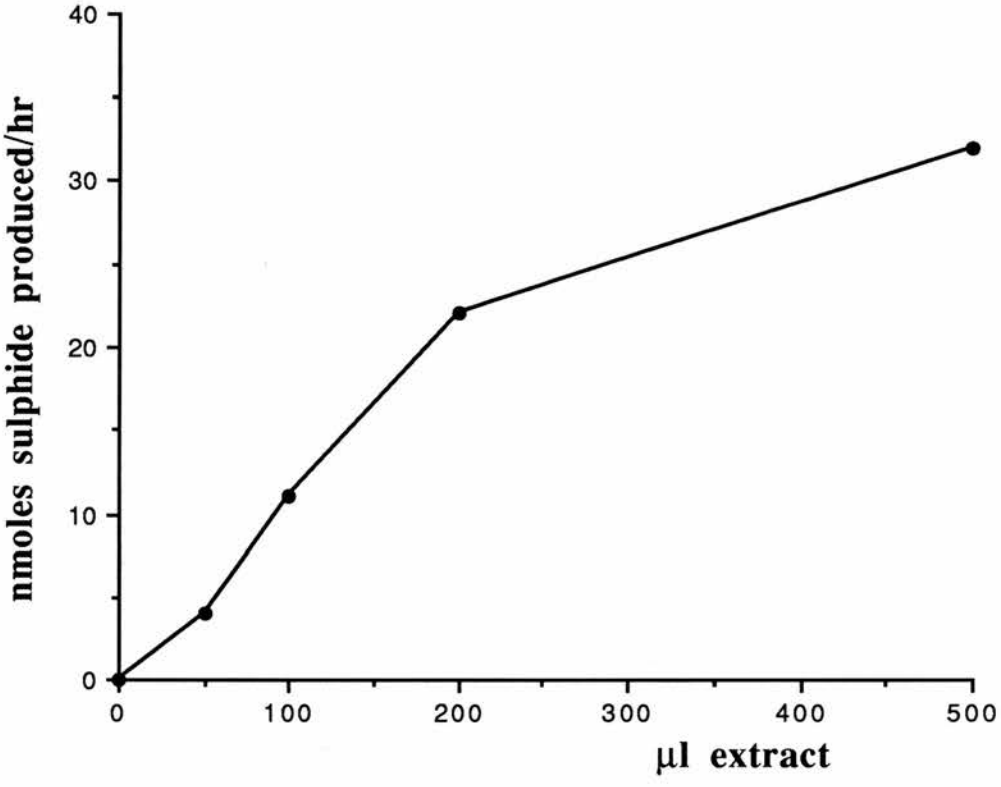
3.1.1.4 Thiosulphate reductase

Using the method of Schmidt *et al.* (1984), in which sulphide production from thiosulphate is determined by the methylene blue method of Siegel (1965), an activity of 110nmoles sulphide produced/mg protein/hr was obtained for crude extract of 14 day old barley seedling leaves. DTE is used as the electron donor and 0.2mM

sodium thiosulphate ($Na_2S_2O_3$) as the substrate. Activity was shown to be roughly proportional to extract volume (Fig 3.6).

The presence of thiosulphate, the enzyme substrate, is reported to progressively inhibit methylene blue formation above 0.2mM (Schmidt *et al.*, 1984). However, when increasing amounts of thiosulphate were added to a solution of known sulphide concentration under the assay conditions concentrations as low as 0.05mM thiosulphate inhibited methylene blue formation. The thiosulphate concentration used in the Schmidt *et al.* (1984) assay method, 0.2mM, caused a 32.4% reduction in absorbance at 670nm compared to zero thiosulphate (Table 3.1). This will result in over estimate of the thiosulphate reductase activity since the methylene blue formation will be influenced by the disappearance of thiosulphate as well as sulphide production.

Fig 3.6 Plot showing the increase in sulphide produced with increasing extract volume in the incubation mixture of the thiosulphate reductase assay



Leaf tissue from 7 day old wild type barley cultivar Golden Promise seedlings was extracted 1g/10ml Tris HCl pH 8. Various volumes of extract were assayed for thiosulphate reductase activity as described in section 2.2.3.3. The results are expressed as the mean of duplicate assays from a single experiment.

Table 3.1 Inhibition of methylene blue formation by increasing concentrations of thiosulphate under the assay conditions for thiosulphate reductase activity

µmoles thiosulphate	% inhibition
0	0
0.01	0
0.02	0
0.05	11.5
0.1	11.5
0.2	32.4
0.5	100

Solutions containing 100 nmoles sulphide/ml and different levels of thiosulphate as indicated above were assayed by the method of Siegel (1965) as described in section 2.2.5.4. The results are expessed as the percentage reduction in absorbance at 670nm compared to the zero µmole thiosulphate assay (1.65 OD units).

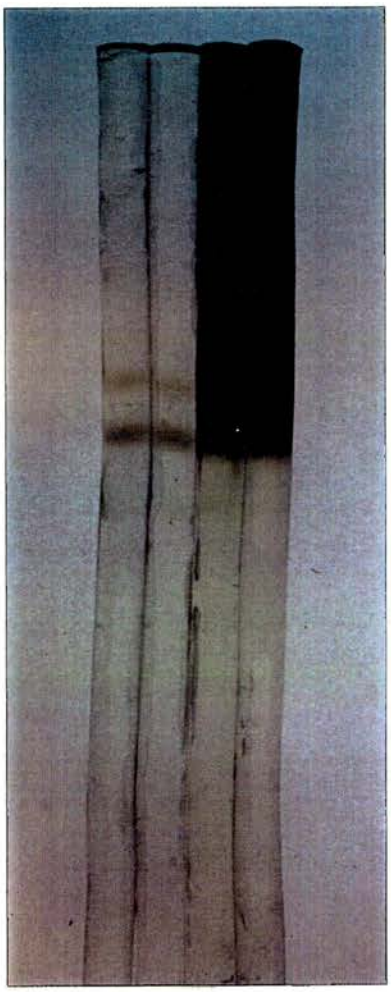
3.1.2 Non-denaturing polyacrylamide gel electrophoresis and the subsequent specific activity staining of the gels

Specific activity staining of non-denaturing polyacrylamide gels is useful as a screening procedure for checking for the presence of functional enzymes in an extract. It was hoped to develop a specific activity staining method for each of the enzymes of the sulphate assimilation pathway. These methods could later be used to check for the presence of the enzymes in putative selenate resistant mutant individuals selected from the selenate screen. Specific activity stains were developed for the pathway enzymes cysteine synthase, thiosulphate reductase, sulphite reductase and ATP-sulphurylase. Since β-cyanoalanine synthase can also catalyse the reverse reaction used in the staining method for cysteine synthase it may also be detected using this method. A modification of this method was developed to allow the detection of β-cyanoalanine synthase activity.

3.1.2.1 Cysteine synthase and β-cyanoalanine synthase

After electrophoresis of crude extract from leaves of barley cultivar Golden Promise wild type, and a two hour incubation of the gel to stain for cysteine synthase activity, one, or more usually two, black bands appeared on the transparent gel. When present the slower migrating of the two bands was less intense than the other (Fig 3.7). Neither band was seen when either the substrate cysteine, or lead acetate, was omitted from the staining mix. No bands were seen if the extract had been boiled prior to loading onto the tube gels. After staining a single band, at the position of the faster migrating band with crude extract, was present on gels loaded with partially purified cysteine synthase extract.

Fig 3.7 Specific staining for cysteine synthase and β-cyanoalanine synthase activity after non-denaturing polyacrylamide gel electrophoresis of barley leaf extract.



1 2 3 4

Leaf tissue from 7 day old wild type barley cultivar Golden Promise seedlings was extracted and subjected to non-denaturing polyacrylamide gel electrophoresis followed by specific staining for cysteine synthase and β-cyanoalanine synthase as described in sections 2.2.4, 2.2.5.1. and 2.2.5.1. All gels were loaded with 200µl extract.

Gels 1 and 2: cysteine synthase activity stain (without NaCN)

Gels 3 and 4: β-cyanoalanine synthase activity stain (0.5mM NaCN)

If 0.5mM NaCN is included in the staining mix then the forward reaction of β–cyanoalanine synthase should proceed according to the reaction;

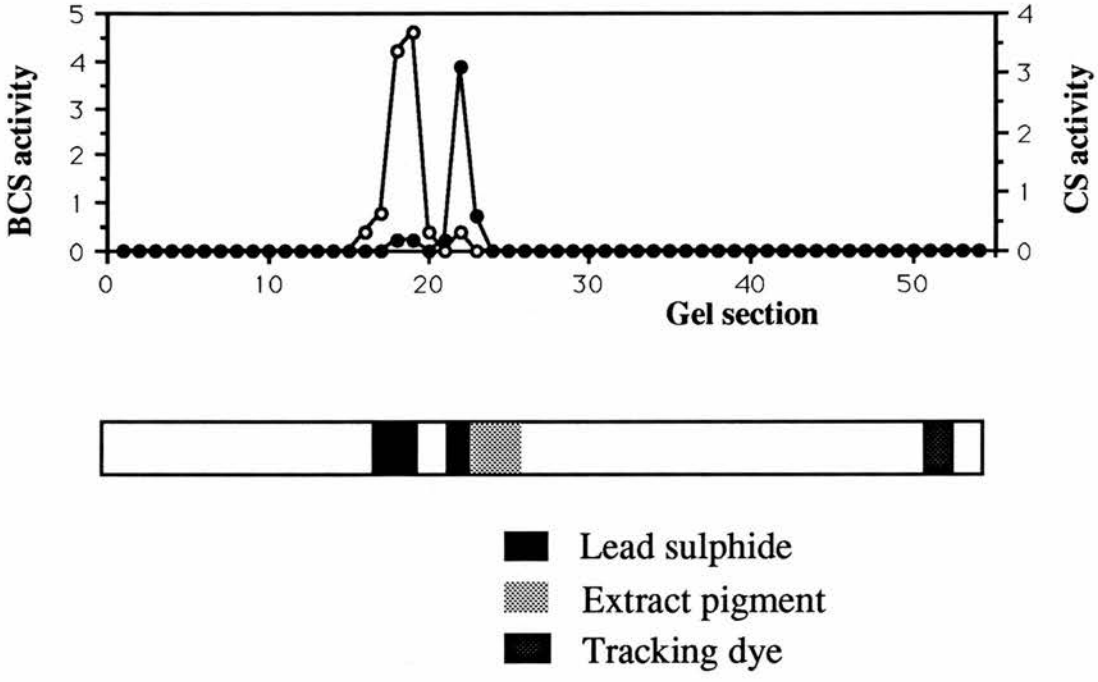
and after staining, bands should indicate the position of the enzyme β–cyanoalanine synthase. Again two black bands were seen on a clear gel. The slower migrating of the two bands was the most intense and appeared almost instantly. The faster migrating band was paler and developed more slowly. No bands were seen on gels loaded with boiled extract, or when cysteine or lead acetate were omitted from the staining mix. When gels loaded with partially purified cysteine synthase were incubated in the presence of 0.5mM NaCN a single pale band was observed at the same position as the faster migrating of the two bands.

The bands observed when cyanide is absent from the staining mixture are due to sulphide formed in the reverse reaction of cysteine synthase, the release of sulphide from cysteine. It may be anticipated that in this case the band intensity due to cysteine synthase will be greater than that due to β-cyanoalanine synthase. In the presence of cyanide β-cyanoalanine synthase can utilise it's normal substrate and the intensity of the band (or bands) at the location of β-cyanoalanine synthase should increase. The reverse reaction of cysteine synthase is also increased in the presence of cyanide (A. Schmidt, pers comm. to J.L. Wray). Hence, we can anticipate the stain with cyanide would show increased band intensity for both enzymes. The increase in intensity of the band(s) at the location of β-cyanoalanine synthase would probably be greater. Based on these suggestions it seems likely

that the slower migrating band is at the position of β-cyanoalanine synthase, which has been shown to exchange sulphide into cysteine (Hendrickson and Conn, 1969), whilst the faster migrating of the two bands indicates the position of cysteine synthase.

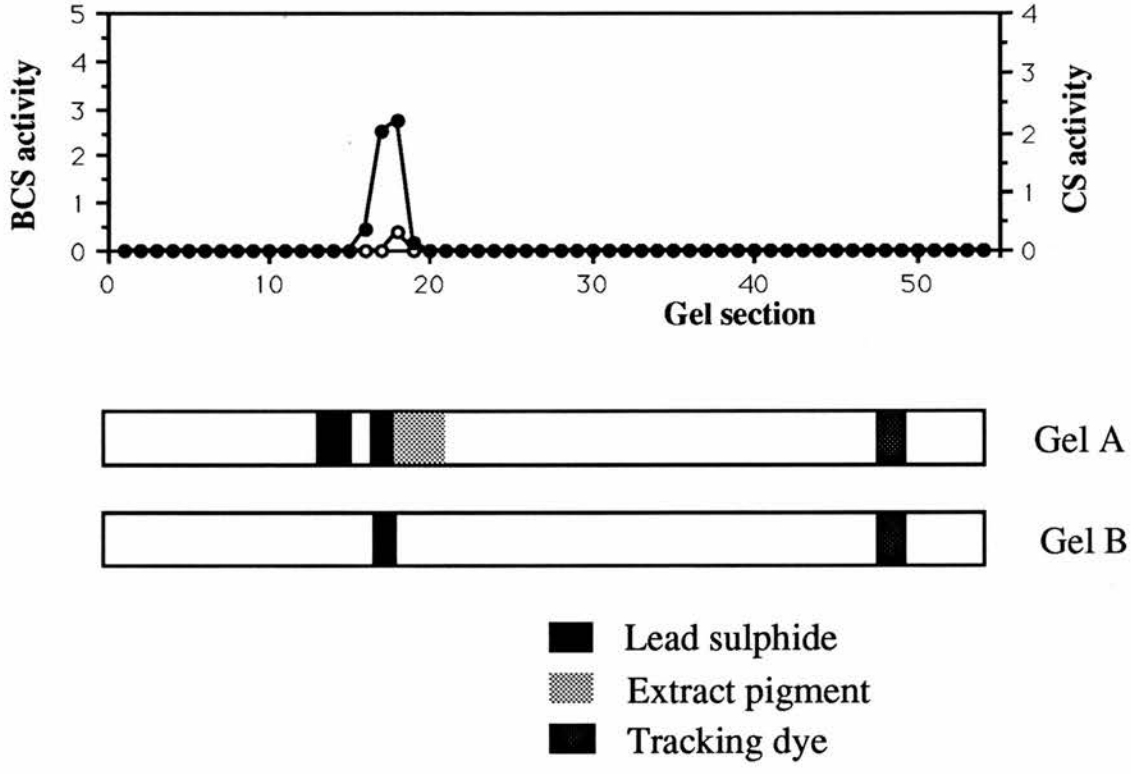
To establish the definite identity of each activity band two experiments were devised. In the first, three gels were loaded with crude extract (containing cysteine synthase activity 3.9 μmols cysteine formed/hr and β-cyanoalanine synthase activity 12.4nmols sulphide produced/min). The gels were electrophoresed as described previously. Two of the gels loaded with crude extract were stained, one in the presence and one in the absence of NaCN. The third gel was left unstained, frozen at -70°C for one hour, then sliced into 2mm sections with a razor blade. Each section was homogenised in 500μl Tris HCl, pH 8.5 and aliquots assayed for cysteine synthase and β-cyanoalanine synthase activity. Fig 3.8 shows a plot of the activities of the two enzymes in each gel section and a diagrammatic representation of the appearance of the gel stained in the presence of cyanide. In the second experiment two gels were loaded with partially purified cysteine synthase (containing cysteine synthase activity 5.6 μmols cysteine formed/hr and β-cyanoalanine synthase activity 0.28nmols sulphide produced/min) and one with crude extract. After electrophoresis one of the gels loaded with partially purified extract and the one loaded with crude extract were stained in the presence of NaCN. The second gel loaded with partially purified extract was left unstained and was sectioned, with each section assayed for cysteine synthase and β-cyanoalanine synthase activity as before. Fig 3.9 shows a plot of the activities of the two enzymes in each gel section and a diagrammatic representation of the appearance of the gels after specific activity staining in the presence of cyanide.

Fig 3.8 Cysteine synthase and β-cyanoalanine synthase activities of sections of a non-denaturing polyacrylamide tube gel loaded with crude extract of barley leaf tissue.



Leaf tissue from 7 day old wild type barley cultivar Golden Promise seedlings was extracted in 0.1M potassium phosphate buffer, pH 7, containing 1mM EDTA and 1mM DTT. Gels were loaded with 200µl of extract and subjected to non-denaturing polyacrylamide gel electrophoresis. One gel was then stained for cysteine synthase and β-cyanoalanine synthase activity, in the presence of 0.5mM cyanide, as described in section 3.1.2.1. The appearance of this gel after staining is shown diagramatically. A second gel was frozen at -70°C for one hour, then cut into 2mm sections. Each section was homogenised in 500µl Tris-HCl buffer pH 8.5 and aliquots assayed for cysteine synthase (●) (µmoles cysteine/gel section/hr) and β-cyanoalanine synthase activity (O) (nmoles sulphide/gel section/min). Activities of each enzyme in each gel section are shown above.

Fig 3.9 Cysteine synthase and β-cyanoalanine synthase activities of sections of a non-denaturing polyacrylamide tube gel loaded with partially purified cysteine synthase from barley leaf tissue.



Two gels were loaded with 40µl partially purified cysteine synthase from barley leaf and one with 200µl of crude barley leaf extract, and subjected to non-denaturing polyacrylamide gel electrophoresis. One gel of each was then stained for cysteine synthase and β-cyanoalanine synthase activity, in the presence of 0.5mM cyanide, as described in section 3.1.2.1. The appearance of these gels after staining is shown diagramatically, gel A was loaded with crude extract and gel B with partially purified cysteine synthase. The second partially purified cysteine synthase gel was frozen at -70°C for one hour, then cut into 2mm sections. Each section was homogenised in 500µl Tris-HCl buffer pH 8.5 and aliquots assayed for cysteine synthase (●) (µmoles cysteine/gel section/hr) and β-cyanoalanine synthase activity (O) (nmoles sulphide/gel section/min). Activities of each enzyme in each gel section are shown above.

In the first experiment the anticipated two bands were observed after staining of gels loaded with crude extract. Two regions of activity were seen for both cysteine synthase and β-cyanoalanine synthase in the homogenised sections of gel. At the position of the slower migrating band on the stained gels 0.4 μmols cysteine/hr cysteine synthase activity and 10.4nmol sulphide/min β-cyanoalanine synthase activity was recovered, whilst at the region corresponding to the position of the faster migrating band on the stained gels 3.92 μmols cysteine/hr cysteine synthase activity and 0.4nmol sulphide/min β-cyanoalanine synthase activity was recovered.

In the second experiment after staining, two bands were observed on the gel loaded with crude extract and a single band was observed, at the position of the faster migrating band on the crude extract gel, on the gel loaded with partially purified cysteine synthase. The sectioned and homogenised gel (loaded with partially purified cysteine synthase) contained one region with both cysteine synthase and β-cyanoalanine synthase activities. This region gave total activities of 4.7 μmols cysteine formed/hr and 0.53nmols sulphide produced/min for cysteine synthase and β-cyanoalanine synthase respectively.

In summary, the slower migrating band, present only in crude extract, contains lower cysteine synthase activity but higher β-cyanoalanine synthase activity than the faster migrating band which is present in both crude and partially purified extracts.

3.1.2.2 Serine acetyltransferase

Attempts were made to modify solution assay methods to allow the development of a serine acetyltransferase activity gel stain. Gels were either incubated with 10mM L-serine, 1mM acetyl CoA and

10mM dithionitrobenzoate (DTNB) in 50mM Tris HCl buffer, pH 7.6 (based on the method of Ascano and Nicholas (1977) and Kredich and Tomkins (1966)), where the disulphide interchange between the CoA liberated in the reaction and the DTNB produces thionitrobenzoic acid (TNB), or with 10mM L-serine and 1mM acetyl CoA in 50mM Tris HCl buffer, pH 7.8 for 90 minutes when dithiodinicotinic acid was added to a concentration of 5mM (based on the assay method of Ngo and Shargool (1974)). Yellow band(s) of TNB and thionicotinic acid respectively were expected but none were seen. No activity stain for serine acetyltransferase was developed.

3.1.2.3 Thiosulphate reductase

Two methods were examined. The first method, based on that of Skyring and Trudinger (1972), precipitated the sulphide produced in the enzymic reaction in the presence of reduced methyl viologen as black iron sulphide. This resulted in the appearance of a single black band on a pale brown gel. The appearance of this band of activity was dependent on the presence of the substrate, thiosulphate, and the electron donor methyl viologen. The appearance of the activity band was completely inhibited by preincubation of the gel with 1mM NaCN and partially inhibited by the preincubation with 1,10 phenanthroline (Fig 3.10). 1,10 phenanthroline has been reported to inhibit thiosulphate reductase activity (Aketagawa *et al.*, 1985). Cyanide inhibits sulphite reductase activity (Krueger and Siegel, 1976 and 1982a) but stimulated thiosulphate reductase activity from Desulfovibrio vulgaris (Aketagawa *et al.*, 1985). A band was still seen when $FeSO_4$ was omitted from the incubation mix however it seems likely that the presence of reduced iron powder allowed the formation of the FeS.

Fig 3.10 Specific staining for thiosulphate reductase activity after non-denaturing polyacrylamide gel electrophoresis of barley leaf extract.

Leaf tissue from 7 day old wild type barley cultivar Golden Promise seedlings was extracted and subjected to non-denaturing polyacrylamide gel electrophoresis followed by specific staining for Thiosulphate reductase as described in sections 2.2.4 and 2.2.5.4. All gels were loaded with 200µl extract.

Gel 1: Complete incubation mix.
Gel 2: Gel loaded with boiled extract
Gel 3: Gel loaded with extration buffer only
Gel 4: Incubation without methyl viologen
Gel 5: Incubation without reduced iron
Gel 6: Incubation without $FeSO_4$
Gel 7: Incubation without thiosulphate
Gel 8: Preincubation with 1mM NaCN
Gel 9: Preincubation with 2mM NaCN
Gel 10: Preincubation with 1mM 1,10 phenanthroline
Gel 11: Preincubation with 2mM 1,10 phenanthroline

The solution assay of Schmidt *et al.* (1984) in which DTE is used as electron donor was modified for use as a gel-staining procedure, again with sulphide precipitated as iron sulphide. After staining by this method the bands which developed were faint, and the number of bands seen varied between one and three. Band number and band position was usually reproducible within an experiment. Appearance of the bands was dependent on the presence of $Na_2S_2O_3$, DTE, and either $FeSO_4$ or $FeCl_3$. When gels loaded with extraction buffer only were incubated in complete incubation mix no bands were seen. However, when the crude extract was boiled before loading onto the gels either one or two bands were seen after staining by this method. The position of these bands did not correspond to any bands seen on gels from the same experiment loaded with unheated extract.

When replicate gels from the same experiment were stained using either the method of Skyring and Trudinger (1972) or that modified from Schmidt *et al.* (1984), the single activity band seen with the former had an R_f value roughly equal to that of the centre activity band of gels stained by the Schmidt method. The bands were too faint to photograph, therefore the R_f values of the observed bands were measured and are given in Table 3.2.

3.1.2.4 Sulphite reductase

Using the method of Skyring and Trudinger (1972) which precipitates sulphide as black FeS, a single band of activity was seen after electrophoresis and specific staining for sulphite reductase of gels loaded with crude extract from wild-type barley (extracted in 0.1M Tris buffer, pH 8.0). The band was absent when either the substrate, sodium sulphite (Na_2SO_3), or methyl viologen was omitted from the staining mix (Fig 3.11). Development of the band was

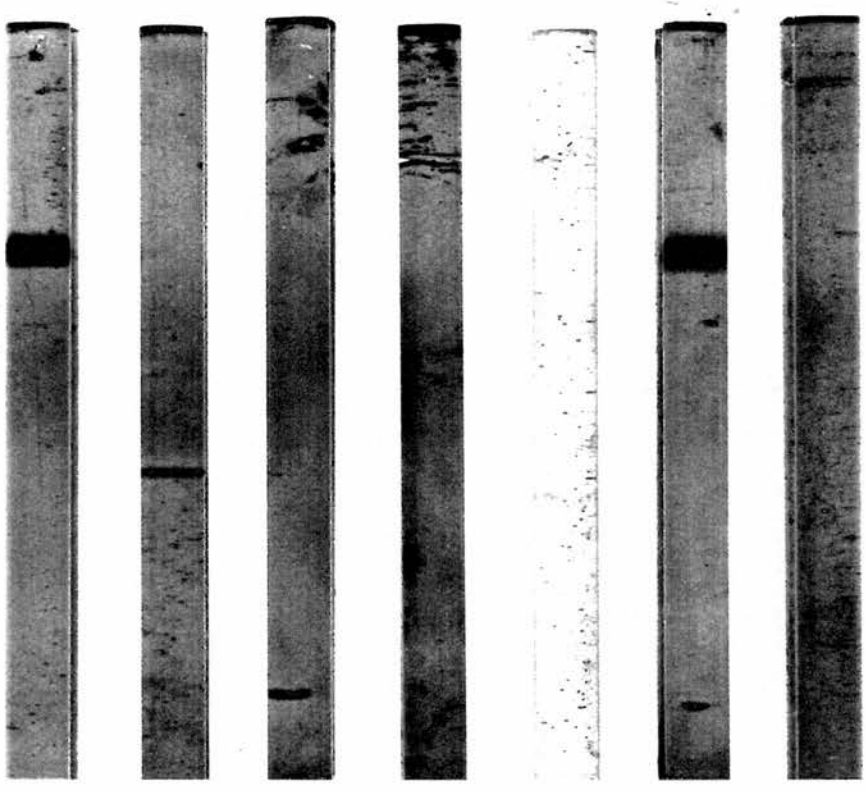
Table 3.2 R_f values of bands observed after specific staining for thiosulphate reductase activity following non-denaturing polyacrylamide gel electrophoresis of barley leaf extract

		Method A					Method B			
Experiment	Gel	1	2	3	4	5	6	7	8	9
1		0.320 0.398 0.680	0.315 0.392 0.669	0.311 0.414 0.674	0.315 0.408 0.685	-	0.403	0.400	0.397	0.403
2		0.350 0.491 0.737	0.466 0.700	-	-	-	0.483	0.475	-	-
3		0.386	0.390	-	-	0.305 0.422	-	-	-	-
4		0.383 0.578 0.695	0.380 0.560 0.686	0.377 0.594 0.692	-	0.295	0.394	-	-	-

Crude barley leaf extract was prepared from 7 day old Golden promise wild type seedlings as described in section 2.2.5.3 and 200µl loaded directly onto gels (1 - 4 and 6 - 9). In experiments 3 and 4 extract was boiled before loading onto gel 5. Gels were subsequently stained for thiosulphate reductase activity by the methods of Schmidt *et al.* (1984) (Method A, gels 1 - 5) or Skyring and Trudinger (1972) (Method B, gels 6 - 9). R_f values for all observable bands are given.

- indicates no gel was loaded.

Fig 3.11 Specific staining for sulphite reductase activity after non-denaturing polyacrylamide gel electrophoresis of barley leaf extract.



Leaf tissue from 7 day old wild type barley cultivar Golden Promise seedlings was extracted and subjected to non-denaturing polyacrylamide gel electrophoresis followed by specific staining for sulphite reductase as described in sections 2.2.4 and 2.2.5.3. All gels were loaded with 200µl extract.

Gel 1: Complete incubation mix

Gel 2: Gel loaded with boiled extract

Gel 3: Gel loaded with extraction buffer only

Gel 4: Incubation without methyl viologen

Gel 5: Incubation without reduced iron

Gel 6: Incubation without $FeSO_4$

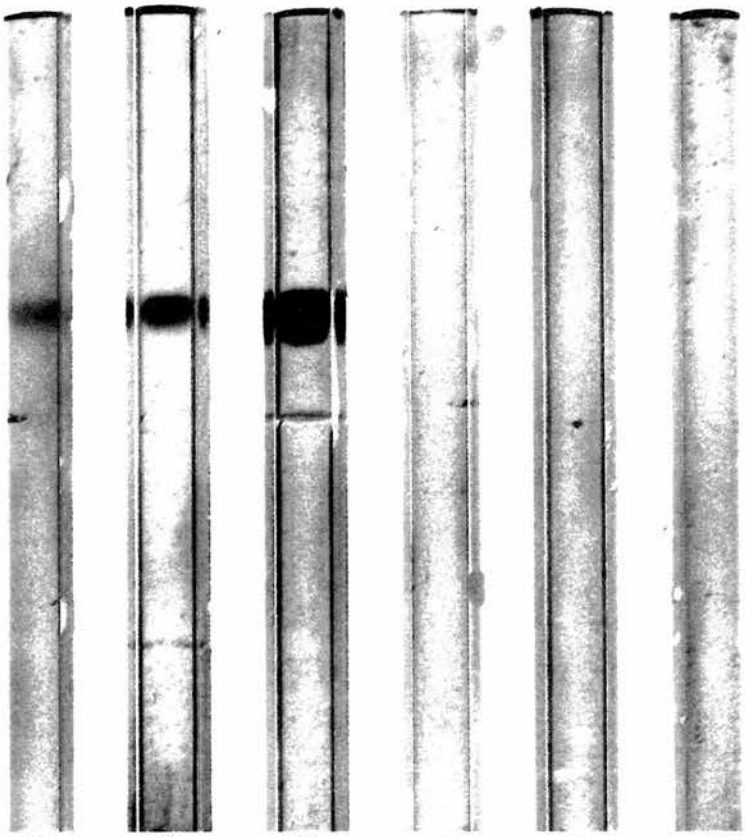
Gel 7: Incubation without Na_2SO_3

prevented in the presence of 1mM NaCN which has been reported to be an inhibitor of sulphite reductase (Krueger and Siegel,1976 1982a)(Fig 3.12). It has been suggested that cyanide binds tightly to the siroheme prosthetic group inhibiting enzyme activity.

When tissue was extracted in 0.1M Tris, pH 8.0, containing 10mM mercaptoethanol or 1mM DTT or 1mM cysteine, a single band of activity was seen after staining. However, when extracts were prepared in 0.1M phosphate buffer, pH 7.8, containing 1mM EDTA both in the presence and absence of 1mM DTT or cysteine, two bands were sometimes seen. For routine specific staining of sulphite reductase activity, 0.1M Tris, pH 8.0 was used for extraction.

Using the method of Skyring and Trudinger (1972) gels were incubated in the presence of thiosulphate and/or sulphite to show the relative positions of thiosulphate reductase and sulphite reductase. A single band was seen in each case. Preincubation of the gels with 1mM NaCN for 1 hour before the addition of the substrate(s) completely inhibited the formation of the activity band (Fig 3.12, thiosulphate only Fig 3.10). Development of the band was partially inhibited by preincubation with 1mM 1,10 phenanthroline (Fig 3.13). 2mM 1,10 phenanthroline had a greater inhibitory effect than 1mM when thiosulphate was the substrate (Fig 3.10, thiosulphate only). Cyanide was expected to inhibit sulphite reductase activity and possibly enhance thiosulphate reductase activity. 1,10 phenanthroline should inhibit thiosulphate reductase activity but its effect on sulphite reductase is unknown.

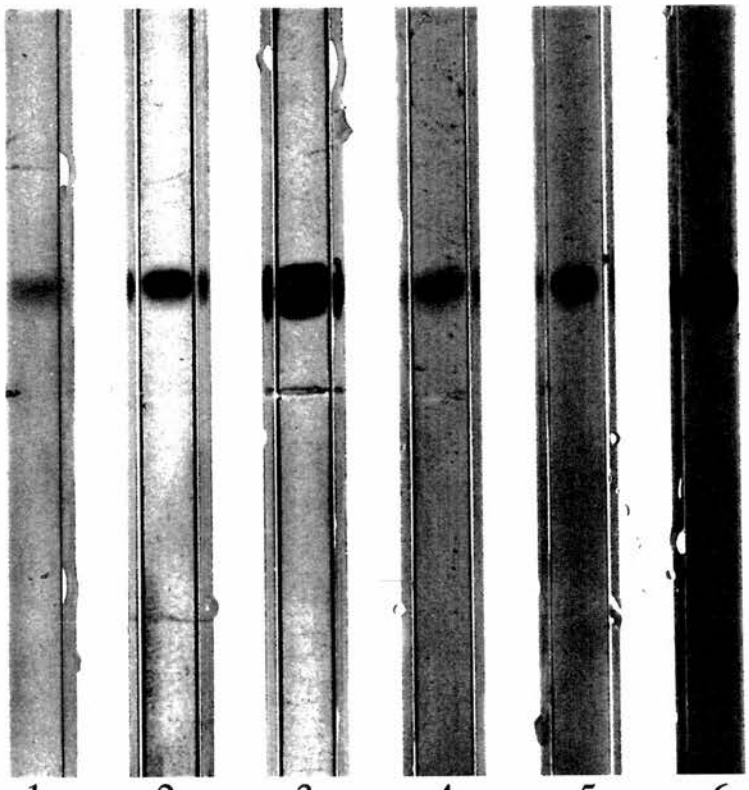
Fig 3.12 Specific staining for sulphite reductase activity vs thiosulphate reductase activity and the effect of preincubation with NaCN on the activities



Leaf tissue from 7 day old wild type barley cultivar Golden Promise seedlings was extracted and subjected to non-denaturing polyacrylamide gel electrophoresis followed by staining using the method of Skyring and Trudinger (1972) with sulphite, thiosulphate or both combined as substrate as described in sections 2.2.4, 2.2.5.3 and 2.2.5.4. All gels were loaded with 200µl extract.

Gel 1: Incubation with sulphite
Gel 2: Incubation with thiosulphate
Gel 3: Incubation with both
Gel 4: Preincubation with 1mM NaCN followed by incubation with sulphite
Gel 5: Preincubation with 1mM NaCN followed by incubation with thiosulphate
Gel 6: Preincubation with 1mM NaCN followed by incubation with both

Fig 3.13 Specific staining for sulphite reductase activity vs thiosulphate reductase activity and the effect of preincubation with 1,10 phenanthroline on the activities



Leaf tissue from 7 day old wild type barley cultivar Golden Promise seedlings was extracted and subjected to non-denaturing polyacrylamide gel electrophoresis followed by staining using the method of Skyring and Trudinger (1972) with sulphite, thiosulphate or both combined as substrate as described in sections 2.2.4, 2.2.5.3 and 2.2.5.4. All gels were loaded with 200µl extract.

Gel 1: Incubation with sulphite
Gel 2: Incubation with thiosulphate
Gel 3: Incubation with both
Gel 4: Preincubation with 1mM 1,10 phenanthroline followed by incubation with sulphite
Gel 5: Preincubation with 1mM 1,10 phenanthroline followed by incubation with thiosulphate
Gel 6: Preincubation with 1mM 1,10 phenanthroline followed by incubation with both

3.1.2.5 ATP sulphurylase

A method of staining native gels for ATP-sulphurylase activity, described in section 2.2.5.5, based on that of Skyring *et al.* (1972) was developed for use with barley extract. The method substitutes molybdate (MoO_4^{2-}) for the natural substrate, sulphate (SO_4^{2-}), forming adenosine 5'-phosphomolybdate (adenosylphosphomolybdate) and pyrophosphate according to the reaction;

$$MoO_4^{2-} + ATP \longrightarrow APMo + pyrophosphate$$

After incubation gels are transferred into 10mM lead acetate in 2% acetic acid and the appearance of a white band of lead phosphate denotes the presence of ATP sulphurylase activity. The assay method was not problem free.

No bands appeared on gels loaded with extraction buffer only or with boiled extract, or on gels loaded with extract and incubated without molybdate. However, the complete range of controls for the activity stains was not possible as gels incubated without ATP turned completely white on transfer to lead acetate. No reason for this could be determined.

Lead acetate reacts with both pyrophosphate and inorganic phosphate. Skyring *et al.* (1972) suggest transferring replicate gels into Fiske-SubbaRow reagent, which specifically identifies inorganic phosphate, and incubating at 50°C until blue bands appear as a means of checking that the reaction product is pyrophosphate. No bands should appear after transfer to Fiske-SubbaRow reagent if the reaction product is pyrophosphate unless pyrophosphatase was included in the incubation mixture. Bands were therefore expected on gels transferred to lead acetate after incubation both in the

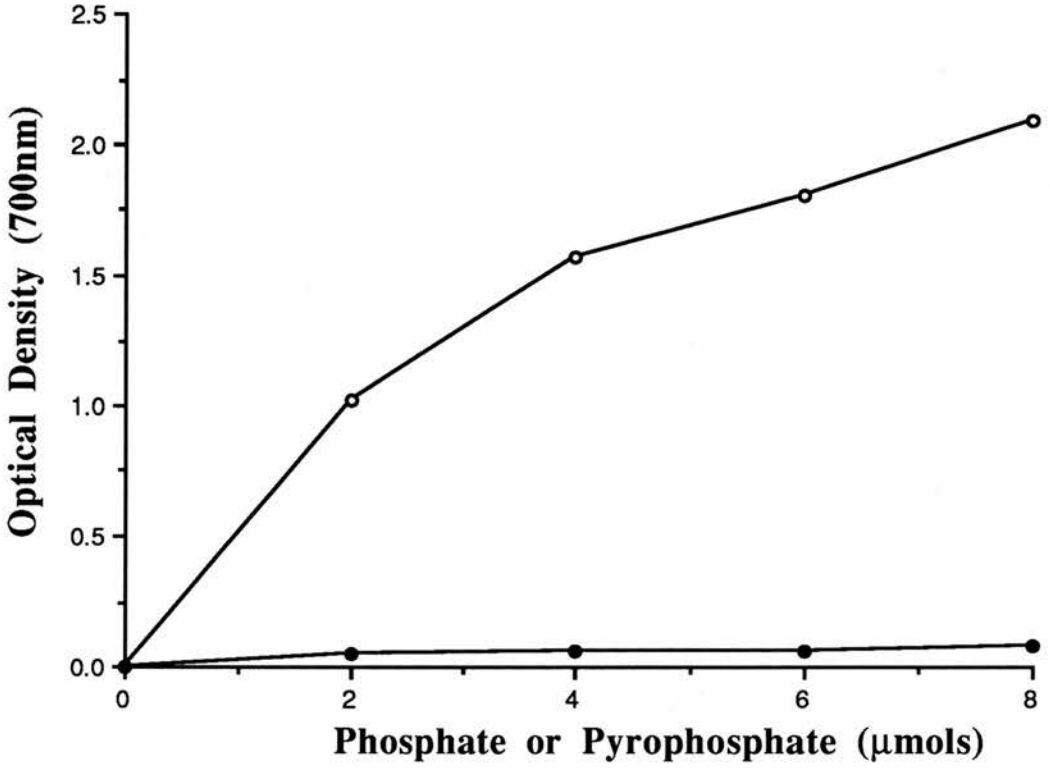
presence and absence of pyrophosphatase, but only after incubation in the presence of pyrophosphatase with Fiske-SubbaRow reagent. In fact, bands were present on all gels. The cause of the unexpected band produced with Fiske-SubbaRow reagent was investigated.

The band could be the result of ATPase activity, forming ADP and inorganic phosphate from ATP and not ATP sulphurylase. If so then the band should not be dependant on the presence of molybdate in the incubation mix. Without molybdate no band appeared with either lead acetate or Fiske-SubbaRow reagent suggesting that ATPases are not responsible for the observed bands.

Fiske-SubbaRow reagent could be detecting the presence of pyrophosphate as well as phosphate under the assay conditions used. Calibration curves for phosphate and pyrophosphate with Fiske-SubbaRow reagent showed a very slight colour change with increasing pyrophosphate concentration but considerably less than with phosphate (Fig 3.14). If the Fiske-SubbaRow reagent did detect inorganic phosphate the bands would have been expected to be more intense in the presence of pyrophosphatase. This was not the case, suggesting the Fiske-SubbaRow reagent was not reacting with pyrophosphate and that the bands were due to inorganic phosphate.

The possibility that pyrophosphate may break down to give phosphate under the assay conditions was investigated. Incubation of pyrophosphate for 30 minutes with acid molybdate at 50°C, the conditions for incubation of the gel after transfer, gave a positive result with Fiske-SubbaRow reagent suggesting that non-enzymatic breakdown of pyrophosphate to phosphate during the incubation with Fiske-SubbaRow occurs. In further tests on gels incubated with Fiske-SubbaRow reagent at 25°C instead of the 50°C suggested by Skyring *et al.* (1972) no band was observed minus pyrophosphatase

Fig 3.14 Phosphate and pyrophosphate standard curves



To check whether the bands seen on non-denaturing polyacrylamide tube gels incubated with ATP-sulphurylase activity gel assay mixture (5mM ATP, 10mM $Na_2MoO_4.2H_2O$ and 5mM $MgCl_2$ in Tris-HCl buffer, pH 7) and then stained with Fiske-SubbaRow reagent could be due to pyrophosphate standard curves were set up with increasing amounts of phosphate or pyrophosphate.

Phosphate or pyrophosphate in 4mls of Tris-HCl buffer, pH 7, 1ml of Fiske-SubbaRow reagent and 400μl acid molybdate solution (see section 2.2.9) were added and mixed well. After 30 minutes standing at room temperature the optical density at 700nm was read. The plot shows the colour reaction of Fiske-SubbaRow reagent with phosphate (o) and pyrophosphate (•).

but a band was seen when pyrophosphatase was included. These results would suggest that pyrophosphate is the product of the reaction. Why the band should appear at 50°C with Fiske-SubbaRow reagent in these experiments and not when run by Skyring *et al.* (1972) is not known.

These results were considered conclusive enough proof that the assay method was detecting ATP-sulphurylase activity for it to be used to analyse M_2 selections from the selenate screen and their M_3 progeny.

3.1.3 Characterisation, localisation and purification of cysteine synthase from barley leaves

Barley cysteine synthase was characterised in a series of experiments. First, how development of activity was affected by the nutrition and growth conditions of the seedlings was examined. Second, the development of activity with seedling age was considered. The subcellular localisation and number of isoenzymes in barley, if any, were also investigated, and finally the enzyme was partially purified. Purified cysteine synthase would be useful in developing the coupled assay systems for sulphite reductase and serine acetyltransferase.

A similar series of experiments would be necessary to characterise the other enzymes of the pathway.

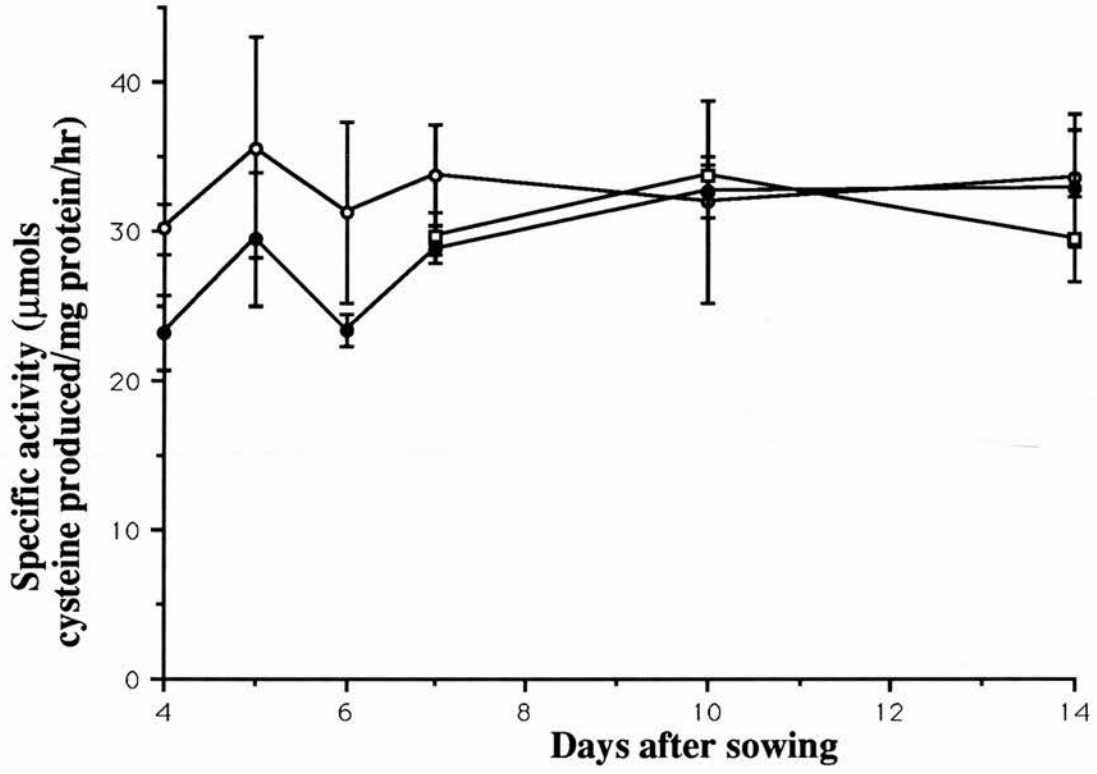
3.1.3.1 Cysteine synthase activity during seedling development

Cysteine synthase activity was measured in seedlings of different ages, grown under differing regimes of lighting and nutrition. Before 4 days after sowing seedlings were not sufficiently developed to allow harvesting of tissue for assay, therefore these experiments were carried out on seedlings from 4 days to 14 days after sowing.

Development of activity was not dependent on light. No significant differences were seen between the cysteine synthase activities of leaf tissue sampled from seedlings grown in the light or in the dark, transfer from dark to light after 6 days did not affect the activity levels (Fig 3.15). No change in the cysteine synthase activity of the seedlings was seen over the 4-14 day sampling period.

Soaking vermiculite in distilled water overnight and the subsequent assay of this water for sulphate content by the method of

Fig 3.15 The effect of lighting conditions on the specific cysteine synthase activity of developing barley cultivar Golden Promise wild type seedlings



Seedlings were germinated and grown in the light (O) or the dark (●), or germinated and grown in the dark until day 6, then transferred to the light (□).

All seedlings were treated with the same nutrient medium, half-strength Hoaglands, containing sulphate and with 20mM KNO_3 added. Leaf tissue was extracted and assayed as described in section 2.2.3.1.

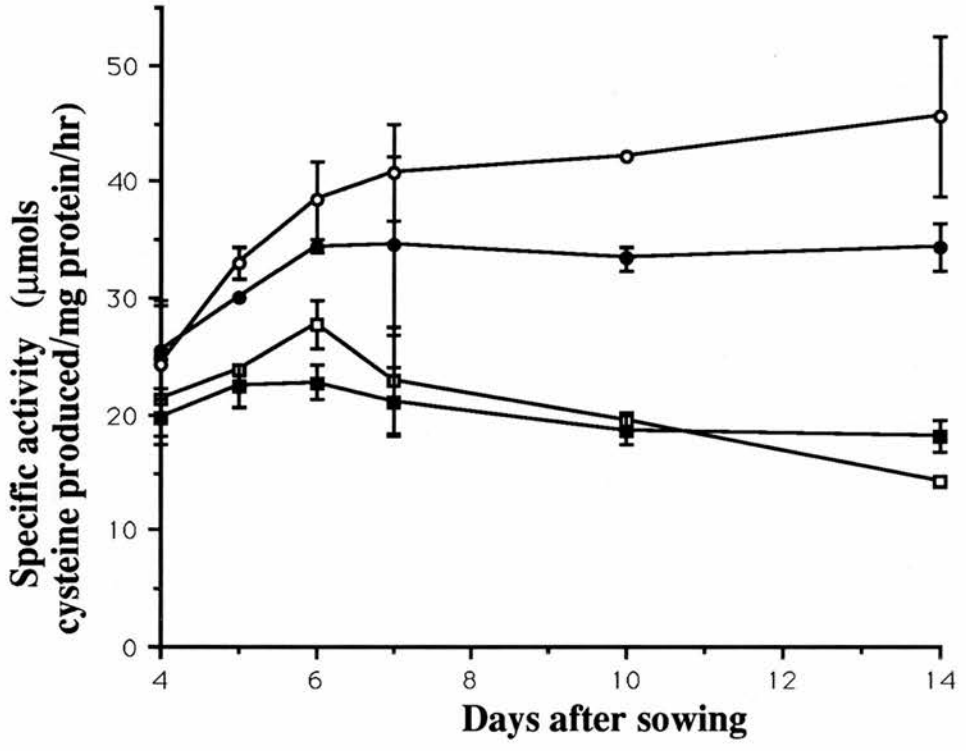
The results are expressed as the mean of 3 separate experiments ± standard error of the mean.

Tabatabai and Bremner (1970) (see section 2.2.10) showed that 1.2L of vermiculite, the volume used/seed tray for sowing, contained approximately 6.2mg sulphur. This compares to 32.9mg sulphur/L modified half-Hoaglands nutrient solution. Therefore even seedlings treated with sulphate-free modified half-Hoaglands nutrient solution had small quantities of sulphate available. To ensure a zero sulphate basal level for experiments where seedlings were grown in the presence and absence of added sulphate, the plants were germinated on muslin draped over metal grids suspended over the nutrient solution. The muslin dipped into the solution and watered the seedlings by capillary action during germination. After germination the seedling roots grew through the muslin into the nutrient solution.

Using this system, seedlings grown with 20mM nitrate added to the nutrient media had higher cysteine synthase specific activities than those grown without added nitrate. The presence and absence of added sulphate in the nutrient media had no effect on seedlings grown without added nitrate. With added nitrate the addition of sulphate decreased the specific cysteine synthase activity (Fig 3.16). The highest specific activity was seen in seedlings grown in the presence of nitrate and absence of sulphate. Seedlings grown without nitrate showed no increase in specific activity over the sampling period. Those grown with added nitrate showed a slight increase from 4-6 days when sulphate was also included in the nutrient medium (this was not seen in the seedlings in the light/dark experiments) and from 4-14 days without added sulphate.

In a separate experiment seed sown in vermiculite was treated with half-strength Hoaglands nutrient medium containing sulphate with (two trays) or without added nitrate (four trays) and germinated in the light. After 8 days two trays of seedlings grown without added

Fig 3.16 Plot showing the effect of sulphate and nitrate starvation on the cysteine synthase activity of barley cultivar Golden Promise wild type seedlings.



Seedlings were grown, in the light, on grids suspended over the nutrient media containing:

No added sulphate or nitrate ($-SO_4-NO_3$) (□)

No added sulphate with nitrate (20mM) added ($-SO_4+NO_3$) (○)

Sulphate (1mM) with no added nitrate ($+SO_4-NO_3$) (■)

Sulphate (1mM) and nitrate (20mM) added ($+SO_4+NO_3$) (●)

as described in section 2.2.3.1.

The results are expressed as the mean of two separate experiments ± standard deviation.

nitrate were treated with nutrient medium containing 20mM nitrate. Leaf tissue samples were harvested immediately after treatment and at intervals up to 48 hours after treatment. As anticipated from the previous results extract of seedlings grown without added nitrate contained lower specific cysteine synthase activity than those grown with added nitrate. The specific activity of seedlings germinated without added nitrate and treated on day 8 increased over that of the untreated seedlings grown without nitrate from 8 hours after treatment. After 48 hours the specific activity of the seedlings grown with added nitrate remained higher than that of the other seedlings (Fig 3.17).

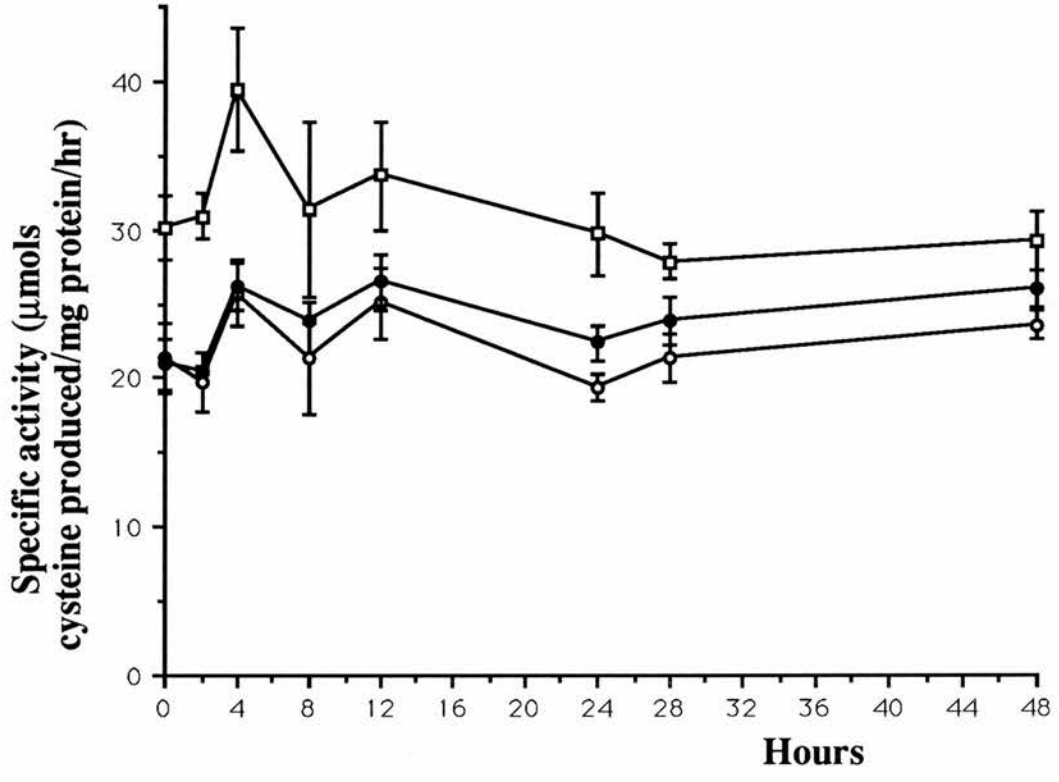
3.1.3.2 Subcellular localisation of cysteine synthase activity

The localisation of cysteine synthase activity in barley leaf cells was investigated using differential centrifugation of ruptured isolated protoplasts.

The method described for the isolation of intact protoplasts from barley seedlings was found to be most effective when the second leaves of 7 day old seedlings grown in compost with a 16 hour day length were used. When vermiculite grown seedlings were used the protoplast harvest was considerably reduced.

The percentage chlorophyll and enzyme activities recovered in each fraction is presented in Table 3.3 for two separate fractionations. In the first fractionation the high proportion of fumarase, a soluble mitochondrial enzyme, and nitrite reductase, a chloroplast located enzyme, recovered in the soluble fraction suggests that most of the mitochondria and some of the chloroplasts are ruptured. P1, the chloroplast enriched fraction is contaminated with peroxisomes as

Fig 3.17 Plot showing the effect of nitrate nutrition on the extractable cysteine synthase activity from barley cultivar Golden Promise wild type seedlings.



Seed was sown in trays of vermiculite, treated with half strength Hoaglands nutrient solution containing no added nitrate (4 trays) (O) or 20mM KNO_3 (2 trays) (□), and germinated in the light. On the eighth day after sowing two trays of the seedlings germinated without added nitrate were treated with nutrient solution containing 20mM KNO_3 (●). 2 x 1g tissue was harvested from each tray at 0, 2, 4, 8,12, 24, 28 and 48 hours after treatment, extracted, and assayed for cysteine synthase activity as described in section 2.2.3.1.

The results are expressed as the mean of the activities for each treatment ± standard deviation.

Table 3.3 Subcellular localisation of cysteine synthase and the marker enzymes; nitrite reductase, fumarase, cytochrome c oxidase and catalase, in fractions obtained by differential centrifugation of mechanically ruptured barley leaf protoplasts.

Fraction	Experiment	Chlorophyll	Enzyme				
			Nitrite reductase	Fumarase	Cytochrome c oxidase	Catalase	Cysteine synthase
P1	1	96.4	41.4	9.9	18.8	50.5	57.1
	2	84.7	46.5	16.5	0.6	6.5	51.4
P2	1	2.5	16.65	14.1	80.3	15.1	2.9
	2	15.3	15.5	43.7	77.2	43.7	10.0
S	1	1.1	41.95	76.0	1.2	34.4	40.0
	2	0	38.0	39.8	22.2	49.8	38.6

Preparation of the protoplasts, centrifugation and measurement of the enzyme activities were carried out as described in section 2.2.6. P1 (the chloroplast enriched fraction) is the resuspended pellet after centrifugation at 15-1600g for 1 minute. P2 (the mitochondria and peroxisome enriched fraction) is the resuspended pellet after centrifugation at 10,000g for 10 minutes. S (the soluble fraction) is the resulting organelle free supernatant. The results are expressed as the percentage of chlorophyll or enzyme activity recovered in each fraction from two separate experiments.

shown by the presence of catalase. Damage to the organelles and contamination of each fraction is less in the second fractionation. However, 40% of the total fumarase activity, a soluble mitochondrial enzyme, is still found in the soluble fraction indicating considerable damage to the mitochondria. About 40% of the nitrite reductase activity is also located in the soluble fraction indicating some damage to the chloroplasts. The chloroplast enriched fraction appears to be much less contaminated with peroxisomes.

In both fractionations the percentage of cysteine synthase in each fraction closely resembles that of nitrite reductase, indicating that in barley leaves cysteine synthase is likely to be located within the chloroplast.

3.1.3.3 Partial purification of cysteine synthase

The purification of cysteine synthase should give information on the number of forms present in barley leaf tissue and allow further studies on the enzyme, including molecular weight determination. The availability of partially purified cysteine synthase would also allow the development of the coupled assay methods for sulphite reductase (von Arb and Brunold, 1983) and serine acetyltransferase (Nakamura *et al.*, 1987).

Investigation showed that 90 percent of cysteine synthase activity is recovered in the 40-60% ammonium sulphate cut (Table 3.4). This was used as the first step in the purification scheme.

The potassium phosphate concentration range used for the gradient on the first DEAE Sephadex A50 column was decided using a series of four 2 ml bed volume columns. Each column was loaded with the same cysteine synthase activity and eluted with buffer containing 200, 300, 400 or 500mM potassium phosphate. The

Table 3.4 Percentage of recovered cysteine synthase activity from increasing ammonium sulphate saturated precipitations.

Percentage Ammonium Sulphate	Percentage Recovered Cysteine Synthase Activity
0-20	0.3
20-40	2.0
40-60	90.5
60-80	7.2

10.7g ammonium sulphate was dissolved in 100mls crude extract, from 10g of 7 day old barley leaf tissue, to give 20% ammonium sulphate. The extract was stirred continuously for 30 minutes then centrifuged at 15,000g in an MSE 18 centrifuge for 15 minutes at 4°C. The pellet was resuspended in a minimum of buffer (11mls), this is the 0-20% cut. The supernatant was adjusted to 40% by the addition of a further 11.5g ammonium sulphate, stirred and centrifuged as before. The pellet was resuspended in 26ml, the 20-40% cut. The supernatant adjusted to 60% with the addition of 12.2g ammonium sulphate. After stirring and centrifugation as before the pellet was resuspended in 25ml, the 40-60% cut, and the supernatant adjusted to 80% with 13.1g ammonium sulphate. The pellet was resuspended in 11.5ml, the 60-80% cut, and the supernatant discarded. All samples including the original crude extract were assayed for cysteine synthase activity. 83% of the activity of the crude extract was recovered in the ammonium sulphate cuts. The percentage of the recovered activity in each ammonium sulphate cut as given above.

percentage of activity recovered from the four columns was 33.6, 78.9, 97.9 and 107.2 respectively. A gradient of 50-500mM potassium phosphate was chosen to ensure that all the cysteine synthase activity was eluted.

A summary of the purification scheme is given in Table 3.5. Activity eluted from the first DEAE Sephadex column in two distinct peaks (Fig 3.18). A small peak (about 10% of the activity) at 215mM potassium phosphate and a larger peak at 470mM potassium phosphate. Only fractions with significant activity from the large peak (fractions 90-145) were pooled and concentrated before loading onto the G100 column. A single peak of activity eluted from this column (Fig 3.19), fractions 20-27 were pooled and loaded directly onto the second DEAE Sephadex column. Again a single peak of activity was eluted as shown in Fig 3.20.

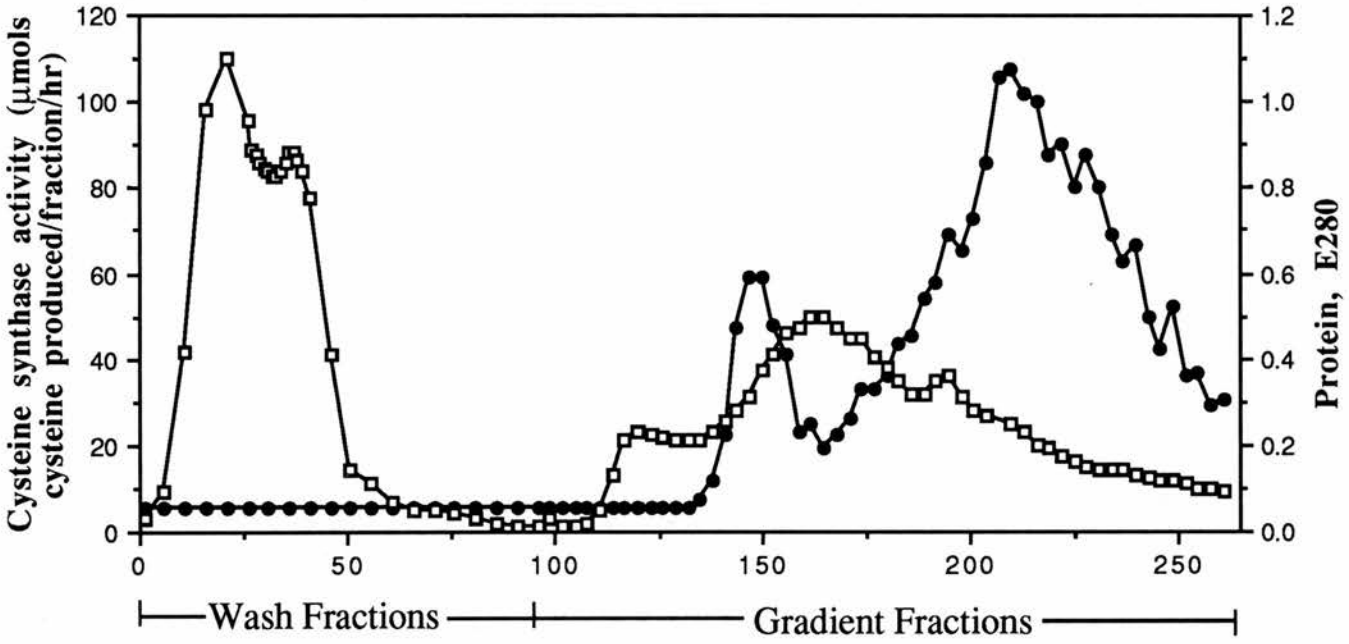
The partial purification procedure afforded *ca* 400 fold purification of cysteine synthase and a yield of 11%.

Table 3.5 Summary of scheme used for the partial purification of cysteine synthase from 7 day old barley leaf tissue

Purification step	Total volume (ml)	Total protein (mg)	Total activity (µmols/min)	Specific activity (µmols/min/mg)	Yield (%)	Purification
Extraction	860	1170.0	160.5	0.137	100	1 0
40-60% ammonium sulphate	208	645.5	168.14	0.260	105	1.9
1st DEAE sephadex A50 pooled fractions	670	144.0	119.71	0.831	74.6	6.06
80% ammonium sulphate	60	54.14	69.33	1.28	43.2	9.34
G100 column	63.5	1.905	25.40	13.33	15.8	97.32
2nd DEAE sephadex A50 column	53.5	0.589	18.19	30.91	11.33	225.62
concentrated pooled fractions	3.4	0.340	17.68	52.00	11.02	379.59

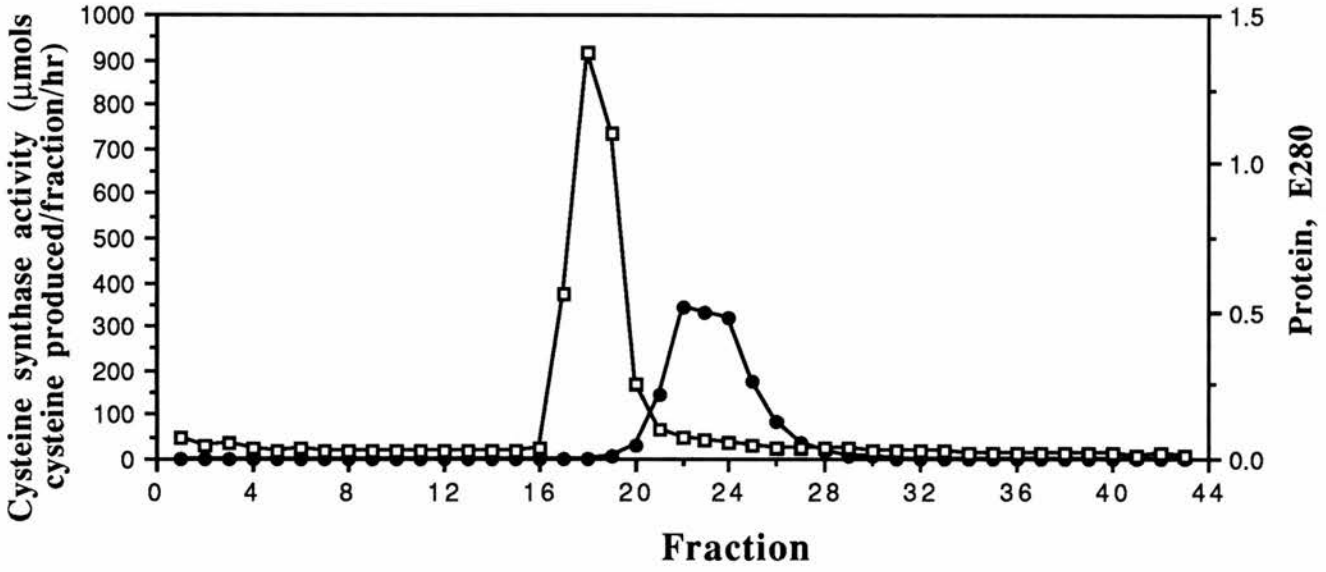
100g of 7 day old barley leaf tissue was extracted in 900ml cysteine synthase extraction buffer, 0.1M potassium phosphate pH 7 with 1mM EDTA and 1mM DTT. After centrifugation at 30,000g (SS34 rotor and Sorval RC5 centrifuge) for 15 minutes at 4°C the supernatant was used as crude extract, the starting point for the purification (see section 2.2.7).

Fig. 3.18 Elution profile of cysteine synthase activity for the first DEAE sephadex column in the partial purification of cysteine synthase from barley leaves.



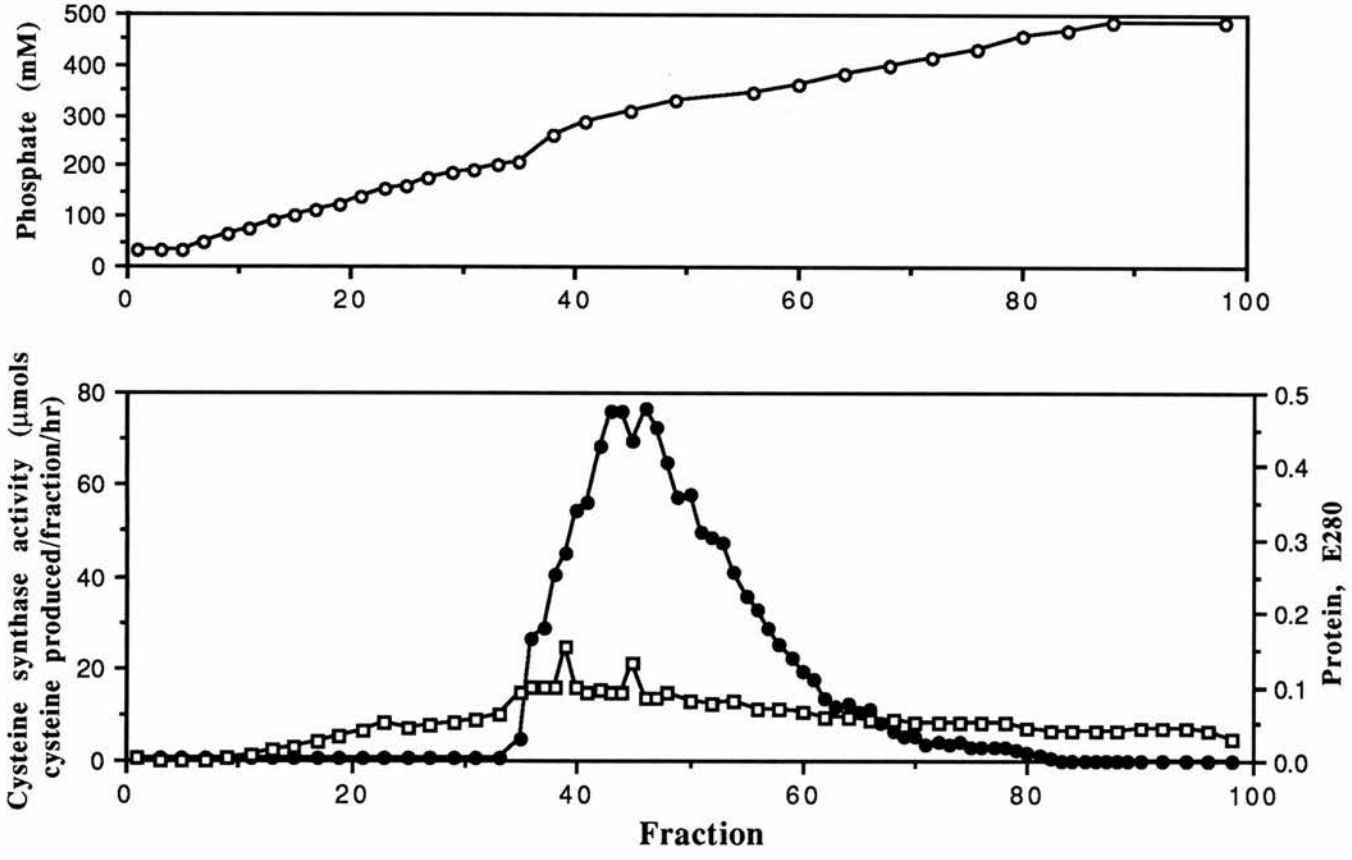
Separation of cysteine synthase activity of barley leaf extract by anion-exchange chromatography using a DEAE Sephadex column (4 x19cm). The dialysate of the 40-60% ammonium sulphate precipitated fraction was centrifuged (15,000g in the MSE 18) and the resulting supernatant applied to the column. The column was washed with 30mM potassium phosphate buffer, pH 8, containing 1mM EDTA and 10mM mercaptoethanol, and then eluted with a linear gradient of increasing potassium phosphate concentration (50-500mM). Wash fractions of 14.4ml and gradient fractions of 12.5ml were collected. Every fifth wash fraction (all fractions between 27 and 39) and every second gradient fraction were assayed for cysteine synthase activity (●) and protein, E280 (□). Fractions 90-145 were pooled and concentrated by ammonium sulphate precipitation before loading onto the G100 column.

Fig. 3.19 Elution profile of cysteine synthase activity from the Sephadex G100 Column step of the partial purification of cysteine synthase from barley leaves.



Separation of cysteine synthase activity of barley leaf extract by gel filtration using a Sephadex G100 column (2.5 x 93cm). Fractions containing significant cysteine synthase activity from the 1st DEAE Sephadex column were pooled, concentrated by 80% ammonium sulphate precipitation and loaded onto the G100 column. The column was eluted with 30mM potassium phosphate buffer pH 8 containing 1mM EDTA and 10mM mercaptoethanol. Fractions of 9.5ml were collected and assayed for cysteine synthase activity (●) and protein, E280, (□). Fractions 20-27 were pooled and loaded onto the second DEAE sephadex column.

Fig. 3.20 Elution profile of cysteine synthase activity from the second DEAE Sephadex column step of the partial purification of cysteine synthase from barley leaves.



Separation of cysteine synthase activity of barley leaf extract using a second DEAE Sephadex column (1.5 x 4cm). Fractions with significant activity from the G100 column were pooled and applied to the column. The column was washed with 30mM potassium phosphate buffer, pH 8, containing 1mM EDTA and 10mM mercaptoethanol and eluted with a linear gradient of increasing potassium phosphate concentration (30 - 500mM). Fractions of 5ml from the wash and 3ml from the gradient were collected and assayed for cysteine synthase activity (●), protein, E280(□) and phosphate (○). Gradient fractions 36-54 were pooled and concentrated.

3.2 Discussion

3.2.1 Solution assays

During the course of the project solution assays for two of the enzymes of the sulphate assimilation pathway, cysteine synthase and thiosulphate reductase, were developed for routine use with barley extract. An assay method for serine acetyltransferase was developed but it requires further modification before it can be used routinely and reproducibly. An assay method for the enzyme β-cyanoalanine synthase was also developed.

3.2.1.1 Cysteine synthase

The conditions for the assay were optimised for use with barley crude extract. 15 minutes at 25°C with 6mM Na_2S and 15mM OAS was used whereas 15 minutes at 35°C with 1mM Na_2S and 20mM OAS was used by Ng and Anderson (1978a). They reported cysteine synthase activities of 32.4 and 46.8 μmols cysteine produced/mg protein/hr for white clover and pea respectively. These are very similar values to the activities seen here in barley of between 16 and 42 μmols cysteine produced/mg protein/hr. Rosichan *et al.* (1983) reported the 45-75% ammonium sulphate cut of crude 6 day old barley leaf extract to contain 30.6 μmols cysteine produced/mg protein/hr. Other plants are reported to have activities in this region including spinach, 10.2 μmols cysteine produced/mg protein/hr (Murakoshi *et al.*, 1985) and 30 μmols cysteine produced/mg protein/hr (Droux *et al.*, 1992), and wheat 1.2 μmols cysteine produced/mg protein/hr (Ascano and Nicholas, 1977).

The observation that cysteine synthase extracted from barley was stable at 50°C for at least 10 minutes (Rosichan *et al.*, 1983) is

supported by the results from this study where cysteine synthase is stable for at least 20 minutes at 50 and 60°C. Bertagnolli and Wedding (1977) however, report the cysteine synthase from two species of *Phaseolus* to be unstable at 40-45°C. Activity was lost rapidly at 70 and 80°C. The expected protection from inactivation afforded by pyridoxal phosphate (Ng and Anderson, 1978a; Rolland *et al.*, 1992) was not seen when tissue was extracted, incubated and assayed in its presence. Although the differences were not significant after 20 minutes at 50 and 60°C there is a possibility that the presence of pyridoxal phosphate may prevent enzyme inactivation over longer incubation times at these temperatures.

Bertagnolli and Wedding (1977) suggest that extraction of the enzyme in phosphate buffer reduces the protection given by pyridoxal phosphate and gives activities an order of magnitude lower than extraction in Tris-HCl buffer, reporting the activity of crude extract from *Phaseolus* to be 268.2-285 µmols cysteine produced/mg protein/hr. The previously mentioned activity for barley of 30.6 µmols cysteine produced/mg protein/hr (Rosichan *et al.*, 1983) was extracted using Tris-HCl buffer. So, for barley at least, extraction in phosphate buffer does not reduce the observed activity.

3.2.1.2 Serine acetyltransferase

The method of Ngo and Shargool (1974) uses dithiodinicotinic acid to measure serine acetyltransferase activity in the germinating seed of different species of rape (*Brassica* spp.). In attempts to utilise this method to determine the serine acetyltransferase activity of barley leaf extract the addition of 0.2ml 6mM dithiodinicotinic acid, as specified by Ngo and Shargool (1974) (final concentration 1mM), always caused the solution to become turbid. This cloudiness of the

solution made reading the absorbance impossible. Reducing the volume of dithiodinicotinic acid added reduced the turbidity of the solution. However, 0.12mM dithiodinicotinic acid alone gave an absorbance at 355nm above 2 OD units. Why it was not possible to reproduce this assay method is unclear.

A second method, described by Nakamura *et al.* (1987), for determining serine acetyltransferase activity was tried. This is a coupled assay where the O-acetyl-L-serine produced from L-serine and acetyl CoA by the action of serine acetyltransferase is converted to cysteine in the presence of added sulphide and cysteine synthase. The method of Gaitonde (1967) is then used to measure cysteine production. Initially cysteine synthase extraction buffer (0.1M K-Pi pH 7, 1mM Na_2EDTA and 1mM DTT) was used in the hope that a single extraction buffer could be found that was suitable for the enzymes of the pathway. No activity was detected. Other workers used potassium phosphate pH 8 with either mercaptoethanol or DTT (added to maintain the reduced state of the sulphydryl groups) as extraction buffer (Nakamura *et al.*, 1987 and 1988; Ngo and Shargool, 1974; Droux *et al.*, 1992).

Inclusion of 20% (v/v) glycerol in the extraction buffer, which Ngo and Shargool (1974) found necessary, did not result in detectable levels of serine acetyltransferase activity. When 5% PVP (Polyclar AT) and 3% BSA were added to the cysteine synthase extraction buffer an activity of 0.03 µmoles cysteine produced/g barley leaf tissue/hr was measured with crude extract alone and 3.75 µmoles cysteine produced/g barley leaf tissue/hr when partially purified cysteine synthase was included in the assay mix. It was not possible to calculate the specific activities because of the inclusion of BSA in the extraction buffer. These activities compared well with those reported

by Nakamura *et al.* (1987) for leek leaf extract (1.38 μmoles cysteine/g fresh weight/hr), spinach leaf extract (2.1 μmoles cysteine/g fresh weight/hr), radish root extract (2.34 μmoles cysteine/g fresh weight/hr) and rape leaf extract (3.3 μmoles cysteine/g fresh weight/hr). However, the activities obtained here, for barley leaf extract, were calculated from cysteine contents at the lower limit of the cysteine standard curve, and the high error level involved in reading these values means the activities are not reproducible.

More modifications to the assay procedure may improve the results further. For example the quantity of partially purified cysteine synthase added could be optimised to ensure that all O-acetyl-L-serine produced is converted to cysteine. As it stands the assay method is not suitable for use as a routine assay for serine acetyltransferase activity.

Two other assay methods have been used by other workers which could be attempted with barley extract. The first method modified by Ascano and Nicholas (1977) from Kredich and Tomkins (1966) is based on the production of coloured thionitrobenzoic acid from dithionitrobenzoic acid and acetyl CoA. The second method involves the conversion of ^{14}C-serine to O-acetylserine, followed by conversion to N-acetylserine by treatment with alkali (Smith and Thompson, 1969). The labelled N-acetylserine is then separated from the ^{14}C-serine using a Dowex column and quantified by liquid scintillation. This labelled method would appear to be time consuming and complicated as well as potentially expensive, making it inappropriate for routine daily use if a simpler and cheaper method could be optimised to give satisfactory results.

3.2.1.3 Thiosulphate reductase

The activity of 110 nmoles sulphide produced/mg protein/hr obtained for thiosulphate reductase activity in crude extract from barley seedling leaf tissue is of the same order of magnitude as the activity reported by Schmidt *et al.* (1984) of 846 nmoles sulphide produced/mg protein/hr for *Chlorella.* Uhteg and Westley (1979) and Chauncey and Westley (1983a, b) also report activities for baker's yeast in this region, of 243 nmoles sulphide produced/mg protein/hr and 146 nmoles sulphide produced/mg protein/hr respectively. Thiosulphate reductase activity from *Desulfovibrio vulgaris* MF is reported to be 243 nmoles sulphide produced/mg protein/hr (Aketagawa *et al.*, 1985).

The assay was not without problems. As noted by Schmidt *et al.* (1984) the presence of thiosulphate above 0.2mM progressively inhibits the formation of methylene blue. The results shown in Table 3.1 show that even at 0.2mM thiosulphate inhibits the formation of methylene blue by 32%. Clearly if this is the case then our observed activity may be an over estimate of the actual activity since reduction of thiosulphate will not only result in an increase in the methylene blue formation due to sulphide production but also a decrease in the inhibition of methylene blue formation by decreasing the thiosulphate concentration. Before this assay would be suitable for routine use more investigation into the effect of thiosulphate concentration would be needed to be certain that recorded activities were entirely due to enzyme activity.

3.2.1.4 β-cyanoalanine synthase

The colorimetric assay method of Miller and Conn (1980) based on the production of methylene blue from the sulphide product of the enzyme reaction was straight forward. It did not require modification other than 20 minutes being chosen as the most suitable assay time and the addition of an extra step to allow any CN gas evolved after the addition of the acid reagents to be removed safely.

The activities obtained, between 72 and 105 nmoles sulphide produced/mg protein/minute, for crude barley leaf extract are higher than the activities reported for other non-cyanogenic plants (Hendrickson and Conn, 1969; Miller and Conn, 1980). Wurtele *et al.* (1985) reported barley seedling leaves to show particularly high activity of β-cyanoalanine synthase, 1.1μmols/minute/g fresh weight (105 nmoles sulphide produced/mg protein/minute equates to 1.4μmols sulphide produced/min/g fresh weight). Apart from barley only the cyanogenic plants (plants with the ability to produce HCN) *Nandida domestica* and *Sorghum bicolor* have been reported to have activities in this region (30 nmoles sulphide/mg protein/min) (Miller and Conn, 1980).

3.2.2 Activity gels

Non-denaturing polyacrylamide gel electrophoresis separates a protein mixture in such a way that subunit interaction, native protein conformation and biological activity are preserved. Separation of native proteins occurs by virtue of their size and charge. Specific staining of non-denaturing polyacrylamide gels for enzyme activity is a useful tool in the analysis of enzymic pathways. They can be used simply to show the presence of a particular enzyme in an extract, or to provide evidence of multiple forms of an enzyme or of different enzymes that can catalyse the same reaction. An estimate of molecular weight may also be obtained but as separation is affected by charge and protein shape the value obtained may not be completely accurate.

It would therefore be advantageous to develop specific activity stains for all the enzymes which could possibly play a role in sulphate assimilation. Although the presence of an enzyme would not prove it's particular role within the plant, once combined with localisation and regulation studies, evidence would mount.

It is hoped to use specific activity staining in the analysis of putative selenate-resistant mutants selected from the screen. By checking for the presence or absence of functional enzymes previously shown to exist in wild type barley, the position in the pathway of the mutation resulting in selenate resistance may be determined.

During the course of the project specific stains were developed for four of the eight enzymes that have been suggested as having a possible role in sulphate assimilation in either the bound or free intermediate pathway. Further modification of the method attempted for serine acetyltransferase may result in development of a usable stain. No activity staining methods for APS sulphotransferase, APS

kinase and PAPS reductase have been reported but it may be possible to develop them.

3.2.2.1 Cysteine synthase and β-cyanoalanine synthase

After non-denaturing polyacrylamide gel electrophoresis two bands, one at the position of cysteine synthase and one at the position of β-cyanoalanine synthase, were expected, and obtained, when gels were stained in the presence of cyanide as described by A. Schmidt (pers. comm. to J.L. Wray). The appearance of two bands on gels stained specifically for cysteine synthase activity (without cyanide) was unexpected. Inclusion of cyanide in the staining mixture had been suggested as a method of increasing the rate of appearance of cysteine synthase activity bands. Gels stained without cyanide required at least two hours incubation with the staining mix for activity bands to become dark enough to photograph. With cyanide present, activity bands appear within minutes. The detection of β-cyanoalanine synthase in the presence of cyanide should not have caused a problem since staining with and without cyanide would indicate which band was due to which enzyme, with β-cyanoalanine synthase activity only detectable in the presence of cyanide.

It seemed likely that the slower migrating of the two bands, the paler one when stained without cyanide and the more intense one with cyanide, was due to β-cyanoalanine synthase activity. β-cyanoalanine synthase has a pH optimum around 9.5, therefore conditions would be favourable for the enzyme in this assay system (Hendrickson and Conn, 1969). β-cyanoalanine synthase has been shown to possess some cysteine synthase activity and can form cysteine from O-acetyl-L-serine and sulphide (Hendrickson and Conn, 1969). Hence, it is

likely that the back reaction forming sulphide and O-acetyl-L-serine from cysteine can also occur.

The faster migrating of the two bands, the more intense band without and the paler one with cyanide, seemed likely to be the result of cysteine synthase activity. To be sure of the identity of each activity band further investigation was necessary.

Sectioning gels and assaying the sections for cysteine synthase and β-cyanoalanine synthase activity provided further evidence for this theory. Sections of the gel loaded with crude extract showed two distinct regions of cysteine synthase activity which overlap completely the two distinct regions of β-cyanoalanine synthase activity seen for the same gel and correspond exactly with the two activity bands on stained gels. The slower migrating band, believed to be β-cyanoalanine synthase, corresponded with a region of high β-cyanoalanine synthase activity and lower cysteine synthase activity. Whilst the faster migrating band, believed to be cysteine synthase, corresponded with a region of low β-cyanoalanine synthase activity and higher cysteine synthase activity. Sections from the gel loaded with partially purified cysteine synthase produced a single region of cysteine synthase activity which completely overlapped the region of β-cyanoalanine synthase activity and coincides exactly with the activity band on a gel loaded with partially purified cysteine synthase and the faster migrating band on a gel loaded with crude extract after staining in the presence of cyanide.

The evidence strongly suggests the presence of a single form of cysteine synthase in barley leaf tissue that can form β-cyanoalanine from O-acetyl-L-serine and cyanide, but at about 0.1% of the rate at which it forms cysteine from O-acetyl-L-serine and sulphide (0.4nmols sulphide formed/min compared with 3.92μmols cysteine

produced/hr (65nmols cysteine produced/min)). Also that this enzyme is responsible for the faster migrating activity band on gels loaded with crude extract. Hendrickson and Conn (1969) reported the cysteine synthase from blue lupin to form β-cyanoalanine from O-acetyl-L-serine and cyanide at one-tenth the rate of cysteine formation. The slower migrating band would then indicate the position β-cyanoalanine synthase, suggesting a single form of the enzyme in barley leaf tissue. This enzyme appears to possess considerable cysteine synthase activity. The total cysteine synthase activity recovered from the β-cyanoalanine synthase band region of the gel loaded with crude extract was 64% of the total β-cyanoalanine synthase activity recovered (0.4μmoles cysteine/hr compared to 0.624μmoles sulphide/hr (10.4nmoles/min)).

β-cyanoalanine synthase is reported to be a mitochondrial enzyme (Hendrickson and Conn, 1969; Wurtele *et al.*, 1985) responsible for one step in the conversion of cyanide into asparagine (Blumenthal *et al.*, 1968). This is possibly a detoxification mechanism. Cyanide may be produced on the breakdown of cyanogenic glycosides (Blumenthal *et al.*, 1968) or during ethylene biosynthesis (Tittle *et al.*, 1990). β-cyanoalanine synthase has been shown to be present in wide variety of plants, both cyanogenic and non-cyanogenic species (Miller and Conn, 1980) including barley (A. Schmidt, pers. comm. to J.L. Wray). The enzymes of the sulphate assimilation pathway are known to be present in the chloroplasts of higher plants (Anderson, 1980; Schmidt, 1986). Cysteine synthase activity however, has also been shown to be present at other intracellular locations. In spinach leaves cysteine synthase activity was reported as located primarily in the chloroplasts and cytosol, but also present in the mitochondria; each organelle possessed a distinct

form of the enzyme which could be separated by anion-exchange chromatography (Lunn *et al.*, 1990). Further investigation of the three isoforms reported in spinach have shown that polyclonal antibodies raised against the chloroplastic form of the enzyme exhibit very low cross reactivity with both the mitochondrial and cytosolic proteins suggesting the various isoforms differ in their primary structure (Droux *et al*, 1992; Rolland *et al.*, 1993). The presence of cysteine synthase activity in the proplastids, mitochondria and cytosol of cauliflower inflorescences has also been demonstrated (Rolland *et al*, 1992). Again these activities could be separated by anion exchange chromatography. If cysteine synthesis is possible in each of the three sites the physiological significance of it is unclear as the plastids are believed to be the sole site for the synthesis of almost all amino-acids (Rolland *et al.*, 1992). It is possible that the cysteine production reported in the mitochondria (Lunn *et al.*, 1990; Droux *et al.*, 1992; Rolland *et al.*, 1993) is due to a cysteine synthase side reaction of β-cyanoalanine synthase and not cysteine synthase. This would be consistent with the suggestion that the chloroplasts are the sole site of sulphate assimilation (Anderson, 1980; Schmidt, 1986). If this were to be the case, β-cyanoalanine synthase would not play a role in sulphate assimilation *in-vivo* but may be influential with activities of crude extracts *in-vitro*.

3.2.2.2 Serine acetyltransferase

Unfortunately no suitable activity stain for serine acetyltransferase was developed. It was not surprising that the method based on that of Ngo and Shargool (1974) was unsuccessful since it was not possible to get the assay to work conventionally. The solution assay which did produced some results, the coupled assay of

Nakamura *et al.* (1987), was less suitable for modification as a specific gel stain as it requires the presence of cysteine synthase for visualisation. Cysteine synthase activity would be separated from serine acetyltransferase activity on the electrophoresed gel, and would therefore need to be added to the incubation mixture along with sulphide, serine and acetyl CoA. Coupling enzymes penetrate the gel poorly so that the final product is only formed on the gel surface (Hames, 1981) but if the product can be visualised coupled systems may work. With cysteine the product however, visualisation would not be possible since the method used (Gaitonde, 1967) requires boiling with acid ninhydrin reagent for the colour reaction to occur. The method of Ascano and Nicholas (1977) using dithiodinicotinic acid was also unsuccessful. It is possible that with further investigation to optimise the conditions for the assays of Ngo and Shargool (1974) and Ascano and Nicholas (1977) one of them may eventually produce a specific gel stain for detecting serine acetyltransferase activity.

3.2.2.3 Thiosulphate reductase

Initially the fact that activity bands for thiosulphate reductase and sulphite reductase shared the same position on the gels caused concern, with the possibility that thiosulphate was degrading to sulphide and sulphite, thereby allowing the detection of sulphite reductase instead. However, Skyring and Trudinger (1972) use this staining method for the detection of both sulphite reductase and thiosulphate reductase activities and report that preparations from *Desulfotomaculum nigrificans* give activity bands with sulphite as substrate but not with thiosulphate, indicating thiosulphate does not undergo non-enzymatic breakdown in the assay conditions. Identical

conditions were used for the incubation of gels loaded with extract from barley leaves, so non-enzymatic breakdown of thiosulphate should not occur. Longer gels were run in an attempt to separate the two activities but this was not possible.

Since the presence of bands could not be observed until after auto-oxidation of the methylene blue, gels were incubated for times previously found to give clearly visible bands. There were differences between the two sets of results, gels incubated with thiosulphate required incubation for five hours, compared to two and a half hours with sulphite, for the activity band to be formed. Auto-oxidation of the methylene blue took longer with sulphite as the substrate.

The second method, modified from the solution assay of Schmidt (1984) in which DTE is utilised as electron donor, was used to try to prove whether the band was indeed thiosulphate reductase. The number of bands seen with this assay method varied (Fig 3.1). The presence of a band on some of the boiled extract control gels is an as yet unexplained anomaly. These bands did not correspond to any of the bands seen with unheated extract. The bands observed after incubation using the method of Schmidt *et al.* (1984) were too faint to photograph. Incubation with the two different assay mixtures caused the gels to swell by different amounts but measurement of the R_f values suggest the band observed after incubation using the Skyring and Trudinger method to be at the same position as one of the bands observed after incubation using the Schmidt method. Thus making it seem likely that this band was in fact due to thiosulphate reductase activity.

Thiosulphate reductase activity was inhibited by 1,10 phenanthroline as expected. Activity was reported to be reduced by

88% in the presence of 1mM 1,10 phenanthroline (Aketagawa *et al.*, 1985). Cyanide also inhibited the formation of an activity band although Aketagawa *et al.* reported a stimulation of activity in solution assay with *Desulfovibrio vulgaris*, the mechanism of which is unclear.

Because it was by far the simpler staining method the Skyring and Trudinger method was used for subsequent investigation of the selenate screen selections and their progeny. This method of specific gel staining for thiosulphate reductase activity has also been used by Aketagawa *et al.*, (1985).

Four distinct proteins, of differing molecular weights, all with thiosulphate reductase activity have been reported in *Chlorella* (Schmidt *et al.*, 1984). These thiosulphate reductase activities were shown to have most activity when DTE was supplied as the artificial electron donor. The possibility exists that if more than one form of the enzyme is present in barley, as suggested by the appearance of multiple bands on gels incubated with the solution assay mix of Schmidt, not all of them can utilise reduced methyl viologen as electron donor. This may explain the appearance of a single band with methyl viologen compared with one to three bands observed with DTE.

Clearly the number of isoenzymes catalysing this reaction needs to be investigated further, and as a consequence the method used for the analysis of M_2 selenate screen selections and their M_3 progeny may have to be changed.

3.2.2.4 Sulphite reductase

The fact that the appearance of the band seen on gels loaded with barley leaf extracted in Tris buffer and incubated after electrophoresis with sulphite as substrate, as described by Skyring and Trudinger (1972), was dependant on the presence of sulphite and reduced methyl viologen suggests strongly that the band represents sulphite reductase activity. This is further supported by the inhibition of band formation by CN. CN has been shown to bind to the siroheme prosthetic group of sulphite reductase totally inhibiting the enzyme from catalysing sulphite reduction (Krueger and Siegel, 1976, 1982a).

When extracts for electrophoresis were prepared in 0.1M Tris-HCl a single band of activity was always the result. Extraction in phosphate buffer was tested to see if activity was obtained in the search for a universal extraction buffer suitable for all the enzymes of sulphate assimilation. This would allow a single extraction to be used to test for the presence of all the enzymes which would be especially useful for the analysis of putative selenate resistant plants selected from the selenate screen. When barley leaves were extracted in 0.1M potassium phosphate buffer, pH 7.8, two bands of activity were seen on the gels after electrophoresis and staining for sulphite reductase activity. Aketagawa and Tamura (1980) and Krueger and Siegel (1982a) have reported sulphite reductase to be capable of existing as either a dimer or a tetramer depending on the ionic environment. Two forms of sulphite reductase were reported in spinach leaf extract, with the subunits of one form proteolytically degraded *in vitro* to give rise to the other form. Both species are catalytically active and both forms have been shown to exist in 50mM phosphate buffer, pH 7.7 (Krueger and Siegel, 1982a). It is possible that the

extraction in 0.1M potassium phosphate buffer, pH 7.8, allows the formation of this second form of sulphite reductase, whilst extraction in 0.1M Tris, pH 8, results in only a single form of the enzyme.

3.2.2.5 ATP sulphurylase

The apparent inconsistencies seen in the ATP sulphurylase specific gel staining method are discussed in section 3.1.2.5 along with our attempts to find an explanation for the blue bands observed with Fiske-SubbaRow reagent when the product, pyrophosphate, should only be detectable with the lead acetate method. It seems that under the Fiske-SubbaRow assay conditions pyrophosphate breaks down to inorganic phosphate. However why this problem was not experienced by Skyring *et al.* (1972) when identical conditions were being used remains unclear. We were satisfied that pyrophosphate was the product of the reaction and that the bands on the gels represent ATP sulphurylase activity. The fact that the appearance of the band was dependant on the addition of molybdate also suggested the band represents ATP sulphurylase activity. The assay was used for analysis of M_2 plants selected from the selenate screen.

Two forms forms of ATP sulphurylase have been reported in Spinach (Lunn *et al.*, 1990). The presence of a single form of the enzyme in barley leaf extract is suggested by the appearance of the single white band of activity seen on the gels stained with lead acetate.

3.2.3 Investigations into the properties of cysteine synthase in barley

Barley leaf cysteine synthase was characterised in a series of experiments. Factors influencing the development of the activity were investigated. The subcellular localisation of cysteine synthase was examined and the enzyme was purified ca 400 fold.

3.2.3.1 Cysteine synthase activity during seedling development

Light was shown not to be important in the development of cysteine synthase activity in barley. Levels remained stable over the period 4-14 days after sowing for plants grown with sulphate and nitrate added to nutrient media, regardless of whether the seedlings were grown in the dark or in the light as seen in Fig 3.15. Great care was taken throughout to ensure that no light reached dark grown seedlings. Neuenschwander *et al.* (1991) report cysteine synthase activity to be unaffected by lighting conditions in *Lemna minor*. Cysteine synthase activity was shown to remain unchanged in dark grown maize leaves after transfer to the light whereas ATP-sulphurylase activity increased (Ghisi *et al.*, 1987). Development of activity of both the enzymes of nitrogen assimilation, nitrate reductase and nitrite reductase, are affected by the intensity of illumination. Nitrate reductase activity remains at a basal levels in dark grown pea leaf extract and nitrite reductase activity of dark grown plants is only 30-50% of light grown plants (Gupta and Beevers, 1983).

Cysteine synthase activity in developing barley seedlings was shown to be influenced by nitrogen deprivation. The specific activity of seedlings grown in the presence of nitrate increased over the 4-14 day period of analysis, whilst the specific activity of seedlings grown

without nitrate remained unchanged (see Fig 3.16) (the observation here that activity increases over time in seedlings grown in the presence of sulphate and nitrate contradicts that seen in the previous experiment where the effect of light was considered). The presence and absence of sulphate in the nutrient regime had no effect on the cysteine synthase activity of plants grown without nitrate. The addition of sulphate seemed to decrease the activity of plants grown with nitrate. The effect of nitrate on activity was shown to be slow. Forty eight hours after the addition of nitrate to 8 day old nitrogen deprived barley seedlings the cysteine synthase activity had increased above that of nitrate starved seedlings but was not as high as in seedlings grown with nitrate present from day 1 (Fig 4.4). These results support those of other workers who report that activity declines in plants deprived of nitrogen (Brunold and Suter, 1984; Smith, 1980). Whilst cysteine synthase is not believed to play a major role in regulation of the sulphate assimilation pathway this would fit with the regulatory coupling of the nitrate and sulphate assimilation pathways proposed by Reuveny *et al.* (1980) discussed in section 1.1.2. If coupled in this way sulphur starvation positively regulates sulphate assimilation when nitrate is not rate limiting and when deprived of nitrate no derepression of ATP sulphurylase occurs.

Cysteine synthase activity is then unaffected by some environmental factors, lighting conditions and sulphate availability, and responds only slowly to nitrate availability. During the course of this series of experiments a slight increase in activity over the 4-14 day period was seen in seedlings grown in the light with sulphate and nitrate present, in the investigation of sulphate and nitrate nutrition (Fig 3.16) although seedlings grown under exactly the same conditions whilst looking at the influence of light showed no such

increase (Fig 3.15). In other plants a response during plant development is evident. Schmutz and Brunold (1982) report that the total cysteine synthase activity of primary leaves of *Phaseolus* increases to a maximum level 10 days after imbibition, then decreases to 50% of that maximum at 17 days. Specific cysteine synthase activities were at an appreciable level 3 days after imbibition and remained constant up to 15 days after imbibition, then rising towards the final period of senescence as the total extractable activity decreases more slowly than the total extractable protein (Schmutz and Brunold, 1982). A developmental response was also seen in the cysteine synthase activity during fruit ripening of bell peppers, where cysteine synthase is suggested to be up-regulated during chromoplast development as part of an active antioxidant production program (Romer *et al.*, 1992). The cysteine synthase activity of barley seedlings during development requires further examination.

The role played by cysteine synthase in the regulation of the sulphate assimilation pathway is likely to be minor.

3.2.3.2 The subcellular localisation of cysteine synthase

The results described here suggest that cysteine synthase in barley is probably located within the chloroplast although the fractionations were not entirely successful. Further work would be required before it was possible to be more certain of the location. Although damage to organelles and contamination between fractions was reduced in the second fractionation they were still considerable. Practice, combined with minor modifications to the technique might result in cleaner fractions.

The location of cysteine synthase reported within other plants varies. Most of the activity in peas and white clover is reported to be

within the chloroplast (Ng and Anderson, 1979a) with at least 20% reported present in the chloroplasts of spinach leaves (Fankhauser and Brunold, 1979). Rolland *et al.* (1992), reported activity in the cytosol, proplastids and mitochondria of cauliflower buds. Activity has also been reported in the mitochondria of spinach and pea leaves (Lunn *et al*, 1990), although Fankhauser *et al* (1976) found no appreciable levels of activity associated with the mitochondria or peroxisomes of spinach. Clearly further clarification of the localisation of cysteine synthase is necessary. As discussed in section 3.2.2.1 it is possible that activity seen by some workers in the mitochondria of higher plants is due to β-cyanoalanine synthase rather than cysteine synthase. In any future work the β-cyanoalanine synthase activity of the fractions should be considered along side the cysteine synthase activity.

Cysteine synthase is present not only in leaves but also in roots (Fankhauser and Brunold, 1979), fruit (Romer *et al.*, 1992) and flower buds (Rolland *et al.*, 1992)of plants. In C4 plants, cysteine synthase activity has been shown to be present in both mesophyll and bundle sheath cells (Passera and Ghisi, 1982)

3.2.3.3 Purification of the barley enzyme

The partial purification scheme outlined in Table 3.5 afforded a 400 fold purification of cysteine synthase with a specific activity of 52μmols cysteine produced/min/mg protein and a yield of 11% from 7 day old barley seedling leaf tissue.

Ammonium sulphate precipitation between 40 and 60% followed by dialysis was found to give the highest yield at the first step. No activity was lost at this step. An ammonium sulphate cut between 45 and 70% was used by Rosichan *et al.* (1983) as their first step in barley

cysteine synthase purification,35-70% with spinach (Murakoshi *et al.*, 1985) and 30-70% with *Brassica juncea* (Ikegami *et al.*, 1988b). A greater purification was seen with spinach and *Brassica juncea* at this point than with barley.

The second step in the purification scheme was ion-exchange chromatography on a DEAE Sephadex A50 column. Activity eluted from the column in two distinct peaks. A small peak, containing around 10% of the total activity, eluted around 215mM potassium phosphate and a large peak eluted around 470mM potassium phosphate. Murakoshi *et al.* (1985) and Ikegami *et al.* (1988b) who also use this as the second step of their purification scheme report only single peaks of activity eluting between 110 and 175mM, and 115 and150mM potassium phosphate respectively. The presence of two peaks suggested the possibility of two cysteine synthases in barley. Although only a single form of the enzyme has been reported previously in barley (Rosichan *et al.*,1983), multiple forms of the enzyme have been reported in spinach (Fankhauser and Brunold, 1979; Lunn *et al.*, 1990; Droux *et al.*, 1992; Saito *et al.*, 1992, 1993), watermelon (Ikegami *et al.*, 1988a) and pea (Ikegami *et al.*, 1987). Based on earlier discussions it now seems likely that the first small peak was the result of β-cyanoalanine synthase activity. β-cyanoalanine synthase has been shown to catalyse the formation of cysteine from O-acetyl-L-serine and sulphide, although at only 1/25th of the rate at which it catalyses the production of β-cyanoalanine (Hendrickson and Conn, 1969), and has been proposed here as responsible for the second activity band seen on non-denaturing polyacrylamide gels loaded with barley leaf crude extract stained specifically for cysteine synthase activity. Determination of the β-cyanoalanine synthase activity of these peak fractions would have been

useful in identifying the cause of the peak. Why only a single peak was reported by Murakoshi *et al.* (1985) and Ikegami *et al.* (1988b) when two peaks were seen on each occasion barley extract was used (4 column runs) is unknown. β-cyanoalanine synthase activity has been demonstrated in *Brassica juncea* and blue lupin (*Lupinus augustifolia*) (Miller and Conn, 1980) therefore since both these purification schemes started with crude leaf extract any cysteine synthase activity from the mitochondrial enzyme β-cyanoalanine synthase should have been detectable. Ikegami *et al.* (1988a) have reported two peaks of cysteine synthase activity from the first DEAE Sephadex column with *Citrullus vulgaris* , an initial large peak followed by a second smaller peak. Two peaks of β-(pyrazol-1-yl)-L-alanine (BPA) synthase were also reported, that were completely overlapped by the two cysteine synthase peaks. The first peak showed high cysteine synthase activity compared to the second peak; the opposite of our situation. Ikegami *et al.* (1988a) suggest that the cysteine synthase isoenzyme A (from the first peak) and BPA synthase from *Citrullus vulgaris* are identical.

The first small peak was not considered further and only fractions from the second larger peak of activity were pooled and taken through the remaining steps of the purification scheme. More than 40% of the total activity extracted from the barley leaves was lost at the next step of the purification, an 80% ammonium sulphate precipitation designed to reduce the volume from the 670ml pooled fractions to a manageable one before loading onto the G100 column. The tolerance of cysteine synthase to ammonium sulphate has been shown to be variable with preparations from *Phaseolus vulgaris* losing 39% of their activity when the ammonium sulphate is increased to 90% compared to only 6% lost with preparations from *Phaseolus*

polyanthus under the same conditions (Bertagnolli and Wedding, 1977)

A G100 gel filtration column was chosen as the next step in the purification scheme as used by Murakoshi *et al.* (1985), Ikegami *et al.* (1988a,b) and Rosichan *et al.* (1983) which should separate proteins of molecular weights between 4000 and 150,000. Cysteine synthase should fall within this fractionation range, with the molecular weight reported between 52,000 in pea (Ikegami *et al.*, 1987) and *Brassica juncea* (Ikegami *et al.*, 1988b), and 68,00 in spinach (Droux *et al.*, 1992). A large purification was seen at this step from 9 fold to almost 100 fold purification.

The second DEAE Sephadex ion-exchange column also resulted in a large purification factor as did concentration using an Amicon stirred cell followed by Amicon microconcentrators. Had the stirred cell been have used instead of the second ammonium sulphate precipitation to reduce the volume for loading onto the G100 column, the activity lost at this stage may have been reduced.

89% of the total initial activity was lost during the purification which is around 20% more than the other workers following similar schemes after the same steps. Replacement of the 80% ammonium sulphate precipitation as mentioned above may increase the yield in future purifications.

The stability of the partially purified cysteine synthase after storage was investigated briefly. Aliquots of the partially purified cysteine synthase were frozen at -20°C and -70°C with and without the addition of 50% glycerol for 25 days, after which time they were thawed and assayed for activity. The partially purified enzyme is more stable in the presence of glycerol retaining almost 90% of the activity after freezing at -70°C with added glycerol compared to 62%

without. At -20°C the loss of activity was greater, with only 4% of the original activity retained without glycerol and 86% retained with glycerol. Droux *et al.* (1992) report a 50% loss in specific activity of purified cysteine synthase after storage at -20°C for one week. Clearly the addition of glycerol is important in preserving the activity of stored partially purified enzyme and should be added to all future preparations.

The preparation was subjected to SDS polyacrylamide gel electrophoresis. The appearance of multiple bands (results not shown) indicated that cysteine synthase had not been purified to homogeneity.

CHAPTER 4

DEVELOPMENT OF A POSITIVE SELECTION SCREEN FOR IDENTIFYING SELENATE-RESISTANT BARLEY PLANTS

4.1 Results

4.1.1 Growth of wild type barley seedlings on different nitrate and sulphate regimes

Before deciding on the conditions with which to begin screening the M_2 barley seed for selenate resistance, a knowledge of the effects of differing nutrient regimes on wild type seedling growth was required.

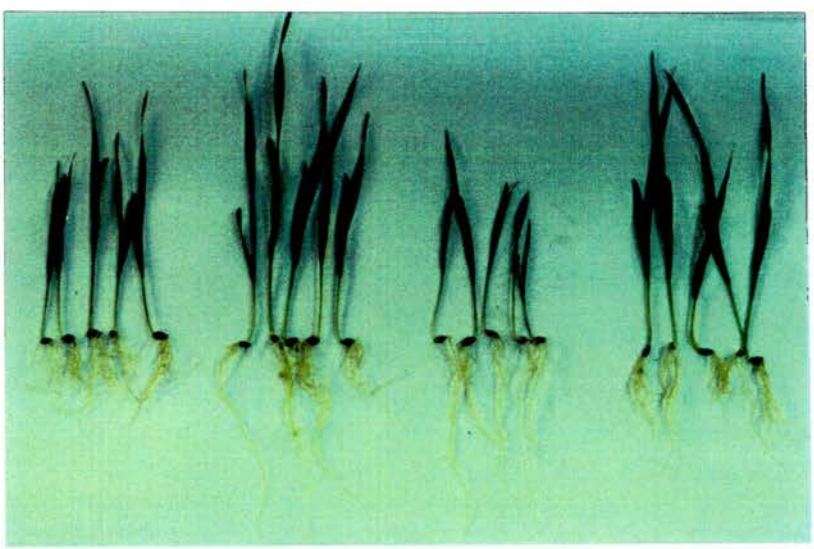
To assess the effect of sulphate and/or nitrate deprivation on the growth of wild type barley cultivar Golden Promise, seed was sown in trays of vermiculite and grown in the light. The trays were treated daily with modified half-strength Hoaglands nutrient medium with or without added sulphate (1mM) and nitrate (20mM). The seedling height and weight were examined 14 days after sowing. Examination showed that the omission of nitrate from the medium reduced plant growth as determined both by their height (Fig 4.1) and fresh weight (Table 4.1). The presence or absence of sulphate had little effect on the appearance of the plants or their weight.

4.1.2 Selenate sensitivity of wild type barley plants

It was necessary to know how different nutritional conditions affect the sensitivity of barley seedlings to selenate so that the screen could be set up using the conditions most likely to allow selenate resistant seedlings to be distinguished from selenate sensitive seedlings.

Seedlings were grown as above (section 4.1.1) until day 6 after sowing, when sodium selenate at concentrations from 0.01-1mM was included in the nutrient medium. Sensitivity of the plants to selenate on the different sulphate and nitrate regimes was assessed on day 14

Fig 4.1 Appearance of 14 day old wild type barley cultivar Golden Promise seedlings grown in the presence and absence of added sulphate and nitrate.



a b c d

Seed was sown in trays of vermiculite, germinated in the light and treated daily with modified half-strength Hoaglands nutrient solutions containing:

a) no added sulphate or nitrate

b) no added sulphate but with KNO_3 (20mM) added

c) added sulphate (1mM) but no nitrate

d) added sulphate (1mM) and nitrate (20mM)

At day 14 plants were removed from the seed trays and the roots gently washed free of vermiculite.

Table 4.1 Fresh weight of 13 day old wild type barley cv Golden Promise seedlings grown in the presence and absence of added sulphate and nitrate.

Nutrient regime	Mean fresh weight mg	(±SD) n=10
$-SO_4$ $-NO_3$	117.2	(43.7)
$-SO_4$ $+NO_3$	278.8	(102.6)
$+SO_4$ $-NO_3$	114.9	(25.1)
$+SO_4$ $+NO_3$	250.5	(49.5)

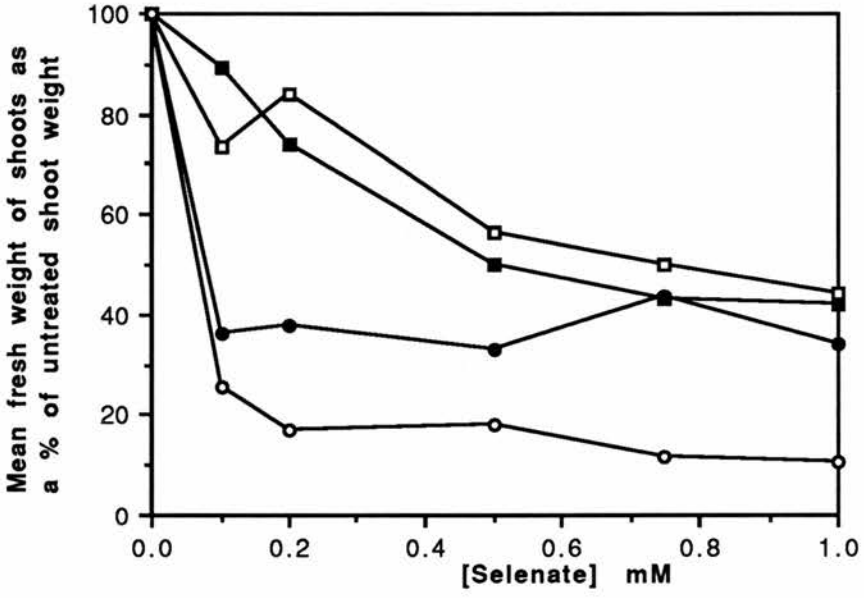
Seed was sown in trays of vermiculite and germinated in the light. Trays were treated daily with modified half-strength Hoaglands nutrient media with or without added sulphate (1mM) and nitrate (20mM). On day 13 after sowing 10 seedlings were carefully removed from each tray and any vermiculite was washed from the roots. After drying the seedlings were weighed.

on the basis of their fresh weights (Fig 4.2), and their appearance relative to appropriate controls which did not receive selenate (Fig 4.3 and Fig 4.4).

Selenate is toxic to barley seedlings. After 6 days exposure to low concentrations of selenate (0.01-0.02mM) seedlings developed brown spots on the leaves followed by chlorosis of the remaining leaf. At higher concentrations these symptoms appeared more quickly and the seedlings then withered and died. The seedlings became increasingly stunted with increasing selenate concentration. Above 0.05mM selenate little or no growth occurred after the inclusion of selenate in the nutrient medium.

Seedlings were most sensitive to selenate in the absence of added sulphate. In both the presence and absence of nitrate, sulphate protected the seedlings against selenate toxicity, but this was most obvious in the plants grown with added nitrate. Sensitivity of seedlings grown with added sulphate was not affected by the presence of nitrate. Although in Fig 4.3 it may look as if the presence of nitrate protects the seedlings, selenate toxicity is assessed by comparison to the control seedlings grown with the same nutrient regime. Control seedlings grown in the absence of nitrate (a) were much smaller and less healthy than those grown with 20mM KNO_3 added (b) as shown in section 4.1.1 (Fig 4.1 and Table 4.1). In all cases sensitivity was the same for plants treated from above with a sprinkler or from below by standing the trays in the nutrient medium. Based on these experiments screening of M_2 barley seedlings for selenate resistant individuals was initiated at a concentration of 0.05mM sodium selenate in modified half-Hoaglands nutrient solution without sulphate but with 20mM nitrate added. After more than 20,000 seedlings had been screened the selenate concentration was increased to 0.1mM and later, after

Fig 4.2 Effect of sodium selenate on the growth of wild type barley cultivar Golden Promise seedlings grown in the presence and absence of added sulphate and nitrate.



Seed was sown in trays of vermiculite, germinated in the light and treated daily with modified half-strength Hoaglands nutrient solutions containing:

a) no added sulphate or nitrate (●) (117.2mg)

b) no added sulphate but with KNO_3 (20mM) added (O) (278.8mg)

c) added sulphate (1mM) but no nitrate (□) (114.9mg)

d) added sulphate (1mM) and nitrate (20mM) (■) (250.5mg)

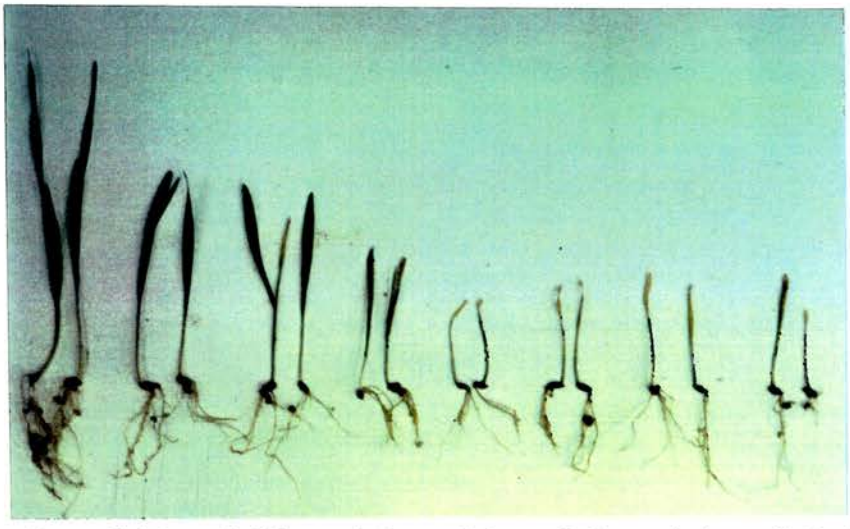
Sodium selenate at 0, 0.1, 0.2, 0.5, 0.7 or 1mM was included in the nutrient media from day 6 after sowing. Mean fresh weights of shoots (n=10) from 13 day old seedlings are expressed as a percentage of the mean control (selenate untreated) seedling shoot weight. The mean weights of the control seedlings are given in brackets.

Fig 4.3 Appearance of 13 day old wild type barley cultivar Golden Promise seedlings, grown in the absence of added sulphate and with and without added nitrate, after 8 days treatment with sodium selenate.

a)

0 0.01 0.05 0.1 0.2 0.3 0.4 0.5mM selenate

b)



0 0.01 0.05 0.1 0.2 0.3 0.4 0.5mM selenate

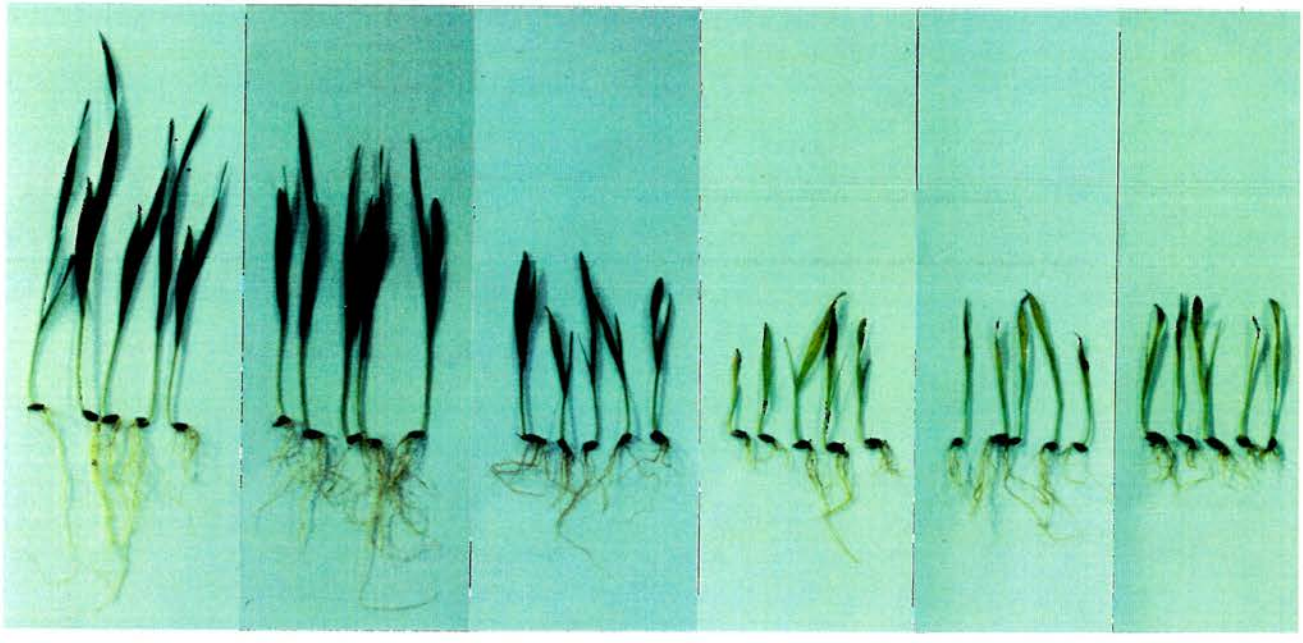
Seed was sown in trays of vermiculite, germinated in the light and treated daily with modified half-strength Hoaglands nutrient solutions without sulphate containing:

a) no added nitrate

b) 20mM KNO_3 added

Sodium selenate at 0, 0.01, 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5mM was included in the nutrient media from day 6 after sowing.

Fig 4.4 Appearance of 13 day old wild type barley cultivar Golden Promise seedlings grown in the absence of added sulphate and with added nitrate after treatment for 8 days with sodium selenate.



Seed was sown in trays of vermiculite, germinated in the light and treated daily with modified half-strength Hoaglands nutrient solution without sulphate but with nitrate (20mM) added.

Sodium selenate at 0, 0.01, 0.02, 0.05, 0.075 and 0.1mM was included in the nutrient media from day 6 after sowing.

At day 13 plants were removed from the seed trays and the roots gently washed free of vermiculite.

another 40,000 seedlings had been screened, further increased to 0.2mM.

4.1.3 Screening for selenate resistant barley plants

4.1.3.1 Assessment

M_2 barley seed of the cultivars Golden Promise, Tweed, Vista and Klaxon were screened for resistance to selenate. Screening was carried out in batches of eleven trays of seedlings. Each tray was sown with sufficient seed to give approximately 200 seedlings after germination. After sowing, the trays of seed were treated daily, for 5 days, with modified half-strength Hoaglands nutrient solution lacking both sulphate and nitrate. On day 6 selenate was included in the medium for 10 of the trays. The remaining tray, the selenate untreated control, continued to receive the nutrient solution without selenate.

When assessed on day 14 all seedlings showed signs of selenate sensitivity compared to the selenate untreated control seedlings. In no case did we see the anticipated situation, similar to that seen when screening barley seedlings for chlorate resistance (Bright *et al.*, 1983), of a green healthy M_2 seedling growing amongst a background of withered yellow or brown selenate damaged seedlings. This made the selection of putative selenate resistant seedlings very difficult. Therefore the healthiest looking seedlings from the selenate treated trays were selected, removed from the vermiculite, and rinsed well in distilled water before transfer into compost. In addition, two seedlings from the selenate untreated control tray were tranferred to compost from each screen batch allowing us to compare the subsequent growth of the selected plants with the untreated plants.

The percentage of seedlings selected from the screen as putative selenate resistant mutants was higher at lower selenate concentrations, and ranged from 0.2% (at 0.2mM) to 5% (at 0.05mM). Over 70,000 seedlings were screened for selenate resistance, with a total of 231 seedlings selected. The numbers of selected seedlings of each cultivar screened at the three selenate concentrations are given in Table 4.2.

4.1.3.2 Chlorophyll-deficient seedlings

The frequency of chlorophyll-deficient seedlings in an M_2 population serves as an indication of the relative success of the mutagenesis of the M_1 seed by quantifying the mutation frequency within that population. The overall frequency of chlorophyll-deficient seedlings in the M_2 seed used for screening was 1 percent. However, different cultivars showed different frequencies (Table 4.3). The overall frequency obtained is comparable to the average frequency of 1.47 percent chlorophyll-deficient barley seedlings derived from an M_2 population treated with 2mM sodium azide in the M_1 and 3.51 percent chlorophyll-deficient barley seedlings derived from M_2 populations treated with 1mM sodium azide in the M_1. This second M_2 population (1mM azide) also contained nitrite reduction deficient mutants at a frequency of 1:17,000 (Duncanson, 1991) .

4.1.3.3 Growth characteristics of the selected putative selenate resistant M_2 plants

On selection each M_2 seedling was labelled with a code made up of the initials of the barley cultivar, the screen batch number and the number of the plant as selected from that batch. For example GP 2:1 would be the first M_2 barley plant of cultivar Golden Promise selected from screening batch 2.

Table 4.2 Summary of the screen for selenate resistant M_2 barley plants

Selenate Treatment	Cultivar	No. of M_2 Plants Screened	No. of Putative Se^r Plants Selected
0.05mM	Golden Promise	15306	55
	Tweed	800	7
	Vista	5015	47
	(Total)	21121	109
0.1mM	Golden Promise	15290	43
	Tweed	12596	16
	Vista	14057	44
	(Total)	41943	103
0.2mM	Tweed	8225	15
	Klaxon	1215	4
	(Total)	9440	19
	Total	72504	231

Seed was germinated in the light and treated daily with modified half-strength Hoaglands nutrient medium containing 20mM KNO_3 but lacking sulphate. On day 6 sodium selenate was included in the medium. Seedlings were assessed for selenate resistance on day 14.

Se^r - selenate resistant.

Table 4.3 The frequency of chlorophyll-deficient seedlings in the M_2 populations of barley screened for selenate resistant individuals

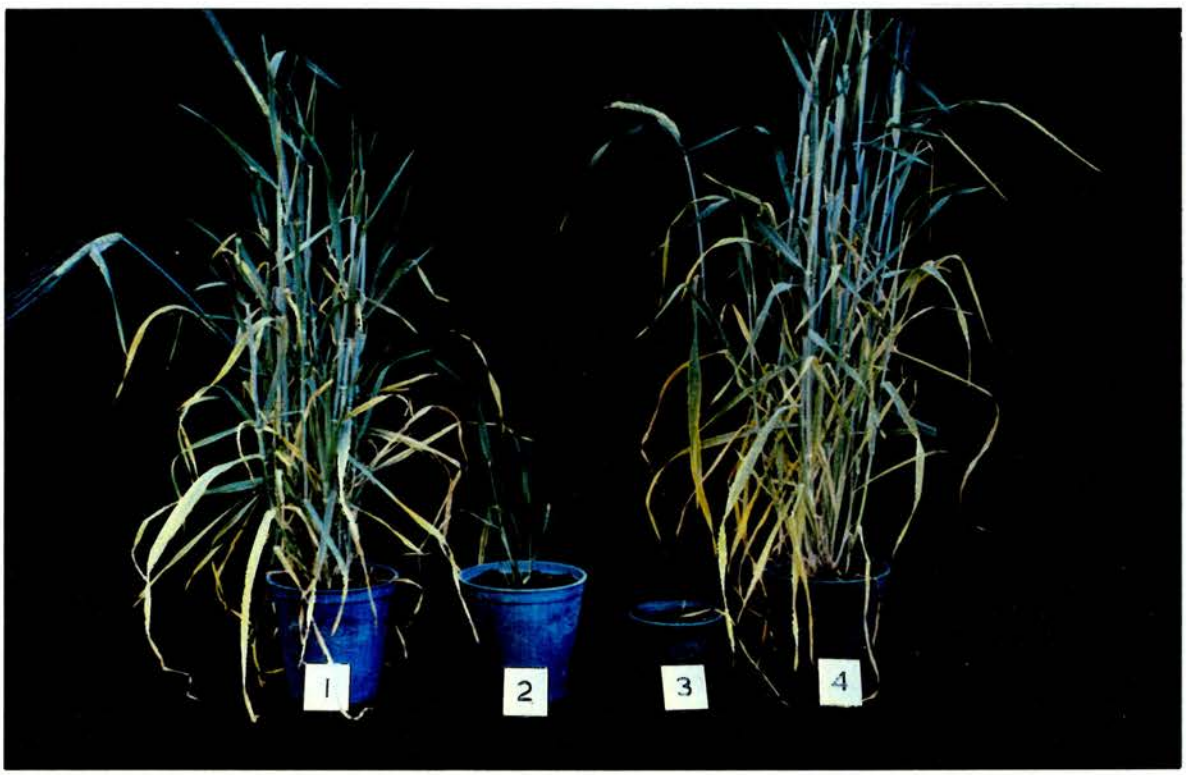
Barley cultivar	Number of plants screened	Number of chlorophyll-deficient seedlings	Percentage chlorophyll-deficient
Golden Promise	30596	440	1.44
Tweed	21621	124	0.57
Vista	19072	144	0.75
Klaxon	1215	23	1.89
Total	72504	731	1.01

M_2 seed, mutagenised with sodium azide in the M_1 were sown for use in the selenate screen. On day 6, prior to the seedlings receiving the selenate treatment, the numbers of seedlings that had germinated and the number of those which were chlorophyll-deficient were counted.

After transfer into compost the selected putative selenate resistant seedlings were monitored daily. Three types of growth behaviour were observed. One class of plants (class I) became progressively brown and withered, and died, without further growth, within three weeks of transfer to compost. A second class of plants (class II) produced new healthy leaves within 14 days of transfer to compost. The older damaged leaves become progressively brown and died. These class II plants subsequently grew phenotypically as the selenate untreated control plants which had been transferred to compost. The third class of plants (class III), which was by far the smallest (a total of 9 plants compared to 105 class I and 117 class II plants) grew slowly. These plants were shorter and produced fewer tillers (0-17 compared to 14-25 for the control plants) and did not flower until 19-28 weeks after transfer to compost, compared to flowering from 9 weeks after transfer for the untreated control plants. Fig 4.5 shows some of the M_2 barley cultivar Vista plants as well as a selenate untreated control plant isolated from the screen in batch 22. These plants show growth characteristics typical of the three classes of selection.

The number of plants of each cultivar selected in each class of selection is summarised in Table 4.4. M_3 seed was harvested from each class II and class III selection that was fertile. However only the class III putative selenate resistant M_2 plants and their M_3 progeny were investigated further.

Fig 4.5 Barley cultivar Vista M_2 selections from batch 22, photographed eleven weeks after selection from the screen.



Batch 22 was screened at 0.1mM selenate. On day 14 of the screen the M_2 seedlings were assessed and those selected as putative selenate resistant individuals were transferred to compost. Their growth was monitored daily.

M_2 selections from batch 22 exhibited class I, class II and class III growth behaviour after transfer. The photograph shows, eleven weeks after selection and transfer to compost :

1 - Selenate untreated control plant

2 - VI 22:2 a class III selection

3 - VI 22:4 a class I selection

4 - VI 22:3 a class II selection

Class I selections died with three weeks of transfer; Class II selections recovered and grew as the selenate untreated control plants and Class III selections grew on slowly.

Table 4.4 Growth of the M_2 selections from the screen after transfer to compost

Selenate Treatment	Cultivar	No. of Class I Plants	No. of Class II Plants	No. of Class III Plants
0.05mM	Golden Promise	23	30	2
	Tweed	4	3	0
	Vista	17	29	1
	(Total)	44	62	3
0.1mM	Golden Promise	25	16	2
	Tweed	10	5	1
	Vista	14	28	2
	(Total)	49	49	5
0.2mM	Tweed	11	3	1
	Klaxon	1	3	0
	(Total)	12	6	1
	Total	105	117	9

After selection from the screen seedlings were carefully washed free of vermiculite and tranferred into compost. The subsequent growth of the putative selenate resistant selections was monitored daily. Three distinct types of growth were seen, and these were used to distinguish classes of selection.

Class I did not grow and died with a few weeks of transfer.

Class II recovered and grew as wild type.

Class III grew on slowly but remained weak and stunted.

4.1.4 Examination of the M_2 class III putative selenate resistant plants and their M_3 progeny

4.1.4.1 Maintenance of the M_2 class III putative selenate resistant selections

In order to determine the ability of individual class III selections to grow on different sulphur sources, rooted tillers of some of the M_2 class III plants were separated from each other and grown on sulphate or cysteine as the sole sulphur source. Plants defective in sulphate assimilation would not be expected to grown well on compost. Transfer to medium with a reduced sulphur source should improve the chances of maintaining mutant individuals.

Ten weeks after selection from the screen and transfer to compost the M_2 selection VI 25:3 was separated into three rooted tillers. One tiller was grown on compost, the other two in hydroponics with either sulphate (1mM) or cysteine (1mM) as the sole sulphur source. The subsequent growth of the individual tillers was monitored. All three tillers grew on slowly to maturity and produced seed. None showed any improvement in growth over the others. The M_2 selection TW 42:1 was divided into two rooted tillers and transferred to hydroponics as above. Neither tiller showed any improvement in growth. Both died before flowering.

Growth of the M_2 class III selections, and the numbers of M_3 seed, if any, that they produced are summarised in Table 4.5.

Table 4.5 The class III M_2 selections. How they were grown after selection, their production of M_3 progeny seed and the viability of that seed.

M_2 Selection			M_3	
Plant	Screen mM selenate	Grown in	Seed available	% germination
GP 2:1	0.1	A	3	0
GP 5:2	0.1	A	Yes	50
GP 8:2	0.05	A*	No	
GP 13:1	0.05	A	Yes	90
VI 21:23	0.05	B	No	
VI 22:2	0.1	B	Yes	50
VI 25:3	0.1	+A,B*,C	Yes	30
TW 34:1	0.1	B	No	
TW 42:1	0.2	+B*,C	No	

Varieties: GP - Golden Promise. VI - Vista. TW - Tweed.
+ Plant split, growth tested on different regimes. * Plant died before reaching maturity.
A - Compost. B - Cysteine hydroponics. C - Sulphate hydroponics.

4.1.4.2 Germination of the M_3 progeny of the class III putative selenate-resistant mutants

M_3 progeny seed were sown in vermiculite, treated with half strength Hoaglands nutrient solution ($-SO_4 + NO_3$) and germinated in the dark. Approximately 4 days after germination the seedlings were transferred to the light. None of the three GP 2:1 M_3 seed germinated. The germination rates for M_3 seed harvested from the other selections varied; 30% for VI 25:3, 50% for VI 22:2 and GP 5:2, and 90% for GP 13:1 (Table 4.5).

M_3 seedlings were individually labelled with a code made up of the code of their M_2 parent, M_3, and their individual number in brackets. For example, VI 22:2 M_3(1) is the number one M_3 seedling from M_2 selection VI 22:2.

4.1.4.3 Growth of class III M_3 progeny

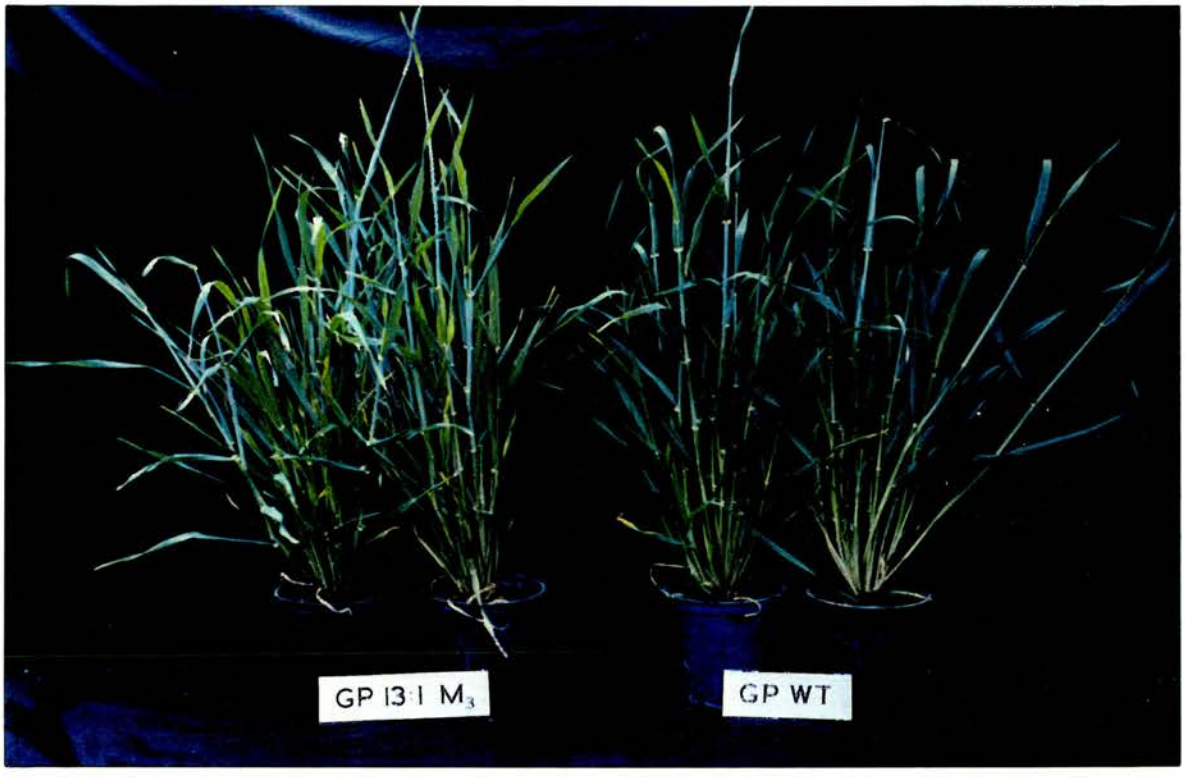
To determine whether the selenate resistant phenotype and growth phenotype exhibited by the Class III M_2 selection were inherited by their M_3 progeny the growth of M_3 seedlings on compost and when exposed to selenate was assessed.

No tests were carried out on the M_3 progeny of GP 2:1 because none of the seed germinated. Some M3 seedlings from the other M_2 selections were transferred to compost after germination in vermiculite, and their growth was monitored. GP 5:2 (Fig 4.6), GP 13:1 (Fig 4.7) and VI 22:2 (Fig 4.8) M_3 seedlings grew as wild type. The growth characteristics of the M_3 progeny of VI 25:3 varied, some of the plants were stunted and appeared to grow more slowly than the M_2 Vista seedling which was used as the control (Fig 4.9). M_2 Vista seedlings taken from the selenate untreated control trays of the screen

Fig 4.6 Growth of M_3 progeny of the M_2 selection GP 5:2 on compost compared to barley cultivar Golden Promise wild type plants used as a control.

Seedlings were germinated in vermiculite and treated with modified half-strength Hoaglands nutrient solution without sulphate but with nitrate (20mM) added. After germination the seedlings were transferred to compost and their growth monitored. Plants were photographed 2 weeks after transfer to compost.

Fig 4.7 Growth of M_3 progeny of the M_2 selection GP 13:1 on compost compared to barley cultivar Golden Promise wild type plants used as a control.

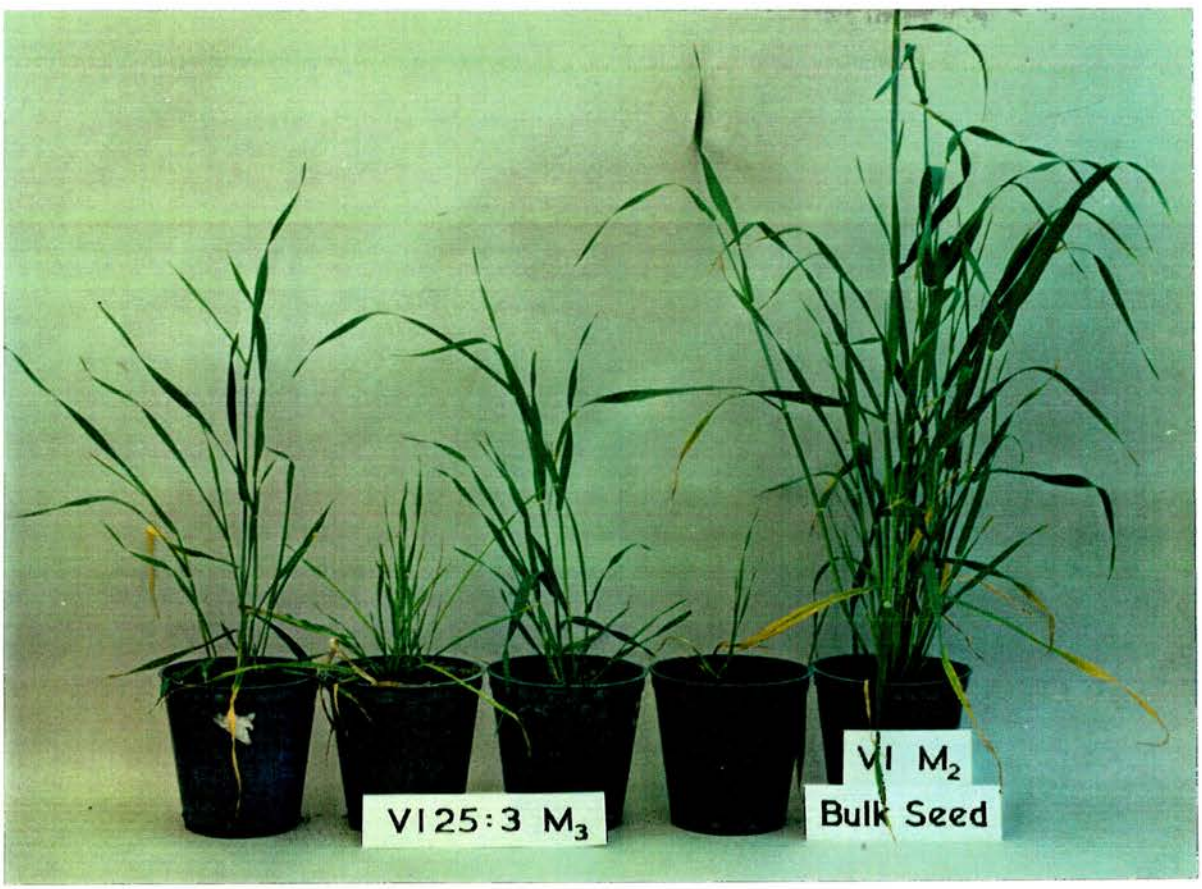


Seedlings were germinated in vermiculite and treated with modified half-strength Hoaglands nutrient solution without sulphate but with nitrate (20mM) added. After germination the seedlings were transferred to compost and their growth monitored. Plants were photographed 10 weeks after transfer to compost.

Fig 4.8 Growth of M_3 progeny of the M_2 selection VI 22:2 on compost compared to M_2 barley cultivar Vista plants used as a control.

Seedlings were germinated in vermiculite and treated with modified half-strength Hoaglands nutrient solution without sulphate but with nitrate (20mM) added. After germination the seedlings were transferred to compost and their growth monitored. Plants were photographed 10 weeks after transfer to compost.

Fig 4.9 Growth of M_3 progeny of the M_2 selection VI 25:3 on compost compared to a barley cultivar Vista M_2 plant used as a control.



Seedlings were germinated in vermiculite and treated with modified half-strength Hoaglands nutrient solution without sulphate but with nitrate (20mM) added. After germination the seedlings were transferred to compost and their growth monitored. Plants were photographed 10 weeks after transfer to compost.

also showed variation in their growth characteristics similar to that of the M_3 progeny of the M_2 selection VI 25:3.

M_3 seedlings of GP 13:1 were also tested for their resistance to selenate (Fig 4.10). No difference was seen between the M_3 progeny of GP 13:1 and Golden Promise wild type seedlings after treatment.

These tests (summarised in Table 4.6) showed that most of the M_3 progeny tested were able to grow on compost like wild type and those tested were selenate sensitive.

4.1.4.4 Enzyme analysis of the class III selections and their progeny

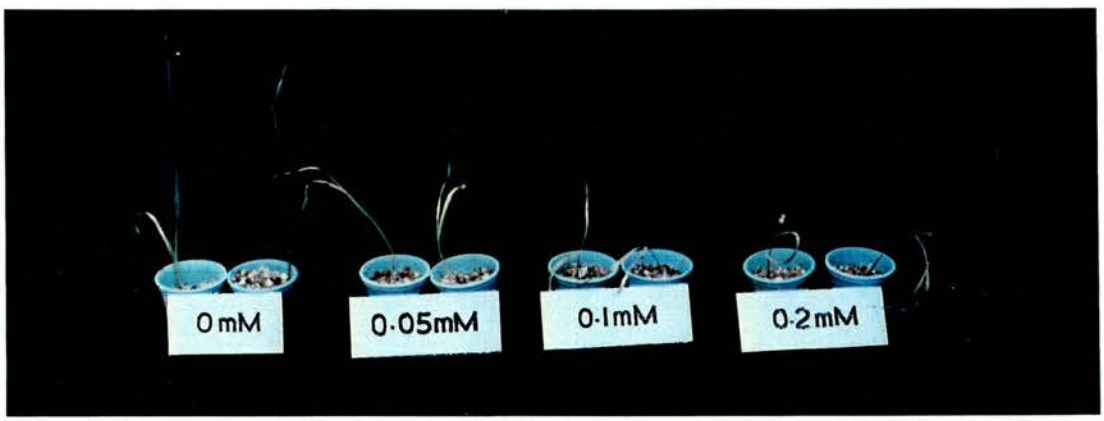
In order to determine whether these selected class III (slow growing) plants did indeed carry a defect in sulphate assimilation, extracts of M_3 progeny seedlings and where possible the original M_2 selection were examined by specific enzyme activity staining after native gel electrophoresis for the presence of cysteine synthase, sulphite reductase, thiosulphate reductase and ATP sulphurylase. The activity bands obtained were compared to those seen for wild type plants and/or an M_2 selenate untreated control for that barley cultivar.

When possible leaf tissue from 7-21 day old seedlings was used for extractions. When this was impossible, as in the case of the original M_2 selections, tissue from new, developing leaves was used.

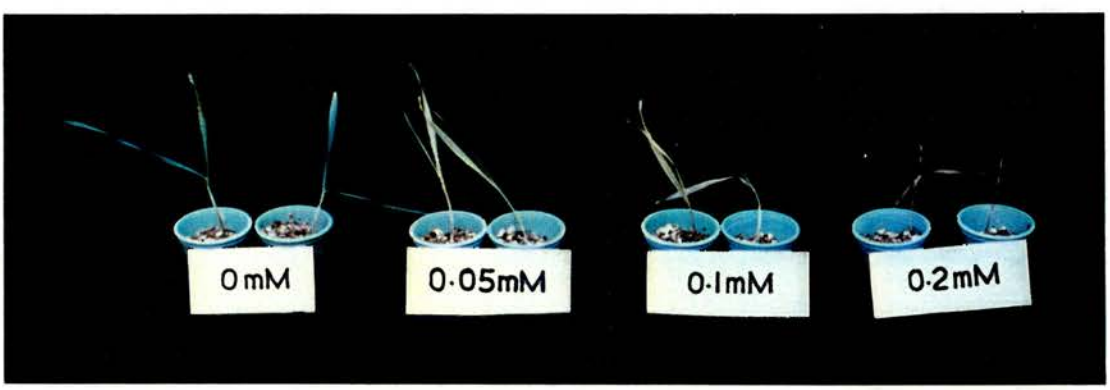
4.1.4.4.1 Enzyme analysis of the class III M_2 selections VI 22:2 and VI 25:3 and their M_3 progeny

M_3 seedlings, grown for use in section 4.1.4.3, from selections VI 22:2 and VI 25:3 were examined for cysteine synthase activity. Extract from two M_3 seedlings from each selection was subjected to non-denaturing polyacrylamide gel electrophoresis followed by

Fig 4.10 Selenate sensitivity of the M_3 seedlings of class III M_2 selection GP 13:1, compared to barley cultivar Golden Promise wild type seedlings.



a) GP 13:1 M_3



b) Golden Promise wild type

Seedlings were germinated in vermiculite and treated with modified half-strength Hoaglands nutrient solution, without sulphate but with nitrate (20mM) added. Sodium selenate at 0.05, 0.1 and 0.2mM was included in the medium from day 5 after sowing. The seedlings were photographed on day 14 after sowing.

Table 4.6 Summary of the tests carried out on the M_3 progeny of class III M_2 selections from the screen

	M_3 progeny		Enzyme activity shown in gel assay of plant extracts				
Class III M_2 selection	Growth in compost	Selenate resistance	Cysteine synthase	β-cyanoalanine synthase	Thiosulphate reductase	Sulphite reductase	ATP sulphurylase
GP 2:1	nt	nt					
GP 5:2	WT	nt	M_3	M_3	M_3	M_3	M_3
GP 8:2							
GP 13:1	WT	None	M_3	M_3	M_3	M_3	M_3
VI 21:23							
VI 22:2	WT	nt	M_2+M_3	M_2+M_3	M_3	M_3	M_3
VI 25:3	Variable	nt	M_2+M_3	M_2+M_3	M_3	M_3	M_3
TW 34:1							
TW 42:1							

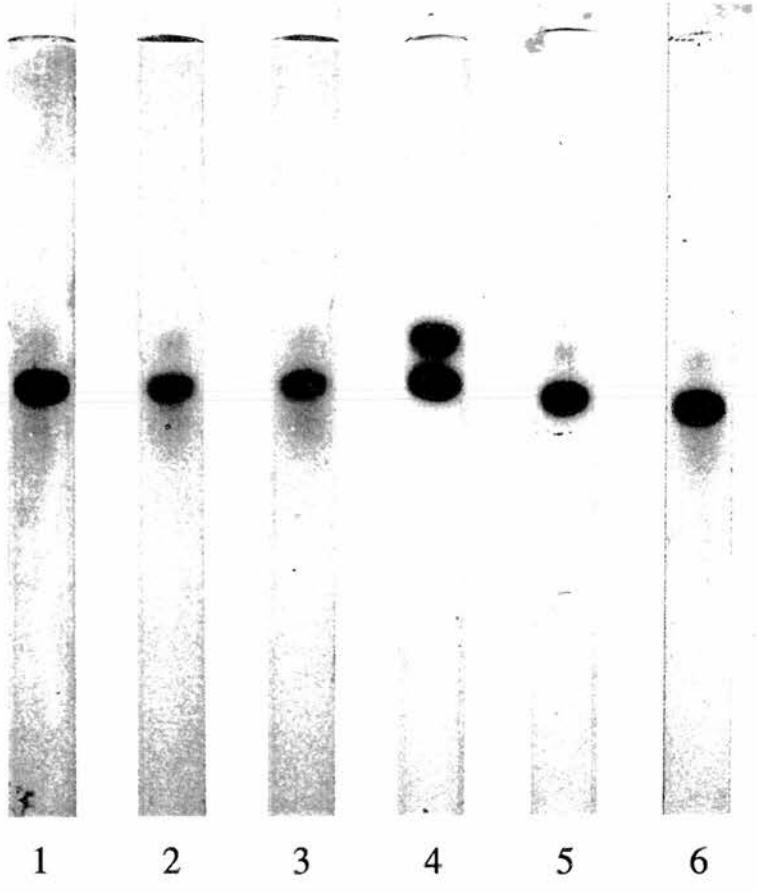
Varieties: GP - Golden Promise. VI - Vista. TW - Tweed.

nt - Not tested. WT - M_3 plants healthy with wild type phenotype.

specific staining for cysteine synthase activity. Two activity bands were seen with extract from both seedlings tested from each selection (Fig 4.11). The faster migrating of the two bands on each gel is at the position of cysteine synthase activity. Cysteine synthase activity bands were of the same intensity on all the gels. However, this assay also shows the presence of the enzyme β-cyanoalanine synthase at the position of the slower migrating of the two bands. The seedling VI 25: 3 M_3(1) (gel 4 on Fig 4.11) produced a more intense band of β-cyanoalanine synthase activity than the other M_3 seedlings, the Vista wild type seedlings or the VI M_2 control seedlings. The β-cyanoalanine synthase activity bands on the other gels were all of similar, lower intensity.

Although β-cyanoalanine synthase is not an enzyme of the sulphate assimilation pathway it has been shown to form cysteine from O-acetyl-L-serine and sulphide in blue lupin at one twenty fifth the rate of β-cyanoalanine formation. A mutation which causes overproduction or increased activity of β-cyanoalanine synthase could therefore potentially influence selenate metabolism. Hence, the apparent difference in β-cyanoalanine synthase activity was investigated further. Extracts from a further two M_3 seedlings from each of these selections and the original M_2 selections themselves were subjected to non-denaturing polyacrylamide gel electrophoresis and staining (Fig 4.12). Very intense bands at the position of β-cyanoalanine synthase activity were seen on the gels loaded with extract from VI 22:2 M_2 (gel 6) and VI 25:3 M_2 (gel 11), the original selections, and seedlings VI 25:3 M_3 (1, 3 and 4) (gels 7, 9 and 10). However, 200µl of extract was loaded onto each gel, rather than a measured amount of protein, and the differing protein content of each extract (see Fig 4.12) accounts for most of the variation in band

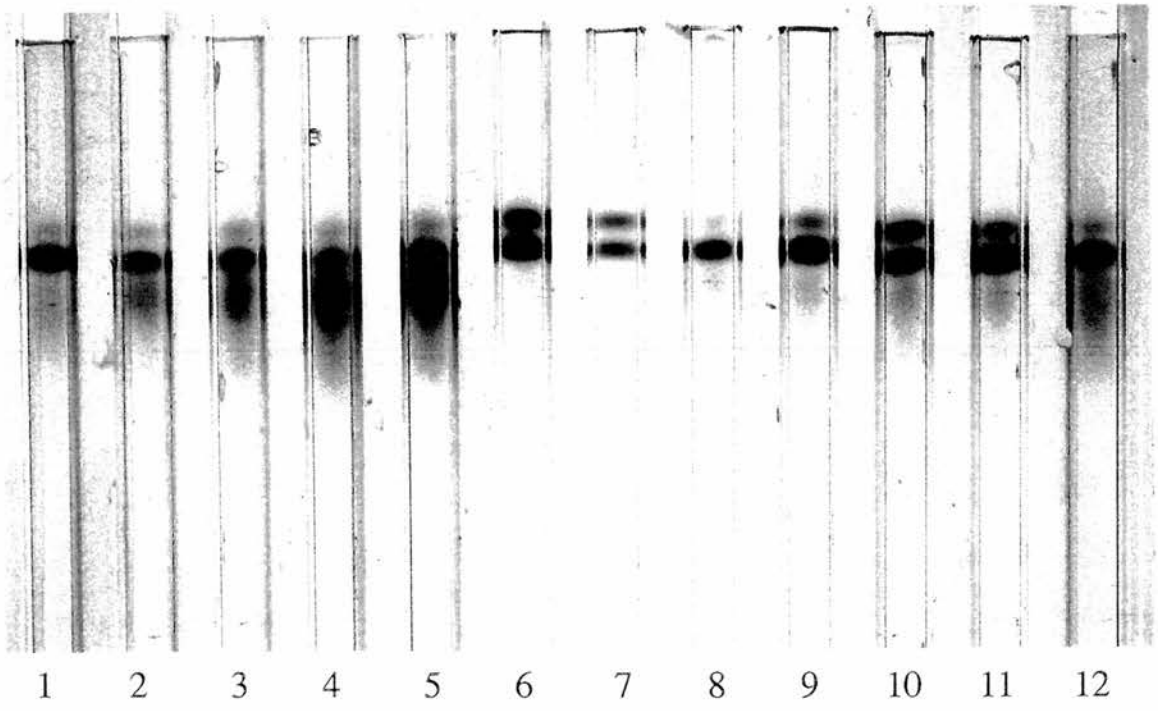
Fig 4.11 Specific staining for cysteine synthase activity after non-denaturing polyacrylamide gel electrophoresis of extracts from M_3 progeny of the selections VI 22:2 and VI 25:3.



Leaf tissue was extracted in 0.1M potassium phosphate buffer pH 7 containing 1mM EDTA and 1mM DTT, 100mg tissue to 1ml buffer, and subjected to non-denaturing polyacrylamide gel electrophoresis followed by specific gel staining for cysteine synthase activity.

All gels were loaded with 200µl extract. Gel 1: Vista wild type, gel 2: VI 22:2 M_3 (1), gel 3: VI 22:2 M_3 (2), gel 4: VI 25:3 M_3 (1), gel 5: VI 25:3 M_3 (2), gel 6: Vista M_2 control.

Fig 4.12 Specific staining for cysteine synthase activity after non-denaturing polyacrylamide gel electrophoresis of extracts from all available M_3 progeny of the selections VI 22:2 and VI 25:3.



Leaf tissue was extracted in 0.1M potassium phosphate buffer pH 7 containing 1mM EDTA and 1mM DTT, 100mg tissue to 1ml buffer, and subjected to non-denaturing polyacrylamide gel electrophoresis followed by specific gel staining for cysteine synthase activity.

All gels were loaded with 200µl extract. Amount of protein loaded is given in brackets. Gel 1: Vista wild type (0.77mg), gel 2: VI 22:2 M_3(1) (0.89mg), gel 3: VI 22:2 M_3(2) (0.67mg), gel 4: VI 22:2 M_3(3) (0.5mg), gel 5: VI 22:2 M_3(4) (0.82mg), gel 6: VI 22:2 M_2 (1mg), gel 7: VI 25:3 M_3(1) (0.45mg), gel 8: VI 25:3 M_3(2) (0.54mg), gel 9: VI 25:3 M_3(3) (1.12mg), gel 10: VI 25:3 M_3(4) (0.9mg), gel 11: VI 25:3 M_2 (0.8mg), gel 12: Vista M_2 control seedling (0.83mg).

intensity. Conventional enzyme assays carried out on these 12 extracts showed considerable variation in both the β-cyanoalanine synthase and cysteine synthase activities of the extracts (Table 4.7). The ratio of β-cyanoalanine synthase activity to cysteine synthase activity also varied between extracts. Those extracts resulting in more intense β-cyanoalanine synthase activity bands on the gels did not have proportionally more β-cyanoalanine synthase activity than the other extracts.

Extract from the M_3 seedlings from VI 22:2 and VI 25:3 produced bands of activity, at the same position and of similar intensity to those from control plant extracts, on gels stained for sulphite reductase (Fig 4.13), thiosulphate reductase (Fig 4.14) and ATP sulphurylase (Fig 4.15). Indicating that each of these enzymes were present and functional in the M_3 progeny of the M_2 class III selections VI 22:2 and VI 25:3.

4.1.4.4.1 Enzyme analysis of the M_3 progeny of the class III selections GP 5:2 and GP 13:1

Extracts from one M_3 seedling from each of the M_2 selections, GP 5:2 and GP 13:1, a Golden Promise wild type plant and a plant grown from M_2 Golden Promise seed used as a control were subjected to non-denaturing polyacrylamide gel electrophoresis followed by specific gel staining for cysteine synthase activity. Bands of activity at the positions of β-cyanoalanine synthase and cysteine synthase were seen on all 5 gels (Fig 4.16). Some variation in the intensity of bands between gels was seen however, band intensity was proportional to the amount of protein loaded onto each gel.

Sulphite reductase and thiosulphate reductase activity were also shown to be present (Fig 4.17 and 4.18) as was ATP sulphurylase

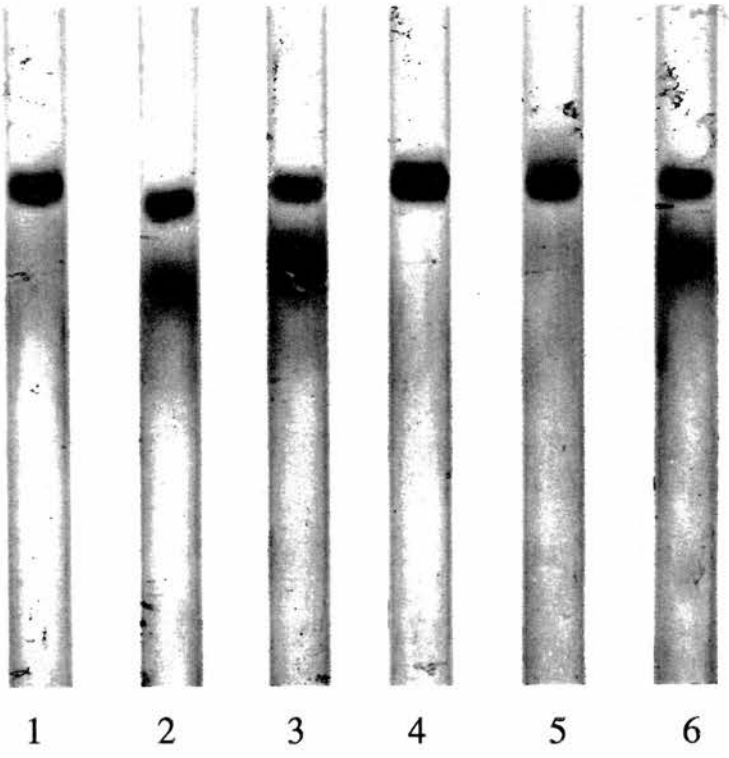
Table 4.7 β-cyanoalanine synthase and cysteine synthase activities of extracts of barley cultivar Vista plants

Plant	β-cyanoalanine synthase activity (nmols sulphide/mg protein/min)	Cysteine synthase activity (μmols cysteine/mg protein/hr)
VI wt	87.66	12.26
VI 22:2 M_3 (1)	126.9	7.73
VI 22:2 M_3 (2)	138.8	10.27
VI 22:2 M_3 (3)	150.0	12.80
VI 22:2 M_3 (4)	111.6	7.61
VI 22:2 M_2	164.3	11.56
VI 25:3 M_3 (1)	173.3	18.31
VI 25:3 M_3 (2)	103.7	12.15
VI 25:3 M_3 (3)	116.1	8.14
VI 25:3 M_3 (4)	211.1	17.24
VI 25:3 M_2	110.0	13.60
VI M_2	96.4	10.80

100mg leaf tissue from individual plants was extracted in 1ml 0.1M potassium phosphate buffer, pH 7, containing 1mM EDTA and 1mM DTT, and assayed for β-cyanoalanine synthase and cysteine synthase activity. Extracts were also subjected to non-denaturing polyacrylamide gel electrophoresis followed by gel staining for cysteine synthase activity (Fig 4.12).

VI M_2 was a selenate untreated M_2 plant of the cultivar Vista used as a control.

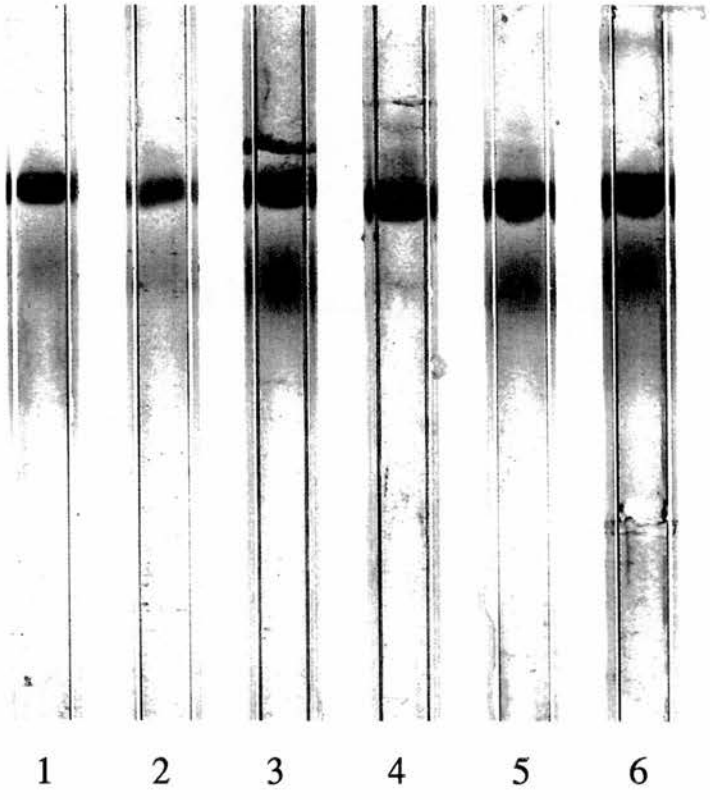
Fig 4.13 Specific staining for sulphite reductase activity after non-denaturing polyacrylamide gel electrophoresis of extracts from M_3 progeny of the selections VI 22:2 and VI 25:3.



Leaf tissue was extracted in 0.1M Tris buffer pH 8.0, 100mg tissue to 1ml buffer, and subjected to non-denaturing polyacrylamide gel electrophoresis followed by specific gel staining for sulphite reductase activity.

All gels were loaded with 200µl extract. The amount of protein loaded is given in brackets. Gel 1: Vista wild type (0.74mg), gel 2: VI 22:2 $M_3(1)$ (0.86mg), gel 3: VI 22:2 $M_3(2)$ (0.54mg), gel 4: VI 25:3 $M_3(1)$ (0.7mg), gel 5: VI 25:3 $M_3(2)$ (0.75mg), gel 6: Vista M_2 control seedling (0.56mg).

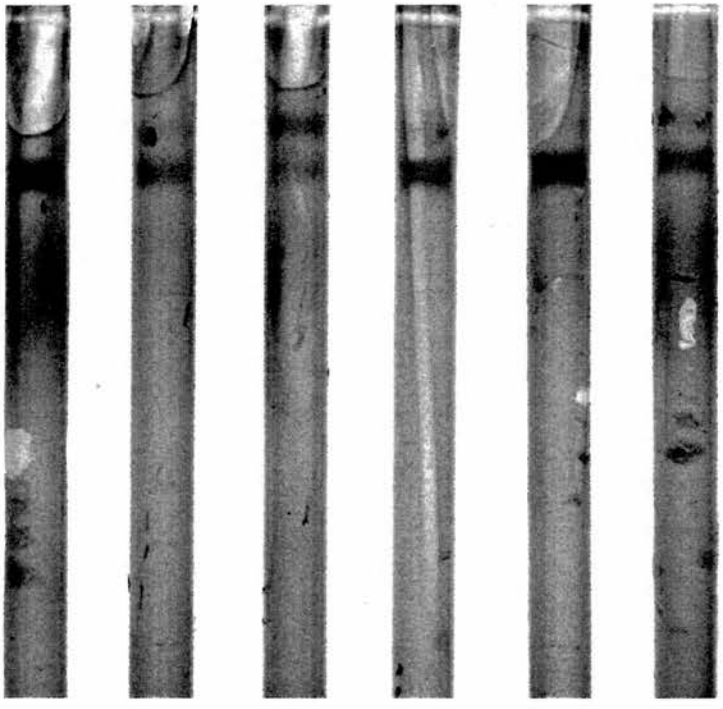
Fig 4.14 Specific staining for thiosulphate reductase activity after non-denaturing polyacrylamide gel electrophoresis of extracts from M_3 progeny of the selections VI 22:2 and VI 25:3.



Leaf tissue was extracted in 0.1M Tris buffer pH 8.0, 100mg tissue to 1ml buffer, and subjected to non-denaturing polyacrylamide gel electrophoresis followed by specific gel staining for thiosulphate reductase activity.

All gels were loaded with 200µl extract. The amount of protein loaded is given in brackets. Gel 1: Vista wild type (0.74mg), gel 2: VI 22:2 $M_3(1)$ (1mg), gel 3: VI 22:2 $M_3(2)$ (0.72mg), gel 4: VI 25:3 $M_3(1)$ (0.6mg), gel 5: VI 25:3 $M_3(2)$ (0.81mg), gel 6: Vista M_2 control seedling (0.72).

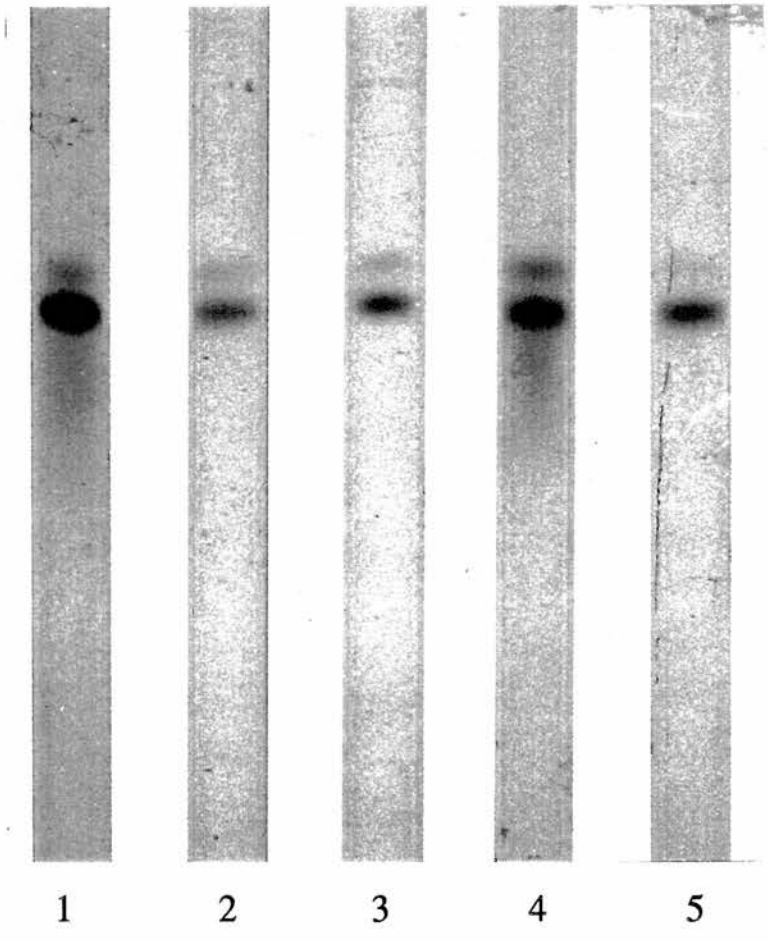
Fig 4.15 Specific staining for ATP sulphurylase activity after non-denaturing polyacrylamide gel electrophoresis of extracts from M_3 progeny of the selections VI 22:2 and VI 25:3.



Leaf tissue was extracted in 0.1M Tris buffer pH 8.0, 100mg tissue to 1ml buffer, and subjected to non-denaturing polyacrylamide gel electrophoresis followed by specific gel staining for ATP sulphurylase activity.

All gels were loaded with 200µl extract. Gel 1: Vista wild type, gel 2: VI 22:2 M_3 (1), gel 3: VI 22:2 M_3 (2), gel 4: VI 25:3 M_3 (1), gel 5: VI 25:3 M_3 (2), gel 6: Vista M_2 control seedling.

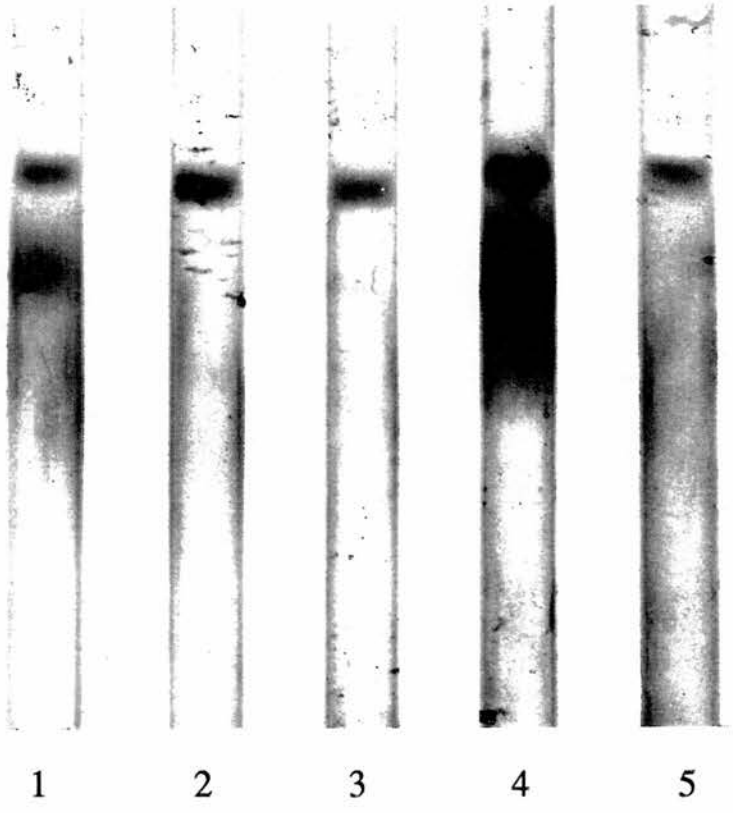
Fig 4.16 Specific staining for cysteine synthase activity after non-denaturing polyacrylamide gel electrophoresis of extracts from M_3 progeny of the selections GP 5:2 and GP 13:1.



Leaf tissue was extracted in 0.1M potassium phosphate pH 7 containing 1mM EDTA and 1mM DTT, 100mg tissue to 1ml buffer, and subjected to non-denaturing polyacrylamide gel electrophoresis followed by specific gel staining for cysteine synthase activity.

All gels were loaded with 200µl extract. The amount of protein loaded is given in brackets. Gel 1: Golden Promise wild type (0.75mg), gel 2: GP 13:1 $M_3(1)$ (0.32mg), gel 3: GP 5:2 $M_3(1)$ (0.35mg), gel 4: Golden Promise M_2 control seedling (0.3mg), gel 5: Golden Promise wild type (0.18mg).

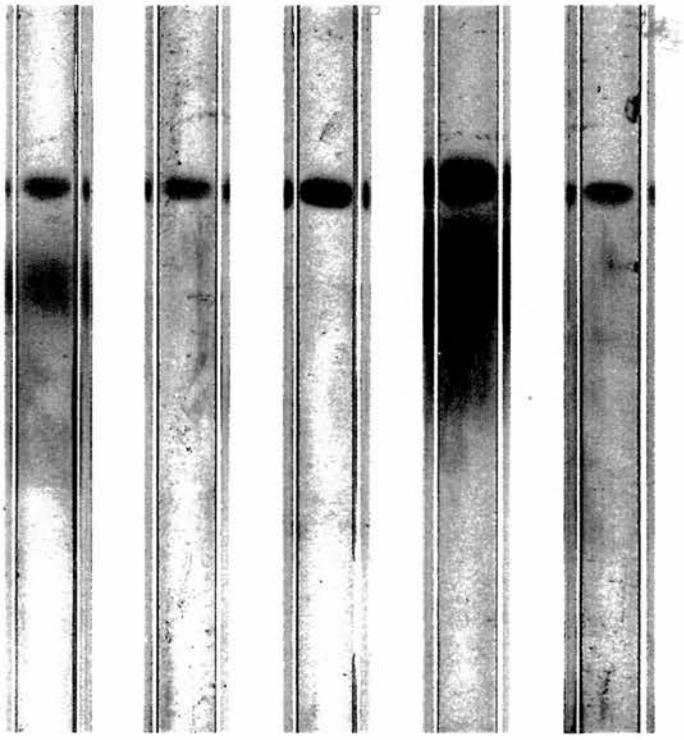
Fig 4.17 Specific staining for sulphite reductase activity after non-denaturing polyacrylamide gel electrophoresis of extracts from M_3 progeny of the selections GP 5:2 and GP 13:1.



Leaf tissue was extracted in 0.1M Tris buffer pH 8.0, 100mg tissue to 1ml buffer, and subjected to non-denaturing polyacrylamide gel electrophoresis followed by specific gel staining for sulphite reductase activity.

All gels were loaded with 200µl extract. The amount of protein loaded is given in brackets. Gel 1: Golden Promise wild type (0.57mg), gel 2: GP 13:1 $M_3(1)$ (0.5mg), gel 3: GP 5:2 $M_3(1)$ (0.52mg), gel 4: Golden Promise M_2 control seedling (0.85mg), gel 5: Golden Promise wild type (0.32mg).

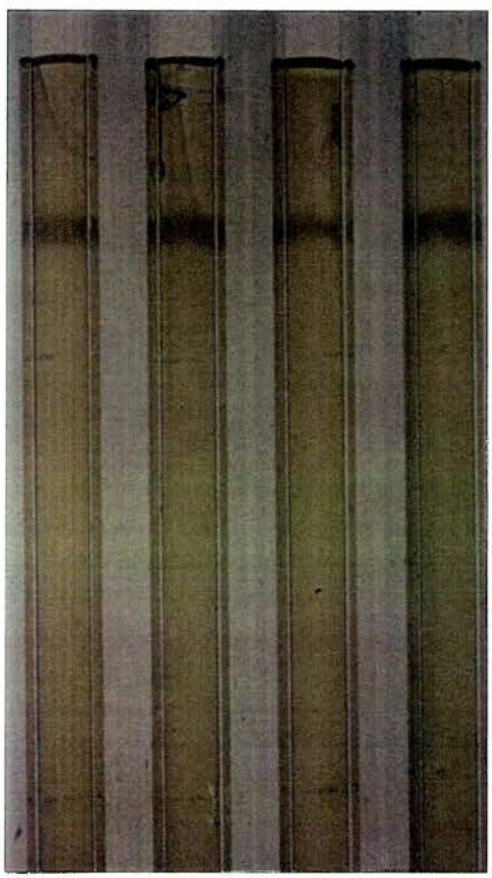
Fig 4.18 Specific staining for thiosulphate reductase activity after non-denaturing polyacrylamide gel electrophoresis of extracts from M_3 progeny of the selections GP 5:2 and GP 13:1.



Leaf tissue was extracted in 0.1M Tris buffer pH 8.0, 100mg tissue to 1ml buffer, and subjected to non-denaturing polyacrylamide gel electrophoresis followed by specific gel staining for thiosulphate reductase activity.

All gels were loaded with 200µl extract. The amount of protein loaded is given in brackets. Gel 1: Golden Promise wild type (0.57mg), gel 2: GP 13:1 $M_3(1)$ (0.5mg), gel 3: GP 5:2 $M_3(1)$ (0.52mg), gel 4: Golden Promise M_2 control seedling (0.85mg), gel 5: Golden Promise wild type (0.32mg).

Fig 4.19 Specific staining for ATP sulphurylase activity after non-denaturing polyacrylamide gel electrophoresis of extracts from M_3 progeny of the selections GP 5:2 and GP 13:1.



1 2 3 4

Leaf tissue was extracted in 0.1M Tris buffer pH 8.0, 100mg tissue to 1ml buffer, and subjected to non-denaturing polyacrylamide gel electrophoresis followed by specific gel staining for ATP sulphurylase activity.

All gels were loaded with 200µl extract. The amount of protein loaded is given in brackets. Gel 1: GP 13:1 $M_3(1)$ (0.18mg), gel 2: GP 5:2 $M_3(1)$ (0.22mg), gel 3: Golden Promise M_2 control seedling (0.19mg), gel 4: Golden Promise wild type (0.26mg).

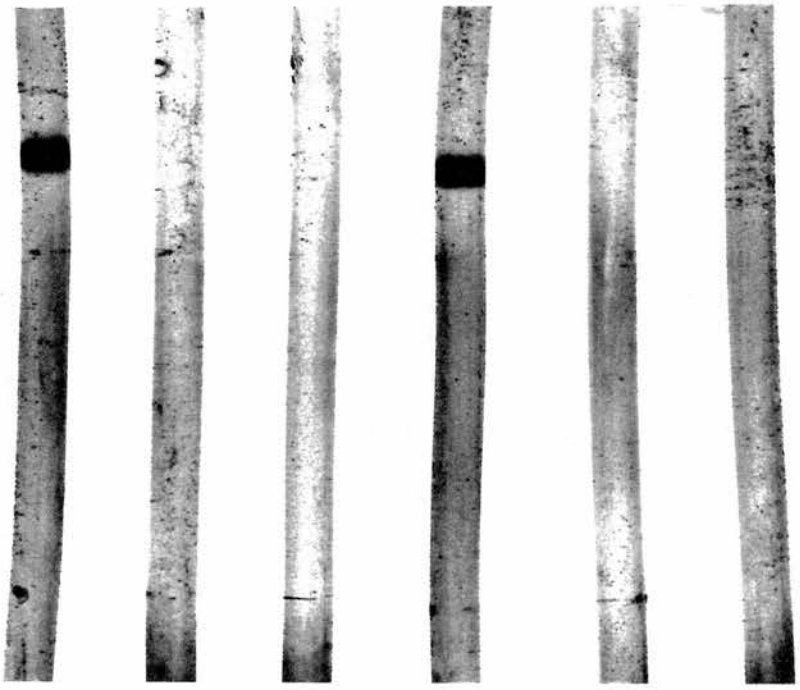
activity (Fig 4.19). Differences in intensity of the bands between extracts with sulphite reductase and thiosulphate reductase may again be explained by the protein content of the extract.

The M_3 progeny of the selections GP 5:2 and GP 13:1 possess cysteine synthase, sulphite reductase, thiosulphate reductase and ATP sulphurylase activity.

4.1.5 STA 3999 - a mutant defective in nitrite reduction

The *nir1* mutant, STA 3999, isolated from the barley cultivar Tweed, is defective in nitrite reduction and lacks detectable NiR cross reacting material (Duncanson *et al.*, 1991, 1993). The enzyme nitrite reductase, like sulphite reductase, possesses sirohaem as a prosthetic group (Krueger and Siegel 1982). In order to test whether the defect in nitrite reduction in STA 3999 is due to a defect in sirohaem biosynthesis, extract from leaves of the mutant was subjected to non-denaturing polyacrylamide gel electrophoresis followed by specific gel staining for sulphite reductase. STA 3999 possessed functional sulphite reductase activity as shown by the appearance of a single band of activity on the gel (Fig 4.20). As with the Golden Promise wild type extracts the appearance of the band was dependant on the presence of the substrate, sodium sulphite and of methyl viologen in the staining mix. The appearance was prevented by preincubation of the gel with 1mM NaCN prior to staining. NaCN is a known inhibitor of sulphite reductase activity (Aketagawa and Tamura, 1980). As a functional sulphite reductase was present in STA 3999 it is unlikely that this plant is defective in sirohaem biosynthesis.

Fig 4.20 Specific staining for sulphite reductase activity after non-denaturing polyacrylamide gel electrophoresis of extracts from the *nir1*, nitrite reductase deficient mutant STA 3999, from the barley cultivar Tweed



Leaf tissue was extracted in 0.1M Tris buffer pH 8.0, 100mg tissue to 1ml buffer, and subjected to non-denaturing polyacrylamide gel electrophoresis followed by specific gel staining for sulphite reductase activity.

Gel 1: Tweed wild type, incubated with sulphite, gel 2: Tweed wild type, incubated without sulphite, gel 3: Tweed wild type, preincubated with 1mM NaCN and incubated with sulphite, gel 4: STA 3999, incubated with sulphite, gel 5: STA 3999, incubated without sulphite, gel 6: STA 3999, preincubated with 1mM NaCN and incubated with sulphite. All gels were loaded with 200µl extract.

4.2 Discussion

4.2.1 Growth of barley seedlings on different nitrate and sulphate regimes

Since both nitrogen and sulphur are required by higher plants for the production of sulphur containing amino acids and ultimately protein, and the production of sulpholipids in the case of sulphur, it could be predicted that deprivation of either of these essential nutrients would affect seedling growth. A limited supply of nitrogen and sulphur are likely to be available in the seed reserves but this supply will be used up during early seedling growth. Continued healthy development of seedlings requires an external supply of both these essential nutrients.

The absence of added nitrate significantly reduced the fresh weight of seedlings both in the presence (114.9mg $-NO_3$ and 250.5mg $+NO_3$) and absence (117.2mg $-NO_3$ and 278.8mg $+NO_3$) of added sulphate ($p \leq 0.0001$) (Table 4.1). Seedlings grown without added nitrate were shorter, weaker looking plants (Fig 4.1). The presence or absence of added sulphate surprisingly had little if any effect on the appearance (Fig 4.1) or their weight (Table 4.1).

Why should nitrogen deprivation have a greater effect on seedling growth than sulphur starvation? Harwood (1980) suggests that sulpholipid formation accounts for about 50% of the plants total sulphur requirements. For protein synthesis the ratio of nitrogen to sulphur is 25:1 (Reuveny and Filner, 1977). These two facts would suggest more nitrogen is required by plants than sulphur. Even if plants require lower quantities of sulphur than nitrogen, sulphur starvation would still be expected to affect seedling growth. However, these initial studies into the growth of wild type barley

seedlings were carried out at the outset of the project before it was realised that the vermiculite used to grow the seedlings contained sulphate. 1.2l of vermiculite was used for sowing each seed tray. This was shown to contain 6.2mg sulphate-sulphur compared with 32.9 mg/l half-strength Hoaglands nutrient medium (section 3.1.1.1). This may have been sufficient sulphur to allow some sulphate assimilation and sulpholipid formation to proceed and together with the assumption that lower levels of sulphate are required may account for the lack of any difference in the growth of seedlings in the presence and absence of added sulphate.

4.2.2 Selenate sensitivity of wild type barley plants

Selenate is toxic to wild type barley seedlings. Treated seedlings showed brown spotting of the leaves, followed later by chlorosis and necrosis. Trials where plants were watered either by immersion of the seed tray or by sprinkler from above showed the same levels of toxicity, indicating that the symptoms were a result of selenate uptake and were not topical.

Selenate concentrations as low as 0.02mM were shown to affect seedling growth (Fig 4.3). The toxic effects have been attributed to the incorporation of Se-containing amino acids into proteins leading to structural changes in proteins affecting their function (Brown and Shrift, 1982; Dawson and Anderson, 1988). Since the regulatory coupling of the two pathways is well documented (see section 1.1.2), it was anticipated that selenate sensitivity would be greatest under those conditions when both nitrogen and sulphate assimilation, and therefore protein production, were greatest. This would theoretically result in the highest levels of Se incorporation into protein.

Seedlings were most sensitive to selenate in the absence of added sulphate and the presence of nitrate. 0.02mM selenate has a marked effect on the appearance of seedlings grown under these conditions (Fig 4.3). When treated with 0.1mM selenate, also -SO_4+NO_3, the mean fresh weight of seedlings dropped to around 20% of that of selenate untreated seedlings (Fig 4.2).

Seedlings grown without added sulphate were more sensitive to selenate than those grown with added sulphate, both in the presence and absence of added nitrate. It may be that sulphate protects the seedlings against selenate toxicity by competing with the selenate, initially for uptake and then for activation by ATP-sulphurylase and subsequent passage along the assimilation pathway to form cysteine. Cysteine will in turn compete with the selenocysteine for incorporation into protein. Selenate sensitivity would therefore be expected to be greatest when the supply of substrates for protein production is plentiful, but with selenoamino acids rather than sulphur amino acids available. Uptake of sulphate and selenate by barley roots is mediated by a single carrier, with the two anions competing for binding sites with similar affinity, the uptake ratio is reported as 1.4 S:1.0 Se (Ferrari and Renosto, 1972). The presence of nitrate and selenate would, by the method of reciprocal regulatory coupling of the two pathways, result in the positive regulation of both pathways. As expected then, selenate was most toxic to seedlings grown with nitrate but without added sulphate. This was chosen as the nutrient regime for use in the selenate screen being the most likely to give the quickest and most definite differences between sensitive and resistant seedlings.

4.2.3 Screening for selenate resistant barley plants

When assessed, all treated seedlings showed selenate sensitivity compared to the selenate untreated controls. The anticipated situation, of a healthy seedling growing amongst a tray of withered yellow or brown selenate damaged plants was never seen. This made the selection of putative selenate resistant seedlings very difficult.

Chlorophyll-deficient mutants were seen at an overall frequency of 1%, compared with 1.47% reported by Duncanson (1991) for a similar population of M_2 barley mutagenised with 2mM azide in the M1, indicating that the azide treatment had been successful. The frequency of chlorophyll-deficient mutants varied between cultivars, with the cultivar Klaxon showing the highest frequency, 1.89%, and Tweed the lowest, 0.57% (see Table 4.3). In the study mentioned above the population of M_2 seed, mutagenised in the M_1 with 2mM sodium azide, giving 1.47% chlorophyll-deficient mutants also contained 0.032% nitrite accumulating seedlings, that is seedlings that, for what ever reason, lacked nitrite reductase activity. Duncanson also showed that an M_2 population, treated with 1mM sodium azide in the M_1, had 3.51 percent chlorophyll-deficient barley seedlings. This second M_2 population (1mM azide) also contained nitrite reduction deficient mutants at a frequency of 1:17,000 (0.017%). The frequency of chlorophyll-deficient seedlings in the M_2 generations appears to exhibit an inverse relationship to the concentration of azide used during mutagenesis of the M_1 generation (Duncanson, 1991) rather than the expected direct relationship. The reason for this decrease in the number of chlorophyll-deficient seedlings is uncertain, but may be the result of increases in mutations elsewhere within the genome modifying the phenotypic effects towards the wild type (Tuleen *et al.*, 1968).

It could be expected that our population of M_2 seed may contain seedlings defective in sulphate assimilation at, at least as high a frequency, possibly higher since mutations could occur at a number of enzymic steps of the pathway. Many possible mutations would be expected to result in individuals lacking a functional sulphate assimilation pathway, including those altering sulphate uptake, and the structural and/or regulatory loci for each enzyme. Selenate is known to be activated by ATP-sulphurylase to form APSe (Wilson and Bandurski, 1958; Burnell, 1981) and cysteine synthase has been shown to catalyse the formation of selenocysteine from selenide (Ng and Anderson, 1978b), so mutations at either of these steps both of which are common to both possible pathways, as well as mutations at the uptake level would be expected to result in selenate resistance. The mechanism for the reduction of APSe to selenite is uncertain, with selenite reduced to selenide in a light dependant reaction involving glutathione reductase (Ng and Anderson, 1978a and 1979). Mutations at enzyme steps between ATP-sulphurylase and cysteine synthase will only confer selenate resistance if these enzymes are actually involved in the reduction of selenate. Bosma *et al.* (1991) have reported that sulphite reductase cannot use selenite in place of sulphite suggesting that it is unlikely that mutants defective at the sulphite reductase step could be isolated by this screen. It is noted that some of the other mutations possible, those which cause over production of one of the enzymes of the pathway for example, would not be identified by this system of screening for selenate resistance.

4.2.3.1 Growth characteristics and maintenance of the M_2 selections from the screen

More than 70,000 M_2 seedlings were screened for selenate resistance. 231 seedlings were selected from the screen as being healthier than the other selenate treated plants. After selection, and transfer to compost, daily monitoring showed three distinct types of growth behaviour. Selections were classified according to their growth following transfer to compost.

The first class of plants (class I) became progressively brown and withered, and died without any further growth within three weeks of transfer. The deaths of these plants is believed to be due to selenate toxicity rather than any inability to grow on compost, with selenate having been taken up in sufficient quantities to be fatal.

A second class of seedlings (class II) selected produced new healthy leaves within 14 days of transfer although the older, damaged leaves continued to become necrotic and died. These plants subsequently grew phenotypically as the selenate untreated controls and obviously had a functional sulphate assimilation pathway. It is believed that this class of selection were sensitive to selenate but had not accumulated it in sufficient concentration to be lethal.

The third class of selections (class III), which was by far the smallest group grew on slowly. It is this class of plants that is considered most likely to be defective in sulphate assimilation.

With a non-functional sulphate assimilation pathway plants would not be expected to grow well on compost since the plants would suffer sulphate starvation whilst levels of the substrate for the defective step in the pathway would build up, possibly to toxic levels. In order to test whether the class III selections would recover if grown on a reduced sulphur source, two of them, VI 21:23 and TW

34:1 were transferred from compost to a sulphate-free hydroponic growth system, similar to the one used to grow nitrite reductase deficient mutants (Duncanson *et al.*, 1991), with cysteine supplied as the sole sulphur source (Fig 4.4). No improvement in the growth of either of the two selections was noted after transfer. Both plants grew on to maturity but did not produce seed. Rooted tillers of two of the selections, VI 25:3 and TW 42:1, were separated from each other and grown in hydroponics with either sulphate or cysteine as the sulphur source. No improvement in the growth of tillers on the cysteine hydroponic regime was observed. In fact the tiller of VI 25:3 grown with cysteine as the sulphur source died before reaching maturity, whereas the tiller grown in hydroponics culture with sulphate as sulphur source and a tiller maintained in compost flowered and produced seed.

Screening was initiated at 0.05mM selenate but the numbers of class I and class II selections, considered to be false positives, taken from the screen was too high. After 20,000 seedlings had been screened the selenate concentration was increased to 0.1mM and later to 0.2mM in an attempt to reduce the numbers of class I and class II selections. It was hoped that this would limit the seedlings selected to class III selections, those considered most likely to carry a defect in sulphate assimilation. Reduced numbers of selections would also allow the seedlings to be transferred from the screen directly into the hydroponic system with cysteine as the sulphur source. This would improve the chances of recovering sulphate assimilation defective mutants. Unfortunately, it was never possible to do this as the numbers of selections remained too high.

Seed produced the class II or class III selections was collected. However, only class III selections and their M_3 progeny were studied further.

Some of the Class III selections did not flower, whilst others which did flower did not set seed. M_3 seed was collected from the class III selections GP 2:1, GP 5:2, GP 13:1, VI 22:2 and VI 25:3, although only three seed were produced by GP 2:1. The infertility of many of the class III selections presents a problem for future genetical and biochemical analysis.

One possible way to overcome the loss of putative selenate resistant lines, due to premature death or infertility of the selected plants would be to use M_2 spikes rather than combine harvested seed. Some of the M_2 seed from an M_2 spike could be sown screened for selenate resistance in the same way as screening for nitrite reductase deficient barley plants from M_2 spikes was carried out by Duncanson *et al.* (1991, 1993). Any spike selected as carrying a homozygous recessive mutant will have a high probability of carrying the mutation in the heterozygous form in at least one of the remaining seed (Duncanson, 1991). Heterozygous M_2 seed on the same spikes as homozygous mutants may be identified by segregation analysis of the M_3 progeny, which should segregate in the Mendelian ratio of 1:3, homozygous recessive:heterozygotes and homozygous wild type individuals. Using this screening system the mutation may be recovered even if the original M_2 selection does not survive. The mutation may be maintained in heterozygous form, and these plants should be able to grow normally with sulphate as a sulphur source. Although this system has many advantages it would very time consuming; allowing fewer seedlings to be screened in the same time period. Whilst in the case of nitrite reductase deficient mutants spike

screening was worthwhile until the selenate screening method has been proved to work it is not a viable proposition here.

4.2.3.2 Genetic analysis

The isolation of whole plant mutants defective in sulphate assimilation should allow genetic analysis of the step of the pathway where the mutation occurs. Cross pollination between selected plants, homozygous recessive for the defect, and wild type barley plants should result in F_1 progeny that are heterozygous for the defect. The ratio with which the F_2 generation, derived from self pollination of the F_1 plants, segregate to produce homozygous selenate sensitive mutants and wild type phenotype plants, both homozygous and heterozygous, should confirm the location of the gene responsible for the defect. If the gene responsible for the defect was nuclear encoded then the Mendelian ratio of 1 homozygous mutant:3 wild type phenotype would be expected. Whereas a chloroplastic location would only allow recovery of the mutants from the maternal line since all organelles are inherited from the maternal parent.

In order to carry out this type of analysis the selected plants must grow to maturity and be fertile. No crossing to wild type was carried out and only 5 of the 9 class III selections were fertile and produced M_3 seed. Any mutation carried by the four selections from which no seed was recovered was lost.

M_2 populations of barley, mutagenised in the M_1 with sodium azide have previously been reported to express side effects from the chemical mutagenic treatment including slow growth, sterility and premature death (Duncanson, 1991). It is uncertain whether these growth characteristics in the selections were side effects of the mutagenesis or a result of a defect in sulphate assimilation. However,

these side effects were seen in the growth of the selenate untreated control seedlings transferred to compost from the screen.

4.2.3.3 Growth of the M_3 progeny of class III selections

Tests showed that most of the M_3 progeny tested were able to grow on compost phenotypically like wild-type plants and those tested were selenate sensitive. No tests were carried out on the M_3 progeny of selection GP 2:1 as none of the seed germinated. Seed from GP 5:2 and 13:1, and VI 22:2 grew as wild type when grown on compost. M_3 seedlings from VI 25:3 showed a variety of growth characteristics, similar to those described as side effects of mutagenesis.

The M_3 progeny of GP 13:1 showed no signs of resistance to selenate compared to wild type seedlings.

The selenate sensitivity and ability of the M_3 progeny to grow normally on compost suggests it is unlikely that these M_2 selections actually carry an inheritable mutation in sulphate assimilation.

4.2.3.4 Biochemical analysis of class III M_2 selections and their M_3 progeny

M_3 seedlings from GP 5:2, GP 13:1, VI 22:2 and VI 25:3 were tested for the presence of four of the enzymes believed to play a role in sulphate assimilation; ATP-sulphurylase, thiosulphate reductase, sulphite reductase and cysteine synthase, and β-cyanoalanine synthase by specific gel staining after non-denaturing polyacrylamide gel electrophoresis. The original M_2 selections, VI 22:2 and VI 25:3 were also tested for the presence of cysteine synthase and β-cyanoalanine synthase. All the enzymes tested for were present in all of the plants.

It is possible that there is a mutation at one of the remaining steps in the pathway, in sulphate uptake, APS sulphotransferase; in the carrier bound pathway, APS kinase or PAPS reductase; in the free intermediate pathway, or serine acetyl transferase, which is common to both pathways. However, the results of the growth and selenate sensitivity tests on the M_3 progeny of the selections make that look doubtful.

4.2.4 Use of the gel assay system in the biochemical analysis of the *nir1* barley mutant STA 3999

The *nir1* mutant, STA 3999, from the barley cultivar Tweed is defective in nitrite reduction. It was isolated from a whole plant screen where individuals were tested for nitrite accumulation after treatment with a nitrate containing nutrient solution (Duncanson *et al.*, 1991; 1993). The mutant had been shown to lack nitrite reductase cross reacting material. The possibility existed that the defect in nitrite reduction was due to a defect in the biosynthesis of the sirohaem prosthetic group. This sirohaem group is a common feature of nitrite and sulphite reductase. Hence, if sulphite reductase is functional in STA 3999 then sirohaem biosynthesis must be functional. Since sulphite reductase activity was shown to be present it is unlikely that STA 3999 is defective in sirohaem biosynthesis.

The potential use of gel assays in the biochemical analysis of whole plant mutants has been demonstrated.

CHAPTER 5

CONCLUSIONS

The primary aims of the project were: 1) to develop a screen to allow the isolation of whole plant barley mutants defective in sulphate assimilation and 2) the subsequent genetical and biochemical analysis of these mutants.

A screen was developed using selenium, an analogue of sulphur, in the form of selenate as the positive selection agent. Selenate was shown to be toxic to barley seedlings. Seedlings were shown to be most sensitive to selenate in the absence of added sulphate and the presence of added nitrate. Sulphate probably protects against selenate toxicity by competing with selenate for uptake by the roots (Ferrari and Renosto, 1972) and for activation by ATP sulphurylase (Burnell, 1981; Wilson and Bandurski, 1958). Cysteine synthase has been shown to catalyse the synthesis of selenocysteine from O-acetyl-L-serine and selenide (Ng and Anderson, 1978b). Competition may also occur at other steps in the pathway although Bosma *et al.* (1991) report that sulphite reductase cannot reduce selenite.

More than 70,000 M_2 seedlings (mutagenised in the M_1 with 2mM azide) were screened for resistance to selenate. The anticipated situation of a healthy seedling growing amongst a background of selenate damaged seedlings was not seen, all seedlings showed signs of selenate damage. 231 seedlings that appeared to be healthier than the rest were selected from the screen, of these nine were classified as class III (slow growing) selections, considered the most likely to possess a defect in sulphate assimilation. Biochemical analysis and selenate sensitivity tests on the M_3 progeny of those class III selections that produced seed suggest that it is unlikely that the M_2 selections carry a heritable mutation in sulphate assimilation. It seems likely that the apparent selenate resistance of the M_2 selections may

have been due to differences in growth rate, possibly side effects from the mutagenesis.

There are many reasons why it may not have been possible to isolate mutants defective in sulphate assimilation. The proposal by Schwenn (1989) during the course of this project that PAPS reductase plays a role in sulphate assimilation as part of a free intermediate pathway lead to the questioning of the widely held theory of a carrier bound pathway. Selection of mutants may still have been possible even if both pathways operated in higher plants where the mutation affected a step common to both pathways or where the *in-vivo* activity of the pathway containing the mutation was much greater than the activity of the pathway in which the mutation was not located. However, reports of multiple forms of the enzymes of the pathways, e.g. two forms of ATP sulphurylase in spinach (Lunn *et al.*, 1990), four distinct proteins with thiosulphate reductase activity in *chlorella* (Schmidt *et al.,* 1984), two forms of sulphite reductase in spinach (Krueger and Siegel, 1982a) and multiple forms of cysteine synthase in spinach (Droux *et al.*, 1992; Saito *et al.*, 1992, 1993; Lunn *et al.*, 1990) and other plants, make it seem increasing unlikely that a single mutation could result in selenate resistance. It is then not surprising that we were unable to isolate selenate resistant barley mutants. We have recently become aware that other workers screening for selenate resistance in *Arabidopsis* seedlings have also not found it possible to isolate mutants using this type of whole plant screen (T. Altmann, Institut fur Genbiologische Forschung Berlin, pers comm. to J.L. Wray).

Conventional enzyme assays and specific activity stains following non-denaturing polyacrylamide gel electrophoresis developed to analyse screen selections were also used to investigate

the enzymes of the two possible pathways of sulphate assimilation in wild type barley. The three enzymes which are common to both pathways, ATP sulphurylase, serine acetyltransferase and cysteine synthase were detected in extract of barley seedling leaves. Thiosulphate reductase, an enzyme of the bound intermediate pathway, and sulphite reductase, an enzyme of the free intermediate pathway, were also detected in barley leaf extract. Specific activity staining for thiosulphate reductase and sulphite reductase activities suggested the possibility of multiple isoforms of these two enzymes in barley leaves. The apparent presence of enzymes unique to each of the possible pathways does not allow any conclusions to be drawn as to which pathway is active *in vivo* in barley. Development of assay systems for the remaining enzymes, APS sulphotransferase, APS kinase and PAPS reductase, may give more insight into the situation, although PAPS the product of APS kinase is believed to be the sulphate source for sulpholipid synthesis in plants (Harwood, 1975; Kleppinger-Sparace and Mudd, 1990) and would therefore be expected to be present even if not implicated in sulphate assimilation.

Associated experiments showed the presence of the enzyme β-cyanoalanine synthase in barley leaf extract. β-cyanoalanine synthase activity was shown to be higher in barley than has been reported in other noncyanogenic plants, supporting the same observation made by Wurtele *et al.* (1985).

Cysteine synthase was characterised further in a series of experiments. Light was shown to be unimportant in the development of activity in barley seedlings. The specific activity of seedlings grown in the presence of nitrate increased over the experimental period (from 4-14 days after sowing) whilst the specific activity of seedlings grown without nitrate remained unchanged. In the absence

of nitrate the addition of sulphate to the nutrient medium did not affect the activity. However, in the presence of nitrate the addition of sulphate reduced the increase in activity. Cysteine synthase activity is then unaffected by some environmental factors and responds slowly to others suggesting the role of cysteine synthase in the regulation of sulphate assimilation is likely to be minor.

Initially the appearance of two bands of activity on non-denaturing polyacrylamide gels stained specifically for cysteine synthase and the elution of two activity peaks from the first DEAE Sephadex A50 column of the purification scheme suggested the presence of two forms of cysteine synthase in barley leaf extract. Subsequent investigations of the activity bands on the gels identified one of the bands to be the result of β-cyanoalanine synthase activity. β-cyanoalanine synthase is a mitochondrial enzymes believed to catalyse one step in the detoxifying conversion of cyanide to asparagine (Blumenthal *et al.*, 1968; Hendrickson and Conn, 1969; Miller and Conn, 1980). It now seems likely that the small peak of activity eluted from the DEAE Sephadex column was also the result of β-cyanoalanine synthase. A single form of cysteine synthase in barley is proposed. This suggestion supports the findings of Rosichan *et al.* (1983).

Differential centrifugation of mechanically ruptured barley leaf protoplasts showed cysteine synthase was likely to be predominantly or exclusively located within the chloroplasts although further modification of the localisation procedures would be necessary before the evidence could be considered conclusive. A solely chloroplastic location for cysteine synthase contradicts recent reports of the enzyme in the mitochondria of spinach and pea (Lunn *et al.*, 1990) and cauliflower buds (Rolland *et al.*, 1992). β-cyanoalanine synthase

activity is suggested as responsible for the low levels of cysteine production seen *in vitro* in mitochondrial fractions by these workers.

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