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Studies on the Peptide Transport System in the Scutellum
of Germinating Barley

by

David J.Walker-Smith, B.Sc. (Dunelm)

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A thesis submitted in accordance with the requirements for the
degree of Doctor of Philosophy in the University of Durham

February 1984

ABSTRACT

Through the use of protein-modification reagents, in particular the thiol-specific reagents N-ethylmaleimide, ρ -chloromercuribenzenesulphonic acid, and phenylarsine oxide, it is shown that in the barley scutellum, the transport of peptides, but not the transport of amino acids or glucose, is specifically thiol-dependent. Furthermore, these essential thiol groups are shown to exist as redox-sensitive, vicinal dithiols which lie at the substrate-binding sites of the peptide transport proteins. A technique for the specific labelling of the vicinal dithiols with radioactive N-ethylmaleimide is described, and microautoradiography of scutellar tissue treated in this way shows these groupings to be mainly located in the plasmalemmae of the scutellar epithelium.

In related work, the importance of arginyl and histidyl residues to both peptide and amino acid transport is shown, although other moieties, e.g., carboxyl ligands, would not seem to be critically involved.

In other studies concerning the general characteristics of these transport systems in barley, the development of peptide and amino acid transport capacity during germination is described, and it seems that initially, peptide transport is likely to be the more important in the nitrogen nutrition of the embryo.

A proton-motive force is implicated in the energisation of transport by the scutellum, and some evidence was obtained to indicate that a proton gradient was necessary to maintain the essential dithiol groups in their functional state.

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Memorandum

Parts of the work presented in this thesis also appear in the following publications:

- 1) Harris, N., Oparka, K.J., Walker-Smith, D.J. : Plasmatabules : an alternative to transfer cells? *Planta* 156, 461-465 (1982).
- 2) Walker-Smith, D.J., Payne, J.W. : Peptide uptake in germinating barley embryos involves a dithiol-dependent transport protein. *FEBS Lett.* 160, 25-30 (1983).
- 3) Walker-Smith, D.J., Payne, J.W. : Characterization of a dithiol dependent transport protein in the scutellum of germinating barley. *Biochem. Soc. Trans.* 11, 800-803 (1983).

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CHAPTER 1

INTRODUCTION



Cereal grains form a main component of the diet of both man and his domestic animals, global production exceeding 1200 million tonnes in 1974 (FAO, 1975). They are grown both as a source of carbohydrate, providing nearly 50% of the world's per capita energy requirement, and as an important source of protein, fulfilling nearly 45% of the world's per capita requirement (FAO, 1977). The major cereal staples for human consumption include wheat and maize, which are grown in temperate climates; rice, which is the preferred crop in damper, tropical areas; and sorghum and millets, important staples in more arid, drier zones. Oats and rye are generally cultivated in colder climates, primarily as food for livestock. Barley is also grown for animal fodder, but since it has additional commercial importance as the raw material of the brewing industry it has been subject to particularly intensive study, especially with regard to the mobilization of the seed storage reserves during germination (the malting process).

As a prerequisite to the detailed studies concerning the transport of these mobilized reserves, which are described later in this thesis, an introductory survey of the literature concerned with the structure of the dormant and germinating cereal grain is presented below, with particular reference to the storage materials and their degradation.

1.1 Gross Morphology of the Cereal Grain

The gross morphology of a dormant barley grain is shown in section in Figure 1.1. The grain, which strictly speaking is not a seed but a caryopsis, has two major parts, the embryo and the endosperm. Both the embryo and the endosperm are enclosed within a testa (seed coat) and a pericarp (fruit wall). These two outer layers are fused together to form the husk. The endosperm comprises both aleurone tissue, and the so-called "starchy endosperm", which is storage material whose breakdown products provide for the growth of the embryo during the early stages of germination. The aleurone, a layer 2-3 cells thick overlying the

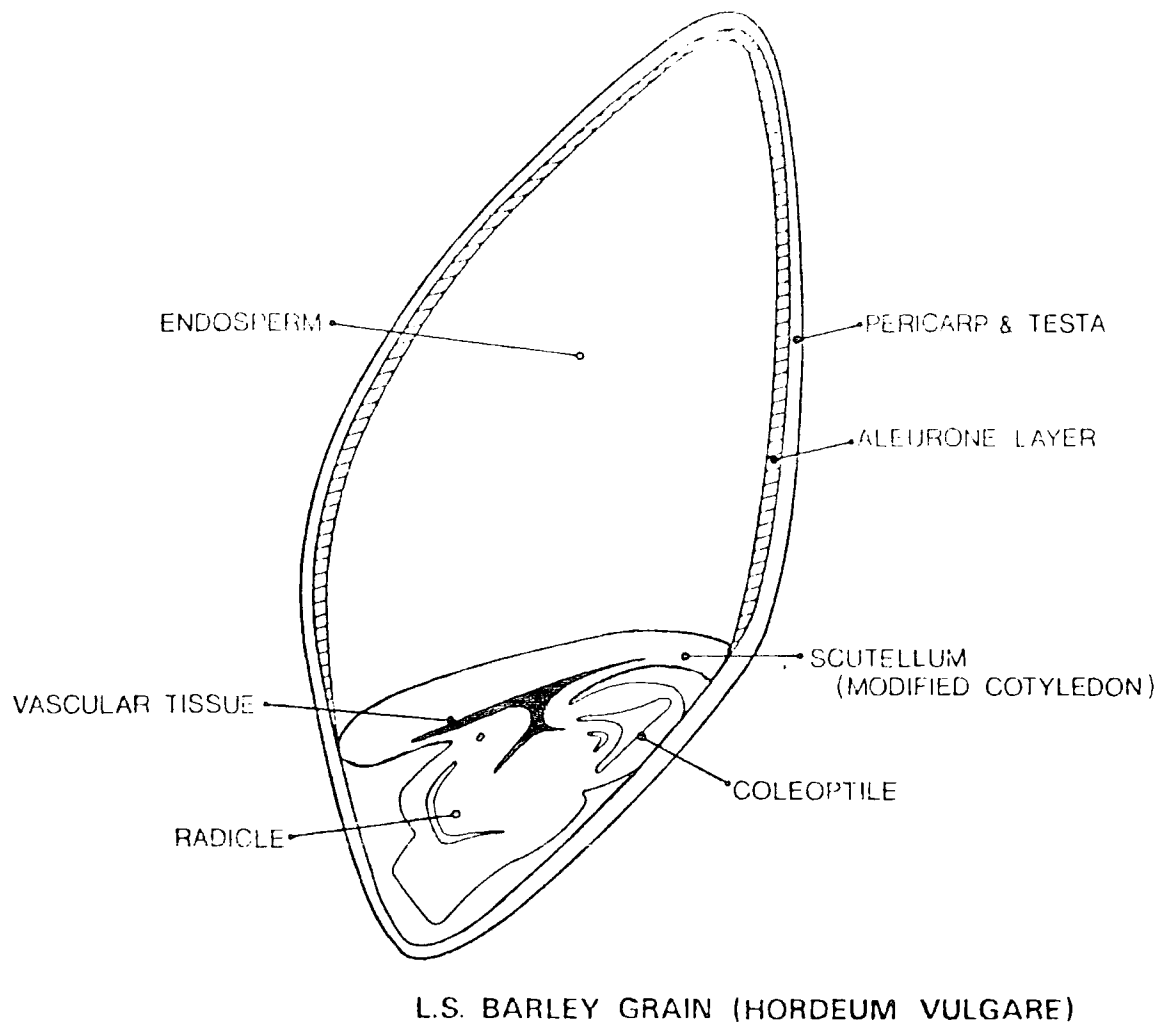


FIGURE 1.1 Longitudinal Section of the Dormant Barley Grain

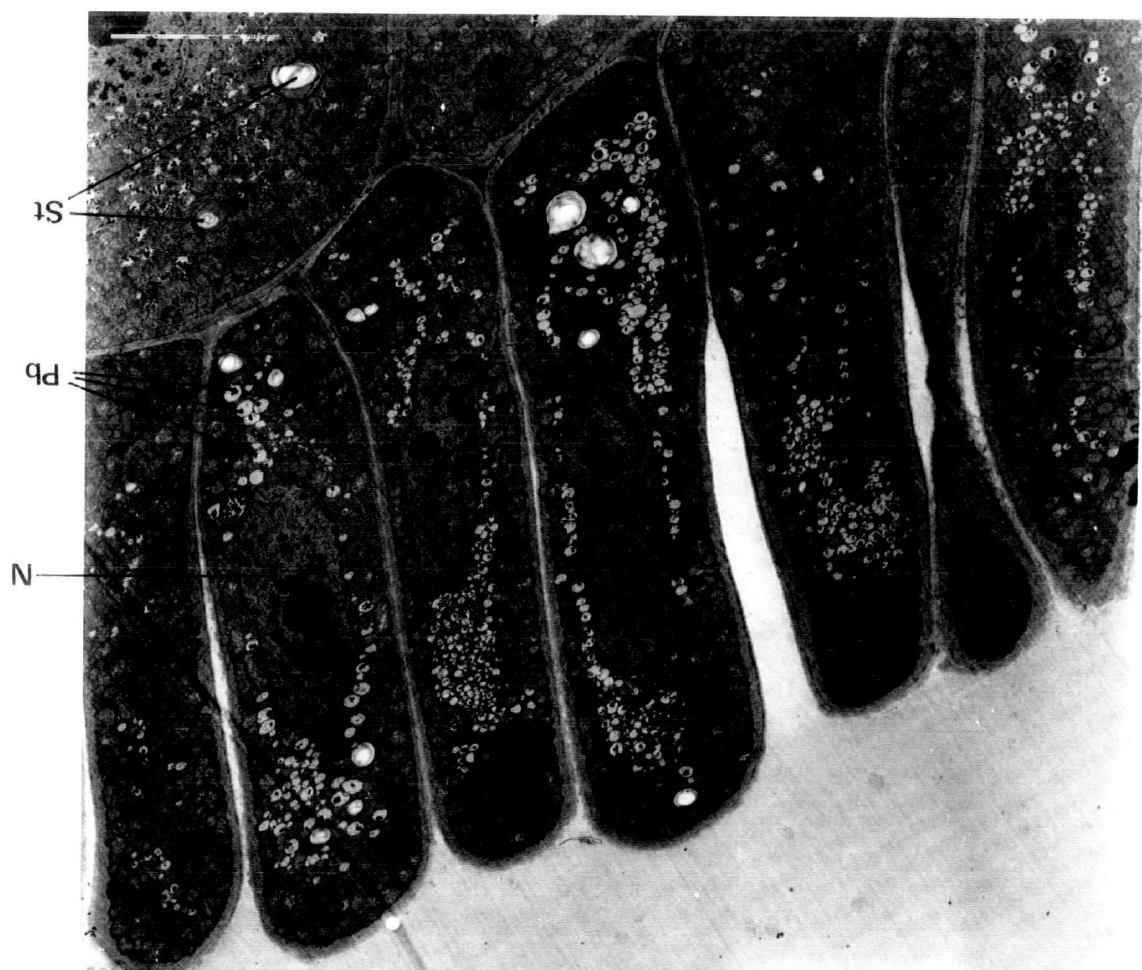
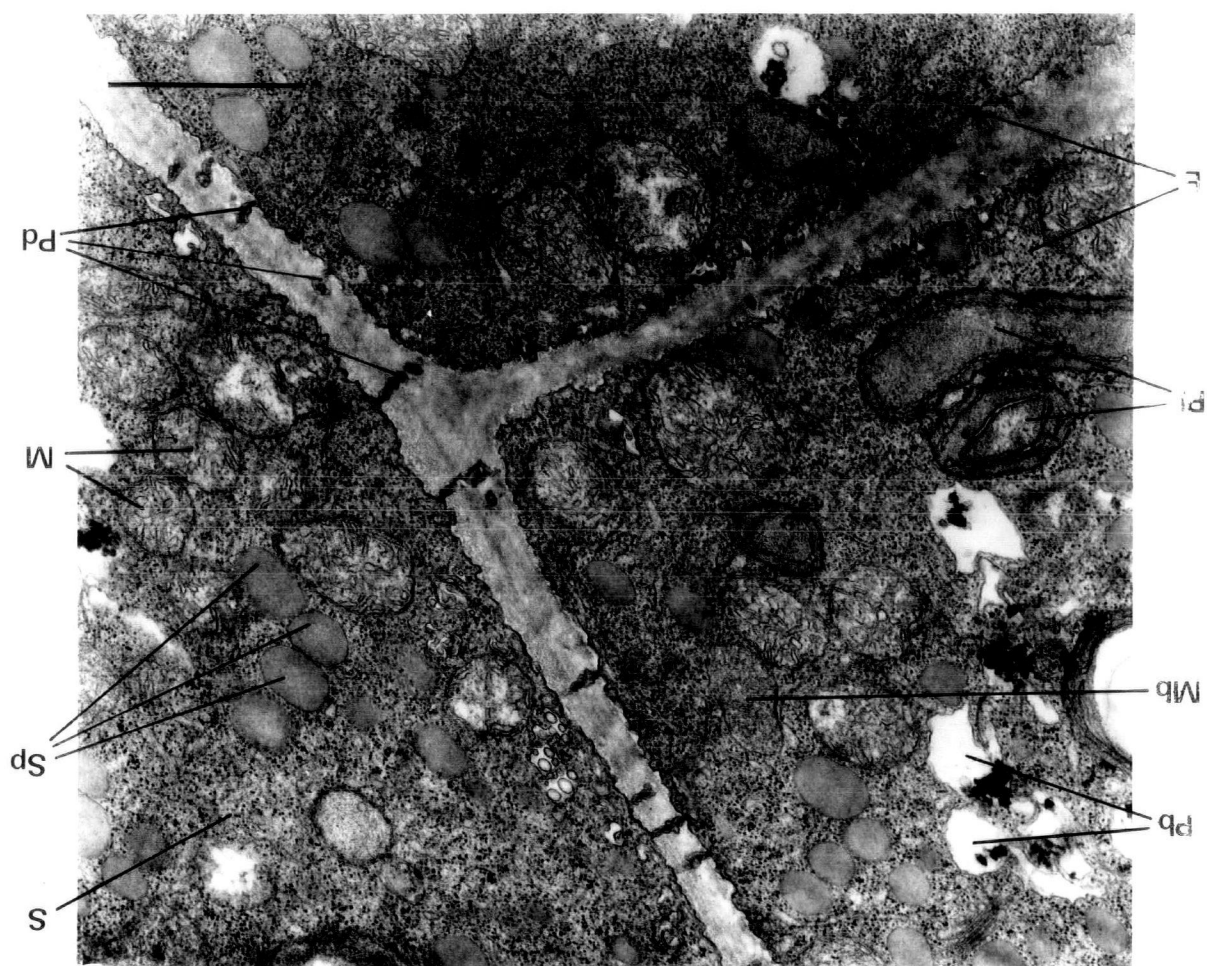
The two main tissues of the grain, the endosperm and the embryo, are separated by the scutellum, a modified cotyledon which functions primarily as a haustorial (absorptive) structure.

starchy endosperm, is the site of production of many of the hydrolytic enzymes responsible for the digestion of the endospermal reserves. The embryo is comprised of a plumule and a radicle, both of which are enclosed in sheath-like coverings, the coleoptile and coleorhiza respectively. The embryonic tissues are connected via the cotyledonary node to a single cotyledon, which in all graminaceous species is highly modified as the scutellum (Avery, 1930). In the cereal grain, the scutellum has a dual role not only as a storage organ (proteins, lipids and also vitamins e.g., 62% of the total thiamine of the wheat seed; MacMasters *et al*, 1971), but also as a haustorial structure with an absorptive surface serving to transfer solutes, required for growth by the embryo, from the starchy endosperm (apoplast) into the symplastic component of the embryo during germination. Since the transfer of material from the apoplast into the symplast occurs at the scutellum, this process must necessarily involve a trans-membrane flux of materials across this boundary. The uptake of solutes such as amino acids, peptides and sugars, via the scutellum into the embryonic tissues, has been shown not to be simply a passive, diffusional flow, but to involve the mediation of specific, membrane-bound carrier proteins located in the scutellar epithelium, which effect transport in an energy-requiring process. The scutellum is therefore unique and of considerable interest; moreover, the experimental ease with which it can be isolated and handled in vitro makes it a convenient, model system with which to study the complexities of membrane transport in higher plants. It is therefore of some relevance to consider the ultrastructure of the scutellum in some detail.

1.2 Ultrastructure of the Scutellum

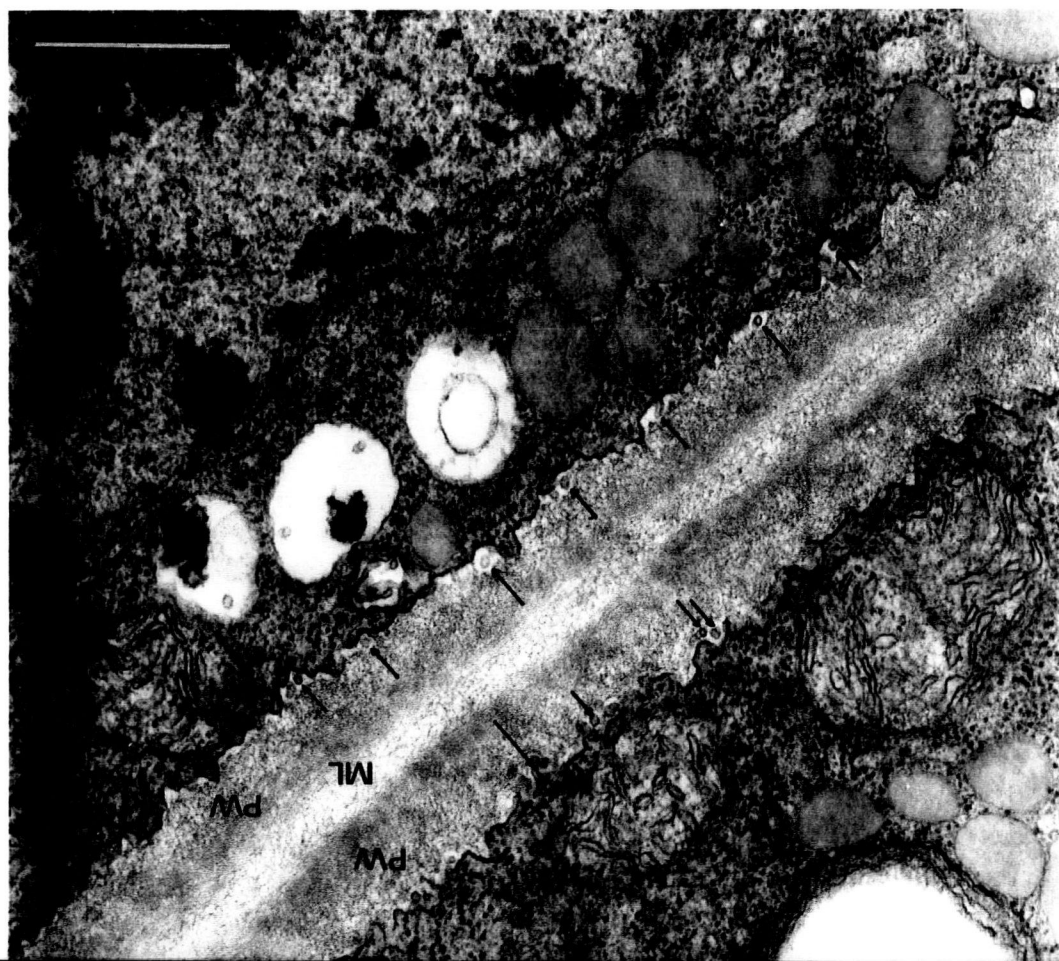
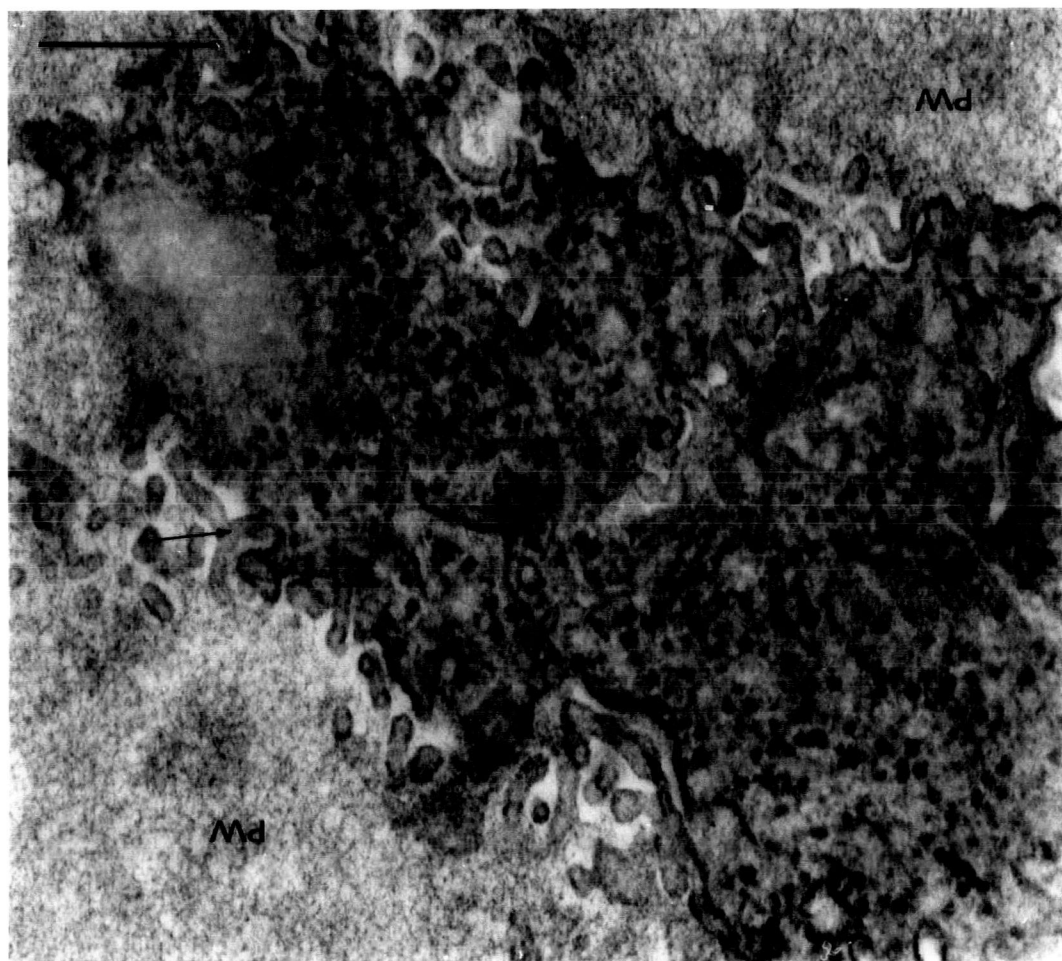
1.2.1 The Epithelium

Early workers such as Brown & Morris (1890) and Sargant & Robertson (1905) first established the role of the scutellum as an absorptive organ in cereal germination, but until relatively recently, it has received



little attention from microscopists and biochemists alike. Electron and light microscopy of the embryos of barley (Nieuwdorp, 1963; Nieuwdorp & Buys, 1964), wheat (Swift & O'Brien, 1972a, 1972b), maize (Zamski, 1973), rice (Bechtel & Pomeranz, 1978), millets and sorghum (Zelezna & Varriano-Marston, 1982) and in a related system, the protodermal cells of the date palm cotyledon (Demason & Thomson, 1981), reveal that in these species, the abaxial scutellar surface (i.e. that abutting onto the endosperm) consists of a single layer of columnar epithelial cells (Figure 1.2). The plasmalemmae of these cells may be highly corrugated. The epithelial cells usually elongate and separate 2-3 days after the start of imbibition (Torrey, 1902; Reed, 1904; Sargent & Robertson, 1905; Toole, 1924) by dissolution of the middle lamella (Swift & O'Brien, 1971), and by increasing access to the absorptive surface area in this way, they come to resemble, in both form and function, the villi of the mammalian intestine. Despite the presence of many hydrolytic enzymes within the starchy endosperm during germination, the cell walls of both the scutellum and the aleurone are themselves remarkably resistant to degradation by these enzymes. The walls of both tissues are rich in the phenolic compound ferulic acid (Smart & O'Brien, 1979b) which may be partly responsible for conferring some protection against enzymic digestion (Smart & O'Brien, 1979c; Fulcher *et al.*, 1972).

The epithelial cells have a prominent nucleus and many ribosomes. They are rich in protein bodies (Section 1.4.1) and spherosomes, the latter being located mainly around the periphery of the cell (Smart & O'Brien, 1979a). Spherosomes are considered to be sites of lipid storage, and contain enzymes associated with lipid metabolism (Yatsu & Jacks, 1972; Semadeni, 1967). The epithelial cells are also rich in mitochondria, the majority of which are aligned close to the plasmalemma (Harris *et al.*, 1982). Their proximity to the membrane is indicative of the high energy demand



that transport incurs, and similar arrangements of these organelles have been observed in other systems where an energy-requiring, high solute flux occurs across a membrane barrier, e.g. grain filling in the rice caryopsis (Oparka et al., 1981) and transport by kidney cells (Gunning & Steer, 1976). The scutellar epithelium is, therefore, modified to perform an absorptive function, and in this regard, the discovery of plasmatabules is perhaps of the greatest interest and is discussed below.

1.2.2 Plasmatabules

Electron microscopy of barley embryos, performed in connection with this thesis (for which I am indebted to Dr.N.Harris, University of Durham), revealed the existence of structures (referred to as plasmatabules) in the scutellar epithelium, whose presence in several other systems had hitherto either gone unnoticed or had been ignored. Plasmatabules are branching, tubular evaginations of the plasmalemma, without any visible core structure, which extend outwards into the cell wall. The scutellar cell walls themselves do not display any extensive ingrowths, which is a characteristic feature of transfer cells. Plasmatabules are located specifically, and in high numbers, in the epithelial plasmalemmae (Figure 1.3) of the barley scutellum. We suggest that, by amplifying the membrane surface area, these structures are short-term, structural modifications associated with the rapid transport of material from the endosperm into the embryo during the early stages of germination (Harris et al., 1982). Plasmatabules serve a specific function in relation to solute uptake, and therefore represent a specialized form of plasmalemmasome. 'Plasmalemmasome' is a general term describing any tubular or vesicular proliferation of the plasma membrane (Harris, 1981; Marchant & Robards, 1968).

Structures resembling plasmatabules are also apparent in published micrographs of the differentiating phloem of cotton (Thorsch & Esau, 1981), Equisetum (Dute & Evert, 1978) and also tobacco callus tissue (Tran Thanh

Van & Chlyah, 1976), although these workers failed to comment upon their presence or their significance. Recently, they have been also observed associated with the plasmalemmae of companion cells abutting the sieve elements of major leaf veins of Lolium temulentum (N. Chaffey, personal communication). Hence, plasmatabules can be contrasted with transfer cells (Gunning & Pate, 1969, Gunning & Pate, 1974) whose presence is usually associated with rather longer-term transport systems. Membrane amplification in the transfer cell is achieved by the convolutions of the plasmalemma corresponding faithfully with highly labyrinthine cell wall ingrowths.

1.2.3 Sub-Epithelial and Vascular Tissue

Beneath the epithelial layer, the remainder of the scutellum is composed of parenchymatous storage tissue, interspersed with vascular strands (Figure 3.4). These parenchymatous cells, which are rich in lipid and protein reserves, are more regularly isodiametric in shape, and those lying directly beneath the epithelial layer are connected symplastically to the epithelial cells by numerous plasmodesmata (Figure 1.2). The large numbers of plasmodesmata in this location provides additional evidence for the bulk, symplastic movement of sugars and amino acids towards the embryo during germination. In the dormant grain, the vascular system is held in a provascular (undifferentiated) state, but mature, functional sieve elements are found in the scutellum just 3-6 hours after the start of imbibition (Swift & O'Brien, 1971). The vascular system consists of a single, large central vascular bundle extending from the scutellar node. The bundle branches and divides many times into a network of curved strands which ramify through the subepithelial parenchyma to run approximately parallel to the scutellar surface (Sargant & Arber, 1915; Avery, 1930; Swift & O'Brien, 1970). The vascular tissue is mainly phloem, and nearly 50% of the sieve elements are unaccompanied by xylem (Swift & O'Brien, 1970).

1.3 The Endosperm

1.3.1 The Starchy Endosperm

The starchy endosperm of the cereal caryopsis is an inert tissue containing large reserves of starch, and, in barley, about 11% protein on a dry weight basis (Dale et al., 1974). This can account for up to 75% of the total protein content of the whole grain. The protein, at least in wheat (Hinton, 1953), barley (Burgess et al., 1982), sorghum and rice (R.Ellis, personal communication), is not evenly distributed, most being located around the periphery of the endosperm. This has important implications with regard to the processing of cereal seed, because a significantly large proportion of total protein may be lost during milling when the grain is dehusked.

1.3.2 The Aleurone

The starchy endosperm is surrounded by a living tissue, the aleurone, which in barley, consists of three layers of cells, but may have more or less depending upon the cereal in question. The aleurone plays an important role in the secretion of enzymes which are responsible for endospermal digestion, but is also a storage tissue and the cells before germination are packed with membrane-bound protein bodies (aleurone grains) and spherosomes (lipid bodies) (Jacobsen et al., 1971).

1.4 Cereal Protein Reserves and Proteolytic Enzymes

1.4.1 Protein Bodies

Within the cells of the scutellum, aleurone and endosperm, the protein reserves are harboured in protein bodies which are usually enclosed by a single unit membrane (Miège, 1982, and references therein) although recent evidence suggests that, in barley, the delimiting envelope may be only partial (Mifflin & Burgess, 1982) or even absent altogether (Mifflin & Shewry, 1979a). The contents of the protein body can appear as a homogenous or granular matrix under the electron microscope, but

occasionally, protein is laid down as an ordered, crystalline deposit, known as a crystalloid, within the general matrix. In addition, protein bodies of the embryo and aleurone, but not the endosperm, generally contain inclusions, or globoids, embedded within the protein reserves (Pernollet, 1978).

Inclusions were first observed by Pfeffer (1872), and contain up to 80% by dry weight of phytic acid (myoinositol hexaphosphate) (Pernollet, 1978). Phytic acid is a cation store, forming a chelated complex with calcium and magnesium (Tanaka et al., 1976, 1977) as well as comprising 70-75% of the total phosphorus of the grain (Peers, 1953).

The ontogeny of the protein body is likely to be different within the aleurone and endosperm, and is a somewhat controversial subject. Protein bodies have been considered as plastid-like organelles capable of the independent synthesis and deposition of proteinaceous material (Morton et al., 1964), or as being derived from the vacuolar system. It is now generally agreed that protein deposition within the aleurone and scutellum does indeed occur within vacuoles (Matile, 1976; Ashton, 1976). However, evidence is currently accumulating in favour of the hypothesis that protein deposition in the endosperm occurs within the lumen of the rough endoplasmic reticulum, whose membranes eventually come to enclose, either partially or completely, the protein body (Harris & Juliano, 1977; Mifflin et al., 1981). In support of this hypothesis, electron microscopy shows polyribosomes to be associated with the outside of the protein body envelope which, in maize, support the synthesis of the storage protein zein in a cell-free translation system (Burr & Burr, 1976). Also, a clear continuity between rough ER and protein body membranes has been demonstrated in electron micrographs (Larkins & Hurkman, 1978), and as mentioned previously the membrane itself may be incomplete, an observation more compatible with the deposition of protein within the ER than within a vacuole, for example.

1.4.2 Classification of Storage Proteins

Cereal storage proteins can be conveniently classified according to their solubility properties, in a range of solvents, as originally defined by Osborne (1907). Thus, the albumins were defined as water-soluble, and are confined largely to the embryo and aleurone. The albumins also include most of the metabolic enzymes. The globulins, soluble in salt solution but not water, are storage proteins located primarily within the protein bodies of the scutellum and aleurone. They have received relatively little attention in the past (Danielsson, 1949), whereas the prolamins (soluble in aqueous alcohol) and the glutelins (soluble only in dilute acids and alkalis), are restricted to the endosperm (Review, Payne & Rhodes, 1982) and have been subject to more intensive research. Glutelins, of particular interest, are large proteins consisting of many subunits held together by disulphide bonds. The glutelin of wheat, (specific name glutenin) has elastic properties important to the making of bread.

The specific names assigned to the prolamins and glutelins of barley are hordein and hordenin respectively. Hordein is the principal proteinaceous reserve of the endosperm, and may account for up to 50% of the total grain nitrogen (Mifflin & Shewry, 1979a). Hordein, which can be extracted successfully from barley flour using isopropanol plus mercaptoethanol (Shewry *et al.*, 1980), has been shown by using polyacrylamide gel electrophoresis to be highly heterogenous in terms of its polypeptide composition. The protein has at least 6-8 individual polypeptide components (Shewry *et al.*, 1977; Doll & Andersen, 1981); these polypeptides fall into two categories on the basis of their molecular weights, solubility properties, amino acid compositions and genetic specification (Kj ie *et al.*, 1976; Shewry *et al.*, 1980, Doll & Brown, 1979). Hordein is rich in proline (7-10%), and glutamine/glutamate (up to 20%), but is

particularly deficient in the essential amino acid lysine (less than 2%) (Shewry et al., 1978, Mifflin & Shewry, 1979b), and for this reason barley is considered to be of low nutritional value. In this regard, some progress has been made either in the selection of mutants whose hordeins are enriched with lysine, e.g., the Risø 1508 mutant of the cultivar "Bomi" (Kjølle & Doll, 1979; Hagberg et al., 1979), or else of mutants richer in hordenins (with a higher lysine content) at the expense of hordeins (Mifflin & Shewry, 1979b).

The barley glutelin, hordenin, which can be extracted in acid-alcohol, or after alkylation following the prior removal of hordein, has proved difficult to characterize because of its insolubility, and fractions of it have a tendency to be contaminated with residual prolamins (Laurière et al., 1976; Brandt, 1975). Barley glutenins appear to be heterogenous with respect to their polypeptide chain composition, but in contrast with the prolamins, are richer in lysine (>4%) but poorer in glutamine / glutamate (10-15%) and proline (7-10%) (Mifflin & Shewry, 1979b).

1.4.3 Proteolytic Enzymes

Germinating barley grains possess several proteolytic enzymes (exopeptidases and endopeptidases), whose concerted action is required to achieve the complete degradation of proteinaceous storage material. Exopeptidases include the amino-peptidases and carboxypeptidases, enzymes which remove single amino acid residues sequentially from the N- and C-termini respectively of a polypeptide chain. Another class of exopeptidase, the peptidases, specifically hydrolyse small di- and tri-peptides to their constituent amino acids. The endopeptidases however, are capable of hydrolysing only the internal peptide bonds of large polypeptides/proteins (Ryan, 1973).

Although the overall activity of each of these enzymes within the whole barley caryopsis is fairly well documented, there are fewer reports concerning the exact localization of a particular proteinase. Moreover, the literature suffers some confusion arising from an inconsistent approach to nomenclature of each enzyme type. However it is now clear that the three main tissues of the germinating barley grain (embryo, aleurone and starchy endosperm) each contain a rather different complement of the different proteolytic enzymes. Several endopeptidases are present within the starchy endosperm (Enari et al., 1963; Enari & Mikola, 1977), the activity of which increases about 20-fold during germination (Mikola & Enari, 1970). It seems likely therefore, that they play an important role in the digestion of the storage proteins, particularly since the low pH optima of these enzymes matches the in vivo pH of the endosperm (pH 4.8 - 5.2). At least three carboxypeptidases, with similar acidic pH optima, are also located predominantly in the endosperm (Visuri et al., 1969; Moeller et al., 1970; Mikola et al., 1971) including a specific proline carboxypeptidase (Mikola & Mikola, 1980). Although aminopeptidase activity has not been detected in the starchy endosperm, this enzyme is, however, present in both the aleurone and scutellum. In addition, these two tissues also contain carboxypeptidase and endopeptidase activity, and it seems likely that these enzymes are involved primarily in making amino acids available for the de novo synthesis of hydrolytic enzymes destined for secretion into the starchy endosperm. Of greatest interest, however, is the high activity of two peptidases, both with alkaline pH optima, in germinating barley (Sopanen & Mikola, 1975; Sopanen, 1976). Both enzymes are found predominantly within the embryo, in particular the scutellar epithelium (Engel & Heins, 1947; Mikola & Kolehmainen, 1972; Prentice et al., 1967). The distribution of these peptidases, and the other enzymes, would suggest that the hydrolysis of the storage proteins may not occur to completion, and that small peptides may be absorbed by

the scutellum prior to complete hydrolysis to their constituent amino acids.

1.5 The Course of Proteolysis

In the first phase of proteolysis, protein reserves in the aleurone (and the scutellum) are hydrolysed to amino acids to provide for the synthesis of the hydrolytic enzymes that are secreted into the endosperm. The protein bodies (aleurone grains) are considered to be autolytic (Adams & Novellie, 1975; Yatsu & Jacks, 1968), and aleurone grains extracted from dormant grains of Hordeum have been shown to contain protease activity (Ory & Henningsen, 1969). As the storage reserves are hydrolysed the protein bodies become vacuolated (Jones, 1969) and may coalesce (Swift & O'Brien, 1972b).

The endosperm-digesting enzymes, whose production and/or release by the aleurone cells is stimulated by gibberellic acid, include α -amylase (Reviews; Ho, 1979; Yomo & Varner, 1971), exopeptidase (carboxypeptidase) (Schroeder & Burger, 1978), endopeptidase (Yomo, 1961; Sundblom & Mikola, 1972), ribonuclease (Bennett & Chrispeels, 1972), and also enzymes required to break down the endospermal cell walls within which the reserves themselves are sequestered e.g., β -1,3 glucarase (Jones, 1971), endoxylanase, xylopyranosidase and α -arabinofuranosidase (Taiz & Honigman, 1976). The scutellum may also play a limited role in the secretion of both amylase (Briggs, 1964) and carboxypeptidase (Ranki et al., 1983; Mikola & Kolehmainen, 1972). The production of several of these enzymes is by de novo synthesis (Chrispeels & Varner, 1967; Jacobsen & Varner, 1967; Dashek & Chrispeels, 1977), through the formation of new, translatable mRNA's within the aleurone (Ho & Varner, 1974; Bernal-Lugo et al., 1981; Mozer, 1980).

In vivo, the scutellum is the site of synthesis of gibberellin, or a mix of closely related giberellins, in particular GA_1 and GA_3 (Radley, 1967; 1969; Macleod & Palmer, 1967) which are released in equal quantities

to both dorsal and ventral sides of the grain (Briggs, 1972). Classically, gibberellin is then thought to diffuse into the aleurone to exert its effects upon enzyme production and secretion (Jones, 1973), although a recent report suggests that other unidentified hormones (not gibberellins) released by the embryo may have importance as the primary stimulus (Atzorn & Weiler, 1983). The exact mode of action of gibberellin is not understood, but intracellular levels of hormone may be regulated by the formation of inactive conjugates e.g., with glucopyranosides. Glucopyranoside-GA complexes have reduced biological activities and have been isolated from plant tissues (Sembdner *et al.*, 1976). Interestingly, radioactively labelled GA exogenously applied to barley aleurone was later recovered in a conjugated form, coupled probably to a small peptide (Nadeau & Rappaport, 1974). Absciscic acid is reported to act antagonistically to gibberellin when applied exogenously (Ho & Varner, 1976; Mozer, 1980), whereas ethylene enhances the effects of the hormone (Eastwell & Spencer, 1982). However, the roles of absciscic acid and ethylene *in vivo* have yet to be demonstrated.

The aleurone represents one of the relatively few plant tissues that secrete large quantities of enzyme extracellularly (these tissues are reviewed in Chrispeels, 1976), but the mechanism whereby enzymes are transported across the aleurone plasmalemmae into the endosperm has not been satisfactorily established, although it is apparently an energy-dependent process (Varner & Mense, 1972). In this regard, most studies have been concentrated upon the secretion of α -amylase. Lysosomes containing α -amylase activity were reported in wheat aleurone (Gibson & Paleg, 1972; 1975) but in studies using immunohistochemical or autoradiographic techniques (Chen & Jones, 1974; Jones & Chen, 1976) no evidence was found for the localization of α -amylase within lysosomes, vacuoles or dictyosomes, implying that the secretion of this enzyme did not occur by means of vesicular fusion with the plasmalemma. However, a possible

explanation for the mechanism of enzymic secretion lies in the fact that α -amylase is known to be synthesised as a large precursor molecule, with a short 'leader' sequence that is subsequently cleaved to yield the functional enzyme (Okita et al., 1979; Boston et al., 1982). It is proposed that this leader sequence, a peptide with a mass of approximately 1500 daltons, may be involved in the specific association of the growing polypeptide with the aleurone plasmalemma, perhaps by a process similar to that envisaged in the signal hypothesis (Blobel & Dobberstein 1975). In this hypothesis, the nascent protein chain is thought of as being able to thread through the membrane to the other side as it is elongated; subsequently the leader is cleaved and the protein assumes its functional tertiary structure. Whatever the mode of secretion across the plasmalemma may be, subsequent movement of the enzyme through the aleurone cell wall is then a simple diffusive process, whose rapidity is dependent upon the thickness of the wall (Varner & Mense, 1972). In this regard, the outer layers of the aleurone walls are reduced in thickness by hydrolytic enzymes released by the aleurone itself (Taiz & Honigman, 1976) leaving an inner layer, rich in ferulic acid, which is resistant to degradation (Fulcher et al., 1972). Apart from α -amylase, it is not known whether the other hydrolytic enzymes, such as the exo- and endopeptidases, are synthesised within the aleurone as precursor polypeptides which might indicate the involvement of leader sequences in their secretion also.

1.6 Peptide Transport in Plants

1.6.1 Introduction

Specific peptide transport systems have been shown to be important in the nutrition of a wide range of bacteria (reviewed in Payne, 1976, 1980), yeasts, including Saccharomyces cerevisiae (Becker & Naider, 1977, 1980; Nisbet & Payne, 1979) and Candida albicans (Lichliter et al., 1976;

Davies, 1980), the mycelial fungus Neurospora crassa (Wolfenbarger & Marzluf, 1974, 1975), and in a variety of mammalian tissues such as small intestine, renal cortex, liver and brain cells (Asatoor et al., 1973; Matthews, 1975; Sleisenger et al., 1976, Matthews & Payne, 1980). In all these cases, peptides are accumulated intact and independently of amino acid transport; uptake is energy-requiring although the mode of energization of each system may be different. Peptides of up to five amino acid residues in length can be transported by most of these systems.

Because of the widespread occurrence of peptide transport systems in nature, it is clearly advantageous for an organism to be able to transport peptides as well as amino acids. Perhaps the main advantage of peptide uptake is energetic, for it appears that equivalent amounts of energy are required to transport equimolar amounts of peptide or amino acid (J.W. Payne, unpublished data); however, such a saving is only likely to be significant in unicellular organisms and will not be so important in the mammalian jejunum or kidney, for example, which represent but small parts of the total body mass. In addition, uptake in the form of peptides can give a more balanced presentation of amino acids, and bacteria for example, will grow better on peptides as the sole nitrogen source rather than an amino acid mix. These apparent benefits of peptide transport would suggest that plants also might well have evolved such systems under certain circumstances. However, because plants are generally autotrophic, the importance of external sources of nitrogen in the form of amino acids or peptides is likely to be limited to highly eutrophic, aquatic environments, or else highly nutrient-deficient habitats where plants have evolved a carnivorous habit (see below). Instead, peptide transport in plants is more likely to play an important role internally in the movement of peptides

intercellularly, or between tissues, particularly when proteins are undergoing turnover. In this regard, protein degradation within germinating seeds, storage organs or senescing tissues may well involve the trans-membrane transport of peptides, especially where a cytoplasmic continuity does not exist and movement of material cannot occur via plasmodesmata, for example.

Over the past decade, some effort has been directed towards the identification and characterisation of amino acid transport systems in plants (reviewed by Higgins & Payne, 1980) but relatively little attention has been paid to the possibility that peptide transport may well be operating also, either in parallel, or else exclusively. A resumé of those peptide transport systems for which some evidence has been found in plants is given below (earlier reviews of this topic are found in Matthews & Payne, 1980; Higgins & Payne, 1980, 1982).

1.6.2 Carnivorous Species

Over four hundred species of carnivorous plants have been identified, most of which generally grow in nutrient deficient habitats and supplement their inorganic nitrogen intake by trapping and digesting small insects. The final products of this digestive process might well be expected to include amino acids and/or small peptides, and at least two species, the sundew Drosera (Simola, 1978) and the pitcher plant Sarracenia (Plummer & Kethley, 1964), are capable of growing on a peptide, or mix of peptides, as the sole nitrogen source. In both cases, peptides were apparently accumulated intact, prior to intracellular hydrolysis to the constituent amino acids.

1.6.3 Aquatic Species

Aquatic environments may be rich in organic nitrogen, particularly the surfaces of eutrophic lakes where the concentration of amino acids can exceed 30 mmol m^{-3} (Gessner, 1959). Many phytoplanktonic and surface-living plants have evolved transport systems to take advantage of these

conditions, and amino acid uptake by several organisms is well documented (e.g., Wheeler et al., 1974, 1977; North, 1975; Cho et al., 1981; Jung & Lüttge, 1980). However, there is a paucity of literature concerning peptide utilization by aquatic species, although Bollard (1966) reported that the algae Chlorella vulgaris and Spirodela oligorrhiza could apparently grow on several defined peptides as the sole nitrogen source.

1.6.4 Peptide Phytotoxins

Many bacterial and fungal phytotoxins have been identified as peptides or peptide derivatives e.g., toxins produced by fungi of the genus Helminthosporium, causing blights in cereal crops (Pringle, 1971; Pringle & Braun, 1958), syringomycin, produced by Pseudomonas syringae, causing canker in stone fruit trees (Prunus spp.) (Backman & Devay, 1971), and tabtoxin, produced by Pseudomonas tabaci, responsible for the "wildfire" disease of tobacco (Taylor et al., 1972). Their chemical structures and modes of action have been the subjects of several reviews (Pringle & Scheffer, 1964; Owens, 1969; Patil, 1974). These toxins may well gain entry to plant cells via hitherto undiscovered peptide transport systems, and, in this respect, the peptide toxins phaseolotoxin and phaseotoxin, both produced by the pathogen Pseudomonas phaseolica, are of especial interest. This organism is the causative agent of bean halo blight of Phaseolus spp. (Mitchell, 1976; Patil, 1974), the symptoms of which are chlorosis and necrosis of leaf tissue. Preliminary evidence has been obtained that the phytotoxins produced by P. phaseolica do indeed gain entry to the host leaf cells through a peptide transport system (Staskawicz & Panopoulos, 1980). Interestingly, strains of Phaseolus resistant to halo blight exist (Omer & Wood, 1969; Russell, 1977), and it would be interesting to determine whether this resistance, at least in part, is achieved by virtue of a deficiency in the peptide transport system.

1.6.5 Transport by Cereal Embryos

1.6.5.1 Early Studies

As discussed previously, the scutellum of the germinating cereal seed has an important role in the absorption of digested endospermal reserves, and it would seem likely that several specific transport systems may well be located here, especially within the epithelium itself. Surprisingly, however, uptake by the scutellum prior to 1977 received very little attention. Since the endosperm of the cereal grain is about 90% starch, the transport of its hydrolytic products, glucose and maltose, was first to be investigated. In wheat and barley, glucose is absorbed by the scutellum, then converted to sucrose within the embryo prior to export to the growing tissues (Edelman et al., 1959). Similarly, the maize scutellum actively transports glucose, probably in a group-translocation mechanism (Roseman, 1968) whereby it is phosphorylated at the plasmalemma (Whitesell & Humphreys, 1972). In addition, maltose is actively absorbed, apparently intact, and also sucrose, although the uptake of the latter is probably illicit transport via the maltose carrier, since the maize endosperm will not contain pools of sucrose (Humphreys, 1973). Sugar transport is likely to be energised by means of a proton gradient (Humphreys, 1975, 1978, 1981).

Most amino acids are available in the endospermal pool in approximately the required ratios for protein synthesis by the embryo, so they are transported there unchanged (Jones & Pierce, 1966). Only two detailed studies of amino acid uptake by the cereal scutellum have been reported. Stewart (1971) described an active glutamine transport system in wheat with a pH optimum of 4.3 - 5.2. The uptake of glutamine was competitively inhibited by the presence of Ala, Cys, Gly, Met, Glu and Ser only, and not by any of the other 'protein' amino acids, suggesting that more than one transport system may exist. Sopanen et al.,

(1980) characterized the transport of leucine by the barley scutellum, and found an active system with a pH optimum of pH 3.5 - 5. Uptake showed multiphasic kinetics. Sopanen did not investigate the absorption of any other amino acids, but leucine transport was competitively inhibited by isoleucine, alanine and glycine, providing indirect evidence for their uptake also.

Thus, it had always been assumed that the complete degradation of endospermal protein occurred, and that amino acids alone were absorbed into the growing embryo. However, a consideration of the enzyme types within the embryo, lead to the conclusion that a specific transport system located in the scutellar epithelium might serve to transport small peptides intact, prior to their hydrolysis within the scutellum. Subsequently, such a system was found in barley and reported by Higgins & Payne (1977), and represented the first unambiguous demonstration of a peptide transporting system in plants. Almost simultaneously, Sopanen et al., (1977) confirmed these preliminary findings, but later work by Higgins characterized the peptide uptake process in rather greater depth as a Ph.D. project at the University of Durham (Higgins, 1979). Transport was shown to be 'active' and carrier-mediated; information about the number of systems, the kinetics and specificity of uptake, and the energy supply was also documented. These aspects are summarized below.

1.6.5.2 Characteristics of Peptide Transport by the Barley Scutellum

Peptide uptake displays many of the accepted criteria defining an active transport process. Thus, accumulation of substrate occurs against a concentration gradient and is inhibited by a range of metabolic inhibitors such as DNP, azide and by anoxia (Higgins & Payne, 1977a; Higgins, 1979). Uptake shows saturable Michaelis-Menten kinetics (Sopanen et al., 1977), which is indicative of a mediated process. In contrast with bacterial systems, where separate di- and oligopeptide

permeases are often found, just one transport system appears to exist in the barley scutellum which can transport peptides of up to five amino acids in length (Higgins & Payne, 1978b), although longer peptides have not been tested. Uptake is independent of hydrolysis and peptides are absorbed intact, but subsequently they are rapidly cleaved (Higgins & Payne, 1978c). In common with many other transport systems in higher plants, uptake shows an acidic optimum (pH 3.8) (Higgins & Payne, 1977b), which corresponds closely to the measured pH of the endosperm in vivo during germination (Kirsi & Mikola, 1971). This low pH optimum, together with the ability to inhibit the system using proton shuttles such as acetate (Higgins & Payne, 1977b), gave preliminary indications that transport was energised by means of a proton gradient in accordance with the chemiosmotic model (Mitchell, 1976). Uptake is not directly dependent upon sodium or potassium ions, but at very high concentrations ($>100\text{mM}$) these cations are inhibitory (Sopanen et al., 1978).

The transporter shows little or no specificity for different amino acid residues, such that all the natural peptides that have been tested are accumulated at approximately similar rates irrespective of their electrical charge, hydrophobic or hydrophilic nature (Higgins, 1979). However, the system is highly stereospecific, in that the uptake of a peptide with a complete D configuration cannot be detected. Some uptake of peptides comprising mixed D and L amino acid residues will still occur, however, but in this regard, transport is most sensitive to the substitution of a D residue at the carboxyl terminus of a peptide (Higgins & Payne, 1978a). Similarly, the chemical modification or 'blocking' of the C-terminus will reduce uptake more effectively than a derivatization of the amino terminus; thus it seems likely that the C-terminus is the more important in conferring affinity of the peptide substrate for the transport systems (Higgins, 1979). Therefore, from the work described above, it is clear that peptide transport

in the barley scutellum is well understood in broad outline. The studies described in this thesis are aimed at characterizing in greater depth rather different aspects of the uptake systems in the barley scutellum, in particular, the nature of the transport proteins themselves. Because of the experimental convenience with which scutella can be prepared, and the relative ease with which substrate accumulation can be measured, the cereal embryo can be considered as an ideal, model system with which to investigate transport mechanisms in higher plants.

CHAPTER 2

MATERIALS AND METHODS

2.1 Introduction

This chapter is concerned primarily with the assays used to monitor amino acid and peptide accumulation by the barley scutellum. These include Dansyl-t.l.c. and fluorescamine techniques, which have already been adapted successfully to follow solute uptake in Hordeum (Higgins, 1979), and the use of radioactively labelled substrates which, generally, have seen a more widespread application in transport studies.

Other general methodologies which were employed routinely are also described e.g., autoradiography and microscopy.

2.2 Materials

2.2.1 Biological Materials

Barley grains (Hordeum vulgare L., cv Maris Otter, Winter) were supplied by the National Seed Development Organisation Ltd., Newton, Cambridge.

2.2.2 Chemicals

Peptides and amino acids were obtained from Sigma (London), Serva and Bachem (through Uniscience Ltd., Cambridge), BDH (Poole, Dorset) and Calbiochem (through CP Laboratories Ltd., Bishop's Stortford).

All protein modification reagents and metabolic inhibitors were purchased from Sigma (London), with the exception of N-Dansylaziridine which was supplied by Pierce-Warriner (U.K.) Ltd., (Chester).

All radiochemicals were purchased from the Radiochemical Centre, Amersham, with the exceptions of Ala - ($U^{14}C$) Ala and Ala-Ala - ($U^{14}C$)Ala which were gifts from Dr.W.J.Lloyd, Roche Products Ltd., Welwyn Garden City.

General reagents were, when possible, of analytical grade and supplied by BDH (Poole, Dorset), Koch-Light Ltd. (Haverhill, Suffolk) and Hopkins and Williams (Chadwell Heath, Essex).

2.2.3 Chemical Nomenclature

Abbreviations for amino acids and peptides are in accordance with the recommendations published by the 1972 IUPAC-IUB Commission (Eur.J.Biochem. 27, 201-207). The nomenclature of isotopically labelled compounds is in accordance with that as published by the IUPAC in 1978 (Eur.J.Biochem. 86, 9-25).

The abbreviations used for all other reagents are listed in Appendix 1.

2.2.4 Instrumentation

Liquid scintillation counting was performed on a Packard Prias Tri-Carb (Model PL/PLD).

Fluorescence assays, using fluorescamine, were performed on a Baird Atomic Fluoripoint (FD 100) spectrofluorimeter.

Spectrophotometric measurements were made on a Pye Unicam model SP8-150.

Electron micrographs were prepared on a Philips EM400 electron microscope (Philips Industries, Eindhoven, The Netherlands).

Light microscopy was performed on a Nikon Diaphot-TMD inverted microscope (Nippon Industries, Japan), or a Leitz Ortholux epifluorescence microscope (Ernst Leitz Ltd., Wetzlar, FRG).

2.3 Seed Growth and Scutellum Preparation

2.3.1 Methods

Barley grains were wetted in 70% (v/v) aqueous ethanol for 10s and surface sterilized for 10 min using sodium hypochlorite (1% w/v available chlorine) and germinated aseptically on 1.2% (w/v) water agar ("Difco Bactoagar", Difco Laboratories, Michigan, U.S.A.) for up to 6 days in a growth room (20°C). ^{12h light/dark cycle} Normally, after 2-3 days germination, scutella were dissected out using a scalpel. The coleoptile and roots were trimmed off at their bases. Scutella were stored before use in oxygenated 50 mM sodium phosphate-citrate buffer, (McIlvaine, 1921) pH 3.8 at 2°C for up to 2h.

Occasionally, following a chemical inhibition or treatment, long term effects upon embryo growth or scutellar transport needed to be monitored. Thus, whole embryos, dissected out between 3-24h following the onset of imbibition, were, after treatment, transferred aseptically to culture flasks containing a suitable nutrient agar to allow any subsequent growth to continue for up to 3 days (Sections 3.7, 4.2.4). Glucose agar (1% w/v) was adequate to maintain good growth of isolated embryos for 3 days but for longer periods a culture medium based on that of White (1934) was used (Appendix 2).

2.3.2 Discussion

2.3.2.1 Seed Growth

Because the rates of peptide and amino acid transport are a function of developmental age (Section 3.5), it is important to attain good reproducibility of seed growth during germination. For this reason agar was chosen as a culture medium (under the conditions specified in Section 2.3.1), rather than water, where germination was found to be less reproducible, caused, most likely, by oxygen limitation. Therefore, reference throughout this study to scutella of a certain age implies a particular stage of seedling development with well defined growth characteristics (Table 3.1).

2.3.2.2 Sterility

Earlier experiments showed that possible interference from contaminating microorganisms was negligible during uptake studies (Higgins, 1979), and was found here never to be a problem during seed germination on autoclaved water agar, although bacterial growth was sometimes evident with the enriched culture media used for the long term growth of isolated embryos. Artifacts associated with excessive use of sodium hypochlorite as a disinfectant have been highlighted (Abdul-

Baki, 1974), but were not evident in the treatment of dormant seeds.

2.3.2.3 Scutella Excision and Storage

For various reasons, 2-3 day old scutella were employed for most of these studies. Thus, 1 day scutella are not only relatively difficult to dissect out but also their transport rates, in particular for amino acids, are not optimal; also, rates of peptide transport decline after 3 days (Section 3.5). For experimental convenience, bulky coleoptile and root tissues were routinely trimmed off. Preliminary experiments showed that amino acid or peptide uptake through these severed surfaces was negligible; thus, uptake of a radioactively labelled peptide from filter paper discs impregnated with substrate was insignificant when the cut surfaces alone, but not the scutellum, was in contact with the paper. Use of light microscopy indicated that, on separation, no visible damage was sustained by scutellar tissue. After dissection, scutella were rarely stored for longer than 2 h, and preliminary work showed that when stored as described, no measurable deterioration of transport occurred for up to at least 6 h following isolation.

2.4 Assay Methods for Peptide, Amino Acid and Glucose Transport

2.4.1 Introduction

In any study involving the movement of substrate across a membrane barrier into whole organisms or tissues, one can measure transport either by monitoring the disappearance of substrate from the medium or from its appearance (either of the substrate itself or a metabolite of it) within the tissue. Both strategies may have advantages or disadvantages depending on the system being studied (Section 2.5.5). In the present study, the accumulation of a radioactive label within scutellar tissue has been used as the main assay for transport.

However, two alternative methods were also used to monitor the

transport of amino acids and peptides, involving the reaction of an amino group on the substrate with a fluorescence label. These fluorescent labelling techniques were used not only to confirm (or otherwise) radiotracer results, but also as methods in their own right, for example where a substrate was unavailable in a radioactively labelled form.

2.4.2 Incubation Media

Typically, up to six scutella were incubated in up to 2 ml of 50mM, preoxygenated sodium phosphate-citrate buffer (McIlvaine, 1921), pH 3.8, in glass vials (50 x 25 mm) on a shaking water bath (100 strokes min^{-1} at 20°C). Following equilibration for 10 min, the assay was initiated by addition of substrate (to a final concentration of 2 mM amino acid or peptide, 20mM glucose, unless otherwise stated). Transport was then monitored as described below.

2.4.3 Assays with Radioactively Labelled Substrates

2.4.3.1 Introduction

To date, studies on amino acid uptake in plants have involved the use of radioactively labelled substrates to monitor transport. The technique is highly sensitive and a wide range of radioactive amino acids are available commercially. As discussed previously (Section 1.6), peptide transport in plants is a neglected field of research, partly because radioactively labelled peptides have not been available, and consequently alternative transport assays have had to be developed.

Despite the relative convenience of using radioactivity, problems associated with the loss of counts via metabolism or exodus of a radioactive label are commonly encountered (Section 3.2), and must be taken into account to ensure that serious underestimates of rates etc., are not recorded.

2.4.3.2 Procedure

Substrates of specified concentration (2mM for amino acids and peptide

20mM for glucose) were normally prepared to a specific activity of $12 \mu\text{Ci mmol}^{-1}$, being labelled either as ^{14}C Carbon or ^3H Hydrogen. Routinely, six scutella were used in an incubation volume of 1 ml, two being removed periodically (usually every 15 minutes following the initiation of assay), transferred to a piece of muslin and washed for at least two minutes with copious quantities of distilled water (500 ml). Radioactivity was then extracted with 0.5 ml, 5M acetic acid in stoppered tubes on a boiling water bath for 20 min. Extracts were added to 5 ml of scintillation fluid (NE 260, Nuclear Enterprises) in polythene vials ("Pico" vials, Packard Instruments). The samples were mixed thoroughly and left overnight to reduce the likelihood of chemi-luminescence before being counted for radioactivity.

2.4.3.3 Chromatographic Identification of Radioactively Labelled Extracts

To follow the metabolic fate of a radioactively labelled substrate within scutellar tissue, the components of an acetate extract were separated chromatographically on thin layers (Sections 3.3, 5.2.3). Following accumulation of radioactivity from a solution of suitable specific activity, scutella were extracted in aqueous acetate (50 - 500 mM, 1 ml) and aliquots were dried down in vacuo. Residues were re-suspended in a suitable volume of water (20 - 100 μl) to bring the activity of the sample to the require value. Samples (5 μl) were loaded onto cellulose thin layer plates (20 x 20 cm, 1mm layer, E. Merck, Darmstadt) and run in n-butanol/acetic acid/water/pyridine solvent (75:15:60:50, by volume) for 3 h.

A radioactivity profile was constructed by scraping off vertical strips of the running track into 20 consecutive 1 cm^2 sections, adding the cellulose to the scintillant, mixing well and assaying for counts as usual. Radioactive peaks were identified by comparison with the positions of known standards run in parallel; their locations were determined either by similarly assaying for radioactivity or by

visualization with ninhydrin-cadmium acetate reagent (amino acids or peptides). Control experiments performed in individual cases estimated the effect, if any, of an unlabelled extract upon the mobility of a radioactive standard.

2.4.4 Fluorescence Assays

2.4.4.1 Introduction

Highly sensitive transport assays, detecting nmol amounts of substrate, are based upon the specific reaction of an amino group on an amino acid or peptide with the reagents dansyl chloride or fluorescamine; the derivatives are fluorescent and can be quantified either after isolation on a thin layer chromatogram, or, for fluorescamine, by assaying samples in solution using a fluorimeter. Fluorescamine and dansyl chloride assays are most effective for monitoring substrate disappearance from an incubation medium; in addition, the dansyl-t.l.c. procedure can be adapted to monitor changes in the levels of one or more amino acids (or peptides) within a tissue extract.

2.4.4.2 Fluorescamine Procedure

At appropriate time intervals throughout the incubation (of 4-6 h duration), 25 μ l samples of the incubation medium, normally containing 10 scutella in 2 ml and up to 50 nmol of substrate, were taken and added to 300 μ l buffer in test tubes (for peptides 50mM Na⁺ phosphate-citrate pH 6.2: for amino acids 0.4M disodium-tetraborate/HCl, pH9), 100 μ l fluorescamine (1.5 mg ml⁻¹ in Analar acetone, made freshly before use) was added whilst mixing rapidly on a vortex mixer, and the final volume made up to 2 ml with more buffer. The tubes were allowed to stand for five minutes before reading on a fluorescence spectrophotometer (390 nm excitation, 480 nm emission). Peptide or amino acid concentrations were calculated by comparison with controls containing zero time samples of incubation media with known amounts of substrate.

2.4.4.3 Dansyl Chloride Procedure

To monitor substrate removal, six scutella were incubated in 1 ml of the standard incubation medium, 5 μ l samples removed (normally hourly for up to six hours), added to 20 μ l distilled water, and a portion of the diluent (20 μ l) dried down in vacuo in small Durham tubes (6 x 30 mm). To each was added 40 μ l sodium bicarbonate (200 mM) and 40 μ l dansyl chloride (2.5 mg ml⁻¹ in Analar acetone) to bring the final pH to about 9.5. The tubes were sealed with parafilm and dansylation allowed to proceed at 40°C for 1 h before again drying down the contents in vacuo and redissolving the residue in 20 μ l aqueous pyridine (50:50 by volume).

To follow substrate accumulation within scutellar tissue, or simply to determine amino acid pools, incubations were carried out as usual and up to six scutella were washed and extracted in 1 ml of 5 M acetate on a boiling water bath for 20 min. A suitable volume of the supernatant solution (usually 50 μ l) was dried down in vacuo before being dansylated as above. As a check on the efficiency of dansylation, 10 μ l of 2mM ornithine was added as an internal standard just prior to extraction and/or dansylation. To analyse dansylated materials, 5 μ l samples of the aqueous pyridine solutions were spotted onto 15 x 15 cm polyamide sheets (BDH Chemicals, Poole, U.K) and chromatographed two dimensionally in each of the following solvent systems in the order given: 1st dimension, H₂O : formic acid (98.5/1.5 v/v); 2nd dimension, acetic acid: toluene (10/90 v/v); 2nd dimension again, butyl acetate: acetic acid: toluene (40/60/2 v/v/v). The plates were dried completely between runs using a hair drier. Derivatives were visualized on the plates under ultra-violet light and quantified where necessary by comparing fluorescent intensities with the fluorescence of known amounts of standards.

2.5 Discussion of Transport Assays Employed

2.5.1 Incubation Media

Conditions employed for transport were essentially those determined as optimal in terms of pH and the type of buffer employed (Higgins, 1979). Routinely, substrate at 2mM was used, which approximates to endospermal concentrations of peptides and amino acids, and lies close to the determined K_t for transport: 1.9 mM for tri-alanine (Higgins, 1979) and 3.4 mM for leucine (Sopanen et al., 1980).

2.5.2 Radioactive Assay

2.5.2.1 Efflux of Radioactivity is Negligible

Several potential problems are inherent in measuring transport by means of accumulation of a radioactive label. For example, loss of accumulated counts through metabolism (e.g. as $^{14}\text{CO}_2$) or by exodus of metabolites into the medium. In barley, metabolic losses may be significant when using certain labelled substrates (Section 3.2) but dansylation studies indicate that exodus of cleaved peptide materials is insignificant at least up to 2h of incubation. In contrast, such exodus can be very important in studies involving microorganisms (Payne & Nisbet, 1980).

2.5.5.2 Efficiency of Scutella Washing

Evidence, both from preliminary experiment and calculation, indicate that passive carryover of radioactive label within the 'dead volume' (cell walls and intercellular spaces) is negligible: thus, scutella dipped into a radioactive solution for 1 min were completely freed of label by the usual washing procedure. Moreover, calculations based upon approximate dead space volumes in the scutellum indicated that the maximum possible carryover, even assuming no washing at all, is barely significant.

2.5.2.3 Efficiency of Scutella Extraction

Evidence indicated that boiling with 5M acetic acid for 20 min was

highly efficient as an extractant; as many counts were released into acetate after 20 min as after 2h by which time the tissue had disintegrated completely. The effectiveness of acetate as an extractant (much lower concentrations, 5-50 mM were subsequently found to be as good) is presumably, in part, by virtue of its ability to act as a proton shuttle destroying any possibility of H^+ -linked maintenance of internal pools against a concentration gradient.

2.5.2.4 Quenching of Radioactivity by an Acetate Extract

Preliminary work showed that the presence of an acetate extract of scutella did have a small but significant quenching effect upon the counting of known amounts of a (^{14}C) label added as an internal standard just prior to extraction (Figure 2.1). Thus, two scutella were extracted in volumes of 5M acetate ranging from 200-1500 μ l, each with the addition of 10 μ l of (^{14}C)Pro ($0.5 \mu Ci ml^{-1}$, 1 mM). Extracts were cooled, added to 5 ml NE260 scintillation fluid, left overnight and assayed for radioactivity. As compared with control vials to which radioactivity but no extract had been added, count recovery was over 90% with added extract up to 800 μ l: beyond this volume severe quenching occurred. The acetate itself is the main cause of quenching and not a component of the extracted material: thus, samples of extract dried down in vacuo to remove the acetate had no effect upon the counting efficiency of added (^{14}C)Pro when the residue was redissolved directly into scintillant. Routinely, 500 μ l of 5M acetic acid were used to extract no more than two scutella, and when calculating rates of uptake, acetate quenching was taken into account.

2.5.2.5 Calibration and Sensitivity

Instrument settings were adjusted to count ^{14}C Carbon for 5 min and a linear relationship was observed between amounts of substrate (up to 500 nmol amino acid or peptide) versus counts per minute (up to 10^6 cpm). Routinely, for the purposes of calculating uptake rates, known amounts

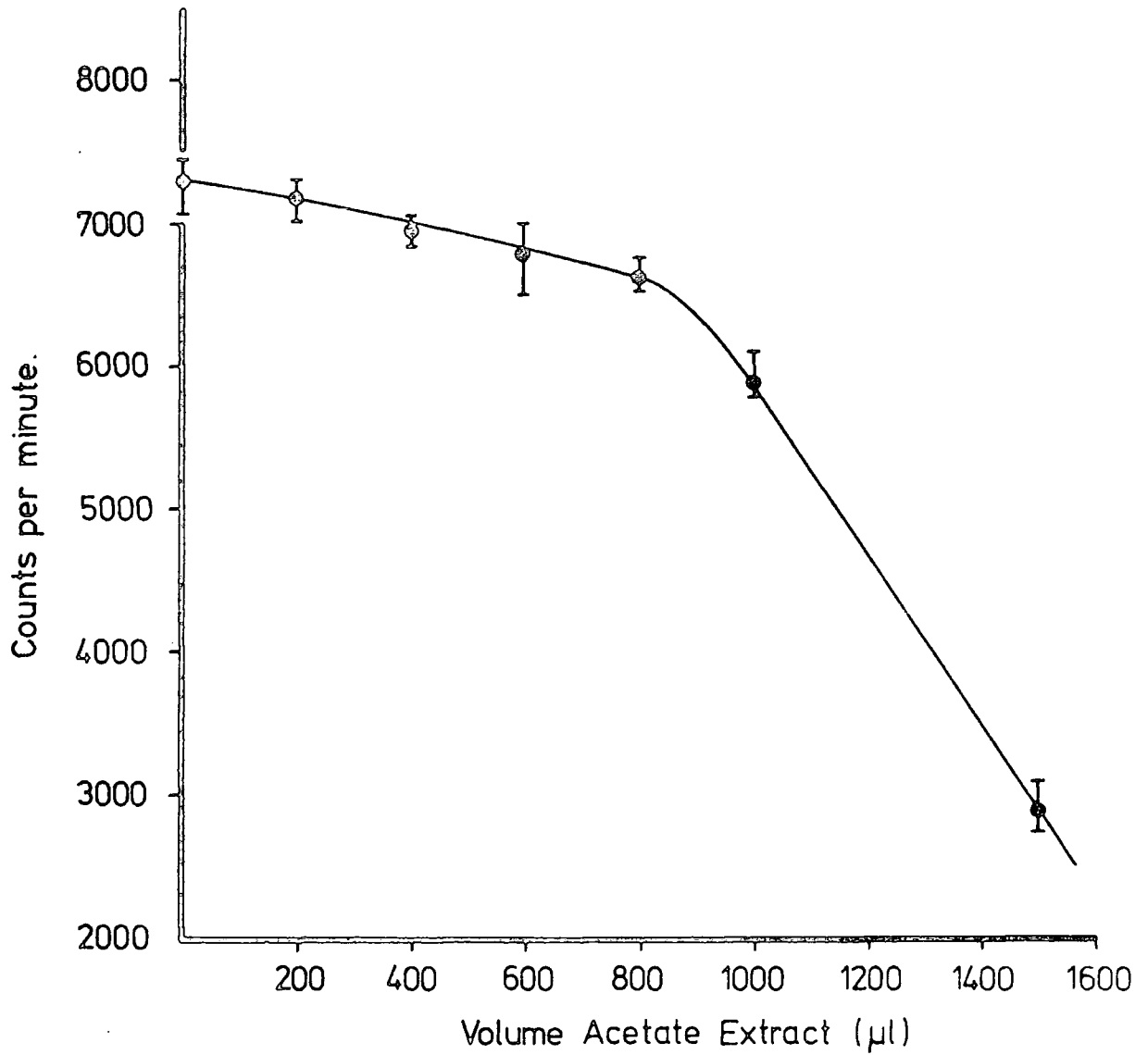


FIGURE 2.1 Quenching of Radioactivity by an Acetate Extract of Scutella

Two scutella were extracted in a range of volumes of 5M acetic acid (200-1500 μl) and standard amounts of (^{14}C)Pro (10 μl, $0.5 \mu\text{Ci ml}^{-1}$) added to each, before assaying for counts in the routine manner. Values given are the mean and range for 3 determinations.

of radioactivity were added to 500 μ l of acetate and counted for radioactivity alongside samples from transport assays. The accumulation of a radioactively labelled substrate, as assayed by its extraction, depends on the specific activity of the substrate, the number of scutella used and the time of incubation, but under standard conditions a minimal rate of about 1 nmol scutellum⁻¹h⁻¹ can be detected. Monitoring disappearance of radioactivity from the medium is less sensitive and was not employed routinely.

2.5.3 Fluorescence Assays

2.5.3.1 Fluorescamine procedure

Fluorescamine (4-phenylspiro - furan-2(3H), 1'-phthalan)-3,3'-dione) developed by Weigle et al., (1972) reacts with primary amino groups of amino acids and peptides optimally at about pH9 and pH7, respectively (Udenfriend et al., 1972) to give fluorescent derivatives. Excess reagent is hydrolysed to give non-fluorescent by-products. The reaction mechanism is complex and not fully understood (Chen et al., 1972). Because it is the non-protonated amino moiety that undergoes the reaction (De Bernado et al., 1974) some measure of discrimination between the assay of peptides over amino acids (but not vice-versa) when present as a mix can be introduced by adjusting the pH. Thus peptides, which have a lower pKa value for the α -amino group, give adequately measurable yields with fluorescamine at pH 6.2 - 6.8, whereas the yields with amino acids are negligible within this pH range. In barley, leakage of amino acids into an incubation medium is minimal and hence any interference from this source is negligible.

2.5.3.2 Calibration and Sensitivity of Fluorescamine Assay

Under the reaction conditions described, a linear relationship exists between fluorescence and amount of substrate of up to 50 nmol peptide per assay tube, with approximately linearity maintained up to

100 nmol (Figure 2.2).

It is inherent in this procedure that small volumes of incubation medium are removed for assay: consequently uptake rates ideally need to be at least $10 \text{ nmol scutellum}^{-1} \text{ h}^{-1}$ in order that the decrease in total amount of substrate present in the incubation medium is large enough to be measured with precision.

It must also be noted that because the volume of incubation medium, but not the amount of biological material, is reduced during an uptake experiment, a multiplication factor must be applied to the apparent amount of substrate present in a sample, which is the ratio of incubation volume just prior to sampling to incubation volume at zero time. This value, less than or equal to one, will minimise any misleading underestimates of rates. This problem is not encountered in the fluorimetric assay of uptake by bacteria or yeasts, because the microorganisms themselves are removed in commensurate amounts from the samples of incubation medium (Nisbet & Payne, 1979) before assay.

In conclusion, fluorescence assays are quite satisfactory for determining overall rates of uptake of amino acid or peptide into barley scutella, but because uptake is generally low (compared with that in microorganisms), they cannot reliably resolve slight changes in rate e.g. brought about by inhibitors.

2.5.3.3 Dansyl Chloride Procedure

The basic methodology employed is adapted from Hartley (1970). Dansyl chloride is more widely known as a reagent used in protein sequencing studies. Amino acid and peptide derivatives were identified on chromatograms from their positions (either after two or three solvents) and, additionally for some their colour, although most amino acids fluoresce green. Two main by-products of dansylation, dansyl hydroxide and dansyl amide, fluoresce blue and blue-green respectively, and provide useful markers.

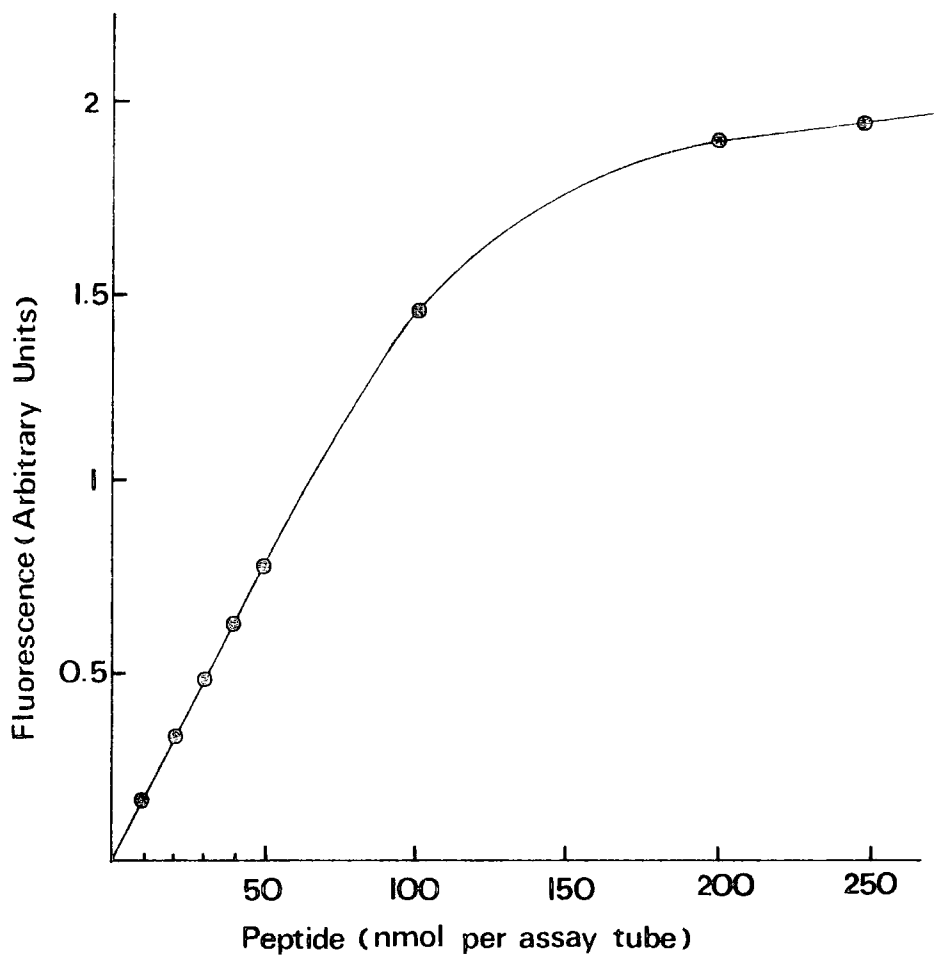


FIGURE 2.2 Effect of Peptide Concentration on Fluorescence Yield in the Standard Fluorescamine Assay

Varying amounts of Gly-Sar were assayed by using the fluorescamine method (Section 2.4.4.2). Values given are the mean of three determinations.

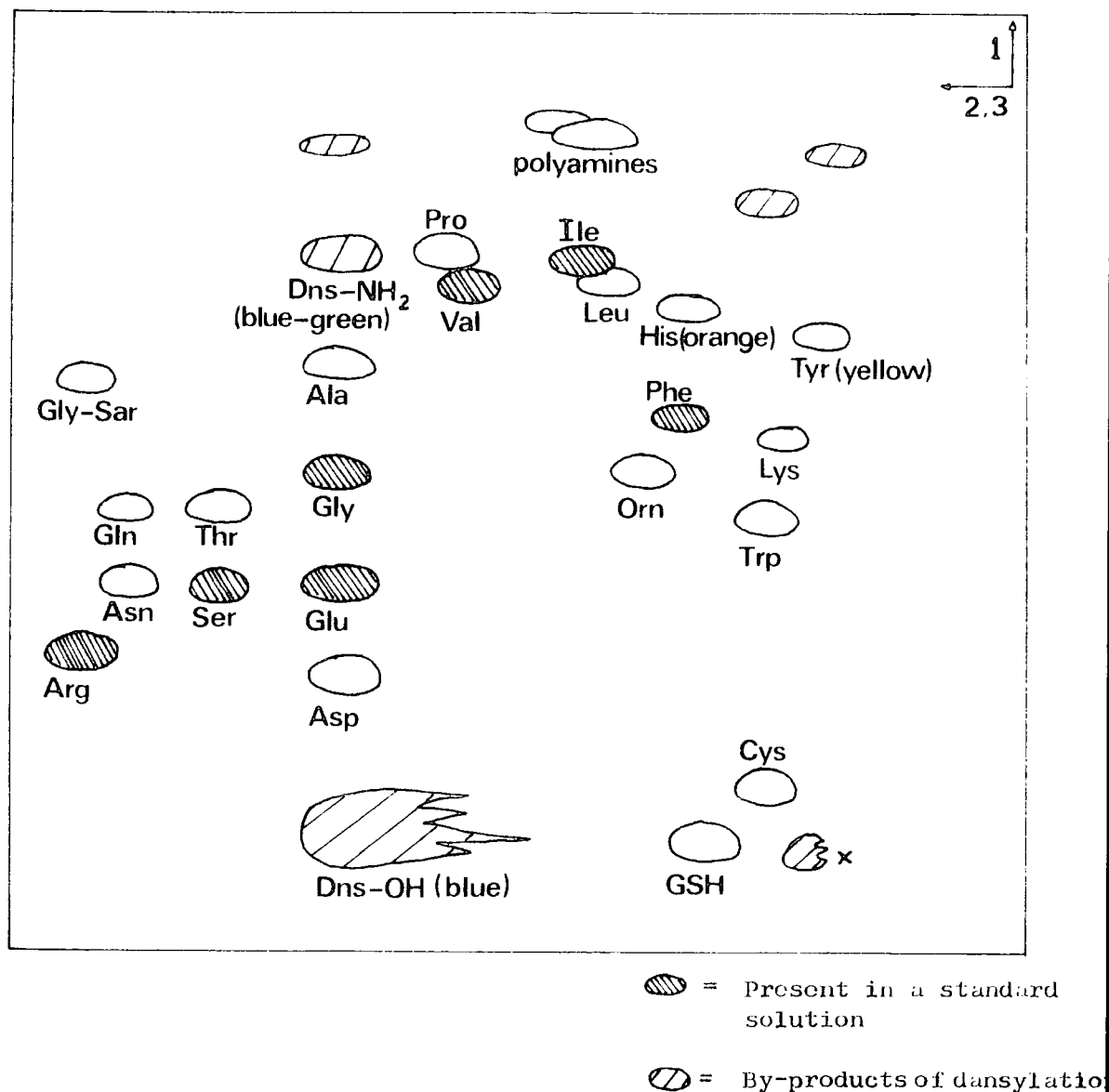


FIGURE 2.3 Chromatographic Locations of Some Dansylated Amino Acids and Peptides

The positions of some dansyl-amino acids and dansyl-peptides after two-dimensional thin-layer chromatography as described in Section 2.4.4.3.

Routinely, to aid identification, a known mix of amino acid derivatives was chromatographed on the reverse of each plate (Figure 2.3). Dansylation studies are most successful when monitoring substrate disappearance from an incubation medium. Usually there are high levels of intracellular peptidase activity such that peptides are rarely found intact within the cells (Section 3.3) and the consequent changes in the internal amino acid pools are difficult to quantify accurately.

2.5.3.4 Calibration and Sensitivity of Dansyl Assay

Quantification of fluorescence was performed visually by comparison with that of known standards, therefore no more than one nmol of an individual peptide or amino acid derivative present on a thin layer plate could be quantified if a linear relationship between apparent intensity versus amount of derivative was to be maintained. Studies using tritiated dansyl chloride in conjunction with a Pannax thin layer scanner (Payne & Bell, 1979) indicated that visual estimation was quite reliable and correspondence with more objective estimates was good. Approximate lower limits of detection are as follows: for monitoring substrate disappearance from an incubation medium, a rate of about $1 \text{ nmol scutellum}^{-1} \text{ h}^{-1}$; for appearance of substrate within scutella tissue, a rate of about $0.1 \text{ nmol scutellum}^{-1} \text{ h}^{-1}$, but these values are dependent upon factors such as the time of incubation, number of scutella employed and substrate concentration.

2.5.4 Expression of Rates of Transport

Routinely, throughout this thesis, uptake rates are expressed as nmol substrate absorbed per scutellum per hour, which is valid as long as experiments are internally controlled. However, the absorptive surface area does not remain constant during development and for studies involving a comparison between tissue of different ages (Section 3.5) uptake rates are quoted as nmol substrate absorbed per gram fresh weight per hour. It must not be assumed from this, however, that transported substrate is uniformly distributed throughout the scutellar mass.

2.5.5 Transport Assays : Concluding Remarks

It is clear from the studies described here and elsewhere that in addition to the well-established use of radioactively labelled substrates, the alternative fluorescence transport assays developed in this laboratory are capable of complementing or confirming data from conventional assays and providing information in their own right. The transport of any amino acid or peptide can be measured by using the fluorescence assays, and these methodologies are invaluable in cases where a particular substrate is not available radioactively, with the exception that fluorescamine does not give fluorescent derivatives with secondary amines such as proline or prolyl peptides. Indeed, the fluorescamine assay has been automated to monitor uptake, on a continuous basis, of peptides or amino acid by microorganisms (Payne & Nisbet, 1981); the output from the flow-through fluorimeter used in this system has been interfaced with a microcomputer allowing detailed kinetic analyses of uptake studies to be performed. The dansyl technique, although rather more laborious experimentally, has the advantage of being able to monitor uptake of more than one substrate simultaneously and can provide, uniquely, information on changes in the amino acid complement both intra- and extracellularly during an uptake experiment. However, for the majority of the work presented in this thesis, when an assay combining convenience with reliability and sensitivity was required, the use of radioactively labelled substrates proved to be optimal.

2.6 Microscopy and Autoradiography

2.6.1 Introduction

Electron and light microscopy, the latter in conjunction with autoradiography, were used to visualize the site of many of the biochemical events discussed elsewhere in this thesis and to relate the structure of the scutellum with its function.

2.6.2 Scutellar Sectioning Procedure

For electron microscopy, scutella were excised, fixed in 2.5% (w/v) glutaraldehyde and 1.5% (w/v) formaldehyde in sodium cacodylate (50 mM, pH 7.0) for 1h. The tissue was then cut into pieces of approximately 1mm³ and fixed for a further 1h. After washing in sodium cacodylate, the tissue was post-fixed in 1% (w/v) aqueous osmium tetroxide for 1h before dehydration by taking it through a series of aqueous alcohol treatments, 25%, 50%, 75%, 90%, 95% (w/v), and 100% ethanol, 10 min in each with two changes at 100%. The tissue was then embedded in Spurr resin (Spurr, 1969) and polymerised overnight at 80°C, prior to sectioning to 80 nm on a microtome. Thin sections were stained with uranyl acetate (1% w/v) and Reynolds alkaline lead citrate, before examination under the EM.

For light microscopy (including sections cut for autoradiography) tissue was prepared as for electron microscopy, with the exception that secondary staining with uranyl acetate and alkaline lead citrate was omitted, and sections were cut to 1 µm routinely. After sectioning, further staining of cell walls was occasionally performed with toluidene blue (O'Brien *et al.*, 1964).

2.6.3 Autoradiography

2.6.3.1 Methods

Scutellar tissue was suitably radioactively labelled (Sections 3.4, 5.1.6), embedded, and sections prepared as described above. Sections were dried onto cleaned microscope slides.

Ilford L4 nuclear research emulsion (Ilford Ltd., Basildon, Essex), which is supplied as gelatin shreds, was melted and kept liquid at 50°C in a water bath before covering the slides with a layer 0.5 - 5 µm thick. Satisfactory thin layers could be produced by spreading the emulsion evenly with a glass rod or by diluting the gelatin with an equal volume of pure distilled water and simply dipping in the slides. The former

method was adequately reproducible and used routinely for most studies. At all times the emulsion was handled only in safelight illumination (Ilford 'S' safelight No.902 - light brown). After leaving the emulsion for 1h at room temperature to dry thoroughly, sections were stored in a light-tight box at 2°C for up to 21 days. Periodically, slides were removed and developed in Ilford 'Phenosol' (8 min at 20°C, with agitation), washed in distilled water and fixed for 15 min (Kodak 'Kodafix'). Slides were rinsed, allowed to dry in air, mounted in oil under a coverslip and examined by light microscopy. Photographs were taken on Ilford XP1 black and white film as soon as possible because some deterioration in the quality of the preparation occurs within 48h.

2.6.3.2 Discussion

Great care must be taken in selecting the conditions for the preparation of a microautoradiograph to ensure that the distribution of silver grains produced in the emulsion on exposure to radioactivity represents faithfully the location of the particular label used. Clearly, greater resolving power is required intracellularly, when the distribution of label within specific organelles or membranes is being investigated, than when gross comparisons are being made between cell or tissue types.

Preparation of specimen

Photographic emulsions are sensitised not only by a radioactively labelled specimen, but also by light, "background" effects and physical stresses. The following steps were taken to reduce these other factors to a minimum.

1. Light. The requirement to perform all autoradiographic manipulations in darkness or for short periods under safelight illumination is paramount and fairly obvious.

2. Physical stressing of the photographic layer can induce artefactual exposure: this can happen when the emulsion, after spreading over the sections, is dried too quickly. Although Ilford incorporate a plasticising

agent into their products, care was always taken to dry the coated specimens slowly. Handling of prepared slides, in particular vertical pressure applied to the emulsion surface, was also kept to a minimum.

3. Background effects. These are of two main types: extraneous radioactivity (including cosmic rays and general environmental radiation) and also the spontaneous degradation of emulsion to give silver grains. These problems are minimized by optimising on the duration of exposure and the development time; in particular, by increasing the amount of radioactivity in the specimen the exposure time can be reduced making background effects negligible.

It can be demonstrated (Caro & van Tubergen, 1962) that, during development, silver grains over a labelled source increase in number and eventually reach a plateau value, whereas background grains increase slowly at first before increasing exponentially. The ideal development time is that which maximises this 'signal to noise' ratio and its determination may often be a case of trial and error. In the autoradiographic studies presented in this thesis a development time of 8 min at 20°C was found to give good results with minimal background interference.

Resolving Power

¹⁴Carbon, used exclusively in these studies, emits β particles with a maximum energy of about 30 keV and maximal range in Ilford emulsion of 3 μ m (Rogers, 1979). Consequently, a labelled source covered by a layer of emulsion is capable of producing a distribution of silver grains with a decreasing, concentric gradation of grain density, such that more silver is likely to be formed nearer the source than at the maximal track length of 3 μ m away. A quantitative feature of this gradation, described under certain specified conditions, is the half distance, or HD value (Salpeter et al., 1974) which is defined as the radial distance from the source delimiting the circular area containing half of the grains

produced by the source. To decrease this HD value (i.e. increase resolution), the covering of emulsion is made as thin as possible to allow β particles to escape from the emulsion without producing more distally located silver grains.

In practice, sections are cut on a microtome to 1 μm and thin emulsion layers are produced by ensuring that interference patterns (coloured blue-purple) are visible in the gelatin once dried. Under these conditions, the HD value for ^{14}C Carbon is 1.1 μm in L4 emulsion (Salpeter, 1979) which is adequate for the studies described subsequently (Sections 3.4, 5.1.6).

With certain tracers, in particular low molecular weight compounds which are unlikely to be in a 'bound' form within the cell, one must be aware of the possible effects of fixation, staining and embedding of the specimen upon distribution of label. The likelihood of such artefacts arising are discussed in greater detail later (Section 3.4.4).

CHAPTER 3

USE OF GLYCYLPHENYLALANINE AND LEUCINE AS MODEL SUBSTRATES
TO INVESTIGATE SCUTELLA TRANSPORT SYSTEMS

3.1 Introduction

The peptide transport system present in the barley scutellum has been well characterized as described in Chapter 1. Thus, data on pH dependency, kinetic studies, the number of systems operating and structural requirements for transport are well documented (Higgins, 1979). Since transport displays saturation kinetics, a requirement for metabolic energy and accumulation against a concentration gradient, the system fulfils these accepted criteria for an active, mediated process. The present study attempted to expand upon the information already available by characterizing the carriers themselves, with respect to those amino acid side chain moieties essential for their integrity and transport function. This was achieved by preincubation of scutella with reagents capable of reacting specifically with selected chemical groupings, prior to a transport assay to monitor any effect. Usually, radioactively labelled Gly-Phe, Leu and to a lesser extent glucose, were employed as 'probe' molecules in these studies in order that a comparison could be made between effects of the pretreatments on amino acid and peptide transport, hence allowing any selectivity of action to be detected. It was therefore considered both prudent and pertinent to evaluate the suitability of Leu and Gly-Phe as representative of their class of compounds and to investigate, also as an exercise in its own right, the metabolic fate and intracellular location of these substrates once transported.

These 'probes' have also been used in studies of the energetics and development of scutellar transport systems, aspects which have received little attention in previous work.

3.2 A Comparison of Transport Assays Using Either a Fluorescence or Radioactive Label

3.2.1 When alternative fluorescence assays are available it is clearly prudent to assess the suitability of the radioactively labelled substrates

used in an uptake study to determine the level of agreement in the measured rates of transport. Studies of this nature are particularly important with respect to Gly-Phe, which is one of the very limited number of peptides which has been commercially synthesised in radioactively labelled form and consequently was used quite extensively. Samples of both ($1\text{-}^{14}\text{C}$)Gly-Phe and Gly-(U^{14}C)Phe were available and a comparison was undertaken between the rates of uptake as measured by using the reaction with fluorescamine, and by using both forms of the labelled peptide.

3.2.2 Methods

The uptake of Leu and Gly-Phe from standard incubation media was monitored either by using fluorescamine, or radioactively using Gly-(U^{14}C)Phe, ($1\text{-}^{14}\text{C}$)Gly-Phe and (U^{14}C) Leu as described previously (Section 2.4), using scutella up to 6 days old.

3.2.3 Results

Rates of peptide uptake as determined by using fluorescamine and by using Gly-(U^{14}C)Phe were in good agreement, and consistently higher than values obtained using ($1\text{-}^{14}\text{C}$)Gly-Phe.

No difference in the measured rate of Leu transport by 1-6 day scutella was observed when assayed either by fluorescamine or by radioactive accumulation (Figure 3.1).

3.2.3.1 Use of Aminooxyacetic acid

When 1 day scutella were preincubated in 5 mM aminooxyacetate (AOA), pH 3.8, 20°C , for 90 min prior to an assay for uptake of ($1\text{-}^{14}\text{C}$)Gly-Phe or Gly-(U^{14}C)Phe, transport of the former was "stimulated" by nearly 80% whereas rates of the latter were unaffected. However, such discrimination between the two different labels is a function of preincubation time in aminooxyacetate; thus, uptake of Gly-(U^{14}C)Phe by scutella germinated for 1 day on agar containing 5 mM AOA was reduced considerably, and ($1\text{-}^{14}\text{C}$) Gly-Phe enhanced only slightly, as compared with untreated controls. However, preincubation with AOA for just 30 min had no detectable effect

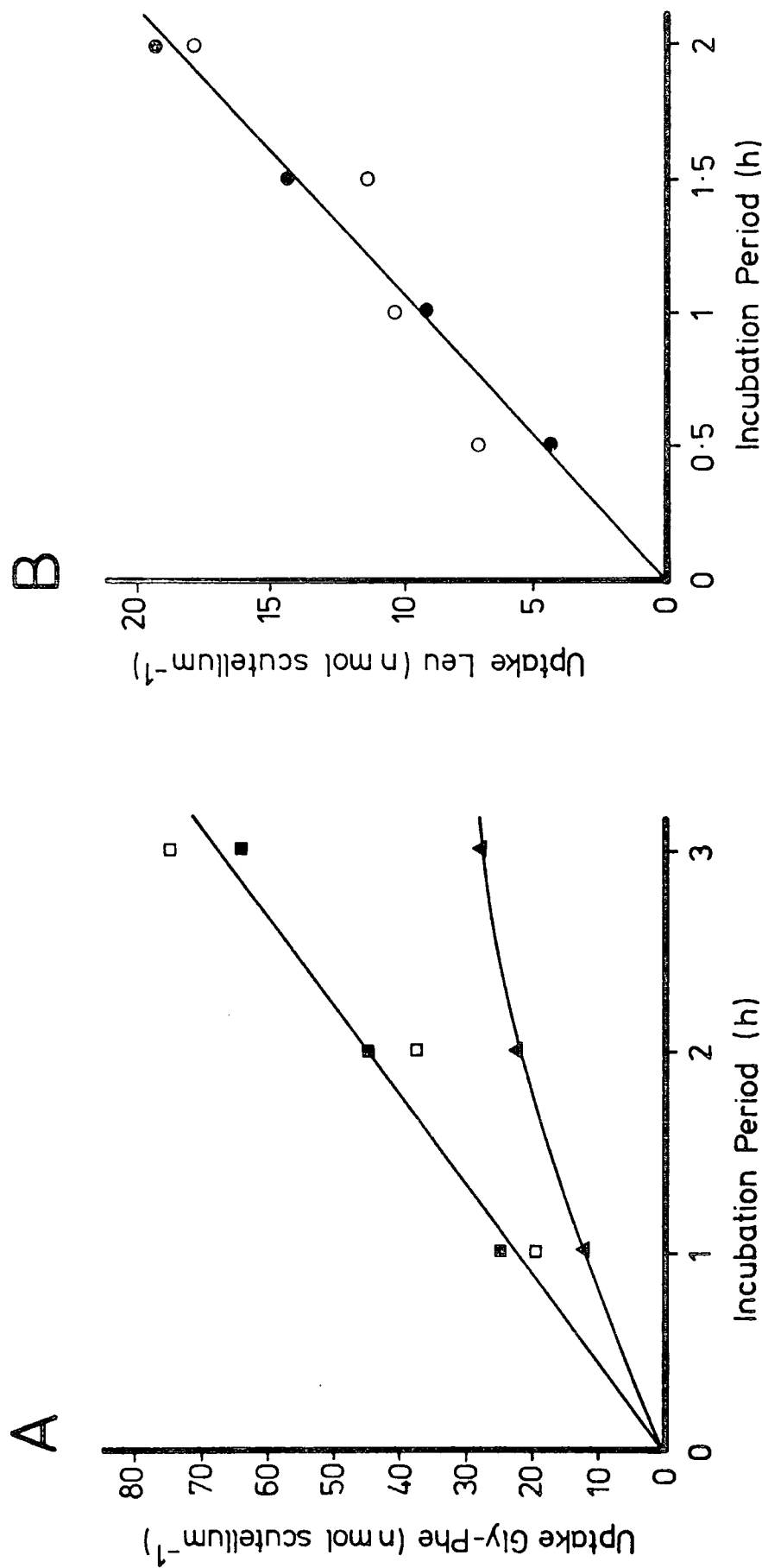


FIGURE 3.1 Comparison of Radiotracer and Fluorescamine Methods for the Assay of Peptide and Amino Acid Uptake

Uptake of Gly-Phe (□), and Leu (○), as assayed by using the reaction with fluorescamine, or else radioactively by using Gly-(U¹⁴C)Phe (■), (1-¹⁴C)Gly-Phe (▲), and (U¹⁴C)Leu (●). All experiments performed with 2 mM substrate using 1 day scutella. All values are the mean of at least 2 determinations.

upon rates of uptake of either label (Figure 3.2).

3.2.4 Discussion

It is likely that the difference in uptake rates observed between the two radioactively labelled substrates arises from the decarboxylation of accumulated glycine, and subsequent loss of counts in the form of $^{14}\text{CO}_2$, leading to an underestimate of transport. Such losses will have a more pronounced effect when, uniquely, the carboxyl grouping is labelled as in $(1\text{-}^{14}\text{C})\text{Gly}$; conversely, if decarboxylation of $(\text{U}\text{-}^{14}\text{C})\text{Phe}$ occurs at all, then proportionately less radioactivity will be lost. A similar phenomenon has been reported in microorganisms during the uptake of radioactively labelled Gly-Phe by E.coli (Payne & Nisbet, 1980) and Staphylococcus aureus (Perry & Abraham, 1979). Gly- $(\text{U}\text{-}^{14}\text{C})\text{Phe}$ was therefore used in preference to $(1\text{-}^{14}\text{C})\text{Gly-Phe}$ in all subsequent transport studies.

The effects of aminooxyacetate would indicate that the decarboxylation is enzymically mediated. AOA is a glycine analogue and a recognised inhibitor of pyridoxal phosphate-dependent enzymes (John et al., 1978); these include transaminases (Wallach, 1961; Hopper & Segal, 1962) and also decarboxylases (Roberts & Simonsen, 1963; Leinweber, 1968).

Decarboxylases are found in microorganisms, animal tissues (see Morris & Fillingame, 1974 for a general review) and plants (Dixon & Fowden, 1961; Mifflin & Lea, 1981). The results described here are consistent with the presence, in barley scutellar tissue, of a glycine decarboxylase inhibitable by aminooxyacetate. Since AOA has other profound metabolic effects, this selectivity is lost in scutella germinated on AOA for 24h when other inhibitions presumably become more important; conversely, preincubation for only 30 minutes with AOA has no measurable effect upon the apparent transport rates of either Gly- $(\text{U}\text{-}^{14}\text{C})\text{Phe}$ or $(1\text{-}^{14}\text{C})\text{Gly-Phe}$, which may represent some limitation on the

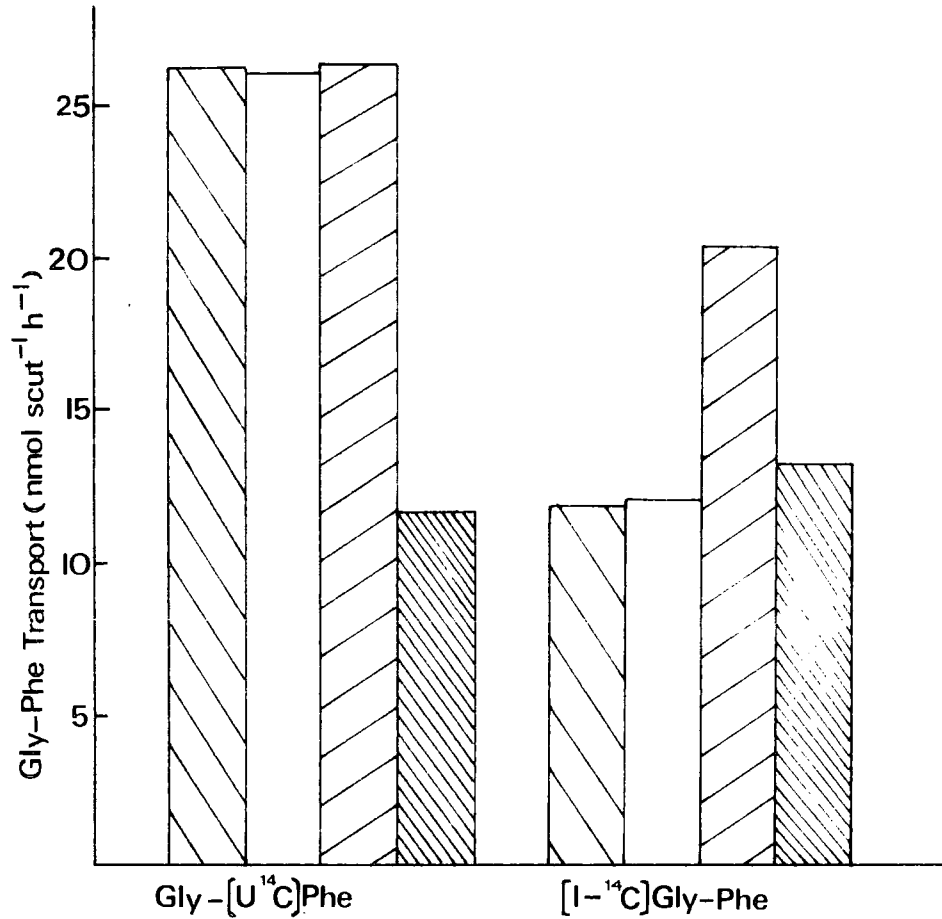


FIGURE 3.2 Effect of Aminooxyacetate on Apparent Uptake of Radioactively Labelled Gly-Phe

Transport of Gly-(U¹⁴C)Phe and (1-¹⁴C)Gly-Phe by scutella germinated on 5 mM AOA for 24 h (▨), or else by 1 day scutella preincubated in 5 mM AOA for 30 min (▧) or 1.5 h (□). ▤ represents untreated control rates.

permeability of the inhibitor itself.

Aglycine decarboxylase is present in plants and functions within the mitochondria of green tissues as part of the photorespiratory sequence (Keys, 1980) by catalysing the oxidative decarboxylation of glycine (formed within the peroxisomes by photorespiration) to serine. A preparation of this enzyme, shown to require pyridoxal phosphate for maximal activity, has recently been isolated from pea leaf tissue (Sarojini & Oliver, 1983). The decarboxylation of glycine is part of the glycollate pathway whereby sugars are regenerated via glycerate, and although it involves the loss of carbon to the gas phase it is nevertheless an integral part of gluconeogenesis (Kisaki & Tolbert, 1970). However, whether this particular enzyme complement is likely to be present in barley scutella and responsible for the observed results remains unclear. Gardeström *et al.*, (1980), reported that only the green, photosynthesising parts of spinach had a glycine decarboxylase, but Kisaki *et al.*, (1971) found decarboxylase activity in non-green tissues, such as etiolated mung bean seedlings, cauliflower and carrot. The scutellar epithelium possesses microbodies (although microscopy cannot successfully identify them as peroxisomes or glyoxysomes) and mitochondria, where glycine formation and decarboxylation respectively may occur (Figure 1.2).

3.3 Chromatographic Separation of Acetate Extracts

3.3.1 Introduction

It is of some interest to establish the nature of the extracted radioactivity, as used in the radiocarbon assay, to determine the extent of metabolism of substrate during the incubation.

3.3.2 Methods

Standard incubation media containing either Gly-(U-¹⁴C)Phe, (1-¹⁴C)Gly-Phe, or (U-¹⁴C)Leu were prepared (1 ml of 2 mM, 0.025 μ Ci ml⁻¹,

in sodium phosphate - citrate buffer, pH 3.8).

2 day scutella (six per vial) were incubated with each substrate for 15 or 60 minute periods after which time they were removed, washed thoroughly and extracted into 1 ml of 100 mM acetate for 4h. Extracts were lyophilized, resuspended in 50 μ l of water and 5 μ l samples run on cellulose thin layers. The plates were sectioned and scraped to build up a radioactivity profile as described previously (Section 2.4.3.3). As an aid to identification, standards of Gly, Phe, Leu and Gly-Phe (5 μ l of aqueous 20 mM solutions) were run in parallel and visualized with ninhydrin spray. Control experiments indicated that when the standards were dissolved in an acetate extract, it had no effect upon their mobilities.

3.3.3 Results

3.3.3.1 Metabolism of Gly-Phe

Analysis of an acetate extraction after 15 and 60 minutes incubation with peptide revealed that most of the radioactivity was present as the hydrolysis products. Thus, after incubation with (1- 14 C) Gly-Phe, (1- 14 C)Gly predominated, with only trace amounts of the intact peptide (Figure 3.3). Therefore, despite the fact that Phe and Gly-Phe co-chromatograph in this particular solvent system, one can be confident that radioactivity extracted after incubation with Gly-(U 14 C)Phe is mainly in the form of labelled phenylalanine (Figure 3.3).

There is no evidence to suggest that within the limits of detection, any significant metabolism of (U 14 C)Phe occurs up to 60 minutes after incubation in peptide; however, since (1- 14 C)Gly is prone to decarboxylation (Section 3.2) and hence the loss of its 14 C tracer, firm statements about its subsequent metabolic fate cannot be made.

3.3.3.2 Metabolism of Leucine

Only (U 14 C)Leu was detected in an acetate extract and no evidence

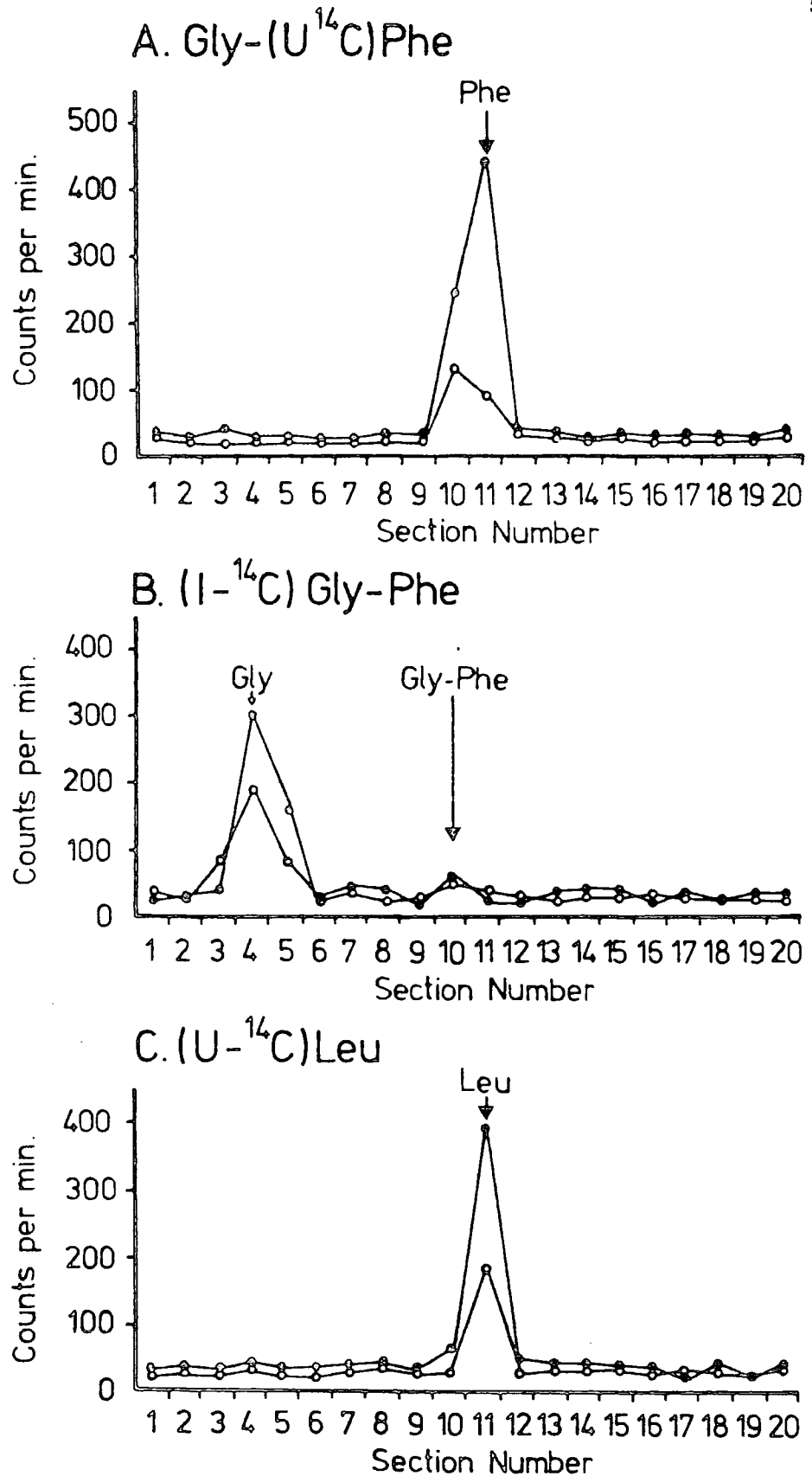


FIGURE 3.3 Chromatographic Analysis of Acetate Extracts Following Uptake of Radioactive Gly-Phe and Leu

Radioactive profiles on cellulose thin-layer plates after chromatography of acetate extracts of 2 day scutella incubated for 15 min (o-o) and 60 min (●-●) with Gly-(U¹⁴C)Phe (A), (1-¹⁴C)Gly-Phe (B), and (U¹⁴C)Leu (C) as described in Section 3.3. The positions of Gly, Phe, Leu and Gly-Phe standards are as indicated.

for its metabolic products was observed for up to 60 minutes of incubation.

3.3.4 Discussion

These studies support the conclusion reached previously using Dansyl analyses (Higgins & Payne, 1978a), that peptides are cleaved very rapidly following transport into barley scutella. However, uptake is independent of hydrolysis, as shown by the accumulation of peptidase-resistant substrates (Higgins & Payne, 1977a). Di- and tri-peptidase activity is low in the endosperm and is largely restricted to the scutellum where the levels of these enzymes are particularly high (Section 1.4.3).

Subsequent to hydrolysis, extracted radioactivity was not observed in any form other than the expected amino acids, above the approximate lower limit for detection (10 μM in the acetate extract). However, despite the fact that amino acid metabolism could not be detected, some incorporation of amino acid directly into protein is likely to have occurred and is readily demonstrable over longer incubation periods in both barley (Pragnell *et al.*, 1966) and oats (Cuming & Osborne, 1978).

3.4 Autoradiographic Location of Transported Gly-(U¹⁴C) Phe

3.4.1 Introduction

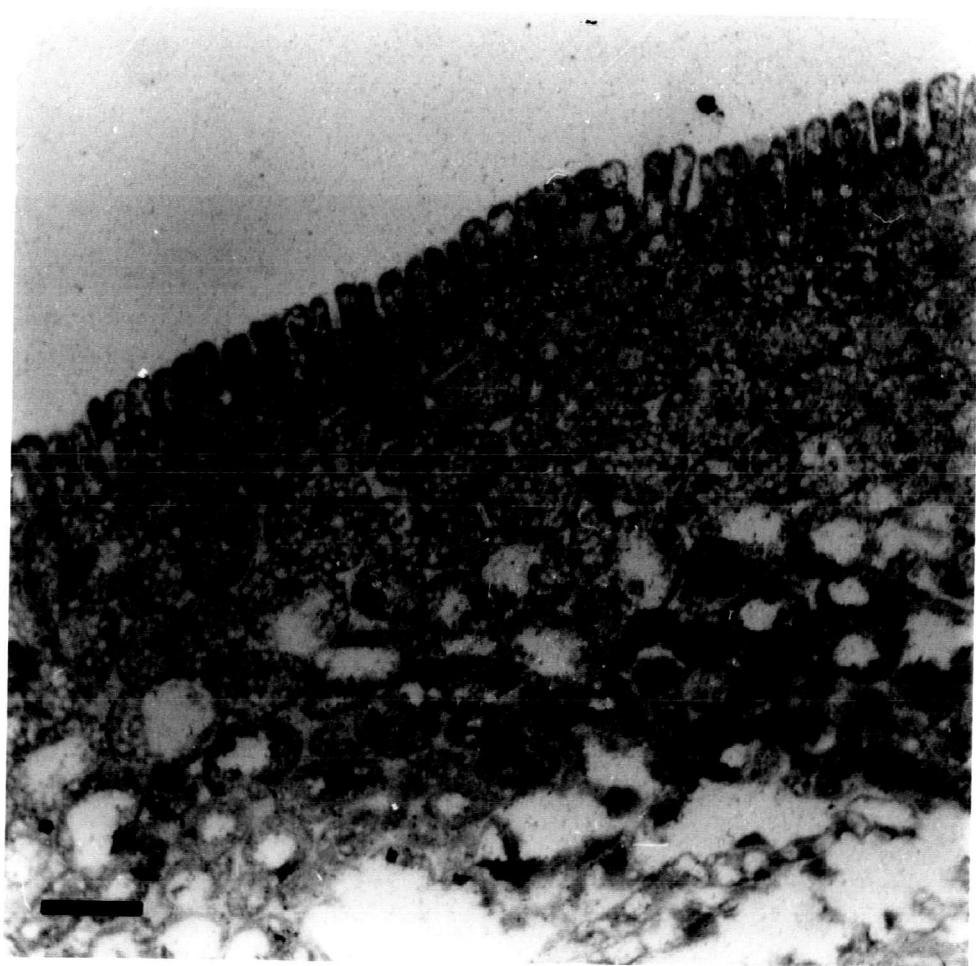
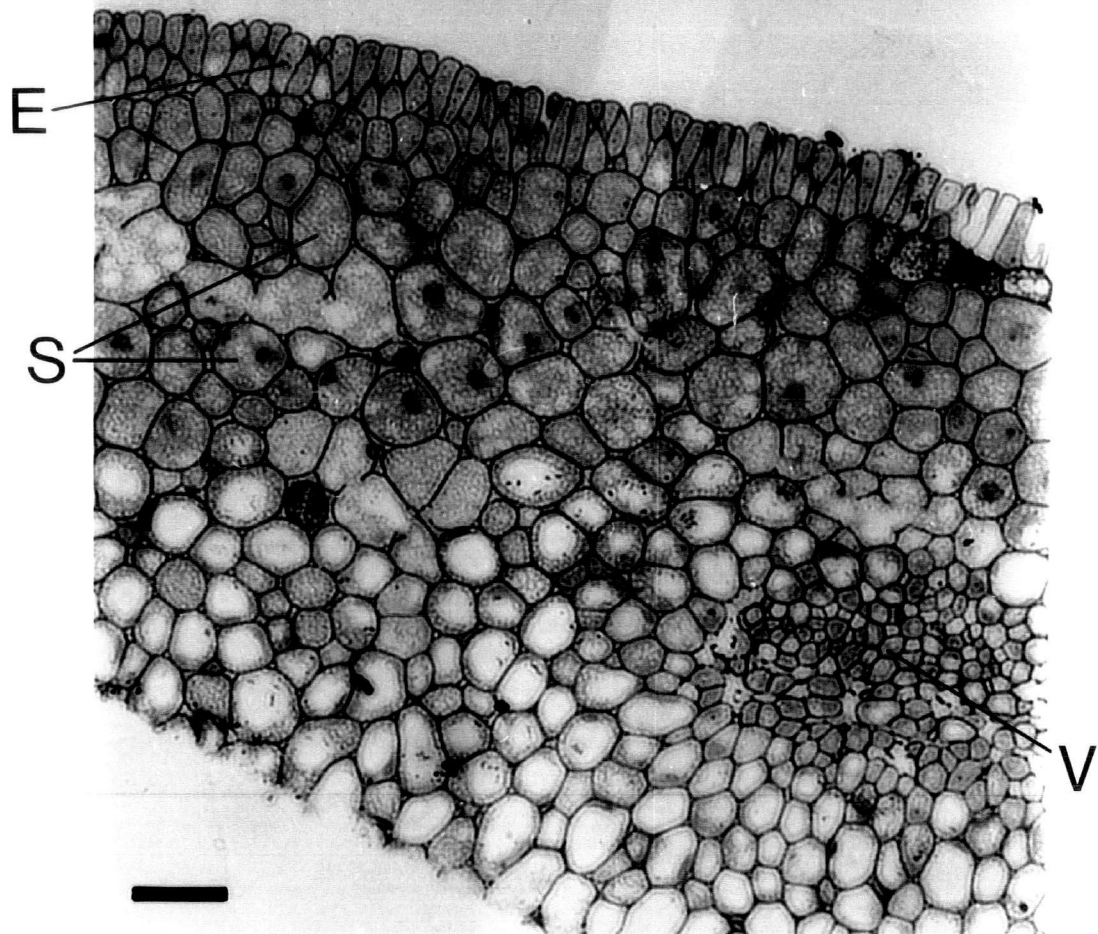
An attempt was made, using light microscopy in conjunction with autoradiography, to locate the distribution of radioactively labelled Gly-Phe after transport by barley scutella.

3.4.2 Methods

3 day scutella were incubated for 15 min in 2 mM Gly-(U¹⁴C)Phe (pH 3.8, 20°C), prepared to a specific activity of 1.22 $\mu\text{Ci } \mu\text{mol}^{-1}$. After washing, scutella were fixed and embedded, prior to covering thin sections (1 μm) on a microscope slide with a 5 μm layer of Ilford L4 autoradiographic emulsion (Section 2.6.3). After exposure for 21 days, slides were developed and fixed as described previously (Section 2.6), mounted in oil under a coverslip and examined by light microscopy. Results were recorded photographically on Ilford XPl film.

3.4.3 Results

The distribution of radioactivity after 15 min incubation is shown



in Figure 3.4. Background exposure is negligible as inferred from the absence of silver grains within the emulsion not overlying the section. Considerable inward movement of tracer is apparent and label is observed not only in the epithelial cells but also in the 8-9 underlying subepithelial layers. Intracellularly, silver grains are restricted largely to the peripheral cytoplasm and absent from the vacuoles of vacuolated cells (but, see Discussion).

3.4.4 Discussion

Since Gly-Phe has been shown to be cleaved very rapidly after uptake, silver grains most likely represent the distribution of free ($U^{14}C$)Phe, which itself is unlikely to have undergone significant metabolic conversion within the time of incubation (Section 3.3.3.1).

Because of the low molecular weight of the tracer it is probable that a large proportion of transported substrate will have been eluted and lost from the section by the preparative methods employed, but enough will have been secured in situ by the cross-linking action of the fixative. It is unlikely that the procedure used had any significant redistributive effect on the label but the selective removal of tracer from locations more accessible and therefore susceptible to elution e.g. surface layers or probably vacuoles, cannot be ruled out (N.Harris, personal communication). After uptake into the symplast has occurred (through transport proteins most probably located mainly in the plasmalemma of the epithelial cells), the rapid intercellular movement of material occurs most likely via plasmodesmata (Figure 1.2) into the vascular system (Figure 3.4) and thence to the growing parts of the seedling. Higgins (1977a) found that the peptidase-resistant peptide, glycylsarcosine, was accumulated to high concentrations in root and shoot tissue of 2 day seedlings after 6 h of uptake, so it is clear that transported material is highly mobile within the embryo.

TABLE 3.1Seedling Growth Parameters

<u>Seedling Age (Days)</u>	<u>Coleoptile Length (mm)</u>	<u>Scutellar Fresh Weight (mg)</u>	<u>Scutellar Surface Area (mm²)</u>
1	1.3	5.1	4.1
2	4.6	7.4	4.7
3	9.2	8.8	5.0
4	19.0	9.3	5.3
5	34.2	9.9	5.9

Growth parameters of seedlings of up to 5 days of age grown on 1.2% w/v water agar at 20°C as described in Section 2.3.1. Values are the mean of at least 10 individual determinations. N.B. The scutellar fresh weight quoted for 1 day seedlings only, represents that of the whole embryo.

3.5 Development of Capacity for Transport of Leu and Gly-Phe in Scutella

3.5.1 Introduction

Because rates of uptake using ($U^{14}C$)Leu and Gly-($U^{14}C$)Phe were in good agreement with results obtained using fluorescamine, these two radioactively labelled substrates were used exclusively in a study of the transport capacity of scutella up to 6 d old, since this topic has received little attention previously.

3.5.2 Methods

Seeds were germinated under the conditions indicated in Chapter 2 (Section 2.3) for up to 6d. Scutella were excised and uptake of Leu and Gly-Phe assayed either by using radioactively labelled substrates, ($U^{14}C$)Leu and Gly-($U^{14}C$)Phe, or by using the fluorescamine method with standard incubation media as described previously (Section 2.4).

3.5.3 Results

Figure 3.5 shows how the uptake of Leu and Gly-Phe changes during the 6 days after the onset of germination. Although based largely on the radioactive assay, confirmatory data were also obtained with fluorescamine. Because scutellar dimensions are not constant with age (Table 3.1), rates in this case are expressed as μmol substrate accumulated per gram fresh weight per hour. Uptake of Gly-Phe is comparatively high just 24 h after the onset of imbibition, reaching a maximal value at 3-4 days and declining thereafter. This pattern is in contrast to rates of leucine transport, which increase less dramatically at first, exceed peptide uptake at day 4 and continue to rise thereafter to achieve rates at least ten fold of those after just 24h germination.

3.5.4 Discussion

The general patterns of developmental rates observed here with Gly-Phe and Leu are in good agreement with developmental data independently obtained for Gly-Gly, Gly-Sar, Leu-Leu (Sopanen, 1979), and Leu (Sopanen et al,

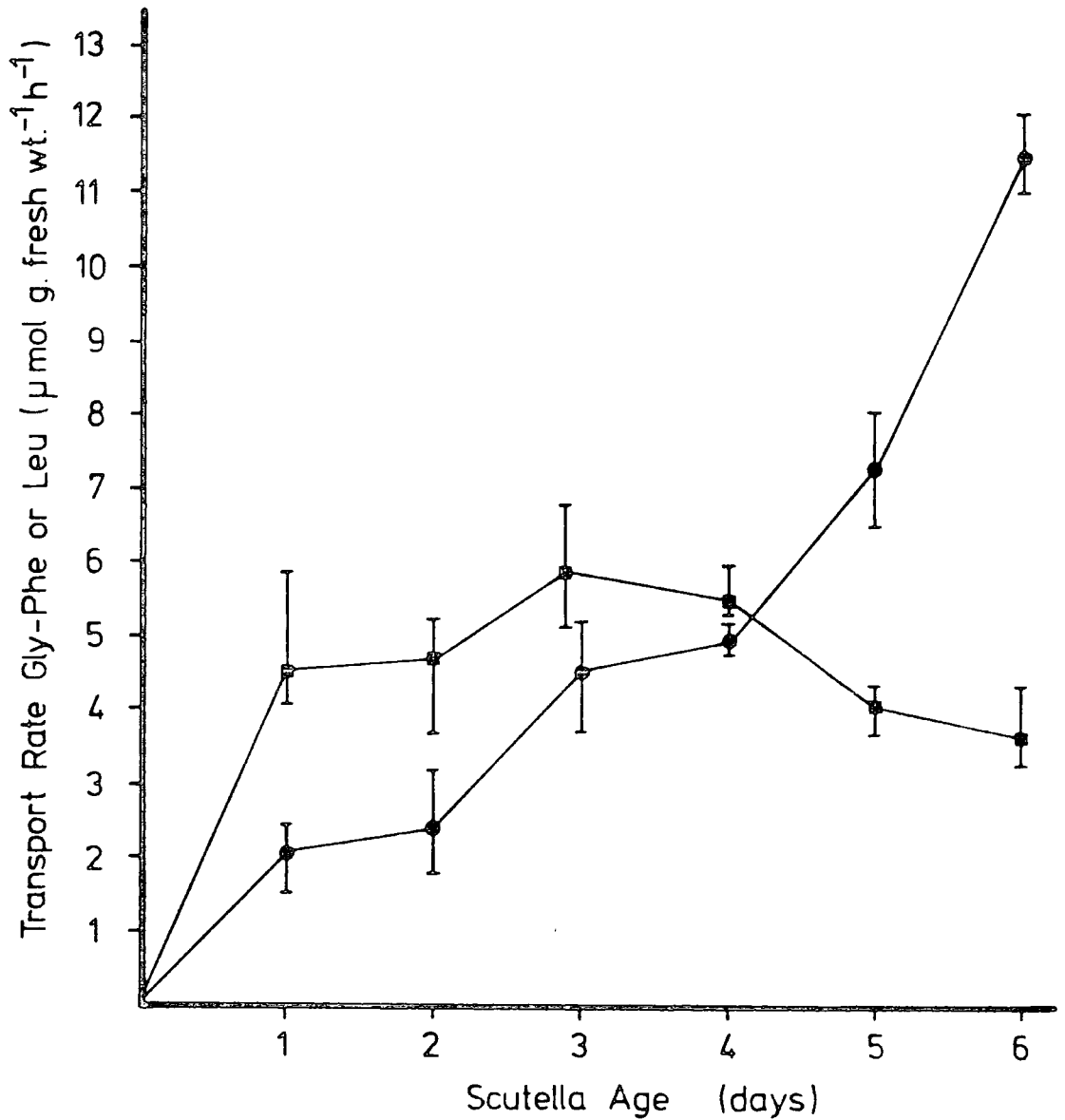


FIGURE 3.5 Development of Capacity for Transport of Gly-Phe and Leu by Scutella

Uptake rates of (U¹⁴C)Leu (●) and Gly-(U¹⁴C)Phe (■) by 1-6 day scutella as assayed by the standard radioactive procedure (Section 2.4.3) from 2 mM solutions. Values given are the mean and range for at least three separate determinations.

1980) in that, as measured in vitro, transport of peptides would appear to be more significant during the early stages of germination, whereas movement of amino acids is greatest into older scutella.

Caution must be taken in extrapolating data such as these to construct a model for the development of uptake in the living system. Although the barley scutellum is capable of transporting a wide range of peptides (Higgins, 1979) and amino acids (Sections 4.2.2, 4.4.2) in vitro, a knowledge of the endospermal 'pool' of organic nitrogen, and ideally kinetics of uptake of each component is required to assess realistically the relative movement of materials in vivo. In this regard, it has been shown that germinating barley seeds do contain peptide and amino acid pools within the endosperm (Higgins & Payne, 1981), whose sizes correlate well with the uptake rates measured here, at least up to 3d of germination.

Thus, three days after imbibition, maximal concentrations of peptide and amino acid of approximately 3-4 mM and 14 mM, respectively, are reached (Higgins & Payne, 1981). Leucine itself reaches a maximal endospermal concentration in barley of 1.4 mM, in the germinating wheat grain about 3 mM (based on the data of Chittenden et al., 1978), and may justifiably be taken as an 'average' amino acid within the endosperm. Gly-Phe was selected primarily because of its commercial availability in the radioactively labelled form, and there is no reason to suspect that it is not a 'typical' peptide within the endospermal pool.

3.6 Effect of Cycloheximide on Transport Development

3.6.1 Introduction

Cycloheximide is a recognised inhibitor of protein synthesis on 80s ribosomes in eukaryotic systems (Lin et al., 1966; Rees et al., 1971), and was used to investigate the extent of de novo synthesis of peptide transport protein in the barley scutellum during germination.

3.6.2 Methods

Grains were germinated for 24h on 1.2% (w/v) agar into which was incorporated cycloheximide to a final concentration of 0.2 mM. Scutella were excised after this period, and assayed for transport of radioactively labelled Gly-Phe and Leu in the routine manner.

3.6.3 Results and Discussion

Grains showed retarded germination in the presence of cycloheximide, and excised scutella displayed very little uptake activity after 24h; transport of Gly-(U¹⁴C)Phe and (U¹⁴C)Leu was reduced to residual values of 2.5 and 0.5 nmol scutellum⁻¹h⁻¹ respectively.

In related studies, Sopanen (1979) reported that treatment with cycloheximide 12h after the start of germination caused a reduction in peptide uptake activity of over 30% during the following 10h. Since cycloheximide is a specific inhibitor of protein synthesis, these observations can be interpreted as a rapid turnover (i.e. synthesis and degradation) of peptide transport proteins occurring within the scutellum. Hence, it can be theorised that the increased capacity for uptake during development may arise as a consequence of the synthesis of more transport proteins (initially, possibly from a pool of preformed mRNA known to be present in dormant embryos of maize, oats and rye (Sanchez de Jimenez *et al.*, 1981; Gordon & Payne, 1976; Caers *et al.*, 1979), rather than an increase in the affinity for substrate (decrease in K_m) of a static carrier complement. However, the possibility that carriers are already present *in situ* but are unable to function because of inhibition of a secondary system vital to their activity (e.g. energy supply), must not be discounted.

3.7 Transport by Cultured Scutella

If peptide transport proteins are being synthesised *de novo*, this feature could offer the potential to radioactively label them with

exogenously supplied radioactive amino acids. In this regard, a preliminary study was undertaken to assess the feasibility of isolating embryos soon after the start of inhibition and continuing their growth on an inorganic, artificial medium (minus amino acids or peptides), and to determine the ability of scutella cultured in this way to subsequently transport amino acids and peptides. Thus, whole embryos were dissected from grains that had imbibed for three hours, and transferred onto nutrient agar (White, 1934) for a further 48h prior to isolating the scutella and assaying for Leu and Gly-Phe transport.

Embryos isolated in this way display growth of coleoptile and roots that is comparable to controls (i.e. intact grains) and, moreover, rates of amino acid and peptide transport by scutella were 75% and 48% higher respectively, than uptake into scutella freshly excised from grains germinated under identical conditions for an equivalent length of time. Recently, these results have been confirmed by Nyman et al (1983), who claims that the increase in amino acid transport capacity by cultured scutella can be inhibited by the addition of cycloheximide or glutamine. It is concluded that the transport of amino acids is regulated by the repression of synthesis of amino acid carrier proteins by glutamine. Glutamine, however, cannot repress the enhanced level of peptide transport by cultured embryos, and the identity of the molecules involved in the regulation of peptide uptake must await further investigation.

CHAPTER 4

THE EFFECTS OF CHEMICAL MODIFICATION OF SCUTELLAR PROTEINS UPON PEPTIDE AND AMINO ACID TRANSPORT

4.1 Introduction

The specific chemical modification of amino acid side chains is a valuable technique in the elucidation of those parts of a protein molecule vital to its continued integrity and function, and has been the subject of several reviews (Cohen, 1968; Sigman & Mooser, 1975; Glazer, 1970).

Clearly, in any modification study, care must be taken in the interpretation of an inhibition. Thus, the inactivation of an enzyme or of a transport protein could be due not only to the direct alteration of catalytically important residues, but also to a disruption of conformation of the active site, either through the steric hindrance of a bulky reagent moiety, or else by a general distortion of the protein molecule. Moreover, in an energy-coupled process such as transport, suitable control experiments must be performed to ensure that a direct effect is being observed and not simply an interference with an electrochemical gradient or ATP supply. The likelihood that the observed inhibitions are secondary in nature can be partially eliminated by the ability to 'substrate screen'; in substrate screening, transport-substrate molecules can confer protection against a modifying reagent, presumably by covering the susceptible amino acid side chains of the active site of the transport protein. However, such protection will not necessarily restrict group modification of non-catalytic residues elsewhere on the protein, and in the studies that are described subsequently, the effect of a reagent upon the transport of a range of substrates, that might reasonably be expected to be mediated by different proteins, was ascertained with the hope of achieving some selectivity of inhibition.

In practice, there is still a rather limited range of compounds known which have the capability of specific amino acid modification, and although several of the available reagents are highly selective, absolute specificity of action is exceptional. In addition, not all the

amino acid residues within a protein will necessarily be available for reaction with a modifying chemical. For example, the successful chemical derivatization of a functional group may be prevented by steric protection of the site by other residues, and similarly, a hydrophobic or particularly electrostatic environment may preclude a successful reaction with certain reagents. Membrane-bound transport proteins are something of a special case in a consideration of group accessibility, because additional factors such as the penetrability of the reagent to sites on the inside of the membrane come into play, and, in addition, the possible lipophilic nature of the carrier proteins may exclude particularly polar reagents from their vicinity. Therefore, despite the fact that many reagents are often pronounced specific or non-specific depending upon their behaviour with simple molecules in a test system, care must be taken in extrapolating this apparent selectivity to the situation in a protein molecule in vivo, where functional groups can have very different reactivities as a result of their micro-environment.

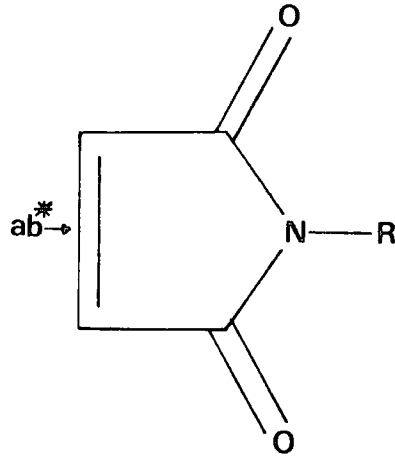
In the studies presented here, the effects of a range of chemical modifiers upon the transport of peptides, amino acids and glucose is described, with a view to the selective inhibition and ultimate isolation of a specific carrier protein.

4.2 Effect of N-Ethylmaleimide on Transport Activity

4.2.1 Introduction - Chemical Properties

N-substituted maleimides, of which N-ethylmaleimide (NEM) is the most widely used, form a family of compounds which react readily with sulphhydryl groups. Structurally, these reagents consist of a chemically reactive maleimide moiety, bonded to a side chain substituent whose structure can be manipulated to confer different physical properties upon the molecule as a whole (Figure 4.1). In this respect, maleimides carrying hydrophilic, bulky side-chains such as glutathione, dextran

A



B

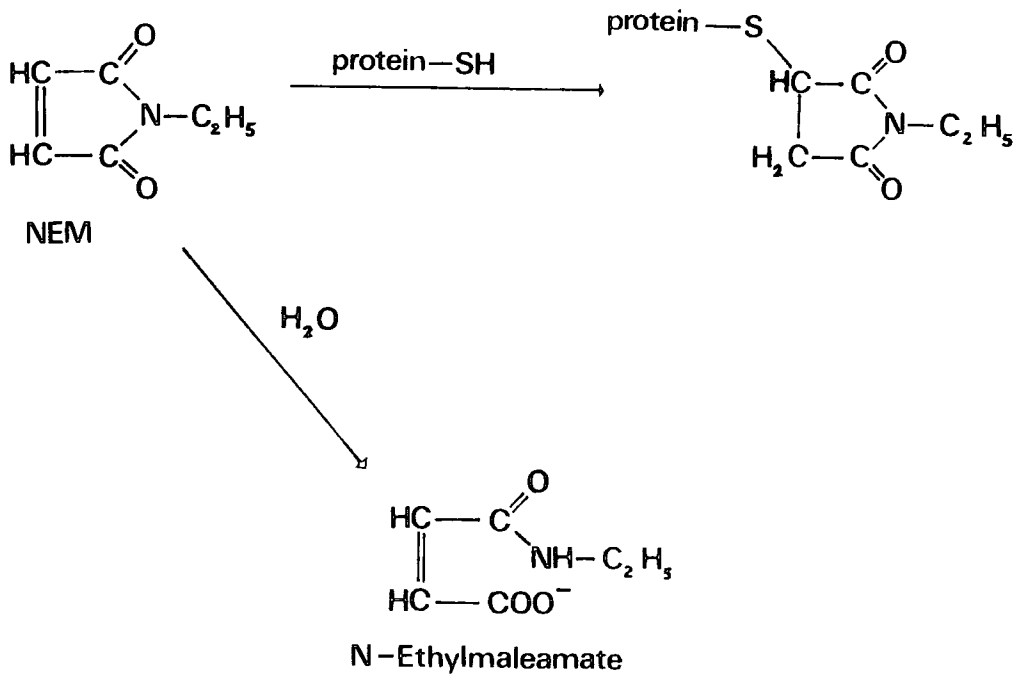


FIGURE 4.1 Maleimide Structure and the Reaction of N-Ethylmaleimide with Sulphydryl Groups

General maleimide structure (A) showing activated double bond (ab*) and position of variable side-chain moiety (R). NEM reacts with protein-bound thiols to form a stable addition product, although a competing reaction involving the hydrolytic ring-opening of NEM can occur, especially at alkaline pH, to form N-ethyl maleamate (B).

(Abbot & Schacter, 1976) or polymethylene-carboxyl groupings (Griffiths *et al.*, 1981) have been synthesised which cannot penetrate membrane barriers. Hence, they can modify only those SH groups exposed at the membrane surface. In contrast, NEM homologues such as N-butylmaleimide and N-benzylmaleimide are more successful in derivatizing SH groups within the more hydrophobic environments of the membrane (Heitz *et al.*, 1968; Lê-Quôc *et al.*, 1981). Fluorescent derivatives e.g. N-(1-pyrene) maleimide (Wu *et al.*, 1976) and coloured maleimides (Witter & Tuppy, 1960) have also been developed.

However, the reagent of greatest versatility is N-ethylmaleimide, whose chemical behaviour, especially in simple molecular systems, has been investigated in some detail (Smyth *et al.*, 1960, 1964; Webb, 1966a). NEM has been used here, in common with many other studies, as a selective reagent for modification of cysteine (thiol) residues. The activated double bond of NEM reacts rapidly, selectively and irreversibly with SH groups in native proteins at pH 5-7 to form a stable addition product (Figure 4.1). Above pH 7, sulphydryl specificity is progressively lost and some reaction with amino groups and with histidine residues may also occur. In addition, the reagent undergoes hydrolysis at alkaline pH to N-ethylmaleamate (60% hydrolysed at pH 8 within 6 hours) but it is fairly stable below pH 7 (Gregory, 1955). NEM is a highly penetrant species, partly because of its small size and uncharged nature, allowing it to traverse membranes (Klingenberg *et al.*, 1974; Gaudemer & Latruffe, 1975) so that, in principle, all parts of the cell are accessible to its action. In as much as most enzymes possess thiol groups, most are susceptible to sulphydryl reagents, and NEM has been shown to inhibit a wide range of cytoplasmic enzymes (Webb, 1966a), except in cases where 'buried' sulphydryl groups preclude successful modification e.g. β -lactoglobulin (Stark *et al.*, 1960).

Similarly, penetration through intracellular membranes can bring about the inhibition of organellar enzymes e.g., rat liver mitochondrial hydroxybutyrate dehydrogenase (Gaudemer & Latruffe, 1975). Finally, there is an extensive literature concerning the effects of sulphydryl reagents, NEM included, on membrane-bound transport systems. This subject is discussed elsewhere in greater detail (Section 5.3).

4.2.2 Kinetics of Inhibition of Transport

4.2.2.1 Methods

In order to measure the inhibition of transport by N-ethylmaleimide, 2 day scutella were preincubated for up to 30 min in NEM (5 mM in 50 mM sodium phosphate-citrate buffer pH 6.8, made freshly before use) at 20°C on a shaking water bath. Scutella were removed, washed, and assayed for transport of the radioactively labelled amino acids ($U^{14}C$)Leu, ($U^{14}C$)Phe, ($U^{14}C$)Gly, ($U^{14}C$)Pro, ($U^{14}C$)Asp, (4,5- 3H)Lys; the peptides Gly-($U^{14}C$)Phe, Ala-($U^{14}C$)Ala (all at 2 mM); and finally for ($U^{14}C$)glucose (20 mM) as described previously. Control scutella were preincubated in buffer only (pH 6.8) for the appropriate time prior to assay.

4.2.2.2 Results

The time-dependent kinetics of inhibition of transport by NEM of a range of substrates is shown in Figure 4.2. Uptake of Ala-Ala and Gly-Phe, which in untreated controls was 50 and 32 nmol scutellum⁻¹h⁻¹ respectively, was inhibited by 90-97% after 4 min preincubation in NEM (Table 4.1). In contrast, the inhibition of transport of several amino acids and of glucose was only 10-38% after the same preincubation period (Table 4.1).

In order to establish that NEM inhibited uptake per se, rather than preventing accumulation by facilitating exodus, a control experiment was performed in which scutella were first preloaded with Gly-($U^{14}C$)Phe from a 2 mM solution for 2h in the routine manner. These were then treated with NEM as above for 4 min, washed, and transferred to phosphate-citrate

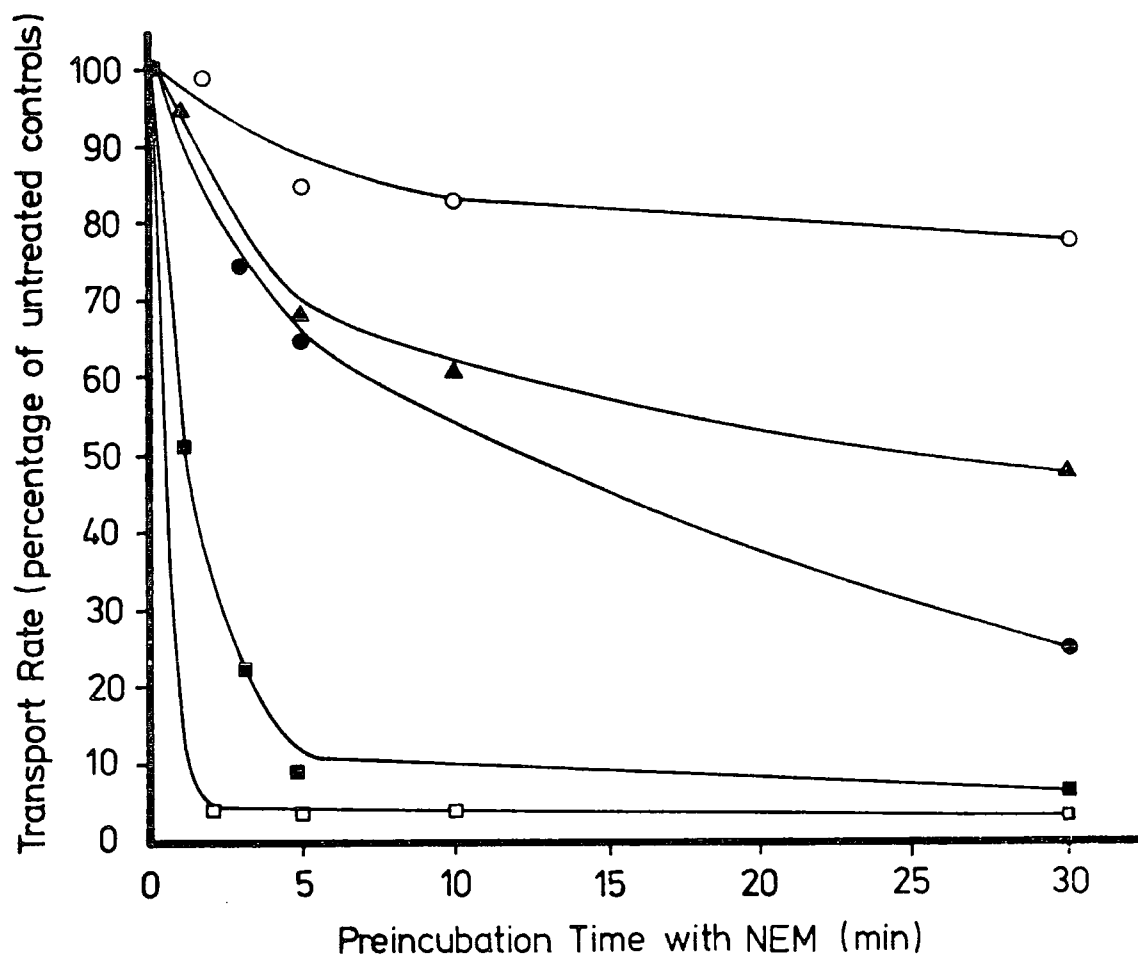


FIGURE 4.2 Kinetics of Inhibition of Peptide, Amino Acid and Glucose Transport by N-Ethylmaleimide

Uptake of Gly-(U¹⁴C)Phe (■), Ala-(U¹⁴C)Ala (□), (U¹⁴C)Leu (●), (U¹⁴C)Phe (O), and (U¹⁴C)Glucose (Δ), by 2 day scutella after preincubation in 5 mM NEM for the indicated times. Rates are expressed as a percentage of untreated controls. Each value is the average of at least 2 separate determinations.

TABLE 4.1Inhibition of Peptide, Amino Acid and Glucose Transport by NEM

<u>Substrate</u>	<u>Control Rate of Uptake (nmol scut⁻¹ h⁻¹)</u>	<u>% Inhibition of Control by NEM</u>
Leu	20	30
Phe	15	10
Pro	22	26
Gly	40	29
Lys	13	38
Asp	8	25
Ala-Ala	50	97
Gly-Phe	32	90
D-Glucose	48	30

Inhibition of uptake (expressed as a percentage of untreated control rates) after preincubation with NEM (5 mM, 4 min) in 2 day scutella. All amino acids and peptides are of the L configuration and present at 2 mM. Glucose was used at 20 mM. Values are the mean of at least two separate determinations.

buffer (pH 3.8) on a shaking water bath for up to 2h, together with control scutella which had been preloaded with peptide but not subsequently exposed to NEM. Scutella were removed every 20 min and assayed for radioactive content by extraction into 5M acetic acid. No significant exodus of counts from the scutella was noted for either the control scutella or those given the prior treatment with NEM, and furthermore, 50 μ l samples of suspension medium removed periodically and analysed by dansylation confirmed that general efflux of pool components was negligible in both cases.

4.2.3 Substrate Screening and NEM Inhibition

4.2.3.1 Methods

1-3 day scutella were preincubated with amino acid or peptide protectant (60-100 mM) for 10 min prior to transfer to NEM (5 mM, pH 6.8) with or without the further presence of competitor (60 - 100 mM) for up to 20 min. Scutella were removed, washed thoroughly and assayed for transport of Gly-(U¹⁴C)Phe and (U¹⁴C)Leu. All incubations were performed on a shaking water both of 20°C.

4.2.3.2 Results

Substrate screening against NEM inhibition of Gly-Phe transport could not be detected despite the use of several potential protectants and a range of preincubation times with NEM (Table 4.2). Similarly, Ala or Val (at 100 mM) could not protect against inhibition of (U¹⁴C)Leu uptake brought about by incubation with NEM for 20 min.

4.2.4 Recovery of Transport Capability after NEM Inhibition

The experiments described below were performed to establish whether the inhibition of transport brought about by NEM was temporary and recoverable, or else was of a more permanent nature.

4.2.4.1 Methods

Whole 1 day embryos were excised and treated with 5 mM NEM for 4 min, pH 6.8, 20°C. After thorough washing, embryos were allowed to

TABLE 4.2

Peptide Substrate-Screening and NEM Inhibition

Treatment Time with NEM	Protectant	Scutellar Age (Days)	Uptake Gly-(U ¹⁴ C) Phe (nmol scut ⁻¹ h ⁻¹)		NEM Alone
			Control (Untreated with NEM)	NEM with Protectant	
40s	Ala-Ala	2	28	16	17
	Gly-Phe			18	
1.5 min	Ala-Ala	3	35	16	15
	Gly-Phe			16	
3 min	Ala-Ala	2	30	9	10.5
	Gly-Leu			8.5	
20 min	Val-Val	3	37	8	8.5
	Gly-Leu			8	

Uptake of Gly-(U¹⁴C)Phe by 2-3 day scutella after preincubation in 5 mM NEM for up to 20 min, with or without added peptide protectant. All protectants were of the L configuration, and all were present at a final concentration of 100 mM, except Val-Val at 60 mM.

recover in buffer (pH 3.8, 20°C) being removed hourly, for up to 5 h, to assay for Gly-(U¹⁴C)Phe transport. For longer recovery periods, embryos were either maintained in buffer (pH 3.8) for 18 h, or else on agar for 72 h, both with a glucose supplement (1% w/v), prior to a peptide transport assay. Peptide uptake by embryos not treated with NEM, but subjected to identical 'recovery' conditions, was assayed in parallel as a control.

4.2.4.2 Results

Typically, a 90-95% inhibition of Gly-Phe transport was achieved by 4 min NEM treatment, and this remained constant irrespective of any post-inhibitory recovery period. Thus, there was no evidence to suggest that uptake rates of peptide increased as a function of recovery time; transport shown by the untreated, control embryos was essentially comparable with freshly excised material.

The physical appearance of embryos excised after 24 h imbibition, treated with 5 mM NEM for 4 min or 30 min, and then transferred to 1% glucose agar for a further 48 h, was also noted. As compared with untreated tissue, embryos were still viable but growth, particularly after 30 min inhibition with NEM was dramatically impaired.

4.2.5 Discussion

It would appear that the rapid and essentially complete inhibition of peptide uptake by N-ethylmaleimide, presumably by direct modification of a transport protein component, is selective. The less dramatic reduction in rates of amino acid and glucose transport achieved under the same conditions may represent secondary inhibition of other processes consequent upon NEM uptake, e.g. energy coupling, but the possibility that there is a much slower modification of more inaccessible sulphydryl groups on these carriers cannot be ruled out. Although the number of amino acid transport systems in barley scutellum is not known, the range of amino acids used, which differ in their chemical nature and might have separate carrier proteins, was sufficient to reinforce the

conclusion that inhibition was specific for the single peptide permease known to be present in barley.

Substrate protection by peptides against NEM inhibition could not be demonstrated, but factors such as the relative accessibility of the susceptible grouping(s) to a highly penetrant reagent, and a protectant limited largely to the outside of the membrane make it difficult to draw firm conclusions about the possible location of the sensitive thiols at a substrate binding site or elsewhere on the protein. Thus, because NEM is a highly reactive species modifying SH groups in a covalent and irreversible reaction, then substrate protection, which is dependent on the loose and transient association of a substrate molecule with the binding site of the transport protein, might be difficult to detect if the sulphhydryl ligands are located at the binding site. Furthermore, even if successful protection is achieved at the outer surface of the membrane where the concentration of competing substrate is high, protection is not guaranteed at other susceptible SH sites to the interior of the membrane which may be exposed to NEM but not to high concentrations of substrate. Therefore, because substrate protection could not be demonstrated, this does not necessarily mean that the sensitive sulphhydryl groups are not located at the active site of the membrane.

The possibility that inhibition of transport is, at least in part, via intracellular action still could not be eliminated at this stage, so aspects of the accumulation of NEM and its attack upon intracellular targets were also studied and are discussed elsewhere (Section 5.2). To gain further evidence for permease interaction, inhibition and protection studies with a non-penetrant sulphhydryl reagent, p-chloromercuribenzene sulphonic acid were carried out (Section 4.3).

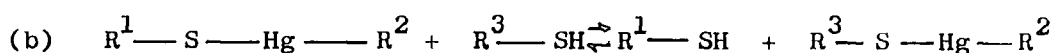
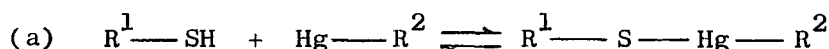
Scutella did not increase their transport activity over the initially inhibited levels within 3 days following NEM inhibition. This is perhaps

not surprising considering the irreversible reaction that the reagent undergoes with sulphydryl groups. Moreover, since it can be demonstrated that NEM rapidly penetrates the tissues (Section 5.2), the intracellular effects of NEM are likely to be widespread, and this could preclude the de novo synthesis or installation of carrier proteins that probably occur during this phase of germination (Section 3.6).

4.3 Effect of ρ -Chloromercuribenzenesulphonic acid on Transport Activity

4.3.1 Introduction - Chemical Properties

To gain further insight into the role of thiol groups in peptide transport, the effects of other sulphydryl reagents, that differed from NEM in their selectivity and membrane permeability, were studied. For example, the organic compounds of mercury are widely employed reagents which react with sulphydryl groups rapidly and specifically at about pH5 to form mercaptides (equation (a)).



The reaction is readily reversible by the addition of excess low molecular weight thiol to regenerate the original SH group (Equation (b)).

Commonly used mercurials are all derivatives of the phenylmercuric ion, and include ρ -mercuribenzoate (PMB), ρ -chloromercuribenzoate (PCMB), ρ -mercuribenzenesulphonate (PMBS) and ρ -chloromercuribenzenesulphonate (PCMB). Most of these compounds are available as the sodium salt and hence dissolve freely in aqueous solution. The unsubstituted phenylmercuric ion is lipid soluble enabling it to rapidly traverse membranes, but the penetrability of its derivatives is reduced considerably by virtue of the charged carboxyl or sulphonate groupings on the molecule (Webb, 1966b). In this regard, PCMB, the mercurial used in these studies as a sulphydryl-specific reagent, is considered to be very poorly permeable; however, limited access to the interior of cells, e.g., in the erythrocyte, may be afforded by anion channels (Rothstein, 1970). Hence, the penetrative properties of PCMB contrast markedly with NEM, and the

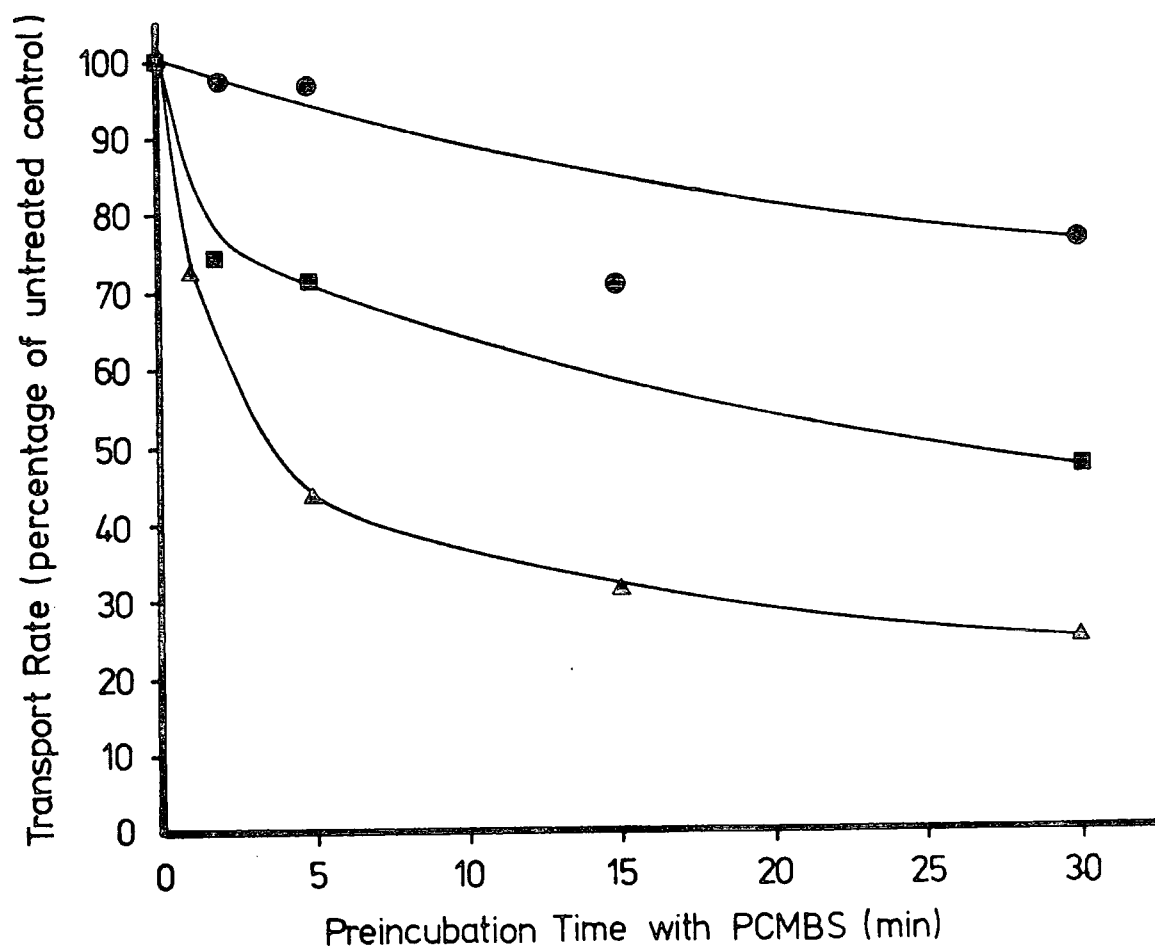


FIGURE 4.3 Kinetics of Inhibition of Peptide, Amino Acid and Glucose Transport by PCMBs

Uptake of Gly-(U¹⁴C)Phe (■), (U¹⁴C)Leu (●), and (U¹⁴C)Glucose (▲) by scutella after preincubation in 1 mM PCMBs for the indicated times as described in Section 4.3.2. Rates are expressed as a percentage of untreated controls. Each value is the average of at least 2 separate determinations.

reagents can be used in conjunction to distinguish between sulphhydryl ligands located on the inside and/or the outside of a membrane (Giaquinta, 1976; Delrot *et al.*, 1980; Despheghel & Delrot, 1983). Therefore, although PCMBs is widely employed in the inhibition of purified enzyme preparations (Velick, 1953; Galanta & Hatefi, 1978; Chen *et al.*, 1964), it finds a particularly useful application in the study of membrane-bound transport proteins, and this is discussed in greater depth elsewhere (Section 5.3).

4.3.2 Kinetics of Inhibition of Transport

4.3.2.1 Methods

Scutella were excised and preincubated for up to 30 min with 1 mM PCMBs, in 50 mM sodium phosphate-citrate buffer, pH 5 at 20°C, prior to washing thoroughly with distilled water and assaying for transport of Gly-(U¹⁴C)Phe, (U¹⁴C)Leu, (both 2 mM), and (U¹⁴C)glucose (20 mM) in the routine manner. Control scutella were preincubated in buffer only at pH 5 for 30 min before assay.

4.3.2.2 Results

The time-dependent inhibition of transport by PCMBs is shown in Figure 4.3. Rates of transport of Gly-Phe, Leu and glucose are expressed as percentages of the uptake in controls not exposed to inhibitor. There is a clear distinction between the kinetics of inhibition of Leu and of Gly-Phe in that transport of the peptide shows an initial, rapid decrease followed by a slower decline for up to 30 min preincubation with PCMBs. In contrast, preincubation of up to 5 min in PCMBs has little effect on leucine uptake and about 80% of the transport activity of controls is still present after 30 min pretreatment with inhibitor. Thus, although the reagent was used as 1mM and overall levels of inhibition are lower, PCMBs resembles NEM at least in its differential effect upon amino acid versus peptide transport; however, glucose uptake, which is largely unaffected by N-ethylmaleimide, appears to be far more sensitive to the

TABLE 4.3

Substrate Protection Against Inhibition of Gly-Phe
Transport by p-Chloromercuribenzenesulphonic Acid

<u>Substrate Added</u>	<u>Percent Inhibition of Peptide Transport</u>
None	72
D-Ala-D-Ala-D-Ala	71
L-Ala-L-Ala-L-Ala	31
D-Ala-D-Ala	70
L-Ala-L-Ala	37
L-Met	73
L-Leu	74
L-Ala	75

2-3 day scutella were incubated with 5 mM p-chloromercuribenzenesulphonic acid for 4 min at 20°C, pH 3.8 with or without added substrate (100 mM). After washing, scutella were used in a standard assay for Gly-(U¹⁴C)Phe transport. Values represent the percentage inhibition of uptake relative to an untreated control.

mercurial. The implications of this are discussed later (Section 4.3.5).

4.3.3 Substrate Screening and PCMBS Inhibition

4.3.3.1 Methods

2-3 day scutella were preincubated in protectant (100 mM amino acid or peptide, 200-2000 mM glucose, pH 3.8) for 10 min prior to transfer to 5 mM PCMBS for 4 min at pH 5 with added competitor (100 mM amino acid or peptide, 200-2000 mM glucose). After washing, uptake of Gly-(U¹⁴C)Phe, (¹⁴C)Leu and (U¹⁴C)glucose was assayed in the routine way.

4.3.3.2 Results

1. Substrate protection of peptide transport

The inhibition of Gly-Phe transport by PCMBS in the presence of a range of competitors is shown in Table 4.3. Di- and tripeptides containing L-residues provided protection. In contrast, the corresponding D-peptide analogues and free L-amino acids were ineffective as protectants.

2. Substrate protection of amino acid transport

Treatment of 2 day scutella with 5 mM PCMBS for 4 min inhibited Leu transport by only 10-15% relative to untreated controls, irrespective of the presence or absence of 100 mM L-Ala, L-Met, L-Leu, Gly-L-Leu or L-Ala-L-Ala. However, since amino acid uptake is largely unaffected by PCMBS, a successful protection attempt might go undetected, hence casting some doubt over the validity of substrate screening experiments in this case.

3. Substrate protection of glucose transport

Glucose at concentrations up to 2M failed to protect against the 70% inhibition (relative to untreated controls) of glucose transport (from 2 mM or 20 mM solutions) achieved by incubation with PCMBS (5 mM for 4 min). As an additional control, preincubation with high concentrations of glucose only (1M for 15 min) did not affect the subsequent uptake of glucose (20 mM) measured over 1h. This eliminated the possibility that protection was occurring but remained undetected due to the inhibitory effect of the protectant itself.

4.3.4 Recovery of Transport Capability After PCMBS Inhibition

Similar experiments to those already described for NEM (Section 4.2.4) were performed with PCMBS, to establish the degree of permanence of the inhibition of peptide transport that is induced by this mercurial.

4.3.4.1 Methods

Whole 1 day embryos were excised and treated with 5 mM PCMBS (in sodium phosphate-citrate buffer, pH 5, 20°C for 7 min). After thorough washing, embryos were transferred onto agar (containing a glucose supplement, 1% w/v) for a 'recovery' period of 30 h, prior to excising the scutella and assaying for radioactive Gly-Phe transport in the routine manner. As controls, untreated embryos were cultured as described above and assayed for peptide transport, or else cultured, and then treated with 5 mM PCMBS (pH 5, 20°C for 7 min) just prior to the transport assay. As additional controls, rates of Gly-Phe transport by scutella freshly excised from intact, 2 day seedlings with or without an identical PCMBS treatment just prior to the uptake assay were also established.

4.3.4.2 Results

The uptake of Gly-(U¹⁴C)Phe by scutella subjected to the various treatments described above is shown in Table 4.4. It can be seen that the rate of peptide transport shown by scutella treated with PCMBS, but allowed subsequent recovery on agar, is essentially comparable with the rates shown by fresh, or cultured scutella not treated with PCMBS. In contrast, cultured or fresh scutella treated with PCMBS just before the peptide transport assay show very little uptake activity. It is clear, therefore, that the inhibition of transport induced by this mercurial is fully restorable after at least a 30 h recovery period.

4.3.5 Discussion

The impermeable nature of PCMBS and the ability of peptides to protect against its inhibition contrasts with the high penetrability of NEM and the failure of peptides to limit its inhibitory effect. These

TABLE 4.4

Recovery of Gly-Phe Transport Activity After PCMBS Inhibition

<u>Treatment</u>	<u>Uptake Rate Gly-(U¹⁴C)Phe</u> <u>(nmol scutellum⁻¹ h⁻¹)</u>
Treat with PCMBS, then culture on agar for 30 h:	36
Agar culture for 30 h only:	30
Fresh scutella, treat with PCMBS:	4
Agar culture, then treat with PCMBS:	5
Fresh scutella, untreated:	33

Uptake rate of Gly-(¹⁴C)Phe by scutella treated with PCMBS (5 mM, pH 5 for 7 min, 20°C) prior to a recovery period on 1.2% w/v water agar (with a 1% w/v glucose supplement). As controls, peptide transport by cultured or freshly excised scutella not treated with PCMBS, and by fresh or cultured scutella treated with PCMBS under identical conditions just prior to the transport assay, is also indicated.

results are consistent with the existence of two types of thiols essential to peptide transport. One set, located either on the inside of the membrane or 'buried' within the lipid bilayer will be sensitive to NEM but not to PCMBS. Because these "internal" groupings would be inaccessible to high levels of substrate (and even after significant accumulation of peptides, it will have been rapidly cleaved and widely distributed), protection against NEM modification cannot be conferred. Another class of sulphhydryls, located on the "outside" of the scutellar plasmalemma, can be modified by both PCMBS and NEM. Substrate screening of these exposed groupings against either inhibitor is feasible, but can be detected only with the mercurial when presumably the internal thiols remain unmodified and functional.

The ability of L-peptides to protect against PCMBS inhibition can be fairly confidently interpreted as the mercurial and substrate competing for same binding site. Because D-peptides and L-amino acids, neither of which are substrates for the transport system, fail to protect, the possibility of an inactivation of PCMBS by the protectant, which could be misconstrued as a positive protection, can be eliminated.

These results, together with the kinetics of inhibition of uptake by both NEM and PCMBS, point towards the existence of a peptide transport protein dependent upon functional thiol ligands present at a substrate binding site. Although there is no evidence to indicate that sulphhydryl groups are important in amino acid transport, firm statements about their role in glucose transport are more difficult to make. Although inhibition of glucose transport by NEM is considered to be slow and secondary in nature, considerable inactivation is achieved by PCMBS. This may be a reflection of the differing reactivities and accessibilities of these inhibitors to the thiols involved, yet paradoxically, NEM is considered to be the more penetrating reagent of the two. Clearly, there is scope here for the further elucidation of the role of sulphhydryl groups in glucose uptake.

Scutella treated with PCMBS can recover peptide transport activity, a feature not shown by NEM treated tissue (Section 4.2.4). This may be a reflection of both the impermeable nature of PCMBS, such that intracellular metabolic processes remain unaffected, and also of the reversible nature of the modification reaction itself, such that the functional sulphydryl groups can be spontaneously regenerated. In contrast, NEM is highly penetrant, and modifies thiols in an irreversible alkylation reaction, so it is perhaps not surprising that treated tissue cannot recover from its action.

4.4 Effect of Phenylarsine Oxide on Transport Activity

4.4.1 Introduction - Chemical Properties

A third sulphydryl reagent, phenylarsine oxide, differs from NEM and PCMBS in that it complexes selectively with vicinal dithiols. Studies were undertaken to determine its effects also upon scutellar transport.

The toxic effects of arsenical compounds upon biological systems have been known for many years and as early as 1909, it was suggested that the particular 'chemoreceptors' involved were either sulphydryl or hydroxyl ligands (Erlich, 1909). The biochemistry of arsenic has been the subject of a recent review (Knowles & Benson, 1983). The trivalent arsenicals, all derivatives of arsine (AsH_3), react readily and highly specifically with thiol groups in a substitution mechanism which is not fully understood (Webb, 1966c). Trivalent arsenicals fall into two categories, the monosubstituted alkyl dihaloarsines (RAsX_2) and alkyl arsenoxides (RAsO), or the dialkyl haloarsines ($\text{R}_1\text{R}_2\text{AsX}$) (Lotspeich & Peters, 1951). Chemically, this distinction is important because the monosubstituted compounds are considered to react reversibly with vicinal, or adjacent dithiols on a protein to form a stable thioarsinite ring structure. Reaction with monothiols does occur but the product is unstable (Figure 4.4). In contrast, the dialkyl haloarsines give

stable derivatives with single cysteine residues only (Webb, 1966c; Whittaker, 1947; Stocken & Thompson, 1946a). The decomposition of cyclic thioarsinites, to regenerate the original sulphhydryl groups, is readily brought about by the addition of excess vicinal dithiol e.g., dimercaptopropanol (Stocken & Thompson, 1946b), dithiothreitol (Konings & Robillard, 1982) or dithioalkanes (Whittaker, 1947) which compete for reaction with the inhibitor. Thus, many enzymes have been classified as dithiol-dependent on the basis of their inhibition by alkyl arsenoxides (Webb, 1966c; Stevenson *et al.*, 1978; Storey, 1964) and more recently, several reports point towards an important role of vicinal thiols in transport processes in particular (Gould, 1978; Robillard & Konings, 1981; Turner & George, 1983). However, the possibility that, occasionally, enzymic inactivation is brought about by the arsenical combining with two somewhat distally located SH groups on a particularly flexible protein molecule, cannot be completely discounted. The ability to restore function through the use of a low molecular weight dithiol as described above, would, however, indicate that inhibition is not achieved through this sort of conformational change of the enzyme molecule, which may often be irreversible in nature.

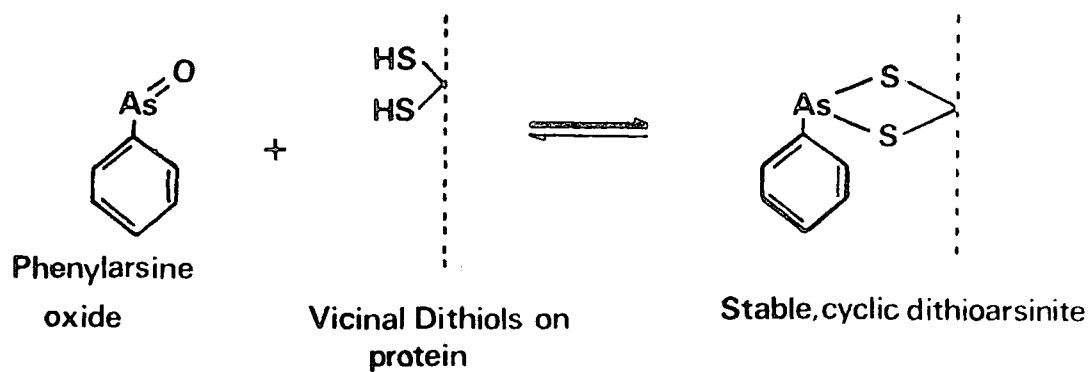
The trivalent arsenical, phenylarsine oxide, PAO (also known as arsenosobenzene or phenylarsenoxide) was selected here to examine the possible role of vicinal thiols in peptide transport by the barley scutellum (Figure 4.4). This inhibitor is only slightly soluble in aqueous solution but because of its hydrophobic, uncharged nature it is considered to be lipid soluble and to possess some ability to pass through cell membranes (Webb, 1966c).

4.4.2 Kinetics of Inhibition of Transport

4.4.2.1 Methods

2-3 day scutella were excised and preincubated in PAO (0.5 mM in 50 mM sodium phosphate-citrate buffer, pH 3.8 at 20°C) for periods of up to 30 min, prior to washing and carrying out a standard assay for peptide, amino acid or glucose transport. Control scutella were

A



B

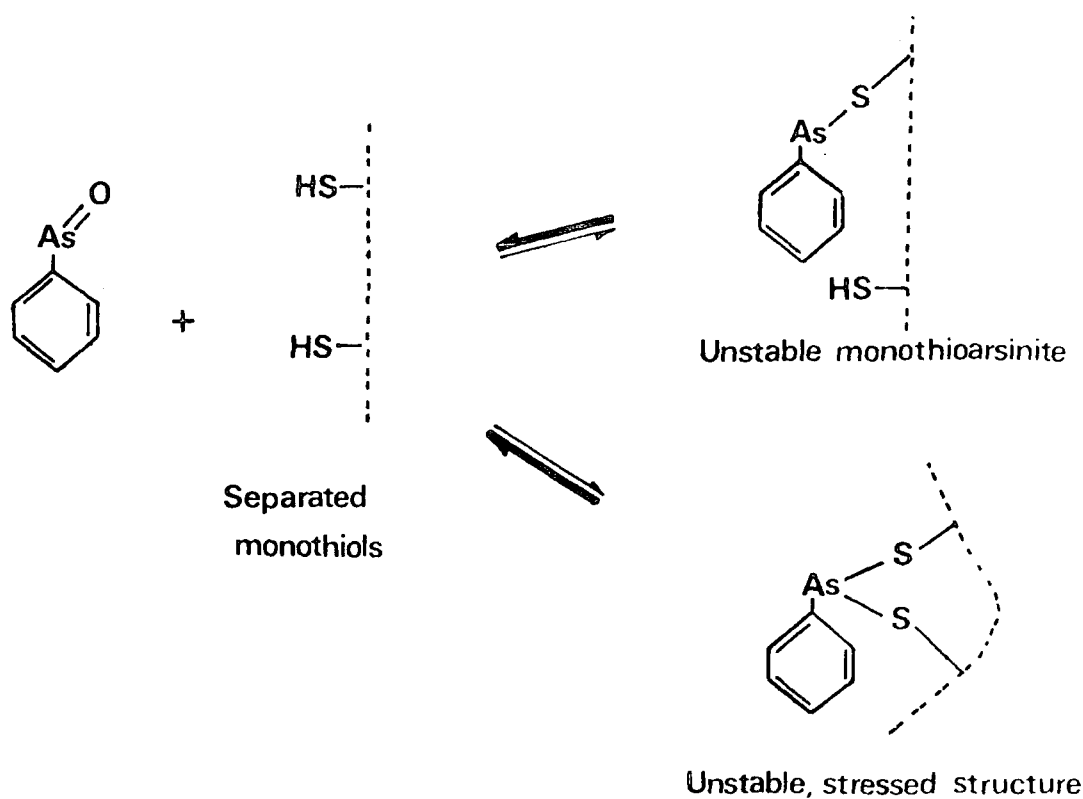


FIGURE 4.4. Reaction of Phenylarsine Oxide with Thiols and Vicinal Dithiols

Phenylarsine Oxide can react with vicinal dithiol groups to form a stable, cyclic dithioarsinite (A), whereas the monothioarsinite produced by reaction with a single thiol group is unstable. Phenylarsine Oxide may in principle combine with two separated monothiol, but the consequent distorted product is only likely to be transient in nature (B).

preincubated in buffer only for 30 min before assay. It must be noted that PAO is only slightly soluble in water, and the concentration quoted (0.5 mM) represents that determined for a saturated solution.

4.4.2.2 Results

The percentage inhibition of transport (relative to untreated controls) of amino acids, peptides and glucose after 4 min preincubation with PAO is shown in Table 4.5, and the time dependent inactivation of uptake is plotted kinetically for a representative range of these substrates in Figure 4.5. The inhibitory effects of PAO are analogous to those of NEM, in that a rapid and selective inactivation of the transport of peptides, but not of amino acids or glucose, is achieved within a 10 min incubation. Interestingly, the transport of Gly-Phe is apparently not inhibited as rapidly or as completely as that of Ala-Ala-Ala. Thus, after 30 min incubation with PAO, 2 day scutella continue to transport Gly-Phe at a rate of $9 \text{ nmol scutellum}^{-1} \text{h}^{-1}$. This value can be inhibited further, however, by a subsequent treatment with DNP (10 μM for 30 min at 20°C) which reduces the rate of uptake to the residual value characteristic of the passive component only ($3\text{-}4 \text{ nmol scutellum}^{-1} \text{h}^{-1}$).

4.4.3 Substrate Screening and PAO Inhibition

4.4.3.1 Methods

2-3 day scutella were preincubated in peptide (60 - 200 mM) for 5 min, and then transferred to PAO (0.5 mM, pH 3.8), with added peptide competitor (up to 200 mM) for 3 min at 20°C . Scutella were removed, washed and assayed for Gly-(^{14}C)Phe transport in the routine manner. Control tissue was treated with PAO under the conditions specified, but without competitor present.

4.4.3.2 Results

The protectants L-Ala-L-Ala, D-Ala-D-Ala (at 200 mM), Gly-L-Leu (at 100 mM) and L-Val-L-Val, D-Val-D-Val (at 60 mM), were all ineffective

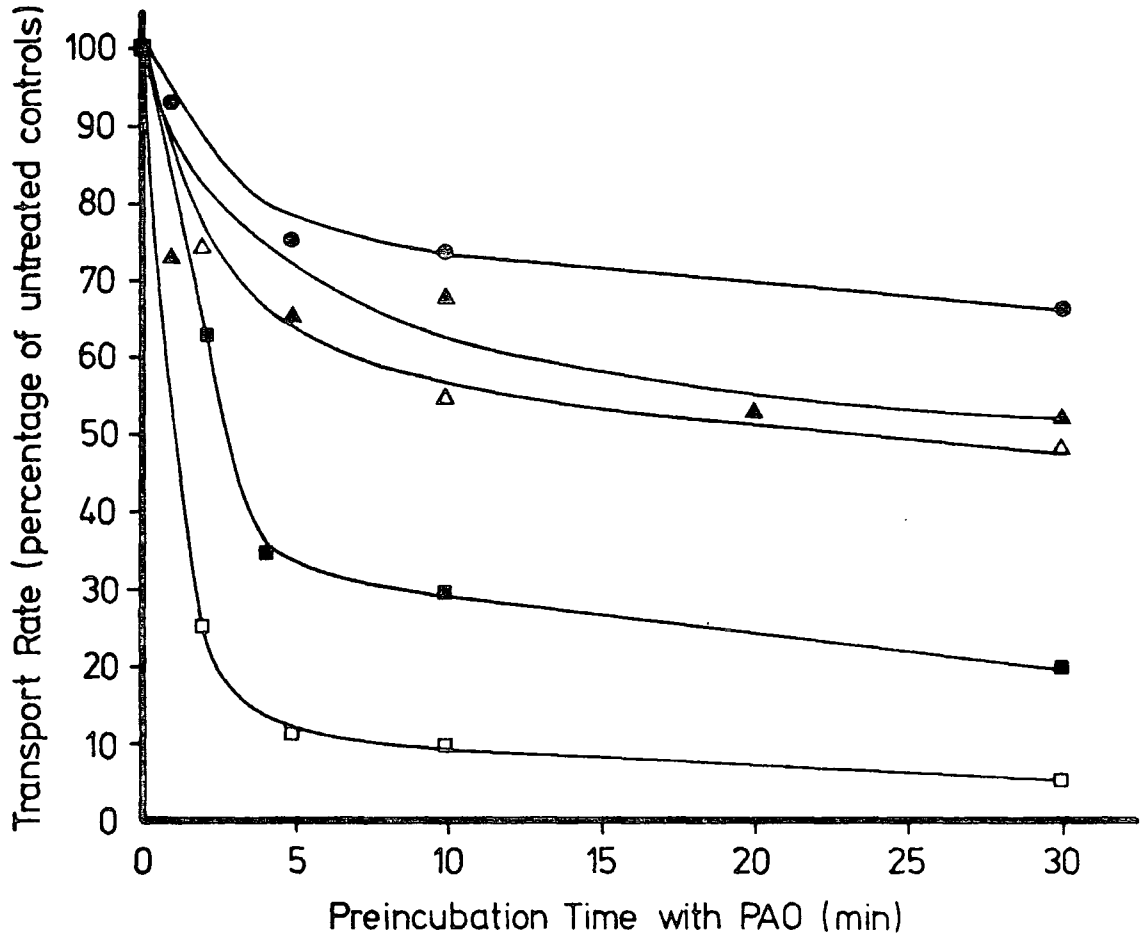


FIGURE 4.5 Kinetics of Inhibition of Peptide, Amino Acid and Glucose Transport by Phenylarsine Oxide

Uptake of Gly-(U¹⁴C)Phe (■), Ala-Ala-(U¹⁴C)Ala (□), (U¹⁴C)Leu (●), (U¹⁴C)Pro (△), and (U¹⁴C)Glucose (▲), by scutella after preincubation in 0.5 mM phenylarsine oxide for the indicated times. Transport rates are expressed as a percentage of untreated controls and each value is the average of at least 2 separate determinations.

TABLE 4.5

Inhibition of Amino acid, Peptide and Glucose Transport
by Phenylarsine oxide

<u>Substrate</u>	<u>Inhibition of Transport by Phenylarsine oxide</u> <u>(Percentage of Untreated Controls)</u>
Gly	22
Pro	26
Asn	32
Lys	30
His	25
Asp	29
Leu	20
Ala-Ala-Ala	89
Gly-Phe	70
D-Glucose	35

Percentage inhibition of transport of substrate (relative to untreated controls) by 2-3 day scutella after preincubation with 0.5 mM Phenylarsine oxide (pH 3.8, 20°C for 4 min). All amino acids and peptides were of the L configuration, and used at 2 mM; glucose was used at 20 mM. Values are the mean of at least two separate determinations.

in screening against PAO inhibition of Gly-Phe transport. Thus, the inhibition of transport was unaffected by the presence of any competitor tested.

4.4.3.3 Discussion

The similarity in the inhibitory effects of NEM and PAO upon peptide, amino acid and glucose transport probably reflects the permeable nature of both these reagents, such that essentially the same sulphydryls are susceptible to modification in each case. As argued previously for NEM, the modification of internal thiol groups by PAO that are inaccessible to protectant would explain the inability to demonstrate successful substrate screening against this inhibitor.

The observations with these several different cysteine-specific reagents point towards a peptide transport system which is dependent upon two classes of sulphydryl groups located at different levels in the scutellar plasmalemmae. It is likely that at least one class of sulphydryl exists as a vicinal dithiol; evidence that this is functional in the reduced form is provided in the next section. Further evidence on the nature of these groups, and for the specificity of inhibition, is provided by the ability to restore peptide, but not amino acid transport, by the use of dithiothreitol after PCMBs or PAO inhibition; these aspects are considered later (Section 5.1.2).

4.5 Effect of Dithiothreitol on Transport Activity

4.5.1 Introduction

Dithiothreitol (Cleland's reagent, DTT) is highly water soluble with a low redox potential, and is capable of reducing cystine residues to the constituent monothiols with great specificity (Cleland, 1964). Hence the use of DTT should establish whether the vicinal dithiols described above are functional as oxidised cystine bridges.

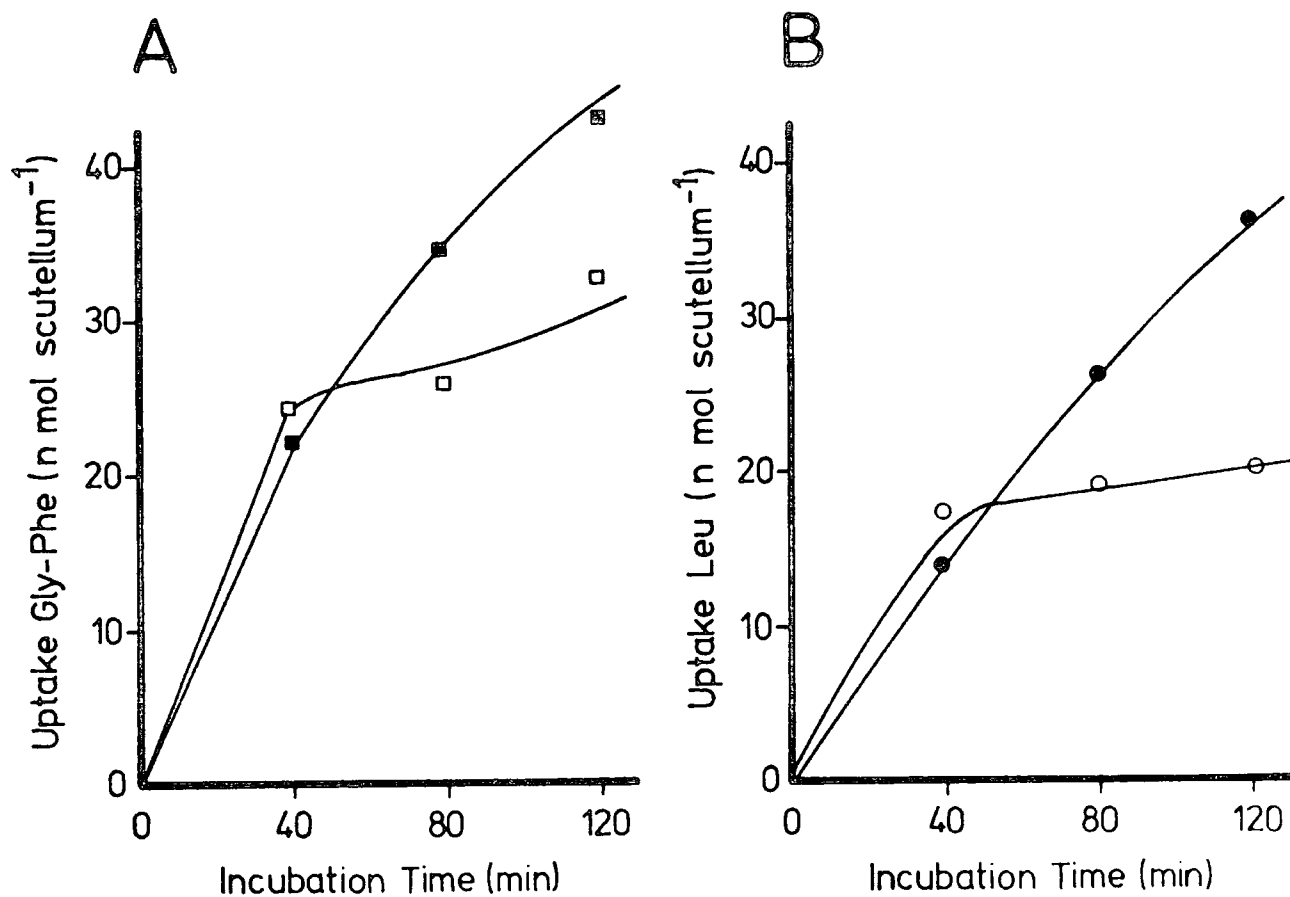


FIGURE 4.6 Effect of Dithiothreitol on Gly-Phe and Leu Transport

Time course of uptake of Gly-(U¹⁴C)Phe (□), and (U¹⁴C)Leu (○) by 2 day scutella after treatment with 10 mM dithiothreitol for 20 min. As controls, the uptake of both Gly-Phe (■) and Leucine (●) after preincubation in buffer only was assayed in parallel.

4.5.2 Methods

2 day scutella were excised and treated with DTT (10 mM in sodium phosphate-citrate buffer, pH 7 at 20°C) for 30 min. Scutella were washed prior to a standard transport assay with radioactively labelled Gly-Phe and Leu. Control scutella were preincubated in buffer alone before assay.

4.5.3 Results

A comparison of the rates of accumulation of Leu and Gly-Phe after DTT treatment with untreated controls reveals that, up to about 40 min, uptake is unaffected but subsequently, further transport into treated tissue is inhibited quite markedly (Figure 4.6). These observations can be interpreted as DTT exerting some secondary, indirect action which ultimately affects substrate transport.

4.5.4 Discussion

Assuming that DTT will have gained some access to the carrier proteins, oxidised disulphides (cystine bridges) would not appear to play a direct role as components of the peptide or amino acid transport systems, and the dithiols inhibitable by NEM, PCMBs and PAO must be functional in the reduced form.

4.6 Effect of Plumbagin on Transport Activity

4.6.1 Introduction - Chemical Properties

Quinones comprise a large family of compounds that can modify both thiol and amino groups in an oxidation or addition process. The oxidation of sulphhydryl ligands occurs via several distinct reaction mechanisms and can be reversed with suitable reducing agents such as dithiothreitol. These aspects and other topics concerning the chemistry of the quinones have been discussed thoroughly by Webb (1966d).

Plumbagin (5-hydroxy-2-methyl-1, 4-naphthoquinone) is a naturally occurring quinone found in plants of the genus Plumbago (the Leadworts). It is lipophilic and soluble in membranes. To date, plumbagin has seen little application to transport studies, but it has been shown to

effectively inhibit the accumulation of lactose and proline in Escherichia coli by oxidising the vicinal dithiols essential to the uptake of these solutes (Konings & Robillard, 1982). Experiments were performed to determine whether plumbagin could inhibit peptide transport in the barley scutellum in the same way.

4.6.2 Methods

3 day scutella were treated for up to 2.5 h in 0.2 mM plumbagin (in sodium phosphate-citrate buffer, pH 3.8 at 20°C), washed, and assayed for transport of radioactive Gly-Phe in the routine manner. Controls were preincubated in buffer only before assay.

4.6.3 Results

The inhibition of peptide transport by plumbagin (Figure 4.7) is considerably slower than that achieved by the other reagents tested previously such as NEM or PAO. In an attempt to recover the inhibited uptake activity, scutella were treated with plumbagin for 2.5 h and then transferred to dithiothreitol (10 mM sodium phosphate-citrate buffer, pH 3.8, 20°C) for 2 h before an assay for Gly-Phe uptake. Under these conditions, no detectable restoration of transport was achieved by dithiothreitol (results not shown).

4.6.4 Discussion

The inability of dithiothreitol to reactivate peptide transport can have at least two interpretations. The inhibitory action of plumbagin may be secondary in nature, such that the vicinal dithiols on the peptide carrier protein are not directly involved, or else dithiothreitol may be unable to gain access to sites modified successfully by plumbagin. However, the ability of dithiothreitol to restore the inhibition of transport brought about by phenylarsine oxide and β -chloromercuribenzenesulphonate (Section 5.1.2) would support the view that the effects of plumbagin are secondary and not reversible with a reducing agent. It must be noted in retrospect however, that the pH value at which

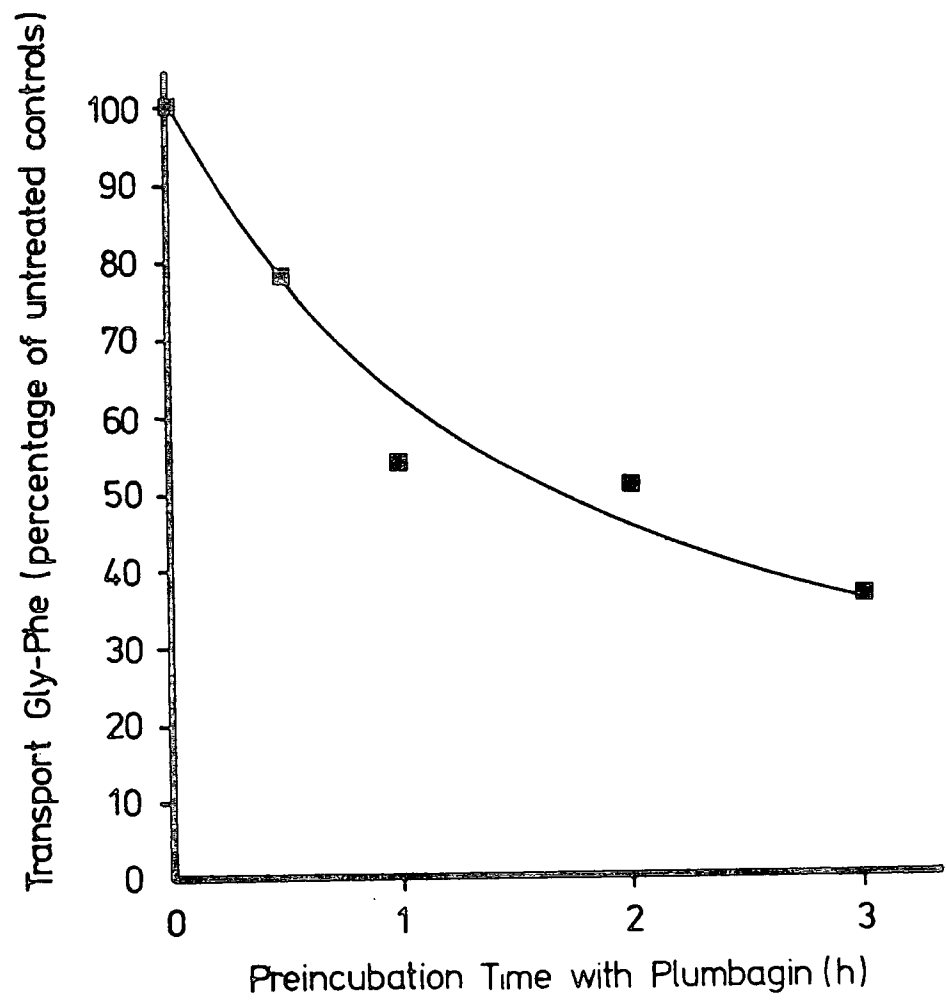


FIGURE 4.7 Kinetics of Inhibition of Gly-Phe Transport by Plumbagin

Transport rates of Gly-(¹⁴C)Phe after preincubation for up to 3 h in 0.2 mM Plumbagin. Values are expressed as percentages of untreated controls.

incubations with plumbagin were performed may not have been optimal for its action. Furthermore, plumbagin is only poorly soluble in aqueous solution, which might have had some bearing on its inability to achieve high levels of inhibition.

4.7 Effect of 1,5-Dimethylaminonaphthalene-sulphoethyleneimide (N-Dansylaziridine) on Transport Activity

4.7.1 Introduction - Chemical Properties

Since thiol groups clearly play an important role in peptide transport by the scutellum, it was considered of some interest to attempt to "visualise" under the microscope the thiol binding sites, by using sulphydryl reagents with a coloured or fluorescent moiety. Several fluorophores have been synthesised for the specific labelling of sulphydryl ligands. Of these reagents, some are autofluorescent, such as N-(1-pyrene) maleimide (Wu *et al.*, 1976; Rasched & Bayne, 1982), and N-dansylaziridine (Scouten *et al.*, 1974), whereas others such as N-(*p*-(2-benzoxazolyl)phenyl) maleimide (Kanaoka *et al.*, 1967, 1968) and N-(1-anilinonaphthyl-4) maleimide (Kanaoka *et al.*, 1973) are only fluorescent after reaction with cysteinyl residues. Of these, only N-dansylaziridine (NDA) is commercially available, and so was chosen for use. The reaction of NDA with sulphydryl groups is selective at pH 6.5 - 8.6, but the rate of derivatization is much slower than that, for example, with NEM (Staples & Reithel, 1976), and incubation times of several hours may be required for complete modification with most proteins (Scouten *et al.*, 1974; Mutus *et al.*, 1981). The largely alkyl and aryl nature of NDA will confer hydrophobicity on the molecule, yet its size may preclude rapid permeation through a membrane barrier. Hence, in this respect, the properties of NDA may be considered as intermediate between those of NEM (highly penetrant) and PCMBBS (non penetrant, hydrophilic). Supporting evidence for this is provided by fluorescence spectra which show that the fluorescent moieties of NDA-labelled proteins within the erythrocyte membrane are surrounded by a hydrophobic environment (Borochoy & Shinitzky, 1976), yet the same report indicates that NDA is poorly soluble in (artificial) bilayers. Therefore

it is likely that, at least in the erythrocyte, some derivatization of thiols within the membrane can occur.

4.7.2 Methods

2-3 day scutella were excised and stored in buffer (2 ml sodium phosphate-citrate, pH 8 containing 20 μ l ethanol, 25°C) for 0 - 4 h prior to transfer and incubation in 0.35 mM NDA (1 ml of sodium phosphate-citrate buffer pH 8, containing 10 μ l of an ethanolic solution of NDA, 10 mg ml⁻¹, 25°C) for up to 4 h. Storage time plus modification time totalled 4 h in each case and finished simultaneously, hence ensuring that all scutella experienced the same exposure to an ethanolic solution. Thus, controls were suspended for 4 h in ethanolic buffer only. After all preincubations, scutella were removed, washed and subjected to standard transport assays using radioactive Gly-Phe, Leu and glucose.

4.7.3 Results

The time-dependent inactivation of Gly-Phe, Leu and glucose transport by NDA is shown in Figure 4.8. Controls in which scutella were suspended in ethanol alone (without NDA) for 4 h, indicated that the alcohol itself had an inhibitory effect over this prolonged preincubation period and reduced transport of Gly-Phe, Leu and glucose by 36%, 40% and 31% respectively. Therefore, suitable corrections were made to the plotted values to allow for the effects of ethanol. Peptide transport is selectively inhibited, but as anticipated, the rate of inactivation is slower than that achieved by NEM or PCMBs.

4.7.4 Discussion

The use of N-dansylaziridine confirms that peptide transport is particularly sensitive to sulphydryl reagents, and potentially, the use of this fluorophore offers an opportunity to selectively label the transport proteins with a view to their extraction and separation, or else their visualization in situ using fluorescence microscopy. These

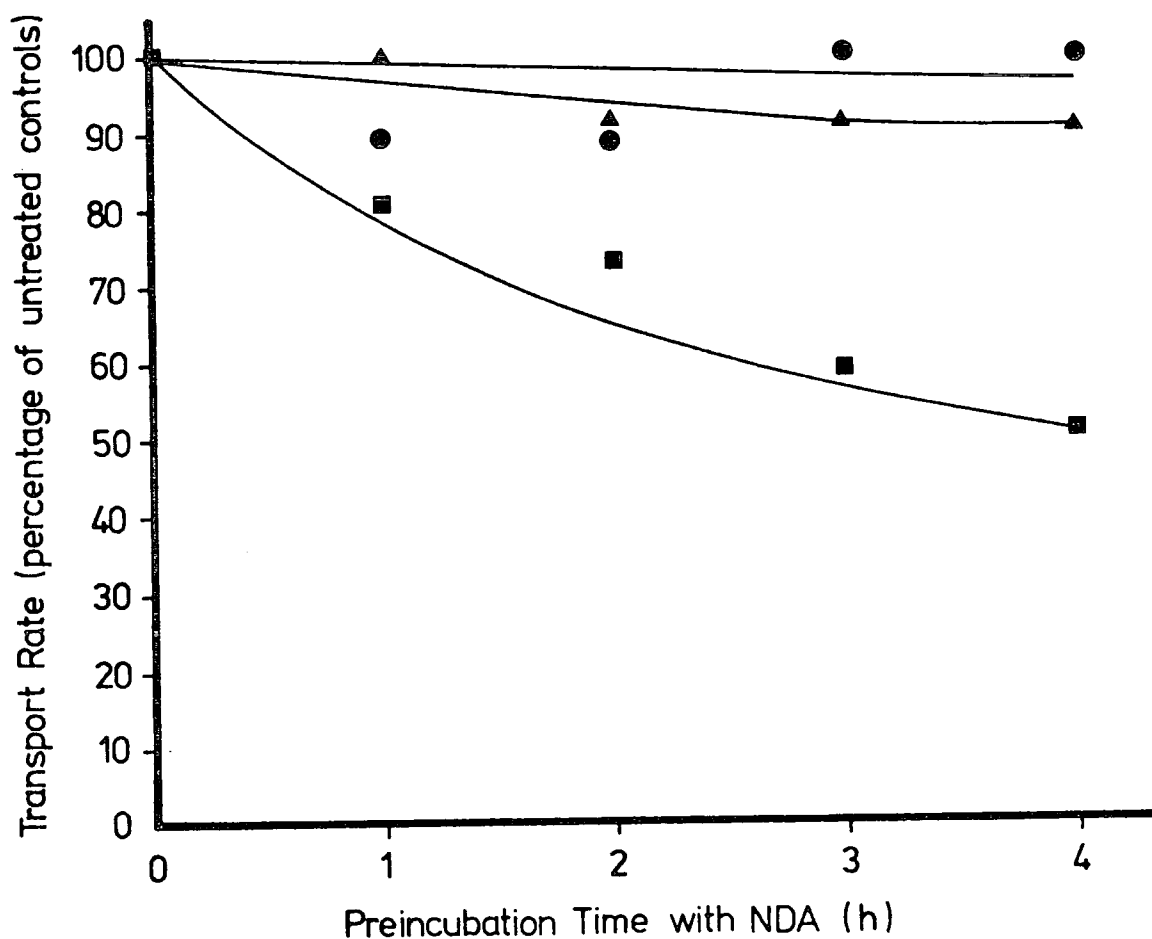


FIGURE 4.8 Kinetics of Inhibition of Gly-Phe, Leu and Glucose Transport by N-Dansylaziridine

Uptake of Gly-(U¹⁴C)Phe (■), (U¹⁴C)Leu (●), and (U¹⁴C)Glucose (▲) after treatment with 0.35 mM N-dansylaziridine for up to 4 h. Rates are expressed as percentages of untreated controls.

possibilities are explored elsewhere (Section 5.1.7).

Substrate protection of NDA inhibition could not be demonstrated (results not shown), but because of the long incubation times involved, such experiments are considered to be unsuitable and largely invalid. This is because substrate screening is essentially a dynamic competition process for access to susceptible sites on the protein, and hence is only likely to be detectable after short periods of time when the protectant can successfully delay group derivatization to a measurable extent.

4.8 Effect of N-Ethyl-5-Phenylisoxazolium-3'-Sulphonate (Woodward's Reagent K) on Transport Activity

4.8.1 Introduction - Chemical Properties

In addition to the several sulphydryl reagents discussed above, other compounds capable of the group-selective modification of different amino acid residues were tested for the inhibition of solute transport by the scutellum.

For example, isoxazolium salts, including Woodward's reagent K (WRK), were originally developed as activators for carboxylic acids in the chemical synthesis of peptides (Woodward & Olofson 1966; Woodward *et al.*, 1966), but their use has since been extended to protein modification studies. WRK reacts specifically with carboxyl groups to form an enol ester derivative over a wide pH range (Bodlaender *et al.*, 1969). Although the penetrability of this reagent across a membrane barrier does not appear to have been discussed in the literature, it is likely that the aromatic nature of WRK will confer some solubility in the lipid bilayer despite the negatively charged sulphonate group which is present on the molecule. WRK has been used successfully in establishing the existence of catalytically essential carboxylate groups in a variety of enzymes, including bovine carboxy-peptidase (Petra, 1971; Petra & Neurath, 1971), yeast phosphoglycerate kinase (Brake & Weber, 1974), chloroplastic

ATPase (Arana & Vallejos, 1980) and ferredoxin-NADP⁺ oxidoreductase (Carrillo et al., 1981).

4.8.2 Methods

2-3 day scutella were preincubated in WRK (5-10 mM in sodium phosphate-citrate buffer, pH 4 for 30 min at 20°C), washed and then assayed for uptake of radioactive Leu, Gly-Phe and glucose (all at 2 mM) in the routine way.

4.8.3 Results

WRK, at the concentrations used (up to 10 mM) did not affect the uptake of any substrate tested, relative to untreated controls.

4.8.4 Discussion

The importance of carboxyl groups to uptake by the barley scutellum cannot be demonstrated here, although it might not be unreasonable that the β and γ side-chain carboxyl groups of aspartate and glutamate residues respectively, that both have pK values of about 4, might be involved in the low pH optima displayed by amino acid and peptide transport. However, the inhibition of uptake by DCCD (Section 6.1.3.4) implies the indirect involvement of carboxyl groups via ATP action, and it must be assumed the accessibility of WRK to these residues is limited.

4.9 Effect of Diethylpyrocarbonate on Transport Activity

4.9.1 Introduction - Chemical Properties

Other amino acid side chains are susceptible to selective derivatization, including the imidazole ring of histidine. Studies were undertaken to establish the role of this residue also in uptake by the scutellum.

Currently, two main techniques are available for the modification of histidine residues. One methodology involves the photo-oxidation of histidine, after first sensitizing the residue with the dye Rose Bengal (Westhead, 1965; Tsai et al., 1982); the other employs diethylpyrocarbonate (ethoxyformic anhydride), as a modifying reagent.

Diethylpyrocarbonate (DEPC) was chosen to evaluate the role of histidine in scutellar transport because of its greater selectivity. Its use in protein chemistry has been the subject of a recent review (Miles, 1977). DEPC will modify the unprotonated form of a range of nucleophilic amino acid side-chains; hence, at alkaline pH, sulphhydryl, guanidyl, tyrosyl and amino moieties are easily derivatized (Burch & Ticku, 1981). However at pH 6-7 the reagent is fairly specific for the imidazole ring of histidine, and forms an N-ethyloxyformyl-histidine complex in a reaction that can be followed spectrophotometrically (Roosemont, 1978). Excess DEPC is hydrolysed to carbon dioxide and water with a half life of 24 min at 25°C. N-ethyloxyformyl-histidine is of limited stability (Melchior & Fahrney, 1970) and its breakdown to regenerate the amino acid is rapidly achieved by the addition of hydroxylamine (Padan et al., 1979).

The use of DEPC has indicated that histidine residues are vital to the activity of many enzymes, including mitochondrial succinate dehydrogenase (Vik & Hatefi, 1981), chloroplastic ribulose biphosphate carboxylase (Saluja & McFadden, 1982), fungal amylase (Kita et al., 1982) and succinyl-CoA synthetase from E.coli (Collier & Nishimura, 1979). DEPC has seen limited application to transport studies but uptake of lactose and of proline by E.coli vesicles was inhibited by the reagent (Padan et al., 1979).

4.9.2 Kinetics of Inhibition of Transport

4.9.2.1 Methods

DEPC was diluted with ethanol (50% v/v) and 5-20 µl of this were added to 5 ml sodium phosphate-citrate buffer (50 mM, pH 6) with vigorous stirring to give standard solutions in the concentration range 3.5 - 14 mM. These were always made up just prior to use. 1-3 day scutella were preincubated in a standard solution at 3.5 mM for up to 1 h at 2°C in an ice bath; because the reagent undergoes some hydrolysis in aqueous solution, those scutella which were treated for longer periods with



inhibitor were transferred to a fresh batch of DEPC after 30 min to continue their preincubation. As controls, scutella were suspended in buffer for 1 h with the addition of equivalent volumes of ethanol only.

After preincubation, scutella were removed, washed and assayed for uptake of radioactive Gly-Phe and Leu in the routine way.

4.9.2.2 Results

The time-dependent inhibition of Gly-Phe and Leu transport by DEPC is shown in Figure 4.9 . Both substrates have similar kinetics of inactivation in that preincubation times of up to 10 min with DEPC bring about a rather gradual reduction of transport activity in both cases, and some 30-40% of the uptake of untreated controls remains after 1 h treatment with the inhibitor.

4.9.3 Substrate Screening and DEPC Inhibition

4.9.3.1 Methods

2-3 day scutella were preincubated in 100 mM peptide or amino acid for 10 min and then transferred directly to DEPC (7 mM standard solution in sodium phosphate-citrate buffer pH 6, at 2⁰C) with added competitor (100 mM) for a further 30 min. Control tissue was treated with DEPC only under the same conditions. After washing, all scutella were assayed for Gly-Phe and Leu transport.

4.9.3.2 Results

The inactivation of radioactive Gly-Phe and Leu transport by DEPC in the presence of a range of competitors is shown in Table 4.6. Peptides of both the L and D configurations, but none of the amino acids tested, were apparently able to confer some protection against inhibition of Gly-Phe uptake. Conversely, Ala and Leu (both L and D forms) were ineffective as protectants of DEPC inhibition of Leu transport, whereas some protection with the peptides Gly-L-Phe and L-Ala-L-Ala was achieved.

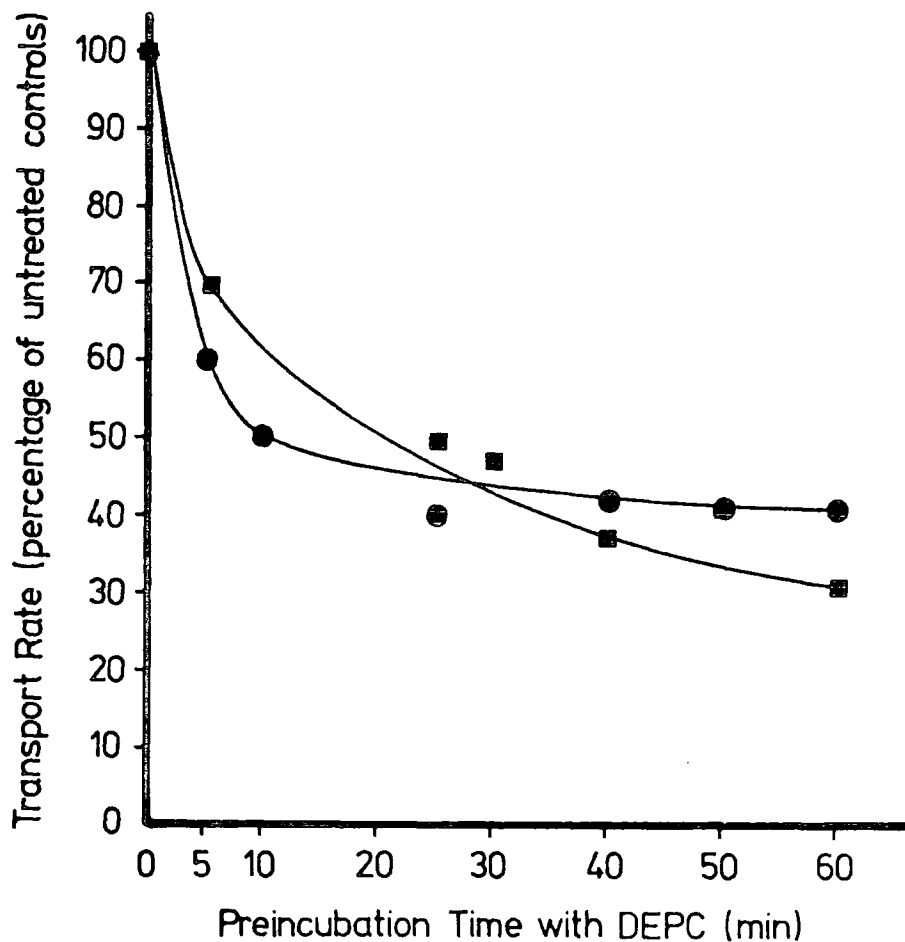


FIGURE 4.9 Kinetics of Inhibition of Gly-Phe and Leu Transport by Diethylpyrocarbonate

Uptake of Gly-(U¹⁴C)Phe (■) and (U¹⁴C)Leu (●) after treatment with 3.5 mM diethylpyrocarbonate for up to 60 min. Each value is the average of at least 2 separate determinations and are expressed as percentages of untreated controls.

TABLE 4.6

Substrate Screening and Diethylpyrocarbonate Inhibition

<u>Protectant</u>	<u>Gly-Phe Transport (Percentage of Untreated Controls)</u>
None	25
D-Ala-D-Ala	69
L-Ala-L-Ala	58
L-Ala-L-Ala-L-Ala	63
D-Ala-D-Ala-D-Ala	52
L-Leu	22
L-Ala	28
<u>Protectant</u>	<u>Leu Transport (Percentage of Untreated Controls)</u>
None	32
L-Ala	30
D-Ala	26
L-Leu	22
D-Leu	26
Gly-L-Phe	62
L-Ala-L-Ala	56

Transport of Gly-(U¹⁴C)Phe and (U¹⁴C)Leu (expressed as a percentage of untreated controls) by 2-3 day scutella after preincubation in DEPC (7 mM, for 30 min, in sodium phosphate-citrate buffer, pH 6 at 2^oC) with or without added peptide or amino acid competitor (100 mM) .

4.9.4 Discussion

Firm statements cannot be made about the direct role of histidine residues in Gly-Phe or Leu absorption by the barley scutellum, in that DEPC does not appear to be discriminatory in its inhibition of peptide versus amino acid uptake, and inactivation of transport in both cases is neither rapid nor complete.

Substrate screening experiments give rise to results which are difficult to explain biologically. It would not be predicted, for example, that unnatural D-peptide stereoisomers could confer protection against the inhibition of Gly-Phe uptake. Moreover, peptides can apparently cross-compete with the DEPC inhibition of amino acid transport, but not vice-versa. As a possible explanation, a chemical interaction between peptides (both D and L forms), but not amino acids, and DEPC would lead to an effective reduction in concentration of the inhibitor, and give rise to elevated uptake rates. Hence the effects of incubating peptide with DEPC could be readily misinterpreted as a successful substrate screening of DEPC inhibition. To test this hypothesis, preliminary experiments were performed in which DEPC (7 mM, pH 6, 2⁰C) was incubated with Ala-Ala (100 mM) for up to 1 h. Samples of this mix were taken at 15 min intervals and used to treat 2 day scutella (30 min at 2⁰C), prior to a standard radioactive Gly-Phe transport assay. In this way, any progressive inactivation of the inhibitory properties of DEPC arising from an interaction with the peptide could be monitored. However, results indicated that no such decrease in the effective concentration of DEPC occurred after preincubation with peptide. Clearly, a greater knowledge of the somewhat complicated chemistry of the reagent is needed before a deeper understanding of these anomalous substrate screening studies can be acquired, and for the time being the protection experiments described here cannot be satisfactorily explained.

4.10 Effect of Phenylglyoxal on Transport Activity

4.10.1 Introduction - Chemical Properties

The modification of arginine residues was also performed as part of the exploratory studies on the nature of the possible groups involved in transport by the scutellum. Arginine residues are considered to be especially important to enzymic function, because the guanidyl group carries a positive charge at most physiological pH values and specific roles have been assigned to arginine in proteins by virtue of this cationic nature. For example, it is often localized at the binding site of enzymes whose substrates or cofactors bear a negative charge. Thus, alkaline phosphatase from E.Coli (Daemen & Riordan, 1974) and molluscan choline acetyltransferase (Mautner et al., 1971) have essential arginine residues at the phosphate and CoA binding sites respectively, both of these species being anionic. Arginine residues may also be numerous on the surface of an enzyme to promote its solubility, e.g. egg white lysosyme (Patthy & Smith, 1975).

Several highly selective reagents are available for the covalent derivatization of the arginyl guanido group. These include 1,2, cyclohexanedione (Toi et al., 1967), butanedione (Riordan, 1973), camphorquinone-10-sulphonic acid (Pande et al., 1980) and phenylglyoxal (Takahashi, 1968). Derivatives of phenylglyoxal have also been developed, e.g. ρ -hydroxyphenylglyoxal, which has a greater solubility in water (Yamasaki et al., 1980), and ρ -nitrophenylglyoxal, which allows a colorimetric determination of arginine residues in a protein to be performed (Yamasaki et al., 1981).

Phenylglyoxal (PG) has been used widely by others, is commercially available, and thus has been used in these studies in an attempt to assess the possible role of arginine residues within the scutellar plasmalemma to transport processes. One guanidyl group reacts with two molecules of phenylglyoxal at pH 7-8 to give a complex ring structure in a reaction

that can be reversed at elevated temperatures (80% decomposed at pH7, 40°C, after 48 h (Takahaski, 1968)). The derivatization is highly specific, although some interaction with amino groups may occur (Takahaski, 1968).

4.10.2 Kinetics of Inhibition of Transport

4.10.2.1 Methods

2-3 day scutella were excised and preincubated in phenylglyoxal (10 mM in 50 mM sodium phosphate-citrate buffer, pH 7.8 at 20°C) for up to 1 h, then washed with distilled water and assayed for transport of radioactive Leu, Gly-Phe and glucose. Control scutella were preincubated in buffer alone before assay.

4.10.2.2 Results

The inhibition of Gly-Phe and Leu transport after preincubation with PG for up to 1 h is shown in Figure 4.10; uptake is expressed as a percentage of the rate of untreated controls. Although the initial kinetics of inhibition are similar for the amino acid and peptide, almost complete inactivation of Gly-Phe uptake is achieved after 1 h preincubation with PG, whereas 50% of Leu transport activity remains after this time. A separate experiment revealed that glucose transport (from a 2 mM solution) was inhibited by 40%, relative to untreated controls, by preincubation in 10 mM PG for 1 h.

4.10.3 Substrate Screening and Phenylglyoxal Inhibition

4.10.3.1 Methods

Scutella were preincubated in amino acid or peptide protectant (100 mM, all of the L configuration) for 10 min, then transferred to PG (10 mM at pH 7.8) in the further presence of protectant (at 100 mM) for 30 min at 20°C. Control scutella were treated with PG under identical conditions but without the presence of competitor. After treatment, scutella were washed and assayed for transport of radioactive Leu and Gly-Phe.

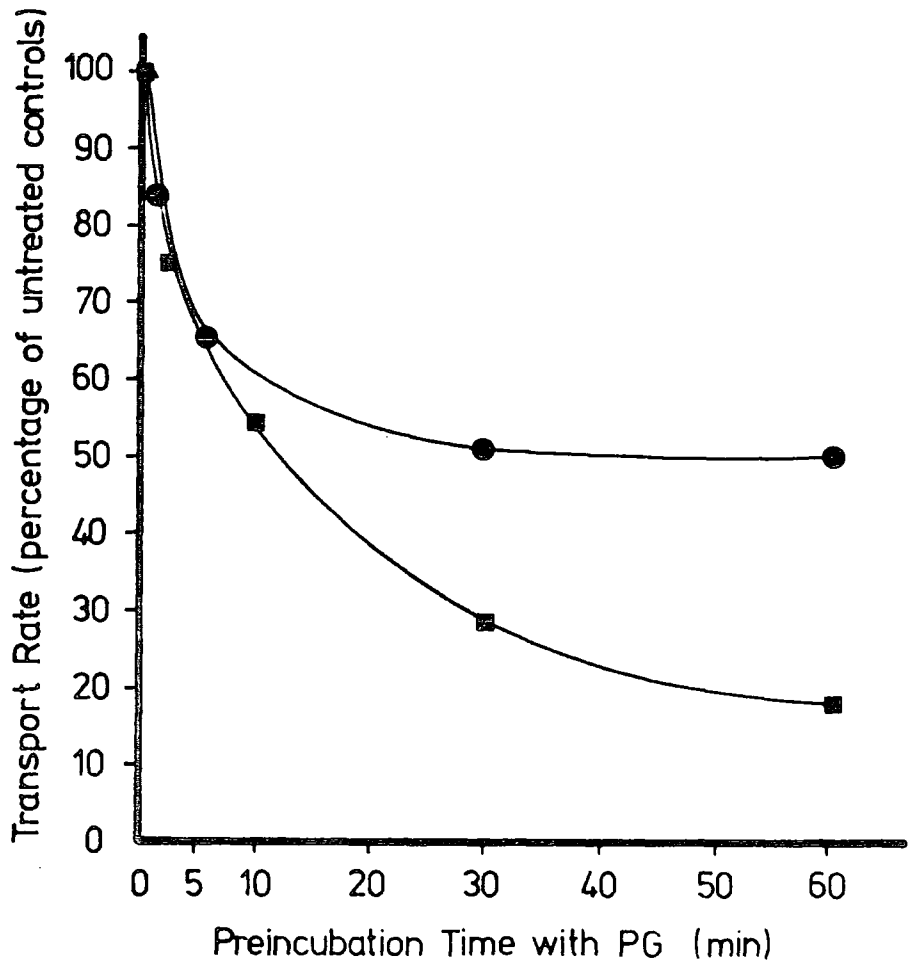


FIGURE 4.10 Kinetics of Inhibition of Gly-Phe and Leu Transport by Phenylglyoxal

Uptake of Gly-(U¹⁴C)Phe (■), and (U¹⁴C)Leu (●) by 2-3 day scutella after treatment with 10 mM phenylglyoxal for up to 60 min. Values are expressed as percentages of untreated controls.

4.10.3.2 Results

Under these conditions, the protectants Ala-Ala, Lys-Lys, Gly-Leu or Met-Ala could not protect against the inhibition of peptide transport brought about by PG. Similarly, Ala, Val and Leu were also ineffective in protecting against PG inhibition of Leu transport.

4.10.4 Discussion

The kinetics of inhibition of transport would indicate that arginine residue(s) may be important in peptide uptake, but substrate protection experiments offer no evidence for the location of the residue(s) at the binding or active site of the carrier protein. However, in order to achieve enough inhibition (relative to untreated controls), such that any protection effect could be detected, it is necessary to preincubate the phenylglyoxal with protectant for long periods of time (30 min). As argued for N-dansylaziridine (Section 4.7.4) a susceptible group will ultimately be modified if left exposed for long enough to an inhibitor irrespective of the presence of competitor, and so it is difficult to be absolutely confident about these results.

The incomplete inactivation of amino acid transport by PG is consistent with the existence of at least two transporters of leucine such that one system is inhibited rapidly, but others are left unaffected and still operative. Little is known of the number and nature of amino acid transport systems in barley scutella, but it is clear from these inhibition data that there is scope here for further study. Although protection experiments could not demonstrate competition between PG and substrate for the same reactive site on the protein, it is nevertheless possible that cationic arginine residues might play a role in the electrostatic binding of peptides or amino acids that carry a negatively charged carboxyl grouping.

CHAPTER 5

FURTHER STUDIES CONCERNING THE SPECIFIC ROLE OF SULPHYDRYL GROUPS IN PEPTIDE TRANSPORT BY THE SCUTELLUM

5.1 Specific Radioactive Labelling of Peptide Transport Proteins

5.1.1 Introduction

The results presented in Chapter 4 give strong indications that the use of thiol reagents offers an opportunity for the selective inactivation of peptide uptake in barley. Further evidence is described below in support of a specific role for sulphhydryl groups in peptide transport, and inhibition of uptake with arsenical and mercurial reagents is shown to be reversible, thus permitting a methodology for the specific labelling of the peptide transport proteins themselves with radioactively labelled NEM.

5.1.2 Reversible Inhibition of Peptide Transport

5.1.2.1 Methods

2-3 day scutella were treated with phenylarsine oxide (0.5 mM for 4 min at 20°C, in sodium phosphate-citrate buffer pH 3.8) or p-chloromercuribenzenesulphonic acid (4 mM for 30 min at 20°C, in sodium phosphate-citrate buffer, pH 5). After washing with distilled water, scutella were transferred to dithiothreitol (10 mM, pH 3.8 at 20°C) for periods of up to 1 h before assaying for uptake of radioactive Gly-Phe and Leu in the routine manner. Transport rates are expressed as a percentage of the rates shown by controls taken through the same incubation but without added inhibitors.

5.1.2.2 Results

The inhibition of peptide transport brought about by PCMBs or PAO (leaving only 15-30% of the transport activity of untreated controls) is reversible by subsequent treatment with dithiothreitol which can regenerate free SH groups (Figure 5.1). Thus, after 1 h in DTT, the restoration of Gly-Phe transport is essentially complete (95-100% of that shown by control scutella). In contrast however, DTT is unable to restore the much smaller reduction in leucine transport brought about by PAO or PCMBs (see inset). Controls, performed here and elsewhere

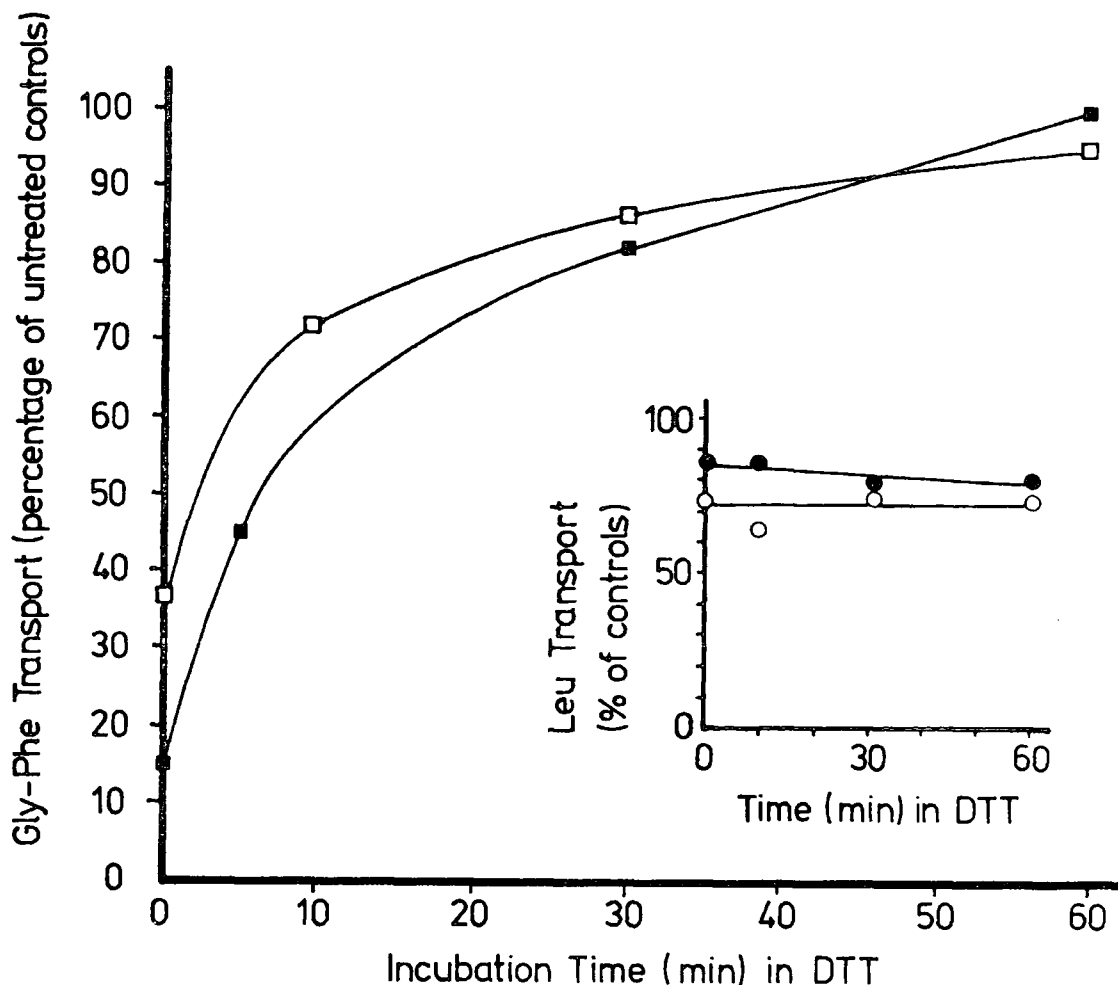


FIGURE 5.1 Effect of Dithiothreitol on the Inhibition of Gly-Phe and Leu Transport Resulting from PAO or PCMBs Treatment

Two day scutella were treated with 0.5 mM phenylarsine oxide for 4 min (□), or 4 mM *p*-chloromercuribenzenesulphonic acid for 30 min (■), washed, and then incubated in 10 mM dithiothreitol for the indicated times before assaying for transport of Gly-(U¹⁴C)Phe. Inset: Scutella were treated with 0.5 mM PAO for 30 min (○), or 4 mM PCMBs (●) for 30 min, washed, and incubated in 10 mM DTT for the indicated times before assaying for transport of (U¹⁴C)Leu. Rates are expressed as a percentage of those shown by scutella taken through the same incubations but without added inhibitors.

(Section 4.5), show that preincubation in DTT alone has no effect upon the uptake of any solute tested.

5.1.2.3 Discussion

Presumably, degradation of the mercaptide or thioarsinite complex (formed by the reaction of sulphhydryl groupings with PCMBS and PAO respectively) using dithiothreitol, re-exposes the original, reduced thiol ligands and concomitantly restores peptide transport activity. The inability to reverse the inhibition of leucine transport would suggest that the effects of PAO and PCMBS in this case are probably of a more indirect nature. These observations offer more support for the occurrence of a dithiol-disulphide conversion playing a central role in peptide uptake.

The reversal of a transport inhibition, induced by mercurials or arsenicals, with low molecular weight sulphhydryls such as dithiothreitol (or its isomer dierythritol) has been reported in various other systems (Nelson et al., 1975; Giaquinta, 1976; Kaback & Patel, 1978; Klip et al., 1979; Lucas & Alexander, 1980), and it is not unreasonable to suggest that, if tested, reducing agents such as these would be effective in most transport processes which are claimed to be directly sensitive to reagents such as PCMBS or PAO.

5.1.3 Protection of Peptide Transport - Dependent Vicinal Dithiols by Phenylarsine Oxide Against NEM Modification

5.1.3.1 Introduction

At the present time, phenylarsine oxide is not available commercially in radioactive form, and so it was decided to develop an alternative procedure to try and selectively radioactively label the vicinal dithiols (including those involved in peptide transport). It was considered that this could be achieved by first masking all vicinal dithiols with phenylarsine oxide, modifying the remaining monothiols irreversibly with NEM, and then re-exposing the vicinal ligands using dithiothreitol to allow their subsequent radioactive labelling with

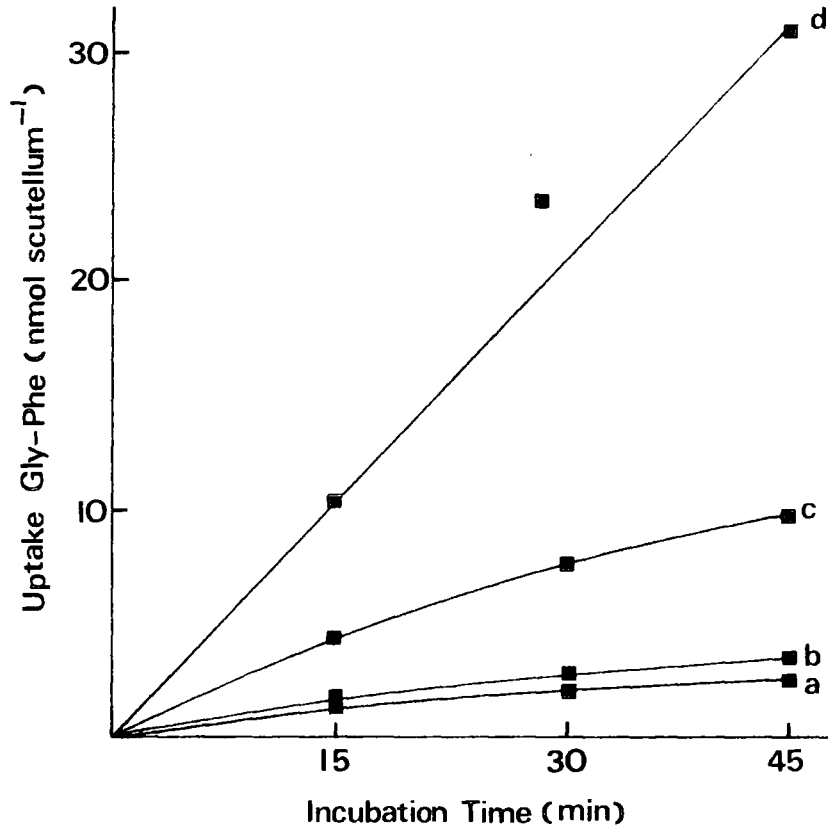


FIGURE 5.2 Protection of Peptide Transport - Dependent Vicinal Dithiols with Phenylarsine Oxide against NEM Modification

Curves represent the uptake of Gly-(U¹⁴C)Phe with time by scutella subjected to the following treatments: phenylarsine oxide, N-ethylmaleimide (a); N-ethylmaleimide, dithiothreitol (b); phenylarsine oxide, N-ethylmaleimide, dithiothreitol (c); control incubated in buffer only (d). Treatment conditions are as described in Section 5.1.3.2.

(^{14}C)-NEM. The success of this methodology was evaluated by monitoring the peptide transport capability of scutella at each stage in the procedure.

5.1.3.2 Methods

2-3 day scutella were treated in four different ways with the following reagents in the order given:

1. Phenylarsine oxide, N-ethylmaleimide, dithiothreitol
2. N-Ethylmaleimide, dithiothreitol
3. Phenylarsine oxide, N-ethylmaleimide
4. Control incubation (sodium phosphate-citrate buffer, pH 3.8
20°C for 1 h)

All preincubations with phenylarsine oxide were at 0.5 mM, pH 3.8 for 4 min, 20°C; with NEM at 5 mM, pH 6.8 for 3 min, 20°C; and with dithiothreitol at 10 mM, pH 3.8 for 1 h, 20°C. Scutella were washed thoroughly between treatments with distilled water and transport of radioactively labelled Gly-Phe then assayed in each case in the routine manner.

5.1.3.3 Results

Peptide transport by scutella subjected to the various treatments is shown in Figure 5.2. Almost complete inactivation of Gly-Phe transport was achieved by combined treatment of scutella with PAO and NEM (curve a), and NEM inhibition alone was not reversible by dithiothreitol (curve b). However, complexing vicinal sites with PAO could protect against subsequent NEM modification, such that some transport activity was demonstrable after the vicinal thiols were re-exposed with dithiothreitol (curve c). Uptake shown by the untreated controls is shown in curve d.

5.1.4 Specific Radioactive Labelling of Vicinal Dithiols

5.1.4.1 Introduction

A quantitative comparison was made between the extent of incorporation of radioactively labelled NEM both into tissue 'masked' with phenylarsine

oxide as described above, and with scutella simply left unprotected from NEM modification. In this way an estimate could be made of the amount of labelled vicinal dithiol, and hence the feasibility of its extraction and further characterization.

5.1.4.2 Methods

1. Labelling of Scutella

2 day scutella were treated with 0.5 mM phenylarsine oxide (pH 3.8, 20°C for 10 min), washed, and transferred to 5 mM N-ethylmaleimide (pH 6.8, 20°C for 10 min). After washing, scutella were then incubated in dithiothreitol 10 mM (pH 3.8, 20°C for 1 h), before labelling with N-ethyl(2,3-¹⁴C) maleimide (5 mM, 2 $\mu\text{Ci } \mu\text{mol}^{-1}$, at 20°C at pH 6.8, 2.5 min). As controls, scutella were treated identically to the above except that initially, instead of incubation with phenylarsine oxide, tissue was suspended in buffer without added inhibitor.

2. Estimation of Bound and Soluble Radioactivity

To estimate the amount of soluble radioactivity within the scutella (i.e. unbound (¹⁴C)-NEM), 2-4 treated scutella were extracted in acetic acid (500 μl of 5M) on a heated water bath for 30 min. Acetate has been shown to be an efficient extractant of unbound N-ethylmaleimide (Section 5.2.3). The acetate extract was added to scintillant and assayed for radioactivity on a scintillation counter in the routine manner. To estimate the residual insoluble radioactivity (including all protein-bound (¹⁴C)-NEM), or else the total radioactive content (bound plus soluble), tissue was left overnight to dissolve completely in tissue solubilizer ('Soluene', Packard Instruments, 500 μl), which was then added to scintillant and assayed for counts also. Suitable controls, in which known amounts of radioactivity were added to equivalent volumes of solubilizer, indicated that the 'Soluene' itself did not have a significant quenching effect.

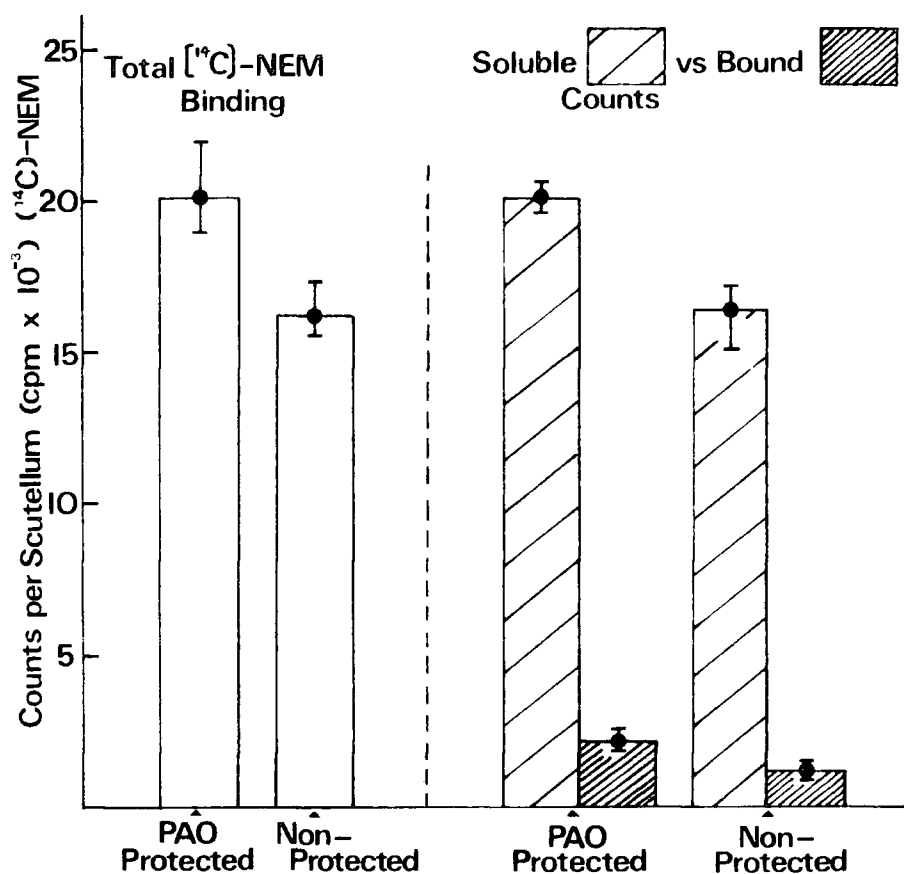


FIGURE 5.3 Specific Radioactive Labelling of Peptide Transport - Dependent Vicinal Dithiols

Bound radioactive NEM (▨) associated with scutella (after removal of all soluble radioactivity with 5M acetic acid (▧) with or without prior treatment with phenylarsine oxide as described in Section 5.1.4. The difference in bound counts between PAO-protected and non-protected scutella can be taken to be specific radioactive binding to vicinal dithiols alone. Values represent the mean and range of three determinations.

5.1.4.3 Results

The bound and soluble counts associated with the treated scutella are represented in a histogram (Figure 5.3). Both phenylarsine oxide-treated and untreated tissue contain large amounts of soluble radioactivity, and smaller amounts of bound label. The small, yet reproducible difference in this bound radioactivity can be taken as representing, specifically, vicinal dithiol groupings which can be protected from NEM modification with phenylarsine oxide.

5.1.4.4 Discussion

These data enable a rough estimate of the amount of peptide transport protein within the scutellum to be calculated (Appendix 3). Several assumptions are made, and therefore it must be stressed that the value obtained will be very approximate but nevertheless the amount quoted (10 μg per scutellum) is likely to be of the correct order of magnitude. Therefore, radioactively labelled vicinal dithiol is likely to be present in sufficient quantity to allow both its detection after extraction, and also its localization in situ within the scutellar epithelium by means of microautoradiography.

5.1.5 Kinetics of Binding of Radioactively Labelled NEM to Scutella

5.1.5.1 Methods

4 day scutella, of approximately the same surface area, were excised and treated in batches of four, with 0.5 mM phenylarsine oxide (sodium phosphate-citrate buffer, pH 3.8, 20°C) for 20 min. After thorough washing with distilled water, scutella were transferred to 5 mM N-ethyl(2,3- ^{14}C)maleimide ($2 \mu\text{Ci } \mu\text{mol}^{-1}$, pH 6.8 at 20°C) for periods of up to 30 min. As controls, scutella were preincubated in buffer only for 20 min, prior to treatment with radioactive NEM under the same conditions. After washing for 3 min with a flow of distilled water, the total radioactive content of treated scutella was determined by dissolving them to completion, in batches of four, in tissue solubilizer ("Solubene", 500 μl).

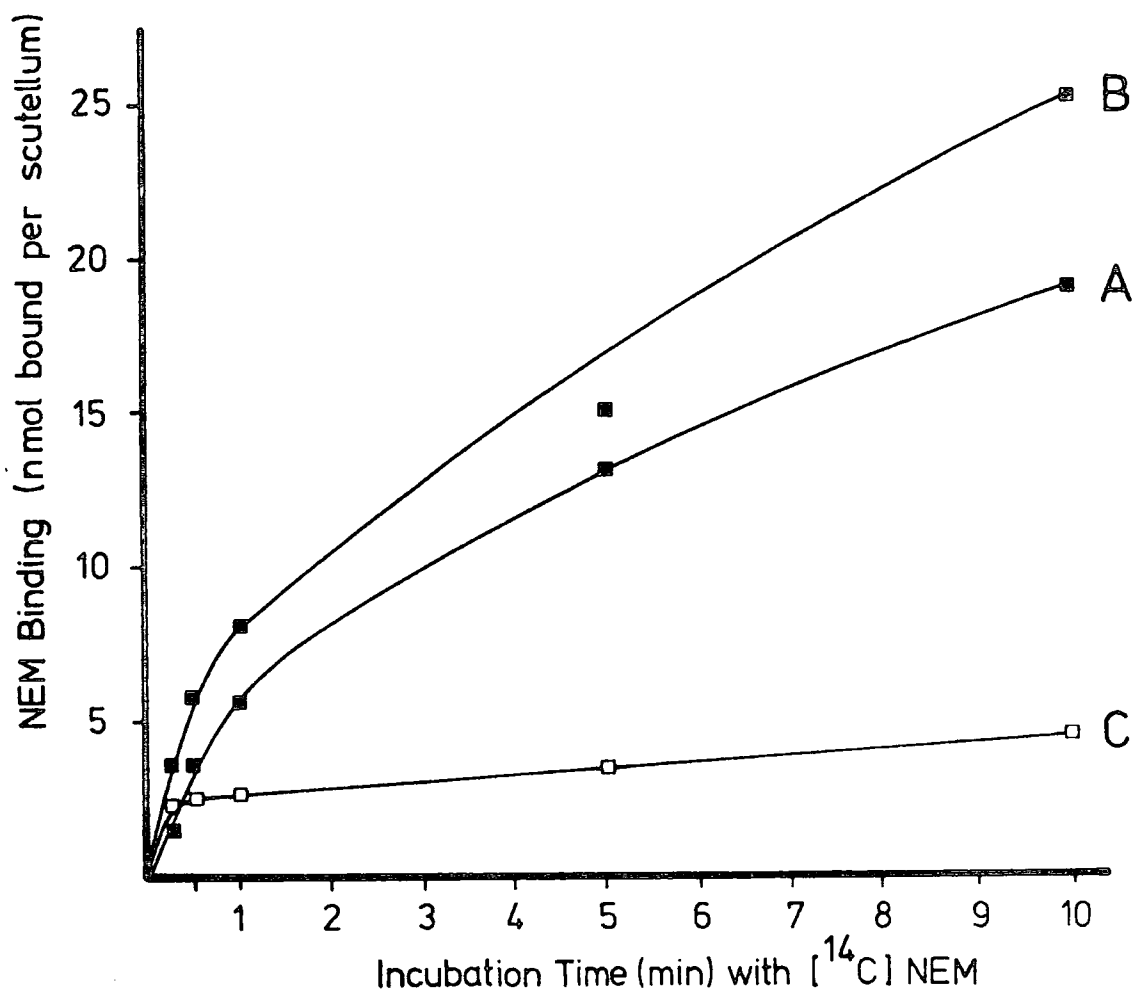


FIGURE 5.4 Kinetics of Binding of Radioactively Labelled NEM to Scutella

Curve A represents the time course of binding of radioactive N-ethylmaleimide to scutella pretreated with phenylarsine oxide (0.5 mM, 20 min); curve B represents the binding of (^{14}C)-NEM used at the same specific activity, to scutella preincubated in buffer only. Curve C represents the difference in these binding characteristics and can be taken to represent the association of (^{14}C)-NEM with vicinal dithiols only.

5.1.5.2 Results

Figure 5.4 shows the kinetics of binding of radioactively labelled NEM to scutella either after previous incubation in phenylarsine oxide (curve A), which protects vicinal dithiols from derivatization, or after previous suspension in buffer only (curve B), such that all SH groups are susceptible to modification. The reduction in radioactive labelling brought about by prior treatment with phenylarsine oxide is shown in curve C, and may be taken to represent binding of radioactive NEM to just the vicinal dithiols.

5.1.5.3 Discussion

The binding of NEM to vicinal dithiols (including those involved in peptide transport) is initially fast (up to 30s), suggesting that the sensitive sites are readily accessible at the membrane barrier. This rapid binding correlates well with the rapid kinetics of inhibition of peptide transport associated with this inhibitor (Figure 4.2). The slower rate of radioactive binding subsequently may be attributed to the modification of other less accessible dithiols e.g., those located intracellularly.

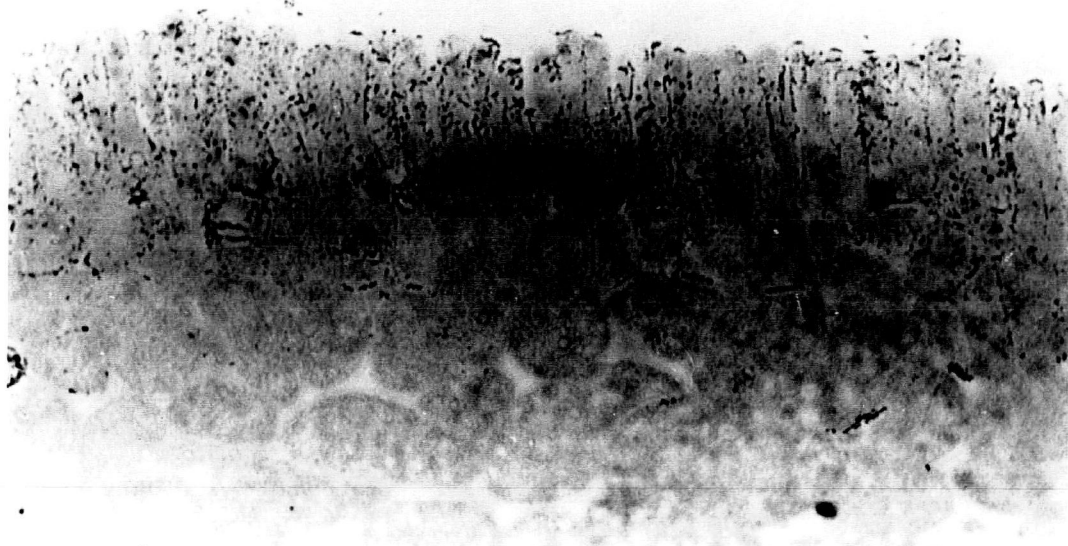
5.1.6 Autoradiographic Location of the Peptide Transport Proteins

5.1.6.1 Introduction

The specific modification of vicinal dithiols with radioactive NEM described in these studies offers the possibility that the exact location of the labelled peptide transport proteins may be visualised microscopically by means of microautoradiography within a thin section of scutellar tissue.

5.1.6.2 Methods

3 day scutella were excised and the vicinal dithiols specifically radioactively labelled as described in Section 5.1.4.2 except that N-ethyl(2,3-¹⁴C)maleimide was used at a higher specific activity of 5 $\mu\text{Ci } \mu\text{mol}^{-1}$. To remove all unbound, intracellular radioactivity,



scutella were treated with three changes of 5 mM acetate (1 ml at pH 3.8, 2 h in each, with thorough washing between changes); acetate at this concentration has been shown to be an efficient extractant of unbound N-ethylmaleimide (Section 5.2.3) without permanently destroying peptide transport activity (Section 6.1.3.1). Scutella were then fixed, sectioned and autoradiograms prepared as described previously (Section 2.6.3).

5.1.6.3 Results and Discussion

The specific localization of vicinal dithiols within the barley scutellum as shown by their modification with radioactive NEM is shown in Figure 5.5. The plasmalemmae of the epithelial cells show most radioactive labelling, but some radioactivity is also present in the cell membranes of the 2-3 underlying subepithelial layers. Although N-ethylmaleimide is a highly penetrant molecule, intracellular labelling e.g., of organellar membranes, is not significant.

5.1.7 Localization of the Peptide Transport Proteins Using a Fluorescence Label

5.1.7.1 Introduction

In principle, the specific radioactive labelling of the peptide transport-dependent, vicinal dithiols as described here should permit their specific labelling with the fluorescent label N-dansylaziridine in an analogous procedure to that described above using N-ethyl(2,3-¹⁴C)maleimide. It was then anticipated that the use of fluorescence microscopy could reveal the distribution of fluorescent label and confirm the results obtained using autoradiography.

5.1.7.2 Methods

3 day scutella were treated with 0.5 mM phenylarsine oxide (pH 3.8, 20°C for 10 min), then transferred to N-ethylmaleimide (pH 6.8, 20°C) for a further 10 min. After washing, vicinal dithiols were re-exposed by treatment with dithiothreitol (10 mM, pH 3.8, 25°C for 1 h). Scutella

were then modified with 0.35 mM N-dansylaziridine for 3 h as described in Section 4.7, before excess reagent was removed from the cell walls by several washes under vacuum in a small Buchner flask (air bubbles that come out of solution within the dead-space of the tissue under reduced pressure provide effective agitation within this 'unstirred' layer). Vertical sections of scutellar tissue 50-100 μM thick were then cut with a fresh razor blade. Sections were mounted in water under a coverslip, and examined by incident-light fluorescence microscopy (excitation wavelength 340 nm). As a control, sections of untreated scutella were examined also.

5.1.7.3 Results

Visualisation of the specific labelling of vicinal dithiols with the fluorophore N-dansylaziridine was unsuccessful due to a high background fluorescence within the tissue. The background fluorescence had two components; an auto-fluorescent property of the cell walls themselves, demonstrable in the untreated sections, and also residual (intracellular) N-dansylaziridine, which could not be removed by washing.

5.1.7.4 Discussion

The high autofluorescence of the cell walls of cereal scutella, which has also been reported elsewhere (Smart & O'Brien, 1979b), would seem to preclude the visual localization of a fluorescent label within the cell membranes. However, to maximise the possibilities of success in future studies, this background fluorescence could be reduced to a minimum by means of a modified procedure in which a different, more intensely fluorescent reagent was used to label thinner tissue sections, cut on a microtome. However, thin sections can only successfully be cut from embedded tissue, but it must be ensured first that the embedding medium itself is not fluorescent, and that it does not quench the fluorescence emitted by the embedded specimen.

5.1.8 Isolation of Radioactively Labelled Protein by Polyacrylamide Gel Electrophoresis

5.1.8.1 Introduction

Membrane-bound proteins, including most of those involved in transport, are usually firmly anchored within the bilayer, and are found in close association with a surrounding 'domain' of lipid molecules, such that their successful extraction into an aqueous medium, and their subsequent isolation, is often quite difficult. The most successful extraction strategy that is generally adopted involves the treatment of the membrane with a detergent solution, such that the lipid domain surrounding each membrane protein is replaced by an aggregation of detergent molecules. The proteins may reside within a micelle or a monolayer of detergent, but whatever form the association between the two takes, the proteins are effectively solubilized and can then be separated and characterized by electrophoretic techniques.

Several detergents are available commercially for membrane solubilization, and have been used in other studies with varying degrees of success. Of these, detergents of the 'Triton' series (isooctylphenoxy-polyethoxyethanol), in particular Triton X100, have probably seen the most use (e.g. Lim & Tadayyon, 1970; Scheule & Gaffney, 1981; LeMaire *et al.*, 1983), but others such as the alkyl glycosides (Baron & Thompson, 1975; Rosevear *et al.*, 1980; Gould *et al.*, 1981, Matsushita *et al.*, 1983), lithium diiodosalicylate (Marchesi & Andrews, 1971) and the cholic acid derivative 'CHAPS' (Simmonds *et al.*, 1980; Hjelmeland, 1980), may be equally effective.

Two of these detergents, Triton X100, and the alkyl glycoside, n-octyl β -D-glucopyranoside, were selected for use in a preliminary study in which an attempt was made to extract the peptide transport proteins specifically labelled with radioactive NEM as described in Section 5.1.4.2, and to separate and isolate them by means of polyacrylamide gel electrophoresis.

5.1.8.2 Methods

with (^{14}C)-NEM exactly as described in Section 5.1.4.2, except that the NEM was used at a higher activity of $5\ \mu\text{Ci ml}^{-1}$. All unbound, unreacted radioactivity was extracted by several changes in 5 mM acetate as described in Section 5.1.6.2. Scutella were then washed thoroughly and transferred to electrophoretic sample buffer (500 μl of 50 mM Tris/HCl, pH 6.8, with added sucrose, 10% w/v) containing either Triton X100, at a final concentration of 1% v/v, or elsen-octyl β -D- glucopyranoside, at a final concentration of 1% w/v. Scutella were extracted in these solutions on a shaking water bath for 3 h at 37°C . Samples of the extract were then centrifuged (12000g, 4 min) and 5 μl aliquots of the supernatant assayed for radioactivity.

Samples of extract (up to 60 μl) were then loaded onto non-dissociating polyacrylamide rod gels (7 x 0.5 cm, 3% w/v acrylamide stacking gel, 7% w/v acrylamide running gel) before performing electrophoresis with a continuous alkaline buffer (25 mM Tris, 200 mM glycine pH 8.3) according to the procedure of Laemmli (1970). Electrophoresis was carried out at 3 mA until the tracking dye (bromophenol blue) had migrated to within 3 mm of the gel base. In order to establish the location of any radioactively labelled species, gels were wrapped in aluminium foil, frozen in liquid air, and then sectioned transversely whilst frozen with a razor blade into approximately 30 sections of 2mm thickness. The gel sections were transferred to scintillation vials, dried overnight at 32°C , and solubilized to completion in 50 μl of 30% w/v hydrogen peroxide, after ensuring that the vials were tightly capped. Scintillation fluid (NE260) was then added to each vial, and each one assayed for radioactive content to allow a radioactive profile to be constructed along the gel.

In addition, the position of extracted proteins on the gels was visualized after staining overnight with Kenacid R250 (0.05% w/v, in methanol: acetic acid: water, 50:7.5:43 v/v/v), and destaining in methanol; acetic acid; water, 40:7.5:52.5 v/v/v.

As controls, 50 μ l samples of a solution of three standard proteins (cytochrome C, bovine serum albumin, and ovalbumin, all 1 mg ml⁻¹ in sample buffer) were electrophoresed under the same conditions, and visualized after Kenacid staining. A sample of radioactive NEM (1 μ Ci ml⁻¹) was also electrophoresed and localized by gel sectioning, solubilization, and scintillation counting.

5.1.8.3 Results

1. Triton Although Kenacid staining revealed that Triton X100 was an effective solubilizer of membrane protein, no radioactive peak was found on the gel, apart from an accumulation of counts at the location of the buffer front (as shown by the marker dye), which corresponded to the position of native NEM after electrophoresis.

2. n-Octyl β -D-glucopyranoside In addition to the presence of radioactivity corresponding to the position of (¹⁴C)-NEM, a single, additional radioactive peak was observed on the sectioned gel, which, by comparison with the positions of the protein standards, had an approximate molecular weight of 30,000.

5.1.8.4 Discussion

Although the studies described here are only of a very preliminary nature, a radioactively labelled protein can be extracted with octyl glucoside, and it is anticipated that further studies might involve both the purification and detailed characterization of the isolated protein, and confirmation of its identity as a component of the peptide transport system. However, it must be noted that in other preliminary experiments, an estimation of the total 'bound' radioactive content of scutella, before and after treatment with detergent (as determined by digestion with 'Solubene' tissue solubilizer) revealed that only some 8-11% of the bound radioactivity could be extracted by Triton or octyl glycoside, despite performing the extraction under rather more rigorous conditions (up to

10% detergent at 60°C). Clearly, further studies are required to optimise on the conditions under which the extraction is performed in order to extract a rather larger proportion of the total bound radioactivity.

5.2 The Role of Glutathione in Transport by the Barley Scutellum

5.2.1 Introduction

The results presented so far do not eliminate the possibility that an intracellular component, containing an NEM reactive sulphydryl group, is involved. In particular, the ubiquitous peptide γ -glutamyl-cysteinyl-glycine, glutathione, has been implicated in transport processes, and it was considered of interest to determine its importance to the scutellar system.

The occurrence and possible functions of glutathione, which can exist in reduced (GSH) or oxidised (GSSG) forms, has received most attention in animal cells and microorganisms (Meister, 1975; Meister & Tate, 1976). However, glutathione is also widespread in plants (Higgins & Payne, 1982; Rennenberg, 1982), where it has been assigned several roles. Its primary function is probably in the maintenance of reducing conditions within the cell necessary for enzymic activity (Gilbert, 1982), especially in the chloroplast (Schaedle & Bassham, 1977), where concentrations as high as 2 mM have been found. Similarly, in its capacity as a reductant, levels of GSH are known to increase in leaf tissue under conditions of frosting (De Kok & Oosterhuis, 1983) or atmospheric oxygen enrichment (Foster & Hess, 1980), and may serve to minimize the effects of harmful oxidation reactions which these stresses induce. In addition, a role for GSH in the long distance transport and storage of sulphur has been reported (Rennenberg *et al.*, 1979; Rennenberg & Bergmann, 1979). However, the implication of glutathione in active transport as part of the γ -glutamyl cycle has generated the greatest interest (Prusiner *et al.*, 1976; Meister, 1980), and although any involvement in the uptake of amino

acids in plants has yet to be demonstrated, several enzymes of the cycle have been isolated from plant material including γ -glutamyl transpeptidase (Thompson et al., 1964), and 5-oxo-prolinase (Rennenberger et al., 1980; Mazelis & Creveling, 1978). In a mechanism completely distinct from the γ -glutamyl cycle, GSH is also reported to be vital to potassium uptake and retention in E.coli (Meury et al., 1980).

Experiments were therefore performed to see if incubation with reduced or oxidised glutathione affected transport, and also to see whether following an NEM treatment, glutathione could have a restorative effect upon subsequent peptide transport by barley scutella (by theoretically replenishing a GSH pool and not by any effect upon modified protein residues). Experiments were also performed to find evidence for the production of an NEM-GSH adduct under the usual incubation conditions.

5.2.2 Effect of Glutathione Upon Peptide Transport by Scutella Treated with NEM

5.2.2.1 Methods

2-3 day scutella were excised and treated with NEM (5 mM at pH 6.8, for 3 min at 20°C) washed, and incubated with either GSH (250 mM) or GSSG (125 mM) (pH 3.8 for 2 h at 20°C) prior to a standard Gly-(U¹⁴)Phe transport assay. In control studies, scutella were pretreated with glutathione alone, or held in buffer for 2h prior to transport assays.

5.2.2.2 Results

Under the conditions studied, neither oxidised nor reduced glutathione could reverse the inhibition of transport caused by NEM (Table 5.1); furthermore, preincubation with GSH or GSSG alone actually reduced uptake of Gly-Phe by comparison with untreated controls.

5.2.3 Analysis of the Intracellular Reaction Products of NEM

5.2.3.1 Methods

1. Acetate extraction

2 day scutella were excised and treated with N-ethyl (2,3-¹⁴C)maleimide

TABLE 5.1

Effect of Glutathione on Inhibition of Peptide Transport by NEM

<u>Treatment</u>	<u>Transport Rate Gly-Phe (nmol scut⁻¹h⁻¹)</u>
Untreated control	26
GSSG Alone	14
NEM Alone	3.4
NEM, then GSSG	5.6
Untreated control	34
GSH Alone	18
NEM Alone	11
NEM, then GSH	8.2

Uptake of Gly-(U¹⁴C)Phe by 2-3 day scutella after incubation in 5 mM NEM (3 min, 20°C, pH 6.8) with or without a subsequent treatment with reduced glutathione (GSH, 250 mM, pH 3.8, for 2 h at 20°C) or oxidised glutathione (GSSG, 125 mM, pH 3.8 for 2 h at 20°C). Sodium phosphate-citrate buffer used throughout. Values are the mean of at least two determinations.

(5 mM, 2 $\mu\text{Ci } \mu\text{mol}^{-1}$ at pH 6.8, 20°C for 4 min) prior to washing thoroughly with distilled water. Scutella, in batches of four, were then extracted by simply suspending in buffered acetic acid (500 μl of 5 mM, 50 mM and 500 mM at pH 3.8) for periods of up to 2 h. Samples of acetate extracts were then added to scintillation fluid and counted for radioactivity. To determine residual ^{14}C content within the tissue after this primary acetate treatment, scutella were transferred to 5 M acetate (500 μl) in stoppered tubes, and boiled for 60 min. Aliquots of this extract were counted as before.

2. Analysis of acetate extract

25 scutella were treated with radioactive NEM, and extracted in aqueous 50 mM acetate (500 μl) for 1 h as described above. Because native NEM is volatile and does not chromatograph successfully on a thin layer plate, its presence in the extract was estimated as a NEM-cysteine adduct. Cysteine itself is not present to any significant extent in the soluble amino acid pool of barley scutellum (Higgins & Payne, 1981). Thus, 50 μl samples of extract were incubated for 30 min with cysteine (20 μl of 20 mM), then evaporated to dryness in vacuo and the residue resuspended in 10 μl H_2O . 5 μl samples were chromatographed on cellulose thin layer plates and sections assayed for radioactivity as described previously (Section 2.4.3.3). Known standards (5 μl of aqueous GSH, Cys, GSH-NEM and Cys-NEM adducts, all at 2 mM) were chromatographed alongside and visualized with ninhydrin. GSH-NEM and Cys-NEM adducts were prepared by incubating GSH or Cys (250 μl of 4 mM) with a large excess of NEM (250 μl of 50 mM) for 30 min at 20°C. A standard solution of N-ethyl (2,3- ^{14}C)maleamate, the hydrolysis product of NEM was also prepared by incubating N-ethyl(2,3- ^{14}C)maleimide (2.5 mM, 1 $\mu\text{Ci mmol}$) in 1M N-ethyl morpholine buffer, pH 8.2 for 24 h. Buffer was removed by evaporation in vacuo, the residue resuspended in water, and suitable volumes analysed chromatographically for radioactivity in the usual way.

5.2.3.2 Results

1. Acetate extraction

The extraction of radioactivity into acetate, from scutella pretreated with radioactive NEM, is shown as a function of time (Figure 5.6). All concentrations of acetate used (5 mM, 50 mM and 500 mM) were equally effective as extractants, and 80-90% of total extraction occurs within the first 30 min. After 2 h, the radioactivity remaining within the tissue probably represents an equilibrium concentration with the external medium, as well as, presumably, protein-bound (^{14}C)-NEM.

2. Analysis of acetate extract

The only radioactive species present in an acetate extract of treated scutella, as determined by comparison with standards, was N-ethylmaleamate, the hydrolysis product of NEM. In contrast, cysteine-NEM adduct was not detected, implying the absence of native NEM in the extract which would have reacted rapidly and completely with added cysteine (Webb, 1966a). Glutathione-NEM adduct was not found, although its amounts may lie beneath the lower limit of detection involved in the methodology, which in this case is a cellular concentration of approximately 100 μM .

5.2.4 Discussion

It is unlikely that glutathione is involved directly in the uptake of small molecules by barley scutellum, and hence the primary target of NEM is probably the transport protein(s) per se. The inhibitory effects of oxidised and reduced glutathione alone may be due to deleterious redox reactions affecting transport directly or indirectly. Although glutathione has not yet been found within the barley scutellum as an adduct with (^{14}C)-NEM, or previously by use of dansylation studies (Higgins, 1979), amounts may be too low for detection. Thus, vacuolar and cytoplasmic concentrations of GSH at 20-60 μM , which have been calculated for tobacco mesophyll cells (Rennenberg, 1982), would

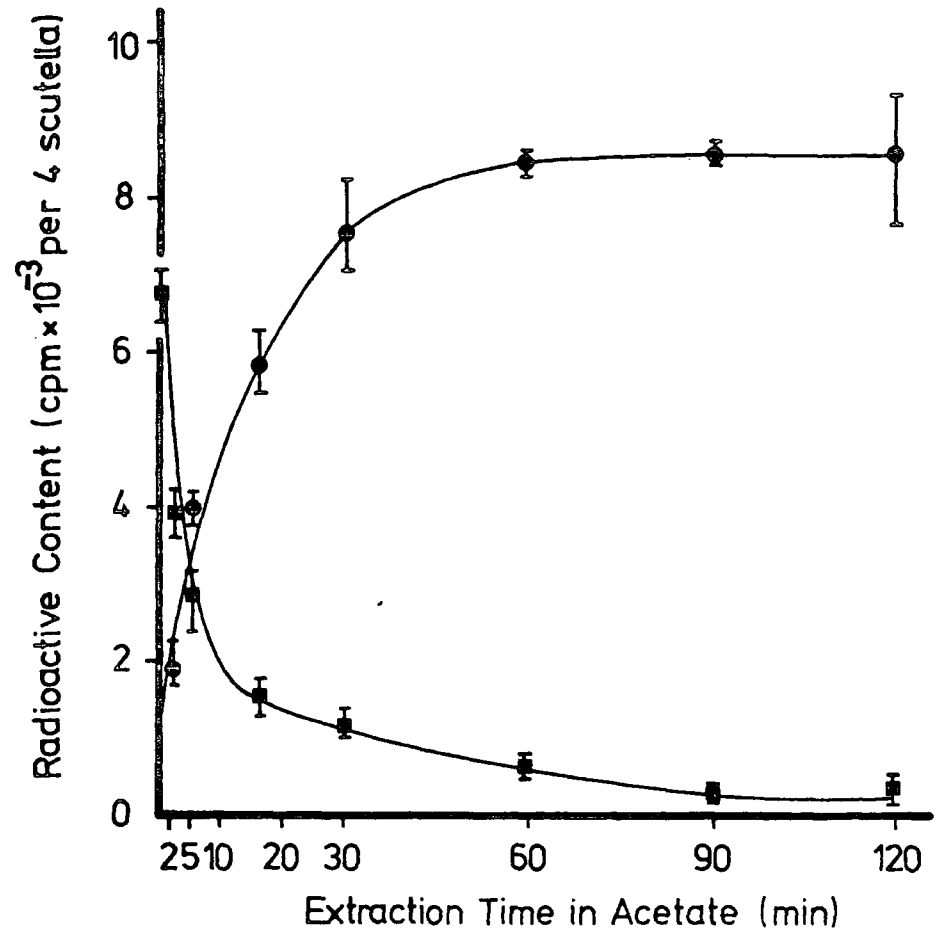


FIGURE 5.6 Extraction of Radioactive NEM from Scutella by Treatment with Acetate

Scutella were treated with (¹⁴C)NEM as described in Section 5.2.3.1 and then incubated with 5 mM, 50 mM or 500 mM acetate (at pH 3.8) for the indicated times before determining both the effluxed radioactivity present within the acetate (mean value indicated thus: ●) and residual radioactivity still remaining within the tissue (mean value indicated thus: ■). The range of values of effluxed and residual radioactivity obtained with all three concentrations of acetate is indicated by bars.

not be detected with the techniques used here, and only just detectable by using the dansylation technique (a minimal detectable concentration of about 30 μM).

The absence of unreacted, native N-ethylmaleimide in the acetate extract may be explained, at least in part, by an alkaline pH within the scutellum leading to hydrolysis of the reagent. An optimal activity at pH 8 is shown by several scutellar peptidases of barley (Sopanen & Mikola, 1975; Sopanen, 1976), and for maize scutellum an intracellular value of pH 8.5 was determined experimentally (Humphreys, 1982). However, an alkaline environment alone is probably insufficient to explain the complete hydrolysis observed and other, unknown factors are probably partly responsible. The extraction of radioactivity by low concentrations of acetate reflects the generally disruptive effect that this inhibitor has upon the properties of the scutellar membranes as an effective permeability barrier, such that an internal pool of N-ethylmaleimide and its derivatives cannot be maintained.

5.3 Final Discussion - The Role of Sulphydryl Groups in Mediated Transport

5.3.1 Introduction

There are still relatively few reagents with the capability of selective modification of amino acid residues. Several of the more important ones have been used here, with varying degrees of success, in an attempted inhibition of transport proteins located within the scutellar epithelium.

The thiol-specific reagents are of greatest interest in that they show rapid and selective inhibition of peptide uptake, and offer an opportunity for the labelling and subsequent isolation of the transport proteins involved. The use of phenylarsine oxide points towards the likelihood of these thiols existing as paired, or vicinal on the peptide carrier, and successful substrate screening against PCMBs inactivation would indicate their location at the binding site of the protein itself.

Over the past five years there has been a proliferation of reports emphasising the importance of sulphydryl groups to mediated transport across a membrane barrier in many systems. This literature is discussed below and includes a consideration of how thiols are thought to function on a molecular basis in the transport mechanism.

5.3.2 Transport Across Plant Plasmalemmae

5.3.2.1 Higher Plants

One aspect of transport to have received some attention recently concerns the mechanism of phloem loading, i.e. the movement of sugars from sites of production in the mesophyll cells of leaves into the sieve elements of the phloem for export (Reviewed in Giaquinta, 1983). Intercellular movement could occur symplastically via plasmodesmata (Cataldo, 1974), or via the apoplast (Baker et al., 1980), which would necessarily involve some mediated transport across at least two membrane barriers. Sulphydryl reagents, including PCMBs and NEM, are highly effective as inhibitors of sucrose and amino acid uptake into the leaf phloem of Vicia faba (Delrot et al., 1980; Despeghel & Delrot, 1983) Beta vulgaris (Giaquinta, 1976, 1977a) and Glycine max (Servaites et al., 1979), indicating an important role of thiol ligands. The non-permeant species PCMBs presumably exerts its effects at the outer surface of the plasmalemma, and provides good evidence for the apoplastic pathway operating in these systems. The sensitive sulphydryl groups are probably located on the carrier proteins themselves (Delrot et al., 1980) but since phloem loading is thought to be proton-linked (Giaquinta, 1977b), the possibility that the mercurial is also inhibiting proton-pumping ATPase molecules must also be considered (Gilder & Cronshaw, 1974).

In related studies, several other transport processes in plants involving intercellular apoplastic movement of materials have been

shown to be thiol dependent. These include the movement of sucrose through the internodal tissue of sugar cane (Bowen, 1972), transport of sugars across the plasmalemma of isolated mesophyll protoplasts (Huber & Moreland, 1981), and sucrose uptake by developing soybean cotyledons (Lichtner & Spanswick, 1981). The glucose and amino acid carriers in the fronds of the aquatic duckweed Lemna gibba also have essential SH groups (Golle & Lüttge, 1983) which are directly inhibitable with mercurous ions.

5.3.2.2 Algae

Bicarbonate transport across internodal plasmalemmae of the alga Chara corallina was markedly inhibited by NEM and inorganic mercury ions (both highly penetrant), but less sensitive to PCMBs (Lucas & Alexander, 1980), implying that functional sulphhydryl groups were located on the inside of the membrane. However, the inhibition of uptake observed may be partly due to the more widespread disruptive effects exerted by these thiol reagents upon the integrity of Chara membranes (Lichtner et al., 1981).

With more specificity, a direct role of thiol ligands in the hexose carrier of the unicellular alga Chlorella has been established. Sucrose transport and uptake can be uncoupled from the energiser using the antibiotic nystatin (Komor et al., 1974). The system is transformed into one of facilitated diffusion, and if the carrier protein is biochemically isolated from its energy supply in this way, a complete inhibition of efflux of substrate from preloaded cells can be achieved with NEM, providing good evidence for direct inactivation of the transport system itself (Komor et al., 1978). Phosphate transport in Chlorella pyrenoidosa is also SH-dependent (Jeanjean, 1976).

5.3.2.3 Cell Cultures

Although the validity of studying the physiology of artificially induced callus cultures may be called into question, several reports have shown that, in cultured cells of Nicotiana tabacum, the uptake of arginine (Berry et al., 1981), cysteine (Harrington & Smith, 1977) and lysine (Harrington & Henke, 1981) is inhibitable by NEM.

5.3.3 Transport Across Fungal Plasmalemmae

Transport of C₄ dicarboxylic acids (Wolfenbarger & Kay, 1973) and glucose (Neville et al., 1971) by the Ascomycete Neurospora crassa are both highly sensitive to sulphydryl modification. The neutral, general, and basic amino acid transport systems of this organism are also inhibited by thiol reagents (Nelson et al., 1975). Although these observations were interpreted as a direct effect on the permeases themselves, the plasmalemma ATPase of Neurospora is also sensitive to cysteine modification (Scarborough, 1977; Brooker & Slayman, 1983), implying that interference with this energy source might be partially responsible for some of the observed inhibitions. In yeasts, leucine transport by Saccharomyces cerevisiae is dependent on thiols, presumed to be located on the inner surface of the plasmalemma, since they are sensitive to NEM but not to PCMB (Ramos et al., 1980; 1983). Peptide transport by Candida albicans is reported to be completely inhibited by 10 mM NEM (Logan et al., 1979).

5.3.4 Transport by Prokaryotes

5.3.4.1 Introduction

Bacteria possess many transport systems which fall into several distinct categories (Rosen, 1978), and although a detailed description of the different systems themselves is not relevant here, many share a sensitivity to sulphydryl modification despite their often different mechanisms of operation. Work, primarily on isolated membrane

vesicles of Escherichia coli, has demonstrated that a dithiol-disulphide interchange, in response to a changing redox environment, is likely to be central to the mechanisms controlling the affinities of a transport protein for a substrate molecule during the uptake cycle of some solutes (Section 5.3.7).

5.3.4.2 PEP-Dependent Hexose Transport

The phosphoenolpyruvate-dependent hexose phosphotransferase system is operative in organisms such as E.coli, Salmonella typhimurium and Staphylococcus aureus, and obligatively couples the transport of sugars to their phosphorylation using phosphoenolpyruvate as the phosphate source. NEM is a potent inhibitor of a membrane-bound enzyme (the E_{II} protein) of this group translocation. However, it has been reported that the maleimide is only effective when added during active phosphorylation, when, presumably, thiols which are normally 'buried' whilst the system is inoperative become exposed by an alteration in the configuration of the enzyme when in its functional state (Haguenauer-Tsapis & Kepes, 1977). It has been suggested that these sulphydryls exist as vicinal dithiols on the E_{II} protein, and that their redox state controls the affinity of the system for substrate (Robillard & Konings, 1981).

5.3.4.3 β -Galactoside Transport

Early work on E.coli established the role of a membrane-bound protein, designated the M protein, in the transport of β -galactosides (e.g. lactose) via the lac-permease. This protein was found to be sulphydryl-sensitive. Specific labelling of the M protein with radioactive NEM facilitated its isolation and characterization (Fox & Kennedy, 1965; Fox et al., 1967). Like the E_{II} component of the phosphotransferase system, the sulphydryl groups on the lactose transporter apparently exist as paired, vicinal dithiols, the redox

state of which alter the affinities of the carrier during an uptake cycle to allow binding, translocation, and release of a solute molecule.

5.3.4.4 Dicarboxylic Acid Transport

Related studies have been made on dicarboxylic acid permeases which show sensitivity to sulphhydryl reagents in E.coli (Murakowa et al., 1972), Pseudomonas putida (Ondrako & Ornston, 1980), and Bacillus subtilis (Fournier & Pardee, 1974), enabling a selective labelling of the carrier to be performed in the last case.

5.3.4.5 Amino Acid Transport

In E.coli several amino acid porters, including those for ornithine, phenylalanine and proline, possess SH groups vital to their activity (Janick et al., 1977) which, in the proline carrier, exist as paired, redox-sensitive dithiols (Poolman et al., 1983). However, other permeases transporting glycine, tyrosine, glutamate, lysine and leucine are not inhibited by modification of cysteine residues (Kaback & Patel, 1978). In this respect, a positive correlation exists between sulphhydryl dependence and resistance to osmotic shock; those systems involving a periplasmic binding protein (i.e. shock-sensitive) are generally less susceptible to NEM inactivation (Janick et al., 1977, Berger & Heppel, 1974). The reasons for this distinction, however, are not clear at the present time.

5.3.5 Transport by Animal Cells

Animal membranes are considerably more diverse in structure and function than those of plants or microorganisms and a representative range of reports covering those animal tissues used most widely in inhibition studies is cited below. For example, thiol reagents, including NEM and mercurials, inhibit amino acid and glucose transport by erythrocytes (Young, 1980; Whitfield & Schworer, 1981; Batt et al., 1978; Smith & Ellman, 1973), but PCMBs in particular is known to have

deleterious effects upon the integrity of the red blood cell membrane and can cause the dissociation of up to 40% of the total bound protein from the lipid bilayer (Carter, 1973) which may have contributed to the inhibitory effect upon transport. Disulphide-dithiol interchange is postulated to play a direct role in the uptake of hexose by white blood corpuscles (thymocytes) (Regan et al., 1981; Kwock, 1981) and by fat cells (adipocytes), (Czech, 1976,1977). Glucose and amino acid transport by intestinal brush border membranes, which in mammalian systems is linked to sodium movement in a cotransport mechanism, is also sensitive to thiol modification, but inhibition is due to a dissipation of the sodium gradient, rather than a direct inactivation of the transport proteins themselves (Will & Hopfer, 1979; Biber & Hauser, 1979; Klip et al., 1979). A disulphide-dithiol interchange is critical to the uptake of glucose by renal brush border membrane (Turner & George, 1983), and a specific labelling of the transporter with radioactive NEM has enabled its isolation and characterization (Poirée et al., 1979). In related studies, a role for thiols in transport by cells in artificial culture, including tumorous lines, has also been established (Hare, 1975; Löffler & Niebch 1976; Dowd et al., 1977).

5.3.6 Transport by Organelles

5.3.6.1 Chloroplasts

The chloroplast envelope is a double membrane, the structure and function of which has been reviewed (e.g. Heber, 1974; Douce & Joyard, 1979). The outer membrane of the envelope is freely permeable to most low molecular weight compounds, whereas the inner membrane is the site of specific transport systems (Heldt & Sauer, 1971), whose activity can control the amounts of various solutes within the stroma. Such control is important because the rate of photosynthesis is regulated by the concentration of many of these metabolites within the chloroplast. Several distinct transport systems are known which operate across the

inner membrane (see below). Although the role of thiols in these carriers has received little attention, PCMBS, the non-penetrant mercurial, has seen the most use because it does not interfere with metabolism inside the chloroplast. It shows some selectivity of inhibition which would imply a direct effect on the transport systems themselves and not on a component (e.g. an energy source) common to all.

A specific phosphate carrier transports inorganic phosphate into the chloroplast in exchange for fixed phosphates, produced by photosynthesis, which are transported out into the cytoplasm (Flugge & Heldt, 1976, 1981; Heldt & Rapley, 1970); the carrier is inhibited by PCMBS. An adenine nucleotide translocator transports ATP into the chloroplast, especially when photophosphorylatory activity is low (Heldt & Sauer, 1971); PCMBS does not affect its activity (Robinson & Wiskich, 1977). A dicarboxylic acid transporter controls the exchange of solutes such as malate and succinate but is not PCMBS sensitive (Werdan & Heldt, 1971). Glycolate, produced by photorespiration, is exported from the chloroplast via a specific glycolate transporter. The system is inhibitable with NEM and the sensitive thiols are probably located at the binding site of the carrier because substrate-screening can confer protection against their modification (Howitz & McCarty, 1983). A specific pyruvate carrier has also been reported, which, in Digitaria chloroplasts, is inhibitable by the SH reagent mersalyl, but not by NEM (Huber & Edwards, 1977).

5.3.6.2 Mitochondria

Although the outer mitochondrial membrane is freely permeable to most solutes, transport across the inner membrane is selective, and, analogously with the chloroplast, several specific transport systems are located here. These are mainly anion/anion exchange systems which are concerned with the maintenance of a regulated intramitochondrial balance of Kreb's cycle intermediates, and include carriers of di- and tri-carboxylic acids, ATP/ADP, and inorganic phosphate. The role of

sulphydryl groups in the mitochondrial transport of anions has recently been reviewed (Fonyo, 1978) and will not be discussed in depth here. To summarise, almost every carrier within the inner membrane is reported to contain thiols essential to activity (e.g., Wiskich, 1974; Klingenberg et al., 1974; Hadvary & Kadenbach, 1976; Klingenberg, 1977; Wohlrab, 1978; Aquila & Klingenberg, 1982). Mercurials and maleimides also profoundly affect the exchange and retention of cations by mitochondria e.g., potassium (Bogucka & Wojtczak, 1979; Brierley et al., 1967, Scott et al., 1970) and calcium (Ramachandran & Bygrave, 1978; Lofrumento et al., 1979). It is proposed that mercurials may exert their effects by exposing latent ionophores within the membrane, which subsequently allow the free flux of cation in and out of the mitochondrion (Southend et al., 1973).

5.3.7 A Model for Thiol-Dependent Transport

The role of sulphydryl groups in solute uptake, in particular a dithiol-disulphide interchange, has been established in many diverse systems throughout the plant, animal and microbial kingdoms, and it seems likely that they place a general, central role in membrane transport processes. The mechanism of any mediated transport system must involve the ability of a carrier to bind a substrate molecule with high affinity (low K_m) on one side of the membrane, and to translocate it across to the other surface. Concomitantly with this translocation, the affinity of the carrier for the substrate must somehow be reduced (higher K_m) e.g., by means of a configurational change of the transport protein, thereby allowing the solute molecule to be released to the other side. Kaback and his co-workers first reported that the carrier protein responsible for lactose uptake by Escherichia coli could exist in an oxidised or reduced form, which involved an SH HS to S-S conversion (Kaback & Barnes, 1971; Kaback & Hong, 1973). The oxidised carrier had a high affinity for lactose, but in the reduced state had low affinity. The carrier

protein itself was envisaged as a respiratory electron transfer intermediate; the affinity of the carrier for a solute molecule was dependent upon its redox state, presumably by means of the configurational change in the protein molecule brought about by a dithiol-disulphide conversion. The redox state of the carrier was considered to be altered by the oxidation of substrate during respiration.

In principle, this model is still thought to apply, except that the carriers are no longer considered as electron transfer intermediates, but as discrete permease proteins (Kaback, 1974), and redox conditions are now thought to be altered in the membrane by means of a transmembrane electrical potential or by a pH gradient, which are generated independently (Reider et al., 1979; Cohn et al., 1981). Over the last few years, studies primarily by Konings and his associates, have clearly established a dithiol-disulphide interchange crucial to the activity of several transport systems in Escherichia coli including phosphoenolpyruvate-dependent hexose uptake (Robillard & Konings, 1981), and the lactose (Konings & Robillard, 1982) and proline (Poolman et al., 1983) permeases. General formulations, together with a model of redox-sensitive transport, have been derived from these studies (Lombardi, 1981; Robillard & Konings, 1982) and are outlined below.

It is proposed that a vicinal dithiol grouping exists at each of two binding sites on the transport protein. These sites are located at either end of a channel, or pore, running through the protein, which traverses the membrane. The binding sites are freely accessible to either the exterior or the interior of the cell respectively. The oxidation states of the dithiols at these two sites are coupled, such that when one is oxidised, the other is reduced, and the affinity of either of the two binding sites for a solute molecule depends upon its redox state. The redox state at each location is altered by the inward movements of protons. Therefore, in a single transport cycle, a substrate

molecule binds to the outer binding site, which is initially in the high-affinity (dithiol) form. Then, proton binding at the exterior causes a dithiol-disulphide conversion, reducing the affinity of this site for the substrate. The proton moves through the membrane, reduces the interior binding site (disulphide→dithiol) thus raising its affinity. Hence, the solute molecule is drawn through the pore. To complete the cycle, the inner site is reoxidised and the solute released to the interior of the cell.

Therefore, the peptide transport proteins of the barley scutellum have features in common with certain of the lactose, hexose and proline permeases of E.coli, in that different classes of sulphydryl groups are involved which exist as redox-sensitive, vicinal dithiols. As discussed in a later section (Section 6.3), the sensitivity of vicinal dithiol groups to sulphydryl modification is dependent upon the presence or absence of a H^+ gradient, which would suggest that in barley also, the affinity of the binding sites is directly affected by the movement of protons across the membrane.

CHAPTER 6

ENERGETICS OF TRANSPORT BY THE BARLEY SCUTELLUM

6.1 Inhibition of Energy Coupling

6.1.1 Reagents Used to Inhibit Energy Coupling - An Introductory Survey

Previous studies have shown that scutellar uptake of peptides and amino acids fulfils the generally accepted criteria for active transport; namely, intact accumulation against a concentration gradient, saturation kinetics and a requirement for metabolic energy. The following studies are concerned with the mechanism whereby metabolic energy is coupled to the uptake of a substrate molecule in the barley scutellum.

To effect transport, a membrane-bound carrier has to have an asymmetrical affinity for the substrate, to permit binding of the molecule on one side, with its subsequent translocation and release, followed by the return of the system to its original state. Maintenance of this asymmetry requires an input of energy, which may be achieved either at the direct expense of ATP or by the establishment of an electrochemical gradient across the membrane. The gradient itself may be generated either through ATP hydrolysis, or through respiratory activity (see Spanswick, 1981; Ferguson & Sorgato, 1982, for reviews). Higgins & Payne (1977b), in preliminary work on the energetics of peptide uptake by barley scutella, tentatively concluded that a proton gradient might be involved, as has been suggested for some other transport processes in plants. Studies here were aimed at characterising this energisation in greater depth, through the use of reagents which are capable of either destroying the proton gradient or inhibiting membrane-bound ATPases.

6.1.1.1 Acetate

Although not widely recognised as a metabolic inhibitor, the possible action of acetate, and other weak acids, to act as disruptors of the proton gradient has been discussed (Heuting & Tempest, 1977; Kaiser & Heber, 1983). The effect is pH dependent; thus, it is the undissociated molecule (predominating below its pK value at pH 4.8), that is presumably

mobile within membranes and acts as a proton shuttle to break down H^+ gradients.

6.1.1.2 Carbonylcyanide-m-chlorophenylhydrazone

Carbonylcyanide-m-chlorophenylhydrazone (CCCP) also acts as a protonophore that destroys proton gradients (Langmüller & Springer-Lederer 1974; Harold, 1972); its effects are often very rapid, e.g. complete inhibition of peptide transport by Streptococcus faecalis and E.coli within 15s (J.W.Payne, personal communication). As well as being active in microbial systems, CCCP has also been found to inhibit many transport processes in higher plants, e.g., leucine uptake by cultured cells of tobacco (Blackman & McDaniel, 1980), sucrose accumulation by vacuoles of sugar beet (Saftner et al., 1983), loading of amino acids and sucrose into soybean phloem (Servaites et al., 1979), and movement of glutamine into tomato internode tissue (van Bel & van Erven, 1979).

6.1.1.3 2,4, Dinitrophenol

The protonophore 2,4, dinitrophenol (DNP) disrupts many plant transport systems dependent on a proton gradient, e.g., inhibition of amino acid uptake by castor bean cotyledons (Robinson & Beevers, 1981), and sucrose uptake by immature barley embryos (Cameron-Mills & Duffus, 1979). Direct effects upon proton movement e.g., across the scutellum of wheat (Mukhtar & Laidman, 1982) and maize (Humphreys, 1975), have also been reported.

6.1.1.4 N,N'-Dicyclohexylcarbodiimide

Electrogenic proton pumps driven by membrane-bound ATPases are ubiquitous, being present in bacteria (Haddock & Jones, 1977), chloroplasts (Nelson, 1976), mitochondria (Senior, 1973, 1979), the plasma membranes of fungi (Scarborough, 1977, 1980; Blasco et al., 1981) and plants (Higinbotham & Anderson, 1974; Hodges, 1976; Dupont et al., 1981; Löppert, 1979). These enzymes are capable (reversibly) of generating a proton

gradient at the expense of ATP hydrolysis. The ATPase enzyme has two parts, a hydrophobic, membrane-bound 'F_o' moiety that acts as the protonophore, and an 'F₁' component that catalyses ATP hydrolysis (Fillingame, 1980). N,N'-Dicyclohexylcarbodiimide (DCCD) inhibits ATPase activity by covalent modification of the carboxyl group of a single glutamic acid residue within the F_o proton channel; however, the reagent can also modify cysteine, tyrosine and amino groups under certain conditions, and there has been some uncertainty over the specificity of this inhibition. Moreover, several reports indicate that the inhibitory effects of DCCD may be due, in part, to stoichiometric binding with the F₁ component also (Yoshida *et al.*, 1982; Ceccarelli & Vallejos, 1983; Kopecky *et al.*, 1982).

6.1.1.5 Sodium azide

The possible action of sodium azide may be three-fold; it is an inhibitor of cytochrome oxidase and membrane-bound ATPase and can also act as a protonophore (Harold, 1972). It is effective in inhibition of plant transport processes e.g., uptake of amino acids into pea leaf tissue (Cheung & Nobel, 1973) and oat mesophyll protoplasts (Rubinstein & Tattar, 1980).

6.1.1.6 Valinomycin

Valinomycin is an antibiotic substance produced by Streptomyces fulvissimus that is freely soluble in membranes and highly effective as a potassium ionophore (Pressman, 1976). The internal dimensions of its cage-like structure (Shemyakin *et al.*, 1969) enable it to act specifically as a potassium shuttle ferrying K⁺ ions across a membrane and equilibrating any gradient that might be present. It is therefore a potent inhibitor of uptake mechanisms involving a potassium flux, and its action was first demonstrated in the inhibition of K⁺ movement across mitochondrial membranes (Moore & Pressman, 1964).

6.1.2 Methods

Typically, 2-3 day scutella were preincubated in inhibitor (buffered to specified pH with sodium phosphate-citrate) for up to 1 h at 20°C, prior to a standard assay for the transport of Gly-(U¹⁴C)Phe, (U¹⁴C)Leu or (U¹⁴C)glucose transport either directly, or after a 'recovery' period of up to 5 h in 2 ml sodium phosphate-citrate buffer pH 3.8, 20°C. As controls, scutella were subjected to a preincubation of up to 5 h in buffer without inhibitor before assay; others were exposed to inhibitor present in both preincubation and assay mix. Stock solutions of CCCP, DNP, DCCD and valinomycin were ethanolic, but ethanol was never present in an incubation at concentrations greater than 0.5% v/v. In these cases, control rates were established by incubations with ethanol alone.

6.1.3 Results

6.1.3.1 Acetate

Figure 6.1 shows the effect of preincubation of 2 day scutella with 5 mM and 50 mM acetate (pH 3.8, for 1 h), with subsequent recovery periods, in a standard Gly-(U¹⁴C)Phe transport assay. Rates of uptake shown by scutella consecutively preincubated in acetate for 1 h, and then assayed for transport in the further presence of inhibitor (at 5 mM or 50 mM), is taken as the maximally inhibited value with no recovery allowed. Recovery periods include the duration of the uptake assay; thus, scutella preincubated in acetate and assayed directly afterwards were, in effect, recovered for 45 min. Rates are expressed as a percentage of those exhibited by acetate-untreated scutella which were preincubated in buffer only, for 4 h, prior to assay. It can be seen that the 80-85% inhibition of peptide uptake achieved by 50 mM acetate is not restored at least 4 h after treatment, whereas considerable recovery of Gly-Phe transport is possible after preincubation with 5 mM inhibitor. Acetate proved to be

somewhat less effective as an inhibitor of amino acid transport; thus 2 day scutella both preincubated and assayed for transport in the presence of 5 mM acetate accumulated ($U^{14}C$)Leu, ($U^{14}C$)Pro and ($U^{14}C$)Phe from 2 mM solutions at rates 55%, 63% and 75% respectively of untreated controls (Table 6.1). At the present time, no satisfactory explanation can be offered as to why acetate is a less effective inhibitor of amino acid transport.

6.1.3.2 Carbonylcyanide- π -Chlorophenylhydrazine

Figure 6.1 shows the response of Gly-($U^{14}C$)Phe transport by 2 day scutella to preincubation for 10 min with 10 μ M and 100 μ M CCCP at pH 3.8, followed by recovery times of up to 4 $\frac{1}{2}$ h (which included the duration of assay). Rates of uptake are expressed as a percentage of rates shown by untreated scutella preincubated in buffer only for 5 h. After the preincubation, some scutella were prevented from 'recovery' by assaying for transport in the presence of 10 μ M or 100 μ M CCCP. It is apparent that CCCP is more effective than acetate as an uncoupler, since lower concentrations and a shorter preincubation time achieve similar levels of inhibition. Scutella still retain the capacity however, at the lower concentration of CCCP, to recover nearly 70% of the transport activity of control tissue within 2 h after removal of CCCP.

Uptake of other substrates, including amino acids and glucose, by 2-3 day scutella was also inhibited quite markedly (Table 6.1) when assayed in the presence of 100 μ M CCCP.

6.1.3.3 2,4-Dinitrophenol

Preincubation of 2 day scutella in buffered 10 μ M dinitrophenol (50 mM sodium phosphate-citrate pH 3.8, 20 $^{\circ}$ C) for 30 min prior to transport assays in the presence of 10 μ M DNP reduced uptake of ($U^{14}C$)Leu (2 mM) to 39%, Gly-($U^{14}C$)Phe (2 mM) to 18%, and ($U^{14}C$)glucose (20 mM) to 28% respectively, of the values of untreated controls.

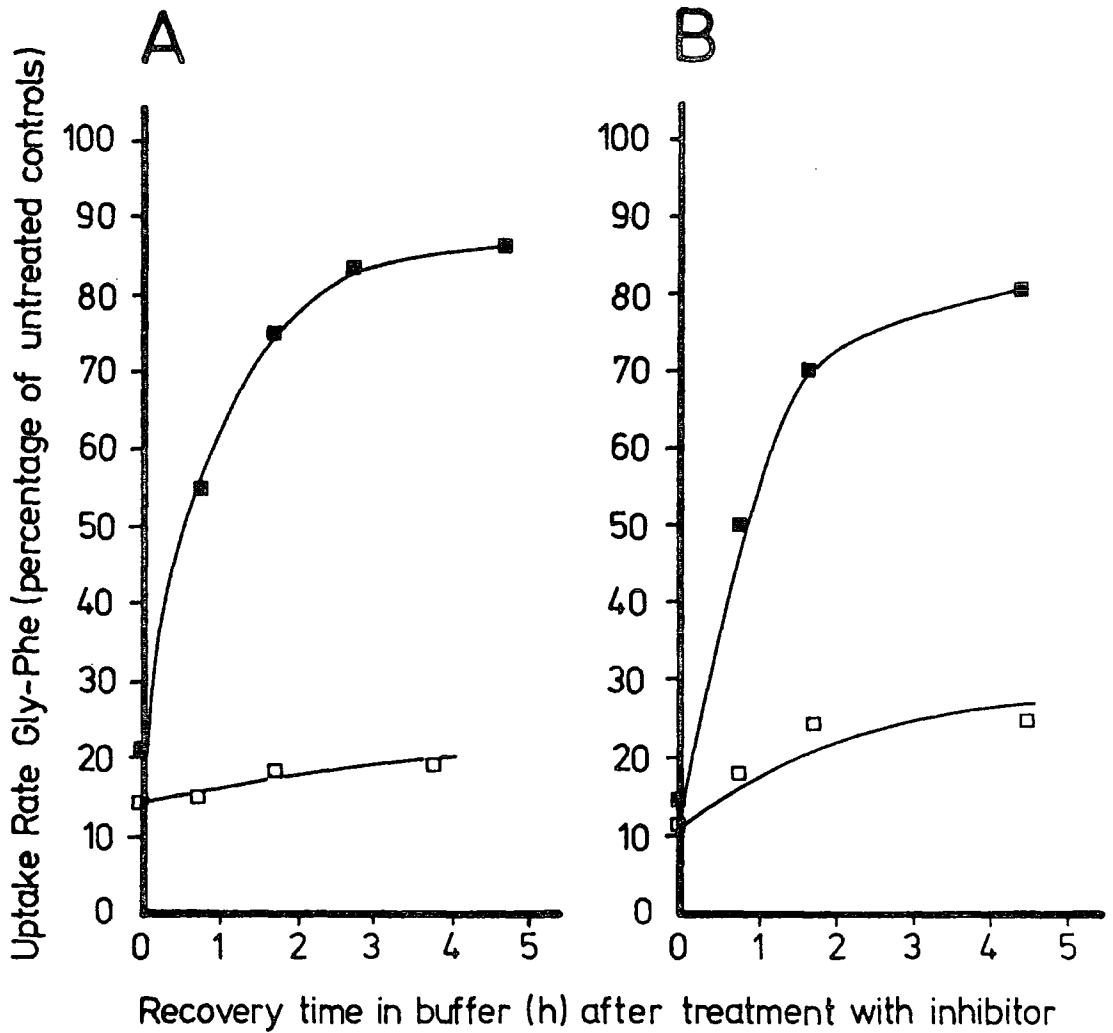


FIGURE 6.1 Recovery of Gly-Phe Transport After Inhibition with Acetate or CCCP

Uptake of Gly-(U¹⁴C)Phe by scutella treated with acetate (A) at 5 mM (■) and or 50 mM (□) for 1 h, pH 3.8, or CCCP (B) at 10 μ M (■) and 100 μ M (□) for 10 min, pH 3.8, before transferring to buffer (sodium phosphate-citrate, pH 3.8) for up to 4 h prior to the transport assay. Rates of uptake are expressed as a percentage of those shown by untreated scutella preincubated in buffer only for 5 h.

TABLE 6.1

Inhibition of Peptide, Amino Acid and Glucose Transport by CCCP and Acetate

<u>Substrate</u>	<u>Control Rate Transport (nmol scut⁻¹ h⁻¹)</u>	<u>Percentage Transport of Control.</u>	
		<u>CCCP</u>	<u>Acetate</u>
Gly-Phe	35	20	21
Ala-Ala-Ala	37	11	ND
Gly	56	17	ND
Leu	20	28	55
Pro	32	31	63
Phe	38	ND	75
Glucose	19	13	ND

Transport of substrate from 2 mM solutions by 2-3 day scutella both preincubated (60 min) and assayed for uptake in the presence of either 100 μ M CCCP or 5 mM acetate. All peptides and amino acids are of the L configuration (ND = Not Determined).

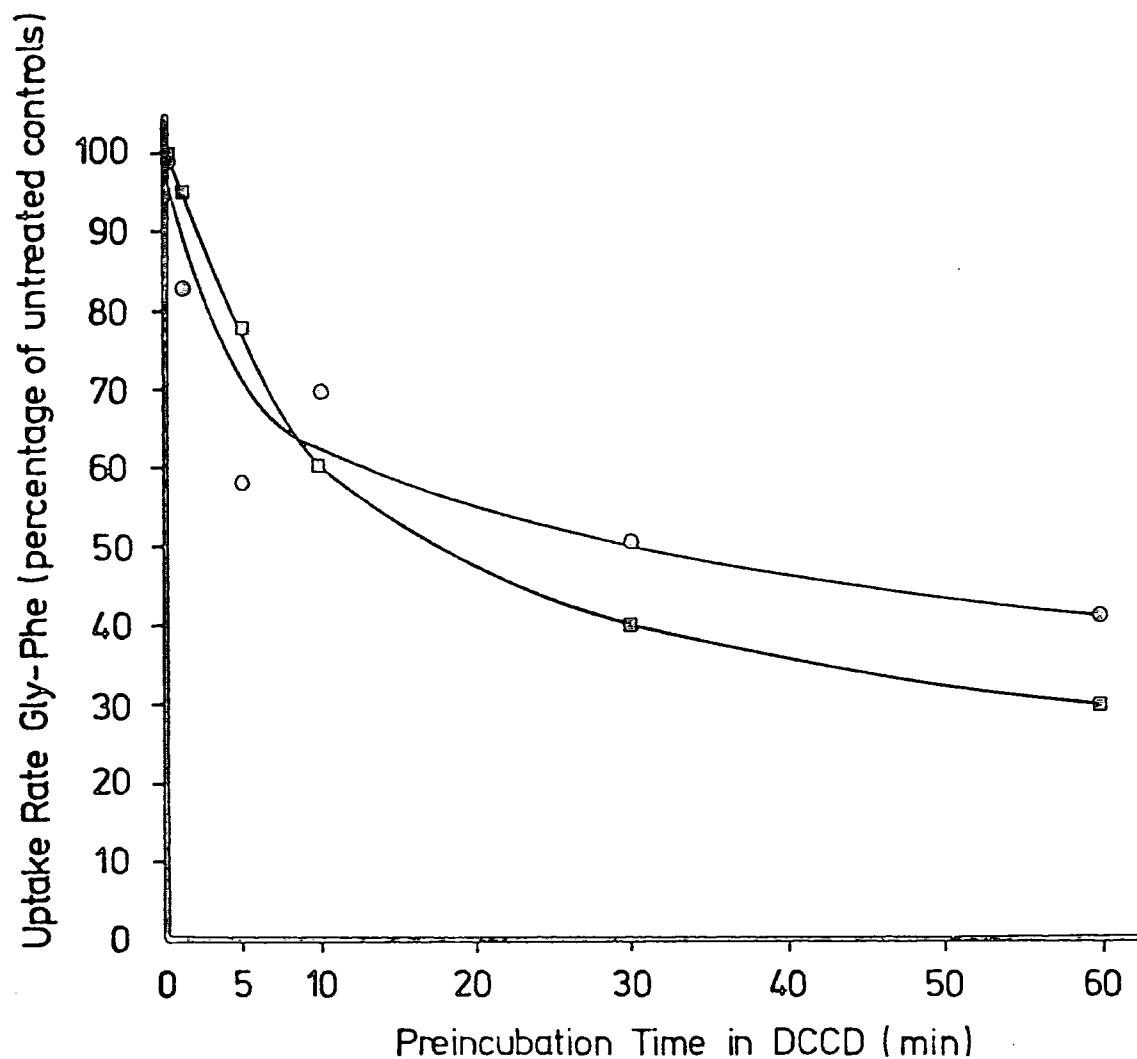


FIGURE 6.2. Kinetics of Inhibition of Gly-Phe and Leu Uptake by DCCD

Uptake of Gly-(U¹⁴C)Phe (□), and (U¹⁴C)Leu (○), by 3 day scutella after preincubation in 2 mM dicyclohexylcarbodiimide for up to 60 min as described in Section 6.1.3.4. Rates are expressed as a percentage of untreated controls.

6.1.3.4 N,N'-Dicyclohexylcarbodiimide

3 day scutella were preincubated in 2 mM dicyclohexylcarbodiimide (DCCD) (20°C , sodium phosphate-citrate pH 6.9) for periods of up to 60 min prior to standard assays for Gly-(U^{14}C)Phe and (U^{14}C)Leu transport. All preincubations were timed to finish simultaneously. Results are expressed in a plot of transport rates (as a percentage of controls in which scutella were preincubated in buffer for 1 h only) versus preincubation time with DCCD (Figure 6.2).

Inhibition of Leu and Gly-Phe uptake follows essentially the same kinetics comprising an initial, rapid action within 10 min followed by a more gradual decline of rates. These results are in marked contrast to the use of an alternative carboxyl reagent, Woodward's Reagent K (Section 4.8) which, when preincubated with scutella for up to 30 min at 10 mM, had no effect upon Leu or Gly-Phe transport relative to untreated controls.

6.1.3.5 Sodium azide

Preincubation of 2 day scutella in buffered 20 μM sodium azide for 1 h (pH 3.8, 20°C) prior to assay for uptake in the further presence of 20 mM azide reduced transport of radioactively labelled Leu (2 mM), Gly-Phe (2 mM), and glucose (20 mM) to 30%, 20% and 37% respectively of the values of uninhibited controls.

6.1.3.6 Valinomycin

Preincubation of 2 day scutella with 100 μM valinomycin for 1 h (sodium phosphate-citrate, pH 3.8, 20°C) did not affect the uptake of Leu (2 mM), Gly-Phe (2 mM) or glucose (20 mM) in a standard, radioactive transport assay.

6.1.4 Discussion

It is clear that the protonophores employed here, acetate, CCCP and DNP are all effective as inhibitors of Leu, Gly-Phe and glucose transport at physiological concentrations (2 mM) implicating the requirement

of a hydrogen ion gradient for uptake. The peptide uptake system is capable of recovering from CCCP and acetate action, suggesting that the scutellar tissue can re-establish a proton gradient following dilution of the inhibitor. Acetate, at low concentrations, has also been shown to be effective as an extractant of accumulated materials (Section 5.2.3). This would imply that the maintenance of internal pools against a concentration gradient is also dependent on the presence of a proton gradient, and acetate equilibrates internal and external concentrations. Thus, in the presence of inhibitors such as acetate, CCCP and DNP, part of the residual rates of uptake observed (15-30% of control values) from 2 mM solutions may be due to a simple, diffusional equilibration of radioactivity. This is borne out by the less effective inhibition of transport by these uncouplers from solutions at higher concentrations. The elevated uptake rates observed at high concentrations arise presumably from diffusion of substrate down a gradient into scutellar tissue, a passive process independent of a proton drive (Section 5.3.5). The possible relationship between the proton gradient and the maintenance of dithiol groupings in the redox state which is vital to the functioning of the peptide transport proteins, is discussed later (Section 5.3.6).

It is known that many plant tissues take up potassium ions in exchange for protons, or vice-versa, in an antiport mechanism to maintain electric neutrality, e.g. in roots (Marrè, 1979). Although the wheat scutellum contains a H^+/K^+ exchange system (Mukhtar & Laidman, 1982), transport in the barley scutellum is independent of exogenously supplied cations (Sopanen et al., 1978), and valinomycin, a potassium ionophore, has been shown here to have no effect on uptake. These observations would imply that if a proton/potassium counterflow also occurs in barley, H^+ efflux is not obligatorily coupled to K^+ influx and electric neutrality must be maintained in some other way. This independence of action has been reported in the antiport of K^+ and H^+ by pea root segments

(Bellando et al., 1979) but Poole (1974), claims that the exchange in beet root tissue is strictly coupled and mediated by the same carrier.

DCCD reduces both Leu and Gly-Phe transport presumably by inhibition of ATPase activity which, in part, is responsible for production of the proton gradient. The ATPases are most probably located in the plasmalemmae of the epithelial cells and supplied with ATP by the many mitochondria that are aligned in close proximity to the membrane (Section 1.2.1). Although DCCD is highly reactive with ATPase carboxyl ligands, the group specificity of its action is not absolute and the possibility of secondary inhibitory effects must be taken into consideration. In contrast to DCCD, Woodward's reagent K does not affect transport of amino acid or peptide. This distinction between the effects of isoxazolium or carbodiimide reagents in the attempted derivatization of carboxyl groups has been observed in other systems (Homandberg, 1982; Ceccarelli & Vallejos, 1983), and probably reflects differing accessibilities of the active site to modification.

6.2 Evidence for a Passive Component to Uptake

The following studies describe the steps that were taken to characterize uptake rates at high concentrations of substrate (up to 100 mM) in an attempt to distinguish between the roles of active versus passive movement, and to describe kinetic parameters of transport in terms of K_m and V_{max} .

6.2.1 Methods

2-3 day scutella were assayed for uptake by extraction of radioactivity in the routine way following incubation in Gly-(U¹⁴C)Phe, (U¹⁴C)Leu, or (U¹⁴C)glucose (1ml of 10-100 mM, 0.5 $\mu\text{Ci ml}^{-1}$, in sodium phosphate-citrate buffer pH 3.8, at 20°C). Uptake of D-Val-D-Val was assayed by a slightly modified fluorescamine method (Section 2.4.4.2) in that 30 scutella were used per incubation, removing 5 μl samples hourly for assaying in the usual way.

6.2.2 Results

Figure 6.3 shows the kinetics of uptake of Leu and Gly-Phe in the concentration range 10 mM to 100 mM by 3 day scutella. Preincubation (for 30 min, 20°C, pH 3.8) and assay for uptake, both in the presence of 10 μ M DNP, reduced transport of 100 mM Leu, Gly-Phe and glucose to 73%, 75% and 71% respectively of the values of untreated controls. Also, preincubation of 3 day scutella for 30 min in 5 mM N-ethylmaleimide (pH 6.8, 20°C) inhibited subsequent uptake of 100 mM Gly-(U¹⁴C)Phe by only 26% relative to untreated controls. This is in contrast to the 90% inhibition achieved under similar conditions when assaying for uptake of 2 mM peptide (Section 4.2.2). Uptake of 60 mM DVal-DVal by 3 day scutella was 100 nmol scutellum⁻¹h⁻¹ as assayed by fluorescamine, whereas the uptake of this peptide at 2 mM, as assayed by using fluorescamine or dansyl chloride, was not detectable.

6.2.3 Discussion

These studies were initiated to determine the active transport of several substrates over a wide concentration range and to derive parameters in terms of K_m and V_{max} . However, it seems that passive uptake, sensitive neither to DNP nor N-ethylmaleimide become prevalent at artificially high non-physiological concentrations of amino acid or peptide, and makes characterization of the kinetics of the active component difficult. Similarly, the experiment with D-Val-D-Val would indicate that uptake at high substrate molarity is not primarily carrier mediated but is probably diffusional in nature. Any concentration-dependent passive component will be operating, although at a less significant level, at the 2 mM substrate concentrations used routinely in inhibitor studies for example, where the active component has been inhibited completely.

It is possible to construct models in which a mediated and non-mediated component are operative and to extract kinetic parameters from the data. A computer program (devised by Dr. J.T. Gleaves, University of

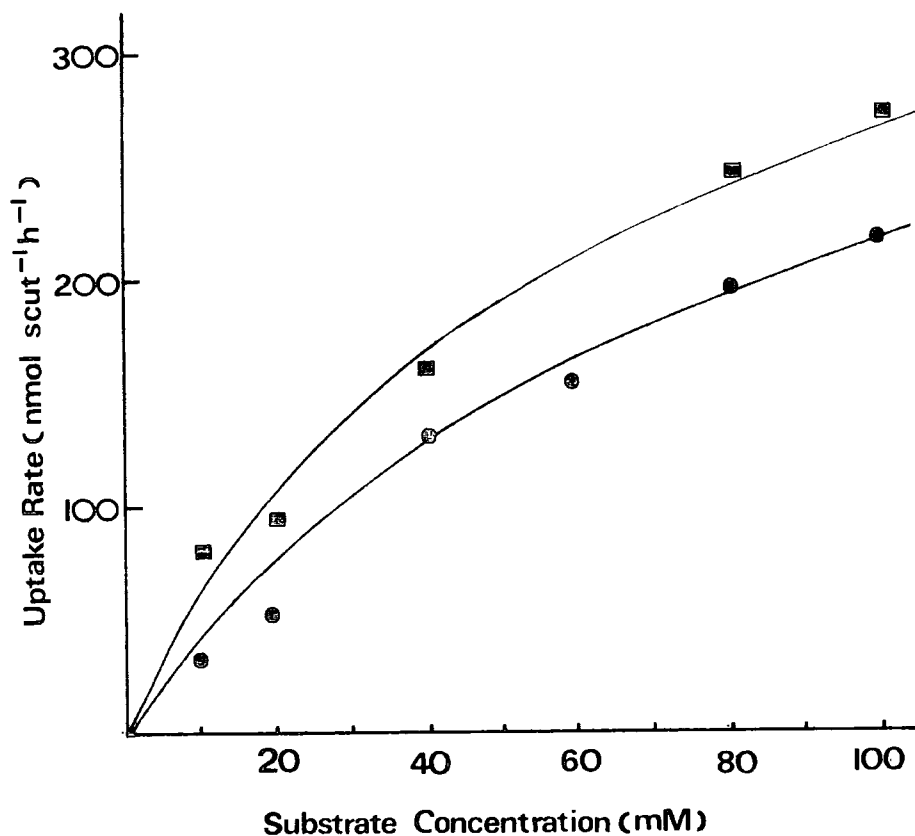


FIGURE 6.3 Transport Rates of Gly-Phe and Leu at High Substrate Concentration

Transport rate of (U¹⁴C)Leu (●) and Gly-(U¹⁴C)Phe (■) by 3 day scutella was monitored from solutions in the range 10-100 mM. Values are the means of at least 3 separate determinations.

Durham) is available for this purpose but in the present studies it was decided not to pursue this line of study extensively.

6.3 Binding of N-ethylmaleimide to Energised and De-Energised Membranes

6.3.1 Introduction

Work described so far in this thesis establishes the critical role of vicinal dithiols to the functioning of the peptide transport proteins. Thus, almost complete inhibition (95%) of peptide uptake can be achieved by pretreating scutella with selective sulphydryl-modifying reagents such as N-ethylmaleimide or phenylarsine oxide, which modify covalently the functional thiol ligands. In studies related to the energisation of solute transport into the barley scutellum, experiments were performed to establish whether the presence or absence of the proton gradient across the scutellar epithelium affected the sensitivity of the vicinal dithiols to sulphydryl-modifying reagents. Hence, the importance of the electrochemical gradient in maintaining sulphydryl groups in their functional state could be assessed. This was achieved by determining the level of binding of radioactively labelled NEM to scutella in the presence and absence of a protonophore.

6.3.2 Methods

2 day scutella were treated as follows to selectively expose the vicinal thiols within the membrane (see also Section 5.1.3): specific modification of vicinal dithiols with phenylarsine oxide (0.5 mM in sodium phosphate-citrate buffer pH 3.8, for 4 min at 20°C); after washing, scutella were transferred to N-ethylmaleimide (NEM), for irreversible modification of all other thiol groups (5 mM in sodium phosphate-citrate buffer pH 6.8, for 10 min at 20°C) prior to treatment with the reducing agent dithiothreitol (10 mM pH 3.8, for 1h at 20°C) to specifically re-expose only those vicinal dithiols modifiable by phenylarsine oxide (these vicinal sulphydryl groups will include those involved in peptide transport). Scutella were then transferred to

carbonylcyanide-m-chlorophenylhydrazone (CCCP, 10 μM , pH 3.8, for 15 min at 20°C) to collapse the proton gradient, hence de-energising the scutellar membranes. The tissue was then transferred, without washing, to a solution of N-ethyl (2,3- ^{14}C) maleimide (5 mM, 1-5 $\mu\text{Ci } \mu\text{mol}^{-1}$, pH 6.8, for 3 min at 20°C) containing CCCP (10 μM) to radioactively label the re-exposed vicinal dithiols. Stock CCCP solutions were ethanolic, and, as controls, scutella were treated identically but with the omission of CCCP from the ethanol. Final ethanol concentrations in all incubation media were 0.5% v/v. Finally, unbound (i.e. soluble) N-ethyl (2,3- ^{14}C) maleimide was extracted with a series of dilute acetate solutions as described previously (Section 5.1.4.2). The residual radioactivity (including that bound to vicinal dithiols) was estimated by dissolving scutella in tissue solubilizer, and the solubilized radioactivity assayed by scintillation counting, again as described previously (Section 5.1.4.2).

6.3.3 Results

The counts per minute associated with energised and de-energised scutella is shown in Table 6.2. The experiment was performed twice using different specific activities of radioactive NEM, and each treatment was performed in triplicate. Within each treatment, radioactive binding is highly reproducible and there is a clear reduction in radioactive labelling associated with the de-energised, CCCP-treated material, as compared with the energised, untreated controls.

6.3.4 Discussion

On the assumption that the difference in radioactive labelling is significant, and that the sulphydryl reagents themselves do not interfere with the production or maintenance of the proton gradient, then the decrease in binding of ^{14}C -NEM to de-energised membranes might be indicative of a shift in the redox state of the vicinal thiols from

TABLE 6.2

(¹⁴C)NEM Binding to Energised and De-energised
Membranes

	De-energised (CCCP Treated)	Energised (Ethanol Control)
	206	235
EXPT A	220	238
	218	239
	594	825
EXPT B	630	811
	587	822

N-ethyl (2,3-¹⁴C)maleimide binding (Expt A, specific activity 1 $\mu\text{Ci } \mu\text{mol}^{-1}$; Expt B, specific activity 5 $\mu\text{Ci } \mu\text{mol}^{-1}$; both 5 mM for 3 min at pH 6.8, 20°C) to vicinal dithiols in the absence (CCCP-treated) or presence (ethanol control) of membrane energisation. Values represent counts per minute associated with four scutella, and were performed in triplicate. CCCP treatment comprised an initial preincubation (10 μM CCCP, pH 3.8, for 15 min at 20°C), prior to transfer to the radioactively labelled NEM with the further presence of CCCP (10 μM).

the reactive dithiol (reduced) condition to the non-reactive disulphide (oxidised) condition. Alternatively, the difference in binding may be due to a structural or conformational alteration of the membrane protein(s) in response to the destruction of the proton gradient, such that the thiol ligands are still in the reactive (reduced) state, but are physically made less accessible to N-ethylmaleimide. Therefore, it would seem that the vicinal dithiols are more sensitive to sulphhydryl group modification when the system is in the energised state. The degree of inactivation of peptide transport itself by sulphhydryl reagents, in relation to the state of energisation of the system, is explored in the next section.

6.4 Inhibition of Peptide Transport by Sulphydryl Reagents in Relation to Membrane Energisation

6.4.1 Introduction

As de-energisation of the membrane apparently decreases the susceptibility of thiol ligands to covalent modification, an attempt was made to determine whether peptide transport itself showed changed sensitivity to inhibition with thiol reagents when the proton gradient had been temporarily destroyed.

6.4.2 Methods

1-2 day scutella were pretreated with CCCP (10 μ M) to eliminate the proton gradient, then incubated separately with the following sulphhydryl reagents: 1. 5mM N-ethylmaleimide (NEM); 2. 0.5 mM Phenylarsine oxide (PAO); 3. 4mM p-chloromercuribenzenesulphonate (PCMBs); all in the further presence of CCCP (10 μ M), for the times and pH's as indicated below:

1. CCCP (pH 3.8, 3 min), NEM + CCCP (pH 6.8, 2 min)
2. CCCP (pH 3.8, 20 min), PAO + CCCP (pH 3.8, 5 min)
3. CCCP (pH 3.8, 4 min), PCMBs + CCCP (pH 5, 4 min)

All scutella were then removed, washed thoroughly and suspended in

buffer (pH 3.8) for 1 h to recover from CCCP action (Section 6.1.3.2), prior to a standard peptide transport assay using radioactive Gly-(U¹⁴C)Phe or Ala-(U¹⁴C)Ala.

Control incubations were performed with each sulphydryl reagent, in which all ethanolic CCCP solutions were replaced with equivalent volumes of ethanol alone. All ethanol concentrations were 0.5% v/v. All solutions were buffered with 50 mM sodium phosphate-citrate, and all incubations performed on a shaking water bath at 20°C.

6.4.3 Results and Discussion

With all three of the sulphydryl inhibitors used, the degree of inhibition of peptide transport achieved was unaffected by the state of energisation of the membrane (Table 6.3). However, a positive effect, even if it occurs, is likely to be difficult to demonstrate. This is because the optimal preincubation times in CCCP and the sulphydryl reagents are difficult to assess. Thus, the preincubation period in CCCP must be sufficient to collapse the proton gradient (and thus in theory to desensitize the thiol ligands) and yet the system must be able to recover after removal of the protonophore. Overexposure to CCCP may lead to permanent inactivation of the transport system. Similarly, overexposure to sulphydryl reagent may still achieve modification of the desensitized thiols despite the presence of protonophore. Therefore, a range of preincubation times in CCCP and sulphydryl reagent were used in this study in an attempt to approach the optimal conditions required to detect a positive effect.

Thus, although CCCP could not be shown to affect the sensitivity of the peptide transport system to thiol modification, the degree of sulphydryl-reagent inactivation has been shown to be dependent upon the state of energisation of the membranes for the following systems: the phosphate carrier in mammalian mitochondria (Lê-Quôc et al., 1977),

TABLE 6.3

Inhibition of Peptide Transport by Sulphydryl Reagents
in the Presence or Absence of Membrane Energisation

	Percent Inhibition of Gly-Phe Transport by NEM	Percent Inhibition of Ala-Ala Transport by PAO	Percent Inhibition of Ala-Ala Transport by PCMBS
De-Energised (CCCP-Treated)	73	72	71
Energised (Ethanol Control)	71	71	72

Percentage inhibition of either Gly-(U¹⁴C)Phe or Ala-(U¹⁴C)Ala uptake (relative to untreated controls) by 1-2 day scutella, preincubated in 10 μ M CCCP (for times indicated in text) before treatment with either 5 mM NEM (2 min), 0.5 mM PAO (5 min) or 4 mM PCMBS (4 min), each containing further CCCP (10 μ M). Scutella were washed and resuspended in buffer (sodium phosphate-citrate, pH 3.8) for 1 h before assaying for peptide transport. As controls, scutella were treated with sulphydryl reagents under identical conditions except that all CCCP solutions were replaced with ethanol.

the leucine permease in Saccharomyces cerevisiae (Ramos et al., 1983), and in Escherichia coli, lactose (Cohn et al., 1981), glucose (Haguenauer-Tsapis & Kepes, 1973, 1977), glucuronic acid (Abendano & Kepes, 1973) and amino acid uptake (Janick et al., 1977).

6.5 Final Discussion - Energisation of Transport in Other Plant Systems

6.5.1 General Considerations

It is generally agreed that of the active, mediated transport systems that operate across the cell membranes of higher plants, most are energised by means of an electrochemical gradient of ions in accordance with Mitchell's chemiosmotic hypothesis (Mitchell, 1966, 1976). In the chemiosmotic model, solute transport is effected in response to the electrical and/or the pH differential to either side of the membrane which exists when a gradient of ions has been produced. The electrochemical gradient itself is produced and maintained by an ion pump using energy derived from ATP hydrolysis. Solute cotransport is associated with the return movement of ions down the gradient, either in the same direction (symport) or in counterflow (antiport). Cotransport has been the subject of several reviews (Crane, 1977; Harold, 1977; Poole, 1978; Baker, 1978; Spanswick, 1981; Ferguson & Sorgato, 1982).

Until quite recently, the exact identity of the ion(s) involved in the electro-chemical gradient in higher plant systems had not been established (Poole, 1974; Higinbotham & Anderson, 1974) but now, several lines of evidence support the view that solute uptake is linked to proton movement by means of the so-called proton-motive force, or pmf. In support of this hypothesis, a plasmalemma-bound ATPase capable of generating a proton gradient has been identified in several plants (for a review, see Hodges, 1976). In addition, the application of sugars or amino acids to plant tissues may often induce a transient depolarisation of the cell membranes, as measured by the use of glass microelectrodes (Novacky et al., 1978), an observation consistent with

the movement of protons from one side to the other (Böcher et al., 1980; Jones et al., 1975; Etherton & Nuovo, 1974; Etherton & Rubinstein, 1978; Jung & Lüttge, 1980). Furthermore, compounds that stimulate proton extrusion, e.g. fusicochin and indoleacetic acid, enhance solute uptake (Lüttge et al., 1981; Marré, 1980; Fischer & Lüttge, 1980; Colombo et al., 1978), whereas protonophores such as CCCP, DNP and acetate, that have the reverse effect and destroy proton gradients, inhibit transport (Section 6.1.1). As additional evidence for the role of hydrogen ions in solute absorption, many transport processes show an acidic extracellular pH optimum (e.g. van Bel & Reinhold, 1975; van Bel & Hermans, 1977; Guy et al., 1979) and uptake may lead to the increased alkalization of the external medium as protons are removed (van Bel & van Erven, 1976; Hutchings, 1978).

6.5.2 Cotransport of Organic Solutes

Using the above criteria as indicative of the trans-membrane movement of hydrogen ions, proton symport is reported to operate in the transport of sugars by a range of plant tissues, e.g., sucrose by maize scutella (Humphreys, 1978, 1981; Humphreys & Smith, 1980), developing soybean embryos (Thorne, 1982), cotyledons of soybean (Lichtner & Spanswick 1981a, 1981b) castor bean cotyledons (Hutchings, 1978; Martin & Komor, 1980; Komor et al., 1980) tomato internode tissue (van Bel & Reinhold, 1975), vacuoles of sugar beet tap root (Saftner et al., 1983), and Chlorella (Komor & Tanner 1974), and glucose by maize roots (Kennedy, 1977).

Similarly, evidence has been presented for proton-linked amino acid transport in soybean embryos (Bennett and Spanswick, 1983) soybean phloem (Servaites et al., 1979), oat mesophyll cells (Rubinstein & Tattar, 1980), oat coleoptiles (Etherton & Rubinstein, 1978), fronds of the duckweed Lemna (Jung & Lüttge, 1980; Fischer & Lüttge, 1980; Lüttge et al., 1981) thalli of the aquatic liverwort Riccia fluitans (Felle, 1981), tomato

internodal tissue (van Bel & van Erven, 1976; 1979) and cultured cells of tobacco (Smith, 1978; Harrington & Henke, 1981). However, in this regard, there is some evidence that basic amino acids are transported via a separate carrier, at least in some plant systems (Kinraide & Etherton, 1978; Kinraide, 1981), and the uptake of arginine by sugar cane parenchyma is not obligatorily coupled to proton movements (Komor *et al.*, 1981). Basic amino acids, which will carry a net positive charge at physiological pH's, could be accumulated in response to the membrane potential alone (interior negative), in a so-called "uniport" mechanism.

6.5.3 Transport of Inorganic Solutes

The proton-linked cotransport of several ions has also been demonstrated in plants, including the symport of chloride (Jacoby & Rudich, 1980), ammonium ions (Dejaegere & Neirinckx, 1978), and the antiport of sodium (Ratner & Jacoby, 1976). However, the role of potassium ions in solute uptake is of the greatest interest, although being somewhat controversial. A potassium-dependent cotransport of sugars has been reported in leaf mesophyll protoplasts (Huber & Moreland, 1981) and Ricinus phloem (Malek & Baker, 1977), and of amino acids in the liverwort Riccia (Felle *et al.*, 1979) and tomato xylem parenchyma (van Bel & van der Schoot, 1980). In other tissues, a counterflow of potassium ions occurs, sometimes only transiently, as a countercurrent to maintain electrical neutrality when proton-linked uptake is operating (Bellando *et al.*, 1979; van Bel & van Erven, 1979; Komor *et al.*, 1980), although as an alternative, protons may be recirculated immediately after cotransport by means of the proton pump (Steinmuller & Bentrup, 1981) to eliminate the need for such a counterion. Clearly, further studies are needed to establish with greater certainty the importance of potassium to solute transport.

6.5.4 Other Systems

Plant transport processes often show multiphasic kinetics (e.g., Lien & Rognes, 1977; Soldal & Nissen, 1978; Shtarkshall & Reinhold, 1974) and it seems that other modes of uptake, that are independent

of a proton gradient and non-saturable, are operative, especially at high substrate concentrations. For example, in addition to a proton-driven component to transport, a non-saturable phase also contributes to uptake in several systems e.g. phloem loading in Beta vulgaris (Maynard & Lucas, 1982), leucine uptake by cultured tobacco cells (Blackman & McDaniel, 1980) and sucrose transport by soybean cotyledons (Lichtner & Spanswick, 1981b). In both soya and Beta, solute movement via the non-saturable system is at least as important as the proton driven system.

These non-saturable systems generally become increasingly significant at higher concentrations of substrate (usually in excess of 50 mM), and in most cases solute uptake is energy-independent (Birt & Hird, 1956; Cameron & Duffus, 1979; Maretski & Thom, 1970; Stewart, 1971; Harrington & Smith, 1977; Steinmuller & Bentrup, 1981; Thorne, 1982) which is indicative of a simple, diffusive process. They are therefore exactly analogous to that described here for amino acids and peptides. However, the non-saturable components of amino acid transport by developing soybean embryos (Bennett & Spanswick, 1983), phloem loading in Beta vulgaris (Maynard & Lucas, 1982) and leucine transport by tobacco cells (Blackman & McDaniel, 1980) are energy-dependent by a process, as yet unknown, distinct from proton cotransport; however, it is possible that this energy-dependence is indirect and represents that required for the maintenance of cell integrity and the ability to retain intracellular solutes.

CONCLUDING REMARKS

Throughout this thesis, attention has been drawn to those topics which could not be investigated in depth as part of this study, but which nevertheless, could provide interesting avenues for future work. In general germs, the transport of organic nitrogen across membrane barriers, as opposed to the long-distance translocation of material via the vascular system, has only recently been the subject of critical study, and transport experiments have largely been confined to uptake into intact tissues or tissue fragments from roots, leaves or internodes. However, studies like this provide little information about the inter-cellular or intracellular movement of nitrogenous material in vivo; solute movement may have symplastic and apoplastic components in the plant, and therefore the exact location of a transport system may be difficult to identify. Considerable movement of nutrients and photosynthate must occur at the cell to cell level, and knowledge of this movement is limited, although there is some understanding of the mechanism whereby the products of photosynthesis are loaded into the leaf phloem (Section 5.3.2.1). Similarly, little is known about the extent of active, trans-membrane transport intracellularly. A particularly important role for the movement of organic nitrogen across the membranes of organelles and vacuolar tonoplasts might be envisaged in any tissue undergoing rapid protein degradation, e.g., during the mobilization of proteinaceous reserves in cereal grains or in legume cotyledons, and also in tissues undergoing senescence.

Compared with studies on amino acid transport, the utilization and transport of peptides by higher plants has been largely neglected, partly because of the need to devise alternative transport assays to the use of radioactively labelled peptides which are unavailable commercially. The peptide transport system in the barley scutellum is the first to be fully characterized in a higher plant and it seems not unlikely that other

peptide uptake systems are yet to be discovered. Surprisingly, amino acid transport by the cereal embryo has still to be thoroughly investigated. Certainly in barley, next to nothing is known of the number or specificities of systems transporting amino acids, although the predominance of certain amino acids within the endospermal reserves might be accompanied by specific carriers within the scutellar epithelium.

One aspect of peptide transport about which nothing is known concerns its mechanism of regulation. Amino acid transport is apparently regulated by the presence of glutamine (Nyman *et al.*, 1983), but the nature of the compounds (if any) controlling peptide transport activity remain to be identified. If transport proteins are laid down *de novo* within the scutellum, then the preliminary experiments on the developing transport systems performed in Sections 3.6 and 3.7 point towards the possibility of incorporating a radioactive label into the proteins as they are synthesised. This might permit a direct, quantitative measurement to be made of transport protein synthesis in response to externally applied influences.

The crucial role of redox-sensitive, vicinal dithiols in peptide transport by the barley scutellum has been established. The system shows parallels with others operating elsewhere, especially the uptake of certain solutes by bacterial cells. It is possible that sulphydryl groups, in particular a dithiol-disulphide interchange, may place a general, central role in membrane transport. In this study, a procedure for the selective radioactive labelling of the peptide transport-dependent vicinal dithiols has been established. Autoradiography of sections of radioactively labelled scutella indicated that these vicinal dithiols were located almost exclusively in the plasmalemmae of the epithelial cells. Radioactive labelling was not observed in any intracellular membranes, e.g. tonoplasts, suggesting that dithiol-dependent transport did not occur into the vacuolar compartment. The specific radioactive labelling

of the vicinal dithiols is clearly a prerequisite for any attempt to extract and to isolate the transport proteins themselves. Preliminary experiments (Section 5.1.8) indicated that protein-bound radioactivity could be extracted with a membrane solubilizer and isolated on a polyacrylamide gel. Further studies are required to optimize the conditions for extracting scutellar proteins, and moreover any extracted radioactivity must first be positively identified as a component of the peptide transport system. It should then be possible to characterize the proteins in depth, ultimately in terms of sequence analysis.

The critical role of thiols in amino acid and glucose transport could not be shown here, although the possibility that essential thiol residues are involved, but are inaccessible to the particular inhibitors used, must not be ruled out. No doubt the use of other sulphydryl-specific reagents, which differ in their penetrability and reactivity, could help resolve this question. Similarly, other reagents with different amino acid sensitivities might be studied, for not all of the reagents currently available with high specificity were used here, notably the toxic serine-specific chemicals. In this respect, the use of photoaffinity labels offers exciting prospects in the field of transport biochemistry. The use of a suitably designed photoaffinity label, especially if it can be synthesised in a radioactively labelled form, allows the opportunity to label with considerable specificity the target transport proteins. Their use should complement and extend the present chemical modification approach.

Studies described in chapter 6 provide good evidence that peptide, amino acid and glucose uptake by the scutellum is coupled to the movement of protons in a cotransport mechanism. The inhibition of transport by DCCD would indicate that the proton gradient is generated, at least in part, by means of ATPase activity. Further research could be

aimed at characterizing the development, localization and activity of the ATPase complexes in the barley scutellum, since these enzymes, generally, have previously received little attention in plants.

Finally, to summarise, the barley scutellum has proved to be an ideal tissue with which to study in some depth the characteristics of peptide transport. Experimentally, scutella are quick and easy to prepare, are highly amenable to use in vitro, and display rates of uptake that are sufficiently rapid to be readily detectable over a conveniently short period of time. Moreover, a clear role can be established for the purpose of the transport system in vivo. It is to be hoped, therefore, that further work on the scutellum will help to shed more light on the complexities of active transport in a higher plant, and hence enable comparisons to be made with the more widely studied mammalian and microbial systems.

APPENDIX 1Abbreviations

AOA	aminooxyacetic acid
NEM	N-ethylmaleimide
PCMBS	p-chloromercuribenzenesulphonic acid
PAO	phenylarsine oxide
DTT	dithiothreitol
NDA	N-dansylaziridine
WRK	N-ethyl-5-phenylisoxazolium-3'-sulphonate (Woodward's Reagent K)
DEPC	diethylpyrocarbonate
PG	phenylglyoxal
DNP	2,4-dinitrophenol
DCCD	dicyclohexylcarbodiimide
CCCP	Carbonylcyanide-m-chlorophenylhydrazone
GSH	glutathione (reduced)
GSSG	glutathione (oxidised)

APPENDIX 2Culture Medium for Isolated Embryos (White, 1934)

	<u>mg per l</u>
$\text{Ca}(\text{NO}_3)_2$	200
MgSO_4	720
Na_2SO_4	200
KCl	60
NaH_2PO_4	16
$\text{Fe}_2(\text{SO}_4)_3$	2
MnSO_4	4
ZnSO_4	2
H_3BO_3	2
Thiamin	0.2
Niacin	1
Glucose	10 g

APPENDIX 3Calculation of the Amount of Peptide Transport
Protein Present Per Scutellum

Scutella were treated with NEM with a specific radioactivity of $2 \mu\text{Ci } \mu\text{mol}^{-1}$. But, since $1 \mu\text{Ci} = 3.7 \times 10^4$ disintegrations per second (dps), then, $1 \mu\text{mol NEM} = 2 \times 3.7 \times 10^4 \times 60$ disintegrations per minute (dpm). If the efficiency of scintillation count of carbon-14 is about 70%, then, $1 \mu\text{mol NEM} = 1.55 \times 10^6 \text{ cpm}$.

From Figure 5.3, the difference in bound counts between phenylarsine oxide-treated, and untreated scutella is 1500 cpm, which represents binding to vicinal dithiol.

Therefore, if $1 \mu\text{mol NEM} = 1.55 \times 10^6 \text{ cpm}$,

Then $1500 \text{ cpm} = 1 \text{ nmol NEM bound per scutellum}$.

Assuming that all vicinal dithiols exclusively represent peptide transport protein, and assuming that each protein has two vicinal dithiol groupings, then 0.25 nmol of peptide transport protein are present per scutellum.

If an average protein molecular weight is taken as 40,000

This represents 10 μg of peptide transport protein per scutellum.

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