Peptide transport by embryos of germinating barley (hordeum vulgare)

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PEPTIDE TRANSPORT BY EMBRYOS OF GERMINATING BARLEY (HORDEUM VULGARE)

by

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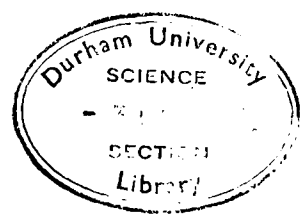

To Elizabeth

ABSTRACT

Two new fluorescent-labelling techniques for studying peptide transport are described.

A peptide transport system has been demonstrated in a higher plant tissue, the scutellum of germinating barley embryos. This system has been extensively characterized, and found to have many similarities to peptide transport systems in microorganisms and mammalian tissues. Evidence has also been obtained for the existence of a peptide transport system in the membrane of an intracellular organelle, possibly the vacuole.

Peptide transport is an active process and appears to require the production of a proton gradient across the plasmalemma. Disruption of the proton gradient not only inhibits peptide transport, but also causes general exodus of amino acids from the embryo and affects amino acid metabolism.

Considerable pools of small peptides have been detected in both the endosperm and embryo of the germinating barley grain. The concentrations of peptides achieved in the endosperm are of the right order of magnitude for the efficient operation of the peptide transport system. It seems that the uptake of small peptides by the scutellum of germinating barley embryos is of considerable importance in the transfer of nitrogen from the endosperm to the embryo during the mobilization of the protein storage reserves.

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MEMORANDUM

Part of the work presented in this thesis has also appeared in the following publications:

1. Higgins, C.F., Payne, J.W.: Peptide transport by germinating barley embryos. Planta 134, 205-206 (1977).

2. Higgins, C.F., Payne, J.W.: Characterization of active dipeptide transport by germinating barley embryos: effects of pH and metabolic inhibitors. Planta 136, 71-76 (1977).

3. Higgins, C.F., Payne, J.W.: Peptide transport by germinating barley embryos: uptake of physiological di- and oligopeptides. Planta 138, 211-215 (1978).

4. Higgins, C.F., Payne, J.W.: Peptide transport by germinating barley embryos: evidence for a single common carrier for di- and oligopeptides. Planta 138, 217-221 (1978).

5. Higgins, C.F., Payne, J.W.: Stereospecificity of peptide transport by germinating barley embryos. Planta 142, 299-305 (1978).

6. Higgins, C.F., Payne, J.W.: The uptake and utilization of amino acids, peptides and proteins by higher plants. In: Transport and utilization of amino acids, peptides and proteins by micro-organisms (Payne, J.W., ed.), J. Wiley & Sons. In Press (1979).

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CHAPTER 1

INTRODUCTION

In the last dozen or so years, the uptake of small peptides has been shown to be of considerable importance in the nutrition of mammals and both prokaryotic and eukaryotic microorganisms. Considering the extensive body of information which is currently available, relating to the occurrence and mechanism(s) of peptide transport in these organisms, it is perhaps surprising that the possibility that plant cells might also absorb and utilize peptides has rarely been considered. However, in view of the wide variety of organisms in which peptide transport is now known to be important, and the several advantages inherent in the absorption of peptides rather than free amino acids (section 1.1.7), it seems likely that, in certain plants at least, peptides will play an important role in the uptake of extracellular nitrogen. This study was undertaken in an attempt to determine a possible role for peptide transport in plants, in particular during the germination of barley grains.

Despite the virtual absence of any literature pertaining to peptide transport in plants, there is a considerable body of information directly relevant to the work described in this thesis. Three important areas of interest must be considered, each of which is reviewed below:

(i) Peptide Transport in Animals and Microorganisms

Clearly, in attempting to demonstrate a role for peptide transport in plant cells, it is pertinent to consider the available evidence concerning peptide transport in other organisms. In particular, it will be of interest to compare the nature and mechanism(s) of peptide transport in plants (if such a system may be demonstrated) with similar systems in other organisms, in relation to the environment in which they operate; differences between species might be expected to reflect specific biological functions. This is especially relevant in the case of germinating barley grains which differ from other species so far examined in that, if a transport system exists, it will operate in a controlled environment. Thus, while protein digestion in the endosperm is no doubt variable, the size, amino acid composition and concentration of peptides presented to the putative transport system will only vary within certain defined limits. This is in marked contrast with the large and often random fluctuations of available peptides which bacteria, fungi and to some extent animals, encounter in their natural environments.

(ii) Plant Peptides

The role of peptide transport in plant systems is clearly related to the availability of suitable substrates.

However, our understanding of the distribution and functions of plant peptides has lagged far behind that in animals and microorganisms; the most recent review on the subject was published more than ten years ago (Synge, 1968). Thus, the literature pertaining to plant peptides is reviewed below, in an attempt to illustrate the potential importance of this group of compounds and consequently, situations in which peptide transport systems might operate.

(iii) Barley

The germinating barley grain was selected as a suitable system, both amenable to study and in which a peptide transport system(s) might be expected to operate. The reasons for this selection are discussed. In addition, the existence of a peptide transport system must be related to its possible function *in vivo*; in this respect an understanding of the mobilization of the nitrogenous storage reserves of the barley grain is also essential.

1.1 Peptide Transport in Animal and Microbial Systems

Although in higher plants 'peptide transport' could imply symplastic cell-to-cell transfer or the long-distance movement of peptides in the phloem and xylem, the use of the term here will be restricted to the transmembrane transport of peptides.

A number of recent and comprehensive reviews have

appeared on the subject of peptide transport in both animal tissues and microorganisms, to which the reader is referred (Matthews, 1975; Matthews & Payne, 1975a; Payne, 1976, 1977; Matthews & Payne, 1979). Consequently, the topic will only be surveyed briefly below; certain aspects will be dealt with in more detail elsewhere, in relation to the barley peptide transport system.

1.1.1 Distinction Between Amino Acid and Peptide Transport: Relationship to Peptidase Activity

Any organism able to utilize externally supplied peptides to support growth must be able to hydrolyse these peptides to amino acids. However, hydrolysis could occur before, during, or after transport across the cellular membrane; a number of theoretical models may be envisaged (figure 1.1). In models A and B peptides are hydrolysed extracellularly, either by free or by membrane-bound peptidases. The cleavage products may diffuse away; subsequent absorption involves the amino acid permeases. Such mechanisms, operative in certain bacteria, do not require the existence of a distinct peptide transport system.

Models C and D are examples of a group translocation mechanism; hydrolysis occurs during transport. They differ in the sequence in which the translocation and

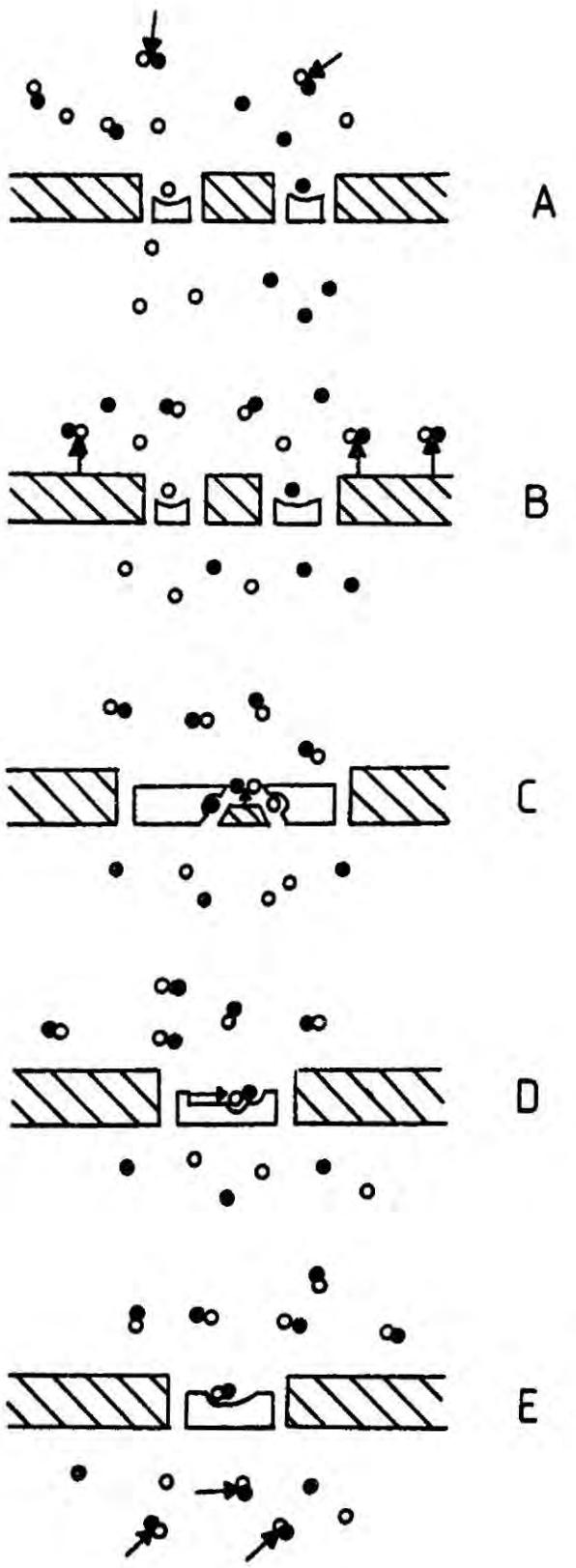


Figure 1.1: Theoretical Models for Peptide Utilization

The cytoplasmic membrane (cross-hatched) contains various carriers (plain). Arrows represent peptidase activity. The cell exterior is above the membrane, the cell interior below. See text for discussion. (Redrawn from Payne, 1977.)

hydrolytic steps occur. In model C, hydrolysis occurs first and the molecular species translocated are the free amino acids. In D, however, the intact peptide is translocated across the membrane, although hydrolysis is obligatory before the peptide may be released inside the cell. Any number of situations intermediate between C and D could occur. These two models do not require the operation of amino acid permeases and may therefore be considered as forms of peptide transport. 'True' peptide transport, however, is illustrated by model E. Peptides are released intact within the cell and only subsequently hydrolysed.

Thus, the ability to utilize a peptide does not necessarily imply the existence of a peptide transport system. Although it is quite often possible to demonstrate that peptide uptake is independent of the amino acid permeases (i.e. models A and B are not operative) it is generally difficult to exclude the possibility of hydrolysis during transport (models C and D) and to prove that the peptide is actually transported intact.

There is now considerable evidence to show that, in many species, peptide uptake is not only independent of free amino acid uptake but also that peptides cross the plasmalemma intact.

(i) The uptake of amino acids is often very much faster when presented as a peptide than in their uncombined form.

(ii) While peptides mutually compete for transport, amino acids do not inhibit peptide transport or vice versa.

(iii) Even when the amino acid transport system(s) is prevented from operating, by saturation with a second substrate, peptides may still be absorbed (Adibi, 1971; Cook, 1973; Cheeseman & Parsons, 1974).

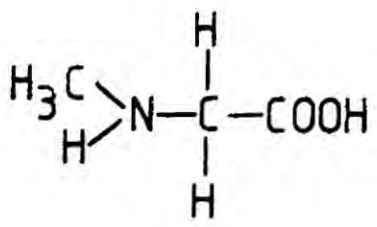
(iv) Extracellular peptidases are absent. While this is the case in many bacteria and yeasts, it is now clear that in the mammalian gut, extracellular or membrane-bound peptidases may contribute significantly to the utilization of peptides (Ugolev & de Laey, 1973; Ugolev et al., 1977).

(v) The most convincing evidence for the distinction between amino acid and peptide uptake comes from the study of transport mutants. In bacteria (Payne, 1968; de Felice et al., 1973; Barak & Gilvarg, 1974) and yeast (Marder et al., 1978; Nisbet & Payne, 1979b), peptide transport-deficient strains are still able to absorb amino acids. Similarly, strains lacking amino acid permeases can utilize peptides (Guardiola & Iaccarino, 1971). In the mammalian gut, certain diseases are associated with the absence of amino acid permeases. In two such cases, Hartnup disease (Asatoor et al., 1970; Leonard et al., 1976)

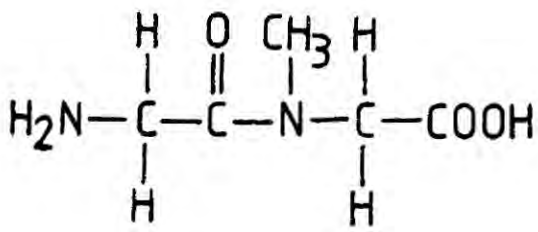
and cystinuria (Asatoor et al., 1971; Hellier et al., 1972), peptides are still absorbed despite the inability to absorb the corresponding free amino acids.

(vi) While it seems clear that peptide transport operates independently of the amino acid permeases, it has proved much more difficult to demonstrate that hydrolysis is not an integral part of the transport process itself. The fact that transport-deficient mutants still possess the normal complement of peptidases indicates the two processes are separate. However, the most convincing evidence comes from the intact uptake of peptidase-resistant substrates. Thus, carnosine and peptides containing N-methylated peptide bonds (sarcosine-containing peptides; fig. 1.2) are absorbed intact by the mammalian gut (Addison et al., 1972, 1975a; Matthews et al., 1974).

Figure 1.2 Structures of Sarcosine and Glycylsarcosine



Sarcosine



Glycylsarcosine

Similarly, sarcosine-containing peptides are taken up intact by bacteria (Payne, 1972a; Payne & Bell, 1979) and yeast (Nisbet & Payne, 1979a). The ability of other peptides to compete for transport with sarcosine-containing peptides indicates that all peptides enter through the same system which at least has the potential of operating independently of hydrolysis.

1.1.2 Occurrence of Peptide Transport Systems

Studies in the animal kingdom have been almost exclusively confined to mammalian systems. Peptide transport has been demonstrated in the intestine of many species and seems to be of considerable importance in the absorption of digested protein (Matthews, 1975). Although much less is known about other tissues, it is clear that peptide transport is not confined to the gut. Slices of many tissues, including liver, kidney (Burston et al., 1977), muscle (Nutzenadel & Scriver, 1976) and brain (Abraham et al., 1964; Yamaguchi et al., 1970), can absorb peptides intact, apparently by an active mechanism. Peptides injected into the blood stream are also absorbed by many tissues (Adibi, 1977). In addition, secretion and absorption of peptides seems to be of importance in the functioning of the renal tubules (see Matthews & Payne, 1975b). Peptides may also exert a considerable influence on the growth of mammalian cells in culture, both specifically and as sources of

indispensible amino acids (Grahl-Nielsen et al., 1974).

As yet, peptide transport has not been described in animal species other than the vertebrates. The only species so far examined, Tetrahymena thermophilia, does not seem to absorb peptides intact (Rasmussen & Zdanowski, 1979).

Fungi are also able to absorb peptides intact. The peptide transport system(s) of the yeast, Saccharomyces cerevisiae, has now been studied in some detail (Becker & Naider, 1979; Nisbet & Payne, 1979a). In addition, peptide transport has been demonstrated in a second yeast, Candida albicans (Lichliter et al., 1976), and the mycelial Ascomycete, Neurospora crassa (Wolfinbarger & Marzluf, 1975a,b).

Many bacterial species have been shown to absorb peptides. Apart from Escherichia coli, which has been most intensively studied (Payne, 1977), peptide transport has been demonstrated in such diverse genera as Salmonella (Ames et al., 1973; Jackson et al., 1976), Leuconostoc (Mayshak et al., 1966), Pseudomonas (Cascieri & Mallette, 1976), and Streptococcus (Law, 1978). However, it should not be assumed that all bacterial peptide transport systems are similar to those of E.coli; a number of differences between species have already become apparent (see below).

1.1.3 Number of Peptide Transport Systems

Cells generally possess several relatively specific amino acid permeases, each capable of handling a limited number of structurally related amino acids. However, the wide variety of peptides which could arise as a result of protein hydrolysis (400 dipeptides, 8000 tripeptides, etc.) makes a similar proliferation of relatively specific peptide permeases extremely unlikely. It seems to be a general rule that most, if not all, peptides are handled by a limited number of transport systems of broad specificity.

E.coli has two separate peptide transport systems, one for dipeptides and one for oligopeptides (Payne, 1968). The oligopeptide system is also capable of handling dipeptides, although rather poorly. However, it is not clear how widespread this duplicity of systems is amongst the bacteria. Although Salmonella (Ames et al., 1973; Jackson et al., 1976) and Pseudomonas (Cascieri & Mallette, 1976) both seem to have a di- and an oligopeptide system, there are indications that Staphylococcus aureus (Kenig & Abraham, 1976; Kenig et al., 1976) and certain Streptococci (Law, 1978) may only have a single system.

In eukaryotes, at least for those so far examined, there is no evidence for separate di- and oligopeptide transport systems. In the mammalian gut the bulk of evidence

indicates that di- and tripeptides are transported by the same system (Sleisenger et al., 1976). Similarly, the yeast Saccharomyces cerevisiae has a single system which handles both di- and oligopeptides (Marder et al., 1978; Nisbet & Payne, 1979a,b). Neurospora crassa also has a single system, although in this species it is apparently unable to handle dipeptides (Wolfinbarger & Marzluf, 1974, 1975b).

While the above-mentioned organisms all possess systems of broad specificity, there have been suggestions that additional systems may also be present. Thus, in addition to the general oligopeptide permease, there have been reports that both E.coli (Barak & Gilvarg, 1975; Naider & Becker, 1975) and Salmonella typhimurium (Jackson et al., 1976) may possess a further oligopeptide system(s) with restricted substrate specificity. Similarly, there have recently been suggestions that more than one system may be present in the mammalian gut (Gupta & Edwards, 1976); a further system specific to peptides of four or more residues has also been implied (Chung et al., 1979). However, these reports require further assessment before the presence of additional transport systems can be fully accepted.

In addition to the general 'nutritional' peptide transport systems which we are mainly concerned with here, the possibility of separate systems for the movement of specific peptides (e.g. peptide hormones) must be borne in mind.

1.1.4 Structural Requirements for Peptide Transport

Considerable effort has been invested in determining the structural requirements for peptide transport. Peptides are particularly suited to such studies since, unlike the substrates for most transport systems, an enormous variety of structures, differing in such parameters as size, shape, hydrophobicity, pK and charge, is readily available, all of which may be handled by the same permease. Information which could potentially emerge from such studies includes the number of transport systems involved, the shape of the 'receptor site(s)' on the putative transport protein, the need for and possible function of co-substrates (e.g. Na^+, H^+), and the separation of the sequential steps of transport (e.g. binding, translocation, release) with the eventual aim of understanding the mechanism by which mediated transport is achieved. In terms of the actual mechanism of peptide transport, it is clearly important to determine the relationship, if any, between peptide transport and peptidase activity (section 1.1.1); the use of chemically

modified peptides has been of key importance here. An understanding of the structural requirements for peptide transport in microorganisms had also led to the design of affinity labels for the peptide permease (Staros & Knowles, 1978) and development of the 'smugglin' concept, the possibility of ferrying normally impermeant molecules into a cell, attached to a peptide (Ames et al., 1973; Fickel & Gilvarg, 1973). This concept has many important applications, especially in metabolic studies (Ames et al., 1973) and the design of specific antimicrobial agents (Lichliter et al., 1976; Allen et al., 1978; Ringrose, 1979).

1.1.4.1 Peptide Chain Length

In all organisms so far examined there seems to be an upper limit to the size of peptide which may be absorbed. In E.coli, the dipeptide system is restricted to peptides of two residues. Similarly, the oligopeptide system, despite its name, is unable to accommodate peptides of more than about five residues. It appears that the cut-off point is dependent, not upon chain length, but the overall hydrodynamic volume (Stokes' radius) of the peptide. This is interpreted as a sieving effect of the outer cell membrane, rather than steric hindrance at the transport site itself (Payne & Gilvarg, 1968b; Decad & Nikaido, 1976).

In eukaryotes the situation is rather less certain. While it is known that both di- and tripeptides may be transported, it is not yet clear whether larger peptides may be handled. Neurospora crassa is apparently unable to utilize peptides with a hydrodynamic volume greater than trileucine. In this case it seems that the restriction is at the transport site itself, rather than by the cell wall (Wolfinbarger & Marzluf, 1975a). However, nothing more specific is known about the maximum size of peptide which can be handled.

In yeasts, it has been reported that peptides with as many as five residues may be absorbed (Naider et al., 1974; Lichliter et al., 1976), although here the picture is complicated by strain differences; certain strains are apparently unable to utilize peptides of more than three residues (Marder et al., 1977). It is conceivable that the yeast peptide transport system is limited to di- and tri-peptides; the utilization of larger peptides may be subsequent to partial extracellular hydrolysis. However, the tetrapeptide Gly-Sar-Sar-Sar is absorbed intact (T.M. Nisbet, personal communication). Assuming that this peptide is handled by the same system as di- and tripeptides, this implies that the yeast peptide transport system is able to accommodate substrates with more than three residues.

In the mammalian gut it has generally been accepted that uptake is restricted to di- and tripeptides (Adibi & Morse, 1977), although these studies have often been based on rather unrepresentative peptides (section 3.2.3.2). Recent suggestions that certain tetrapeptides may be absorbed quite possibly represent an entirely separate system (Chung et al., 1979).

There have also been a few reports that certain bacteria (Pittman et al., 1967) and mammalian cells (Grahl-Nielsen et al., 1974) may be able to absorb larger peptides, of up to ten residues. However, such situations do not seem to be the general rule; besides it has yet to be demonstrated that uptake is intact, rather than dependent upon extracellular hydrolysis.

1.1.4.2 Amino Acid Side Chains

In order to obtain efficient utilization of a mixture of protein-derived peptides, it might be expected that peptide transport systems will lack specificity for the amino acid side chains. Studies involving competition for transport, mutants for the transport system, and the uptake of chemically modified peptides, have shown this is indeed the case. However, while a vast array of peptides may be transported by the same system, it is becoming clear that the nature of the amino acid side chain can have a

significant effect on the binding affinities (K_t) and the maximum rate of uptake (V_{max}) (Yang et al., 1977; Nisbet & Payne, 1979a; Payne & Bell, 1979).

1.1.4.3 N- and C-Terminal Groups

Removal or substitution of either the N-terminal α-amino group or the C-terminal α-carboxyl group of a peptide generally has a marked influence on its uptake (see section 3.2.1.3). Many early studies reported an absolute requirement for one or both of the terminal groups. However, such conclusions were mainly based on growth tests which simply indicate whether uptake is faster or slower than the minimum rate required to support growth. With the advent of more sensitive techniques it is now becoming clear that, while the terminal amino and carboxyl groups of a peptide are both of considerable importance in determining transport capabilities, neither is absolutely essential for transport to occur.

1.1.4.4 Peptide Bond

An α-peptide bond is generally required for transport (section 3.2.4); peptides containing β-, γ- or ε - linkages are excluded. Similarly, substitution of the bond (e.g. methylation) considerably reduces the rate of peptide uptake.

1.1.4.5 α-Hydrogen Atom

In bacteria, the only organisms studied, it seems that the hydrogen atom on the α-carbon is not essential; peptides lacking the α-hydrogen are still taken up, probably by the same permease as other peptides (Young et al., 1964; Smith et al., 1970).

1.1.4.6 Stereospecificity

Peptide transport generally shows a marked, but not absolute requirement for peptides containing L-amino acids (section 3.2.5.3). However, the effect of a D-residue often depends on its position in the peptide. For example, there is a certain amount of evidence in both bacteria (Becker & Naider, 1974) and yeast (Becker & Naider, 1979) to support the suggestion (Payne & Gilvarg, 1971) that the presence of a D-isomer at the C-terminus of a tripeptide should not prevent transport.

1.1.5 Energy Coupling

Peptide transport is an active process. In addition to the inhibitory effects which many metabolic inhibitors (e.g. azide, anoxia, DNP) exert on transport, there is evidence that substrates can be accumulated against their electrochemical gradient in an unmodified form. While most peptides are hydrolysed so rapidly that intact uptake cannot be demonstrated, certain peptidase-resistant substrates

(e.g. Gly-Sar[a]) may be accumulated intact, against a concentration gradient, by bacteria, yeast and mammalian tissues (section 1.1.1). Moreover, peptidase-deficient mutants have been used to demonstrate the intact accumulation of physiological peptides (Jackson et al., 1976; Yang et al., 1977).

The mechanism whereby metabolic energy is coupled to peptide transport is unclear (see also section 3.3.3.5). In the only bacterial species studied, E.coli, it appears that transport is energized directly by ATP (Cowell, 1974; Payne & Bell, 1979). ATP-dependent transport systems are usually associated with a periplasmic binding protein. Although peptide transport is apparently 'shock-sensitive' (Cowell, 1974), a binding protein has not yet been isolated. It remains to be seen whether or not peptide transport is unique in this respect.

Concentrative peptide transport in the mammalian gut is Na^+-dependent (Rubino et al., 1971; Addison et al., 1972; 1975a). It seems likely that a peptide-Na^+ co-transport system will operate, as for free amino acid uptake, although as yet there is no direct evidence to

(a) Abbreviations for amino acids are according to the 1972 IUPAC-IUB recommendations (Biochem. J. 126, 773-780). The L-stereoisomer is implied throughout, unless otherwise stated. All other abbreviations employed in this thesis are listed in appendix 1.

support this view.

Meister has proposed that a number of enzymes known to be present in certain mammalian cells might operate in a cycle (the γ-glutamyl cycle) which could transport amino acids and/or peptides across the cell membrane by a group-translocation process (Meister, 1973; Meister & Tate, 1976; Prusiner et al., 1976). Although by no means proven, evidence is accumulating in favour of this hypothesis, at least for certain amino acid transport systems.

Two further mechanisms, specific to peptides, by which transport might be energized have been proposed; uptake might be linked to amino acid exodus or to peptidase activity. It is conceivable that an antiport mechanism operates such that one molecule of peptide is exchanged for one molecule of an amino acid (Brock & Wooley, 1964; Sigrist-Nelson, 1975). Such a mechanism would be energetically favourable, although there is no evidence available to support this view. It is also feasible that free energy released during the hydrolysis of a peptide bond might be harnessed to the transport process. Two mechanisms can be envisaged:

(i) Hydrolysis simply serves to maintain a concentration gradient of peptide across the membrane; peptides enter the cell by passive diffusion down this gradient.

(ii) The free energy of hydrolysis could be coupled directly to translocation in a peptidase-linked permease (Parsons, 1972).

In those transport systems characterized so far, evidence that peptide uptake is independent of peptidase activity eliminates these possibilities. However, they should be borne in mind when characterizing new systems.

1.1.6 Regulation of Transport

Little information is available concerning the mechanism(s) by which peptide transport is regulated. Indeed, it may be that transport is not regulated *per se*, the intracellular pool of each amino acid being maintained at a constant level by specific exodus (Payne & Bell, 1977c).

Regulation of transport could operate at two levels:

(i) Variation in the amount of permease per cell. It is generally considered that peptide transport is constitutive; the level of permease per cell remains constant. However, it has recently been suggested that this view may not be strictly accurate, at least in *S. typhimurium* (Alper & Ames, 1978).

(ii) Variation in the activity of the permease. The activity of many transport systems is subject to feedback regulation. However, feedback inhibition by peptides seems unlikely as little or no intact peptide accumulates within the

cell under physiological conditions. Regulation by a particular amino acid also seems improbable for a permease of such broad specificity, although a general mixture of amino acids might exert some regulatory influence (Payne & Bell, 1977c).

1.1.7 Advantages of Peptide Transport

The widespread occurrence of peptide transport systems, in organisms encountering many different forms of organic nitrogen, suggests that there might be certain advantages in transporting peptides rather than free amino acids. This position is accentuated in Bacteroides ruminicola which will only absorb peptides; amino acid permeases are apparently absent (Pittman et al., 1967). It has been known for many years that peptides are often better sources of nitrogen for bacterial growth than free amino acids. Early suggestions that a specific peptide growth factor, 'Streptogenin', might be responsible have not been substantiated. It is now generally accepted that the nutritional superiority of peptides can be explained simply in terms of the advantages of absorbing peptide-bound amino acids rather than the unbound residues (see Matthews & Payne, 1975c). A number of benefits that might be derived from the transport of peptides can be envisaged, although little evidence is actually available to support any of these views:

(i) It is a general observation that peptide-bound amino acids are absorbed more rapidly than the equivalent free amino acids. Although obviously an advantage, it is not yet clear why this should be the case; a satisfactory explanation awaits a clearer understanding of the mechanism of peptide absorption.

(ii) The need for extracellular hydrolysis is eliminated. It would seem to be more efficient, at least when amino acids are scarce, to hydrolyse peptides within the cell, rather than to rely on extracellular hydrolysis, when many of the resultant amino acids will be lost to the surrounding medium. In addition, the extracellular medium may be unsuitable for efficient operation of peptidases, especially in species living in extreme or variable environments.

(iii) In certain circumstances, one amino acid can affect the cellular concentration of another, either by inducing the synthesis of the appropriate degradative enzymes or by repressing their common permease. Thus, an excess of a single amino acid can effectively inhibit protein synthesis by reducing the level of a second limiting amino acid. Peptide transport overcomes the possibility of specific permease repression. In addition, the rate at which amino acids are released by peptide hydrolysis is often too slow for the synthesis of degradative enzymes to be induced.

(iv) Probably the most important benefit to be gained from peptide transport is the conservation of metabolic energy. This is important, as a high proportion of any cell's energy expenditure is channelled into the uptake of metabolites.

Two possible, though unsubstantiated, means by which peptide transport might conserve energy have already been discussed (section 1.1.5); uptake could be linked to amino acid exodus or to peptidase activity. It also seems likely, at least for molecules of similar charge, that the transport of amino acids and small peptides will require the same amount of energy per molecule. Thus, uptake of a dipeptide will halve the energy requirement per amino acid residue. Similarly, the uptake of charged amino acids may be energetically expensive; uptake of the same amino acids combined as neutral peptides could prove economical.

1.2 Plant Peptides

1.2.1 Introduction

Plant peptides can be conveniently divided into two categories: (i) Peptides with a specific structure or function. Such peptides commonly contain unusual amino acids or peptide linkages, not found in proteins or peptides derived from them. (ii) Peptides produced as intermediates in the synthesis and degradation of proteins. This 'peptide pool' might be expected to consist of a mixture of small peptides with an overall amino acid composition similar to that of the parent proteins. A rapid turnover of the pool might be anticipated.

The literature concerning plant peptides is widely scattered and an exhaustive coverage will not be attempted here. Rather, a general survey will be made to illustrate the specific functions which peptides may perform in the plant cell, together with any information pertaining to the existence of a 'peptide pool'. The subject is to be reviewed in more detail elsewhere (Higgins & Payne, 1981). Several reviews covering early studies have also appeared (Bricas & Fromageot, 1953; Synge, 1959, 1968; Waley, 1966).

For present purposes, the arbitrary division between peptide and polypeptide will be drawn at a molecular weight of about 1500. In addition, no account will be taken of

cellular compounds containing peptide linkages, but otherwise bearing little or no resemblance to protein-derived peptides (e.g. the peptide alkaloids).

1.2.2 Peptides with a Specific Structure or Function

In addition to their nutritional role, peptides serve many other important functions in both animals and microorganisms (Matthews & Payne, 1975b). In contrast, little is known concerning the specific roles of peptides in plant tissue. However, an ever increasing number of such compounds are being identified and there is no reason to suppose that peptides will prove any less important in plants than in other organisms.

1.2.2.1 Glutathione and Other γ-Glutamyl Peptides

Glutathione (γ-L-Glu-L-Cys-L-Gly) can occur in a reduced or an oxidized disulphide form. The two are readily interconverted by numerous redox reagents or the enzyme glutathione reductase.

Both glutathione and glutathione reductase appear to be ubiquitous. They have been identified in monocots (Conn & Vennesland, 1951; Tkachuk, 1970), dicots (Mapson & Isherwood, 1963; Rennenberg, 1976) and algae (Tsang & Schiff, 1978); in mitochondria (Young & Conn, 1956), chloroplasts (Foyer & Halliwell, 1976; Schaedle & Bassham, 1977) and the cytoplasm (Wirth & Latzko, 1978). In

addition, the enzymes for glutathione synthesis have been isolated from plant tissues (Webster, 1955).

Many functions have been proposed for glutathione, often associated with the maintenance of optimal redox conditions within the cell (see Meister, 1975; Meister & Tate, 1976; for general reviews). These have generally been based upon the activity of glutathione in animal and microbial cells, although a number of functions specific to plant cells have also been proposed. However, despite much speculation, its role *in vivo* remains very much in doubt.

When considering the role of glutathione in plant tissues, it is interesting to note that a homologue of glutathione, γ-L-Glu-L-Cys-β-L-Ala (homoglutathione) has been isolated from *Phaseolus* (Carnegie, 1963a,b). It is present at twenty times the level of glutathione and shows similar patterns of activity. It may therefore serve the same function(s) as glutathione in certain plant tissues.

Glutathione is also a key compound in the γ-glutamyl cycle (section 1.1.5) which has been suggested as a possible mechanism by which amino acids and/or peptides might be translocated across the cell membrane. It is interesting to speculate that a similar group translocation mechanism might operate in plants. A number of enzymes of the cycle have been isolated from higher plant tissues (Messer & Ottesen,

1965; Goore & Thompson, 1967; Mazelis & Creveling, 1978). In addition, the complete cycle seems to operate in marine phytoplankton, which are known to be extremely active in amino acid transport (Kurelec et al., 1977).

Glutathione may also be involved in the synthesis of the numerous γ-glutamyl peptides which have been isolated from plant tissues. Many of these peptides contain non-protein amino acids and occur in considerable quantities, especially in seeds and storage organs. This suggests a role in the storage of nitrogen and/or sulphur (Thompson et al., 1962; Virtanen, 1965), yet most of the known γ-glutamyl peptides could not adequately fulfil such a role.

Certain γ-glutamyl peptides might serve a protective function. The toxin of Lathyrus seeds is a γ-glutamyl peptide (Schilling & Strong, 1955). Hypoglycin, the toxic non-protein amino acid of Blighia fruits, is also maintained in the cell as a γ-glutamyl peptide (von Holt et al., 1956). Similarly, the lachymatory and oderiferous products of onion and garlic are often linked as γ-glutamyl peptides which are hydrolysed by a specific peptidase on crushing the tissue (Austin & Schwimmer, 1970; Whitaker, 1976). However, the function, if any, of the majority of such peptides remains obscure.

1.2.2.2 Algal Peptides

A number of specific peptides have been isolated from seaweeds (Bricas & Fromageot, 1953; Miyazawa, 1971). Eisenine, a tripeptide from *Eisenia bicyclis*, has the structure L<Glu-L-Gln-L-Ala (Ohira, 1940; Ohira *et al.*, 1955a,b; Kaneko *et al.*, 1957). The pyroglutamate residue was probably formed from glutamine during extraction. A similar peptide, fastigiatin (L<Glu-L-Gln-L-Gln), has been isolated from *Pelvetia* (Dekker *et al.*, 1949) and a tripeptide containing alanine and glutamic and aspartic acid from *Undaria pinnatifida* (Kwon & Lee, 1960; Lee *et al.*, 1962). Further reports of peptides, isolated from brown (Channing & Young, 1953; Morita, 1955; Takagi *et al.*, 1973), green (Miyazawa *et al.*, 1976) and red seaweeds (Young & Smith, 1958; Miyazawa & Ito, 1974), have also appeared. These peptides generally contain arginine and/or the acidic amino acids. No function for such compounds has yet emerged.

A number of freshwater algae also contain peptides. L-Pro-L-Val-diketopiperazine and L-Pro-L-Leu-diketopiperazine are released by species of *Scenedesmus* (Luedemann *et al.*, 1961). Many green algal peptides are rich in arginine. L-Arg-L-Gln has been identified in *Cladophora* (Makisumi, 1959); at least seven small peptides containing arginine, glutamic and aspartic acid have been isolated from *Chlorella*

(Kanazawa, 1964; Kanazawa et al., 1965) and several more from Euglena (Schantz et al., 1975). It is possible that these peptides replace the arginine-rich histone known to be absent from certain green algae.

Blue-green algae have long been known to release small peptides, although the chain lengths have never been accurately determined (Fogg, 1952; Jones & Stewart, 1969). Often a very high proportion of the total assimilated nitrogen is excreted in this form. The structures of these compounds are unknown, although it has been suggested that they may be as small as tripeptides (Walsby, 1974). Blue-green algal blooms may also release toxins, at least some of which are peptides (Stewart, 1979); the toxic peptide of Microcystis has a molecular weight of 1300-2600 (Bishop et al., 1959).

1.2.2.3 Peptides in the Phloem and Xylem

Phloem contains high levels of free amino acids (see Higgins & Payne, 1979). Early reports of peptides in the phloem have largely been explained on the basis of the high levels of protein now known to occur in phloem exudate (Eschrich & Heyser, 1975). Only two peptides have definitely been identified in the phloem, alanylaminobutyric acid and glycyl-ketoglutaric acid (Pate, 1976a).

The xylem contains much lower levels of nitrogen than the phloem yet peptides have been reported in the xylem

exudates of many species (Wolffgang & Mothes, 1953; Pollard & Sproston, 1954; Bollard, 1957, 1960; Virtanen & Miettinen, 1963; Fejér & Kónya, 1958; Khachidze, 1975). However, some of the techniques employed may have identified protein material rather than small peptides. More specifically, Pate (1965) has reported that glutathione, present in the xylem, may be involved in sulphur transport, and Miettinen (1959) showed that alanine absorbed by pea roots may be incorporated into a specific peptide, transported to the stem, and then degraded.

Thus, there is little evidence of an important role for peptides in the phloem and xylem, although such a possibility cannot yet be discounted (section 6.6.2.1).

1.2.2.4 Peptides in Symbiotic Associations

In many symbiotic associations, nitrogen is fixed by one symbiont and rapidly transferred to its partner. Although in many cases it seems that nitrogen is transferred as amino acids or in inorganic form (Smith, 1974, 1975; Pate, 1976b), there have been indications that peptides might be involved.

Peptide-like material has been detected in the root nodules of clover (Butler & Bathurst, 1958) and Alnus (Leaf et al., 1958) although its nature was not investigated in detail. In three species of the lichen, Peltigera, the blue-

green algal symbiont (Nostoc) releases peptides in vitro and in vivo which may be utilized by the fungal partner (Millbank, 1974, 1976). These peptides are 2-10 residues long and similar to those released by the free-living alga. However, in liverwort thalli, symbiotic Nostoc apparently releases only a very small proportion of its fixed nitrogen as peptide, the majority being free NH_4^+ (Stewart & Rodgers, 1977). The cause of this difference and the possible role of peptides in nitrogen transfer between symbionts deserves more attention (section 6.6.1.2).

1.2.2.5 Peptide Hormones

Although a number of peptides affect plant growth at very low concentrations (section 1.2.2.6), no peptide has yet been identified in healthy plant tissue which regulates growth under normal conditions. This is despite the wide variety of regulatory and hormonal functions served by peptides in animal tissues (Berson & Yalow, 1973; Vale et al., 1977), and some considerable effort invested in the isolation and identification of plant hormones.

Most reports of peptide hormones have turned out to be peptide-bound derivatives of known plant growth regulators, and speculations that the proposed flowering hormone, florigen, might be a peptide (Collins et al., 1963) have not, as yet, been confirmed.

Auxins: The in vivo conjugation of IAA with peptides has been reported (Jerchel & Staab-Müller, 1954; Winter & Street, 1963), although it is not clear how large these peptides are or whether covalent bonding is involved (Winter & Thimann, 1966). Such complexes may represent 'protein-bound' derivatives isolated by a number of other groups (Siegel & Galston, 1953; Zenk, 1964), or may be artefacts of the isolation procedure. However, it is well known that exogenously supplied IAA can be rapidly conjugated in a peptide linkage with aspartic or glutamic acid, especially by legumes (Andreae & van Ysselstein, 1956, 1960). These derivatives, which also occur naturally (Klämbt, 1960), are hormonally inactive and believed to participate in the regulation of auxin activity. Reports that they possess auxin activity themselves may be the result of hydrolysis (Thurman & Street, 1962). Unfortunately, the presence of many non-peptide derivatives of IAA in plant cells make a precise role for the peptide conjugates difficult to define.

Ethylene: Methionine is generally considered to be the biological precursor of ethylene in plant systems (Yang et al., 1967). However, the production of ethylene from methionyl peptides by pea extracts is much more rapid than from methionine itself and has a more physiological pH optimum (Ku & Leopold, 1970). Thus, one or a number of peptides may be the biological precursor(s) of ethylene.

Gibberellins: Although no GA-amino acid or GA-peptide derivatives have been positively identified in plant tissues, their existence has been postulated on theoretical grounds (Sembdner, 1974) and a GA-peptide derivative has been tentatively identified (Nadeau & Rappaport, 1974). Formation of this inactive derivative is enhanced by ABA, consistent with the inhibitory effects exerted by ABA on GA activity. Synthetic amino acid or peptide derivatives of GA_3 show very little gibberellin activity and cannot be hydrolysed by plant tissues (Sembdner et al., 1976). It has therefore been proposed that such derivatives serve to inactivate, and thus regulate, the levels of the hormone in plant tissue.

1.2.2.6 Other Peptides

Two specific peptides have been purified from higher plant tissues. Evolidine, a cyclic heptapeptide (cyclo-L-Ser-L-Phe-L-Leu-L-Pro-L-Val-L-Asn-L-Leu), was identified in Evodia xanthoxyloides (Eastwood et al., 1955; Law et al., 1958), and the tetrapeptide methyl ester, L-Pro-L-Leu-L-Phe-L-Val-OMe, in linseed oil (Kaufman & Tobschirbel, 1959). Their functions remain unknown.

Peptide-nucleotide complexes have often been isolated from plant tissues (see Waley, 1966; Synge, 1968). As yet none has been fully characterized and little is known concerning

their functions, although they often promote plant growth and have been implicated in protein synthesis (Dudchenko & Sytnik, 1974; Volodin, 1975; Goryunova et al., 1977). At least some of these compounds seem likely to be tRNA-peptide complexes, removed from the ribosome during extraction.

Peptides also seem to be associated with certain plant diseases, exerting hormone-like effects on plant growth. It is not always clear whether, in cases of infection, such peptides are produced by the host or the pathogen. Infection of potato leaves by the leafroll virus induces the production of a specific peptide (Reindel & Bienenfeld, 1956), and Chang has reported the presence of a peptide specifically in tissues infected with crown gall or tomato nematode gall (Chang et al., 1975; Chang & Lin, 1977). Lycomarasmin, a dipeptide produced by Fusarium, induces tomato leaf curl and wilt (Robert et al., 1962), and malformin, a cyclic peptide (cyclo-D-Cys-D-Cys-L-Val-D-Leu-L-Ile) produced by Aspergillus, causes curvature of bean and corn roots at very low concentrations (Bodanszky & Stahl, 1974). Islandi-toxin, produced in Penicillium-infected rice is also a cyclic pentapeptide (Bamburg et al., 1969). Along similar lines, a β-alanine-containing peptide produced by pea roots under conditions of high salinity is also an extremely active inhibitor of plant growth (Prikhod'ko et al., 1975, 1978).

1.2.3 Peptides as Metabolic Intermediates: The Peptide Pool

The concept of a peptide pool is not new (Waley, 1966). However, although it has been widely assumed that peptides will be produced as intermediates in protein metabolism, the possibility that these intermediates might accumulate to form a significant pool has rarely been considered. There seem to be a number of reasons for this neglect. Firstly, although it is accepted that peptides will be produced as intermediates during protein synthesis and degradation, it is often felt that they themselves will be metabolised so rapidly that a significant pool will never accumulate. Secondly, in the absence of any obvious function, an incentive for investigation is lacking. The present demonstration of peptide transport in plants provides one possible function. Thirdly, there are methodological problems. In a peptide pool, any single defined peptide will be present in vanishingly small amounts. Thus, the pool as a whole must be detected, often a difficult proposition in the presence of large quantities of protein and free amino acids.

All plant cells are likely to exhibit protein turnover (Huffaker & Peterson, 1974) and might therefore be expected to contain a peptide pool, however small.

There is a certain amount of circumstantial evidence to

support this view although few of the reports of peptide pools can be regarded as conclusive. This is generally a reflection of the rather uncritical approaches which have been employed in the past (see section 5.1). Even those relatively few cases in which peptides have been more rigorously identified do not give an adequate picture of the nature of the peptide pool; the peptides have rarely been characterized and the methods of extraction and purification seem unlikely to result in a complete or representative sample of the total pool.

Tissues of Rapid Protein Synthesis: Early suggestions that peptides might be directly incorporated into protein led to a number of studies of 'peptides' in growing tissue. For example, a large 'peptide' pool was reported to increase twenty-fold during the elongation of developing root tips (Morgan & Reith, 1954). Similarly, 'peptides' were reported in ripening pea cotyledons during protein synthesis. This pool decreased as protein synthesis reached completion (Raake, 1951). 'Peptide fractions' have also been reported in growing pea seedlings (Lawrence et al., 1959; Beevers & Guernsey, 1966; Prikhod'ko et al., 1978) and barley embryos (Folkes & Yemm, 1958), although they are apparently absent from maize (Ingle et al., 1964).

None of the above reports can now be interpreted as conclusive evidence for a peptide pool, although this is certainly a possible explanation for many of the data. Even a more recent study, reporting a pool of peptides in the axis of germinating soyabeans (Duke et al., 1978) is inconclusive; peptides were only separated from proteins on the basis of ethanol solubility. More critically, peptides have been identified in the growing leaves of barley (Hendry & Stobart, 1977), although in this case little attempt was made to characterize the pool.

Mature Tissues:Tissues in which protein synthesis and degradation are minimal have been least thoroughly studied. Most reports of peptides are rather vague. Thus, ten peptides of 3-12 residues were reported in cottonseed (Yuldashev et al., 1970) and about twenty peptides of less than ten residues in celery root (Curi et al., 1973). The stems of several species may also contain a wide range of peptides (Chang et al., 1975) and it has been suggested that salt-tolerant plants contain a considerable peptide pool (Prikhod'ko et al., 1979). Reports of specific peptides, Ala-Gly in rice (Tsumura et al., 1977) and both Ala-Ala and Gly-Ala in Leptadenia (Dhawan & Singh, 1976), may represent part of a peptide pool or may serve more specific functions.

Carnegie (1961) has reported a pool of small peptides in ryegrass leaves. Although probably rather unrepresentative of the total peptide pool, this remains one of the few convincing reports of a peptide pool to date. A similar pool in bean leaves (Bagdasarian et al., 1964) may represent rather larger polypeptides.

Tissues of Rapid Protein Hydrolysis: Apart from a single report of peptides in senescing barley leaves (Hendry & Stobart, 1977), germinating seeds have received most consideration.

During germination of Vicia, a large pool of soluble, non-protein nitrogen was found in the cotyledons (Boulter & Barber, 1963). Although not positively identified as peptide material, the pool contained at least two defined peptides, one a dipeptide of glycine and alanine, the other containing alanine, aspartic acid and cysteine; it therefore seems likely that much of the rest of the pool also consisted of peptides. Similar fractions, possibly containing peptides, have been reported in the cotyledons of germinating peas (Lawrence et al., 1959; Beevers & Guernsey, 1966), castor beans (Stewart & Beevers, 1967), and cucumber (Becker et al., 1978), although no such pool was detected in germinating soyabean cotyledons (Duke et al., 1978).

Peptide pools may also be produced in the endosperm of

germinating cereal grains. In barley (Folkes & Yemm, 1958) and maize (Ingle et al., 1964; Oaks & Beevers, 1964) a large pool of soluble nitrogen (probably consisting largely of peptides) increases to a maximum and then decreases as germination proceeds. In addition, 'peptides', as well as free amino acids, leach from isolated maize endosperm (Oaks & Beevers, 1964). Barley endopeptidase is known to cleave the storage proteins to fragments of molecular weight less than 3000 in vitro (Popov et al., 1976), and during the sprouting of wheat, high molecular weight proteins disappear while low molecular weight material (probably peptides) appears (Hwang & Bushuk, 1973). A hexapeptide has been isolated and partially characterized from wheat (Bieber & Clagett, 1956) and a pool of peptides, averaging 6-8 residues in length, has been reported in wheat flour (Grant & Wang, 1972). In addition, during the malting and mashing of barley considerable amounts of peptide are produced. This almost certainly reflects the in vivo production during germination (MacWilliam, 1968; Stewart et al., 1974). The presence of a peptide pool in barley endosperm might also be predicted from the activity and localization of the various proteases and peptidases (section 1.3.4.5).

The above reports cannot be considered as conclusive evidence for the presence of a peptide pool, even in such intensively studied systems as germinating seeds. However, the results are generally compatible with such a concept; it seems probable that the application of a more rigorous methodology will show the existence of a peptide pool in many, if not all tissues.

1.2.4 Peptide Transport in Plants

1.2.4.1 Algae

Intact peptide uptake has never been demonstrated amongst the algae. Although peptides apparently stimulate the growth of certain algal species (Jankevicius et al., 1972), and Chlorella is able to utilize several dipeptides as a nitrogen source (Bollard, 1966), the form in which peptides are absorbed has not been investigated; uptake might be subsequent to extracellular hydrolysis. However, it does seem likely that certain algae will possess peptide transport systems; this possibility has yet to be pursued (section 6.6.1.1).

1.2.4.2 Higher Plants

Although the uptake and utilization of free amino acids by higher plant cells has been investigated in some detail (see Higgins & Payne, 1979, for a general review), peptides have suffered relative neglect.

It has been known for many years that peptides can affect the growth of plant tissues cultured *in vitro*. For example, acid hydrolysed casein (no peptides) often enhances growth while enzymic casein hydrolysates (containing peptides) can show inhibitory effects (Riker & Gutsche, 1948; Nitsch & Nitsch, 1957; Staba, 1962; Furuhashi & Yatazawa, 1970; Sood, 1975). Moreover, several individual defined dipeptides are capable of providing the sole source of nitrogen for plant growth: duckweed fronds (Bollard, 1966), *Atropa* callus (Salonen & Simola, 1977) and the sundew (Simola, 1978b). In none of these cases was any attempt made to determine whether the peptides were hydrolysed before or after absorption.

The only true, though rather fortuitous demonstration of intact peptide transport in plants has come from studies on the absorption of nutrients from the pitchers of the carnivorous plant *Sarracenia flava* (Plummer & Kethley, 1964). Three peptides, DL-Ala-DL-Asp, DL-Ala-DL-Met and DL-Ala-DL-Leu, were not hydrolysed by the pitcher fluid but taken up intact into the leaves where they remained uncleaved for as long as 4-5 days. No attempt was made to characterize the mechanism by which these peptides were absorbed. The detection of these peptides intact was probably the result of employing peptides containing both D- and L-amino acids;

all stereoisomers of the dipeptides, except the LL-form, might be expected to be resistant to hydrolysis.

Thus, the information presently available concerning peptide transport in plant tissues is strictly limited. However, a number of situations in which such systems might be of importance can be envisaged; these are discussed elsewhere (section 6.6).

1.3 Barley

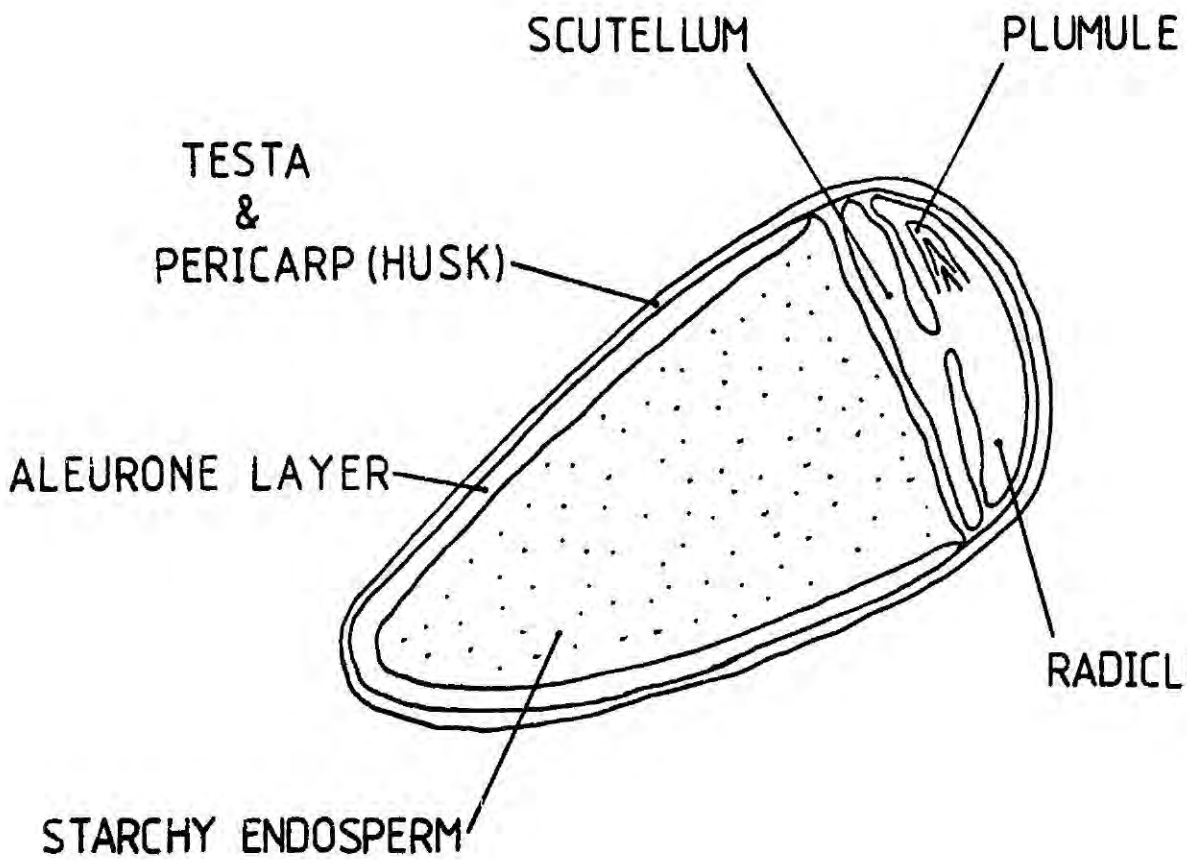
It is well known that in both monocots (Folkes et al., 1952; McConnell, 1957; Folkes & Yemm, 1958; Ingle et al., 1964) and dicots (Oota et al., 1953; Larson & Beevers, 1965; Stewart & Beevers, 1967; Basha & Beevers, 1975), large amounts of nitrogen are transferred from the endosperm/cotyledon to the growing embryo during germination. Thus, germinating seeds seem to be one of the most likely sites for the operation of a peptide transport system in plant tissues.

The physiology and biochemistry of seed germination, particularly nitrogen metabolism, have been studied in a range of different species (see Mayer & Poljakoff-Mayber, 1975; Ashton, 1976; Beevers, 1976; Bewley & Black, 1978, for general reviews). However, the structure and metabolism of monocots, in particular the cereals, makes them especially suitable for the intended investigations. Barley has been subject to particularly intensive study, as a result of its importance to the brewing industry, and the mobilization of the protein storage reserves in barley is probably better understood than in any other species. This is clearly an advantage in assessing a possible role for peptides in the transport of nitrogen during germination.

1.3.1 Structure

The structure of a barley grain is illustrated in figure 1.3. The grain is surrounded by a husk which is readily removed after a few hours imbibition.

Figure 1.3 Structure of the Dormant Barley Grain



The husk has two main layers, the testa and the pericarp, which are fused together; thus, barley grains are not true seeds but caryopses.

The bulk of the grain is occupied by the starchy endosperm, a dead tissue, which is surrounded by a few layers of living cells, the aleurone layer. The majority of

the storage reserves of the grain are located in these two tissues. In any discussions below, the term endosperm will be used to include both the starchy endosperm and the aleurone layer.

The remainder of the grain, about 25% by volume, is occupied by the embryo. Apart from the primordial roots and shoots, the cereal embryo contains a third major tissue, the scutellum. This is believed to be derived from one of the cotyledons, highly developed as an haustorial organ (Avery, 1930). The surface of the scutellum, abutting onto the endosperm, is well adapted for absorptive purposes (Sargant & Robertson, 1905). There is a single, specialized epithelial layer, the cells of which elongate and separate from each other during germination to form villi-like structures (Nieuwdorp & Buys, 1964; Zamski, 1973). In addition, the surface of the scutellum folds to form numerous invaginations, greatly increasing the overall surface area.

During germination, all compounds stored in the endosperm must be absorbed by the scutellum before they can be utilized by the embryo. The scutellum is readily separated from the endosperm without causing any significant damage to the epithelium. Thus, uptake by the scutellum can be studied <u>in vitro</u> without the presence of cut or

damaged tissue. This is in marked contrast to most plant transport studies where excised or fragmented tissue must be used.

1.3.2 Storage Protein

Barley grains contain about 13% protein on a dry-weight basis; about 85% of this protein is stored in the endosperm (Dale et al., 1974). Approximately one third of the endospermal protein is located in the aleurone layer, the remainder being distributed throughout the starchy endosperm (Mikola & Kolehmainen, 1972).

Seed proteins are still subdivided on the basis of their solubility characteristics as described by Osborne (1924). The four major fractions are all present in barley, occurring in the following proportions: albumins (water soluble, 13%); globulins (soluble in saline, 12%); glutelins (soluble in acid or alkali, 25%); prolamines (soluble in ethanol, 50%) (Whitehouse, 1973). The specific names for the barley glutelins and prolamines are hordenin and hordein, respectively.

Although the barley storage proteins have only been poorly characterized, it is clear that they are highly heterogeneous (Enari, 1966, 1968). Even hordein, the major storage protein, which might reasonably be expected to be

homogeneous, seems to have at least 6-8 individual polypeptide components (Shewry et al., 1977). The four protein fractions also have very different amino acid compositions; of particular interest is the high level of proline in hordein (Folkes & Yemm, 1956; see also section 5.4.4).

1.3.3 Protein Bodies

A high proportion of the barley endosperm proteins seem to be located in small, discrete membrane-bound organelles, the protein bodies or aleurone grains (Paleg & Hyde, 1964; Jones, 1969a; Jacobsen et al., 1971). The storage proteins of the cereals are specifically located within these organelles. Thus, in maize, zein (prolamine) is sequestered within the protein bodies while the glutelins remain free in the cytoplasmic matrix (Christianson et al., 1969; Nielsen et al., 1970). Similarly, in barley, hordein is the major component of the protein bodies (Tronier et al., 1971). Protein digestion in many species is believed to occur partly, if not wholly, within the protein body itself (sections 4.4.8; 6.6.2.3). In this context it is interesting to note that acid protease activity has been associated with the protein bodies of barley (Ory & Henningsen, 1969).

1.3.4 Protein Digestion

The transfer of nitrogen from the endosperm to the embryo of barley grains during germination is a well documented process (Folkes et al., 1952; Folkes & Yemm, 1958; Metevier & Dale, 1977). Movement is most rapid between days 2 and 6 of germination; by day 6 more than 85% of the endosperm nitrogen has been transferred to the embryo. Most of the remaining nitrogen can be accounted for by the non-digestible husk (Metevier & Dale, 1977).

The bulk of the nitrogen stored in the endosperm is in the form of protein. Before it can be utilized to support embryo growth, the storage proteins must be hydrolysed to amino acids. The important question here is, where does hydrolysis take place: are the proteins completely degraded within the endosperm or does hydrolysis only reach completion within the embryo? This latter possibility would entail the presence of a peptide transport system in the scutellum of the germinating embryos.

The enzymes involved in protein digestion have been studied in considerable detail. However, two circumstances have led to some confusion. The first is terminology; different investigators often give the same enzyme, or group of enzymes, entirely different names. For example, the acid

endopeptidases of barley (Burger, 1973) have been referred to as proteases (Burger et al., 1966), proteinases (Enari et al., 1963), sulphydryl endopeptidases (Enari & Mikola, 1967), peptidyl peptide hydrolases (Burger et al., 1970a) and even peptidases (Jones & Pierce, 1967a). Secondly, the persistent use of artificial substrates has presented difficulties, both in comparing results from different research groups and in attempting to understand the function of the enzymes in vivo. Very few studies have been made using natural substrates, yet it is clear that the ability to hydrolyse an artificial substrate is not necessarily representative of an enzyme's function in vivo (Beevers, 1968; Cameron & Mazelis, 1971; Spencer & Spencer, 1974; Baxter et al., 1978). For example, the 'typical' trypsin and chymotrypsin substrates, BAPA and BAEE, are hydrolysed rapidly by barley extracts, not by endopeptidases but by a peptidase and carboxypeptidase respectively (Burger et al., 1968; Mikola et al., 1971). Similarly, misleading pH optima have been obtained; wheat carboxypeptidase has an optimum at pH 5.5 on the standard artificial substrates, yet on purified wheat proteins the optimum is as low as pH 4.2 (Preston & Kruger, 1976b). Considering the importance of these enzymes in the brewing industry, it seems that adoption of a consistent approach is long overdue.

For present purposes the barley proteolytic enzymes will be considered in four convenient groups:

(i) Endopeptidases: enzymes able to cleave internal bonds of proteins yet unable to attack small peptides.

(ii) Carboxypeptidases: enzymes which sequentially remove amino acids from the C-terminus of a polypeptide.

(iii) Aminopeptidases: enzymes which sequentially remove amino acids from the N-terminus of a polypeptide.

(iv) Peptidases: enzymes which cleave small peptides yet are inactive against polypeptides/proteins.

1.3.4.1 Endopeptidases

A considerable number of endopeptidases are present in germinating barley grains (Enari et al., 1963; Burger et al., 1966, 1970a). Although these enzymes have not been completely characterized, most, if not all, fall into two major categories (Enari & Mikola, 1967):

(i) The sulphydryl or acid endopeptidases. These account for more than 90% of the endopeptidase activity of barley; at least three such enzymes are present (Burger, 1973).

(ii) Metal-activated endopeptidases, which function in less acid conditions (pH 5.5 to pH 8.5).

The endopeptidases are predominantly located in the starchy endosperm (Enari & Mikola, 1977) where the activity increases at least twenty-fold during germination, paralleling

the disappearance of protein from the endosperm (Jacobsen & Varner, 1967; Bhatty, 1969; Mikola & Enari, 1970; Harvey & Oaks, 1974b; Feller *et al.*, 1978). Maximum activity, on both natural and artificial substrates, is achieved under acidic conditions. Thus, proteolysis is optimal at pH 3.9 in barley (Enari & Mikola, 1967; Burger *et al.*, 1970a; Sundblom & Mikola, 1972), pH 3.8 in maize (Harvey & Oaks, 1974a,b; Abe *et al.*, 1977), pH 3.8-4.4 in wheat (Wang & Grant, 1969) and pH 4.0-4.5 in rye (Nowak & Mierzwińska, 1978). These values are in close agreement with the pH of barley endosperm, which is reported to decrease from pH 4.9 to pH 3.6 during germination (Kirsi & Mikola, 1971). It therefore seems clear that the endopeptidases play an important role in the degradation of the barley storage proteins.

1.3.4.2 Carboxypeptidases

Carboxypeptidases are generally assayed by their activity on N-carbobenzoxy- (Z-) dipeptides. Three such enzymes are known to be present in barley, although as many as five have been reported (Ray, 1976). Two of these carboxypeptidases, assayed using Z-Phe-Ala and Z-Phe-Phe as substrates, have been substantially purified and show optimal activity at pH 5.2 and 5.7 respectively (Visuri *et al.*, 1969; Moeller *et al.*, 1970; Yabuuchi *et al.*, 1973;

Ray, 1976; Baxter, 1978). A third, rather less active enzyme is also present, optimally active on Z-Pro-Trp at pH 4.8 (Mikola et al., 1971).

The carboxypeptidases all release C-terminal amino acids from proteins (Moeller et al., 1970; Mikola & Pietilä, 1972; Mikola et al., 1972), including cereal storage proteins (Preston & Kruger, 1976b, Baxter, 1978) and polypeptides with as few as a dozen residues (Yabuuchi et al., 1973). However, they show no endopeptidase activity and are unable to cleave small unsubstituted peptides (Visuri et al., 1969; Moeller et al., 1970; Preston & Kruger, 1977; Baxter, 1978).

The carboxypeptidases show a similar distribution to the endopeptidases, being predominantly located in the starchy endosperm. Their pH optimum is similar to the pH of this tissue in vivo. Again, activity increases rapidly during germination in parallel with protein digestion (Mikola & Kolehmainen, 1972; Mikola et al., 1972; Kruger & Preston, 1976, 1977; Feller et al., 1978), indicating an important role for carboxypeptidases in the degradation of the endosperm storage proteins.

1.3.4.3 Aminopeptidases

At least four aminopeptidases are present in barley, optimally active on amino acyl-β-naphthylamides at

pH 7.2 (Burger et al., 1970c; Kolehmainen & Mikola, 1971; Hejgaard & Bøg-Hansen, 1974). These enzymes are also active on unsubstituted di- and tripeptides at pH 6.0 (Kolehmainen & Mikola, 1971). The aminopeptidases are absent from the starchy endosperm and, although present in the aleurone layer and scutellum, their activities here remain constant throughout germination (Mikola & Kolehmainen, 1972). It therefore seems unlikely that these enzymes play an important role in the degradation of the barley storage proteins. However, during the early stages of germination they may function in the scutellum and aleurone layer in making amino acids available for the de novo synthesis of hydrolytic enzymes (see section 1.3.4.6). The only tissues in which aminopeptidases are relatively active are the roots and shoots of the embryo; it seems likely that here the enzymes are involved in protein turnover (Collier & Murray, 1977).

1.3.4.4. Peptidases

Two peptidases have long been known to be present in barley, characterized by their activities on Leu-Gly-Gly and Ala-Gly respectively, at high pH (Linderstrøm-Lang & Sato, 1929). Both these enzymes have now been extensively purified. The first is very similar to mammalian leucine aminopeptidases, active on di- and tripeptides at pH 8.0 (Sopanen & Mikola,

1975); the second, a specific dipeptidase, has a similar pH optimum (Sopanen, 1976).

Burger and his colleagues have also purified two peptidases from germinating barley grains although the relationship of these enzymes to the peptidases mentioned above is unclear. The first, peptide hydrolase A, is active on the 'trypsin-substrate' BAPA (Suolinna et al., 1965; Burger et al., 1968). However, it will only hydrolyse di- and tripeptides; true endopeptidase activity is lacking (Burger et al., 1968; Moeller et al., 1969; Mikola et al., 1971). The second peptidase, peptide hydrolase C, is one of at least four enzymes characterized by their activity on ANA (Burger et al., 1970c). Again, while able to hydrolyse a range of dipeptides this enzyme lacks any activity on proteins (Burger et al., 1970b).

Engel & Heins (1947) first showed that peptidase activity is predominantly located in the embryo of barley grains, particularly the scutellar epithelium. This has been confirmed by more recent studies. Thus, the 'leucine aminopeptidase' and dipeptidase purified by Sopanen are both absent from the starchy endosperm, though abundant in the scutellum where their activity increases as germination proceeds (Mikola & Kolehmainen, 1972). Similarly, peptide hydrolases A and C are restricted to

the embryo, especially the scutellum (Prentice et al., 1967, 1969).

1.3.4.5 The Course of Proteolysis

From the foregoing description of the activity, specificity and localization of the various barley enzymes it is possible to speculate on the course of proteolysis during germination. Two stages are apparent:

(i) An initial small-scale degradation, required to produce amino acids for the de novo synthesis of further hydrolytic enzymes (section 1.3.4.6). This takes place in the aleurone layer. It seems likely that all four main groups of proteolytic enzymes are involved; all are present in the aleurone of ungerminated seeds.

(ii) Bulk hydrolysis of the storage proteins. The majority of the storage proteins of barley grains are located in the starchy endosperm. The only enzymes which seem to be important in this tissue are the endopeptidases and carboxypeptidases. The inability of these enzymes to hydrolyze small peptides, together with the absence of peptidase activity from the starchy endosperm indicates that a mixture of both amino acids and small peptides will accumulate in this tissue during germination. Indeed, there is a certain amount of evidence that this is the case (section 1.2.3).

The bulk of the peptidase activity of the barley grain is located in the scutellum. These enzymes seem certain to play an important role in the breakdown of the storage proteins; their activity increases in parallel with proteolysis, specifically in the epithelial layers adjoining the starchy endosperm. It therefore seems probable that peptides, as well as amino acids, will be absorbed by the barley scutellum during germination; subsequent hydrolysis occurs within the embryo.

1.3.4.6 Regulation of Proteolysis

The production of substrates (peptide or amino acid) for absorption by the scutellum will clearly depend upon the activity of the appropriate proteolytic enzymes. Regulation of proteolytic activity may therefore limit the availability of nitrogen to the growing embryo.

During germination, endopeptidase activity increases considerably in the starchy endosperm (section 1.3.4.1). This increase is regulated by gibberellic acid released from the embryo, although the mechanism by which this is achieved is far from clear (Yomo, 1961; Briggs, 1963; MacLeod *et al.*, 1964). However, it seems that most, if not all the additional endopeptidase activity is the result of *de novo* synthesis in the aleurone layer; the enzyme is subsequently released into the starchy endosperm (Jacobsen

& Varner, 1967; Sundblom & Mikola, 1972).

Carboxypeptidase activity shows a similar increase (section 1.3.4.2). Again this seems to be the result of secretion from the aleurone layer (Schroeder & Burger, 1978), although the scutellum might also be involved (Mikola & Kolehmainen, 1972). This process is not yet understood. However, there are indications that it differs from the regulation of endopeptidase activity (Schroeder & Burger, 1978).

The possibility that endopeptidase activity is regulated by protease-inhibitors should also be considered. Barley contains both trypsin inhibitors (Mikola & Suolinna, 1969; Mikola & Kirsi, 1972) and inhibitors of various microbial proteases (Mikola & Suolinna, 1971). These inhibitors have no effect on the activity of endogenous barley proteases, and their levels remain relatively constant throughout germination. It therefore seems unlikely that they are involved in the regulation of proteolysis (Kirsi & Mikola, 1971). However, a third group of inhibitors, also present in barley grains, does inhibit the activity of the endogenous proteases (Burger & Siegelman, 1966; Kirsi & Mikola, 1971). These inhibitors rapidly disappear on germination, in parallel with the increase in proteolytic activity (Mikola & Enari, 1970). Although the loss of inhibitor is too small to account

for the massive increase in proteolytic activity, the possibility that these inhibitors play a role in the initial regulation of germination cannot be ignored.

1.3.5 Absorption of Amino Acids and Peptides by the Scutellum

Although the absorption of sucrose and other sugars by the maize scutellum has been studied in some detail by Humphreys and his co-workers (see Humphreys, 1978), little is known about the mechanism(s) by which nitrogenous metabolites are absorbed.

Most amino acids are present in the storage proteins of the endosperm in approximately the ratios required for protein synthesis by the embryo, and may therefore be transported to the embryo unchanged (Jones & Pierce, 1966, 1967b). Besides, metabolic interconversions are unlikely to occur in the 'dead' starchy endosperm. Thus, unlike dicots (section 6.6.2.2), nitrogen transport does not seem to be restricted to one or two specific amino acids. Only in the shoots and roots of older seedlings, which have begun to assimilate inorganic nitrogen, does glutamine become the predominant form of transported nitrogen (Yemm & Willis, 1956; Oji & Izawa, 1972; Margaris & Thanos, 1974). Certain amino acid interconversions and transaminations do occur during barley germination, but these seem to occur after transport and within the growing embryo (Folkes & Yemm, 1958; Joy & Folkes,

1965; Pragnell et al., 1969).

It has almost always been assumed that the transfer of nitrogen from the endosperm to the embryo occurs as free amino acids. Thus, the possibility of peptide uptake by the scutellum has never been investigated. However, there is a certain amount of evidence to indicate that the scutellum is able to absorb free amino acids.

Isolated monocot embryos are able to absorb and utilize externally supplied amino acids to support growth and protein synthesis (Kent & Brink, 1947; Harris, 1956; Folkes, 1959; Oaks & Beevers, 1964; Norstog, 1973). In addition, (^{14}C)-amino acids may be taken up by isolated embryos and incorporated into protein, although some inter-conversions may occur within the embryo, prior to incorporation (Joy & Folkes, 1965; Oaks, 1965b; Pragnell et al., 1969).

In the only detailed study of the scutellar absorption of amino acids, Stewart (1971) claimed that glutamine uptake was a mediated process. Uptake had a broad, acidic pH optimum, showed specificity for L-amino acids and was inhibited by sodium ions. Only certain other amino acids competed with glutamine for uptake, suggesting that a number of systems may exist to accommodate all twenty protein amino acids.

Little information is available concerning the regulation of nitrogen transfer from the endosperm to the embryo. It seems that in barley, apart from the initial activation and synthesis of proteolytic enzymes in the endosperm, there is little or no further regulation of nitrogen transfer across the scutellum. Although in high-nitrogen strains the absolute rate of nitrogen transfer is increased, this seems to be due to an improved availability of substrate rather than the activation of transport. This in turn is related to the higher concentrations of protein in the endosperm, as opposed to the activation of proteolytic enzymes (Dale *et al.*, 1974; Metevier & Dale, 1977). Although the *de novo* synthesis of amino acids by the embryo is inhibited by an external supply of amino acids (Joy & Folkes, 1965; Pragnell *et al.*, 1969), there seems to be little or no feedback inhibition of nitrogen transfer or peptidase/protease activity, either by accumulated amino acids (Jones & Pierce, 1966, 1967b) or externally supplied nitrogen (Dale *et al.*, 1974). However, in maize the situation may be different; exogenous amino acids apparently reduce the rate of nitrogen loss from the endosperm (Oaks, 1965c) and the embryo can regulate proteolysis (Harvey & Oaks, 1974a,b).

Thus, the transfer of nitrogen from the endosperm to the embryo of barley grains during germination is still a

poorly understood process. However, evidence for the presence of peptides in the endosperm (section 1.2.3), and from the localization of peptidases (section 1.3.4.5), suggests that peptides may be important in this process. This study attempts to define a role for peptides in the absorption of nitrogen by the scutellum and to characterize the transport system(s) involved.

CHAPTER 2

METHODOLOGY: TWO NEW FLUORESCENT-LABELLING TECHNIQUES FOR MONITORING PEPTIDE TRANSPORT

2.1 Introduction

A number of techniques have been developed in the past for the study of peptide transport in both prokaryotes and eukaryotes, yet they all have considerable limitations, especially when considered in relation to possible studies in higher plants. Methods which have been employed can be divided into two broad categories: (i) indirect methods, in which an organism's response to a particular peptide is monitored; (ii) methods in which movement of the peptide is monitored directly.

2.1.1 Indirect Methods

The most commonly employed indirect method for studying peptide transport has been the growth of amino acid auxotrophs of various microorganisms on peptide substrates containing the required amino acid. Such methods have been employed in bacteria (Gilvarg & Katchalski, 1965; Payne, 1968) and both unicellular (Becker et al., 1973) and multicellular fungi (Wolfinbarger & Marzluf, 1974). The poor sensitivity achieved by such methods may be improved by monitoring the synthesis of protein (Payne & Bell, 1977a) or specific enzymes (Cascieri & Mallette, 1974; Bell et al., 1977) as an alternative to overall growth. However, apart from their lack of sensitivity, such indirect methods are inherently difficult to interpret; it is generally unclear

whether the growth response to a particular peptide is actually representative of the rate of peptide transport. Many other factors such as peptidase activity, amino acid metabolism and specific growth promotive/inhibitory properties of the peptide could affect the rate of growth. In the presence of extracellular peptidases such methods are wholly unsuitable.

In addition, such indirect methods are not applicable to higher plant (or animal) systems. Not only are amino acid auxotrophs unavailable, but the relatively slow rates of growth make such methods generally impractical; the growth of one higher plant, Drosera, on various peptide nitrogen sources has been monitored using 6-month incubation periods (Simola, 1978b)!

2.1.2 Direct Methods

Methods for studying peptide transport which allow the movement of peptide to be monitored directly are far less ambiguous, and generally more sensitive, than the indirect methods employed; consequently the actual mechanisms of transport may be investigated in more detail.

The most widely employed method for studying transport processes in both pro- and eukaryotes is the uptake of radioactively labelled substrates. Similar techniques have also been applied to the investigation of peptide transport,

for example, in bacteria (de Felice et al., 1973; Jackson et al., 1976), fungi (Wolfinbarger & Marzluf, 1975b; Becker & Naider, 1977) and mammalian tissues (Rubino et al., 1971; Sleisenger et al., 1976). However, although extremely sensitive the use of radiotracers has a number of limitations:

(i) It is difficult to monitor uptake and exodus simultaneously.

(ii) It is not possible to follow the simultaneous uptake of a number of different substrates.

(iii) Identification of the metabolic products of the substrate is a problem.

(iv) The rates of peptide transport can often be misleading. Transport is generally monitored by the accumulation of radioactivity within the cell. However, since the development of the dansyl chloride method (section 2.4.2) it has become apparent that amino acids derived from absorbed peptides may undergo rapid exodus. Thus, radioactivity will be returned to the medium, often resulting in a considerable underestimate of the rate of peptide uptake.

(v) Radioactively labelled peptides are essential yet are no longer commercially available.

Other direct methods have been employed in mammalian systems in the absence of suitable radioactive peptides.

These involve monitoring the disappearance of peptide from the incubation medium or the accumulation of peptide (or free amino acids derived from it) within the tissue. Peptides and amino acids are separated chromatographically and detected using ninhydrin, generally on an automatic amino acid analyser (Matthews et al., 1968; Burston et al., 1972). Although such methods are applicable to multicellular systems they suffer from being slow, expensive, and in particular from their poor sensitivity.

Thus, in the absence of any suitable techniques which might be adapted to the study of peptide transport in higher plants, two new fluorescence assays, using dansyl chloride and fluorescamine, were developed. The standard procedures employed throughout this study are described below. Subsequently, the development of these methods, their advantages, disadvantages and applicability to other situations are discussed.

2.2 Materials

Biological Materials: Barley grains (Hordeum vulgare L. cv. Maris Otter, Winter), obtained from the National Seed Development Organization Ltd., Newton Hall, Cambridge, were used throughout this work unless otherwise stated. The huskless variety, Himalaya, used in certain experiments was the kind gift of Dr. D. Burston.

Maize (Zea mays L.), oats (Avena sativa L.), wheat (Triticum aestivum L.) and castor beans (Ricinus zanzibariensis) were from the Tyneside Seed Stores Ltd., Gateshead, while sorghum (Sorghum bicolor) was provided by the Tropical Products Institute, Oxford.

Peptides and amino acids were all of the L-isomer unless stated otherwise. Glycylsarcosylsarcosine and glycyl-sarcosylsarcosylsarcosine were the kind gift of Prof. D.M. Matthews. All other peptides and amino acids were purchased from Vega-Fox Chemicals and Cyclo Chemicals (through Cambrian Chemicals, Croydon), Serva and Bachem Fine Chemicals (through Uniscience Ltd., Cambridge), Sigma (London) Ltd. or B.D.H. (Poole, Dorset).

Where necessary peptides were checked for purity using dansyl chloride (section 2.3.2.1).

Dansyl chloride and standard dansyl-amino acids were purchased from B.D.H.

Fluorescamine was a gift from Dr. P.S. Ringrose, Roche Products Ltd., Welwyn Garden City.

Polyamide sheets were from B.D.H. or Pierce and Warriner Ltd. (Chester).

General Reagents were of analytical grade or the highest grade commercially available.

Instrumentation: Fluorescamine derivatives were quantified in solution using either a Perkin-Elmer 1000 Fluorescence Spectrophotometer or a Baird Atomic Fluoripoint Recording Spectrofluorimeter, model FP 101.

Quantification of dansyl derivatives on thin-layer chromatograms was performed using an automatic thin-layer plate scanner (model FP 115; Baird Atomic) attached to the Fluoripoint spectrofluorimeter.

2.3 Methods

2.3.1 Embryo Culture

Barley grains were wetted for 10s in 70% aqueous ethanol, surface sterilized using sodium hypochlorite (1% w/v available chlorine) and germinated in sterile water (previously saturated with oxygen) at 25°C for 24 h on a shaker (100 strokes/min).

When seeds were germinated for longer periods the sterile water was replaced with freshly oxygenated water every 24 h. In certain experiments (indicated in the text) barley grains were germinated on 'Alkathene' polyethylene granules (I.C.I) at 28°C in a dark spray room, with water misting for 5 min every hour.

Embryos were separated from the germinated grains and six transferred to each incubation tube (225 x 15 mm) containing 1 ml of pre-oxygenated medium. Incubations were at 25°C on a shaker (100 strokes/min). Incubation media consisted of 50 mM sodium phosphate:citric acid buffer (McIlvaine, 1921), pH 3.8, containing the appropriate peptide, amino acid or other

supplements. In certain experiments (especially where only small amounts of peptide were available) only three embryos were used per incubation tube (75 x 100mm) containing 0.5 ml of medium. Results obtained were essentially the same as with larger volumes though slightly less reproducible.

Standard incubation periods employed were 6-8h.

2.3.2 Monitoring Peptide Uptake

Three basic methods were used to monitor peptide uptake. Disappearance of peptide from the incubation medium was followed using either dansyl chloride or fluorescamine to determine peptide concentrations. In addition, barley embryo extracts were analysed using dansyl chloride to determine the accumulation of intact peptide, or of amino acids derived from the peptide by hydrolysis.

2.3.2.1 Dansyl Chloride Procedure

The medium was sampled at regular intervals throughout incubation and the peptide concentration determined as follows: Samples (normally 20 µl) were diluted appropriately with distilled water, aliquots (20 µl; containing 1-5 nm of peptide) added to Durham tubes (6 x 30 mm) together with an ornithine standard (10 µl; 0.125 mM), and evaporated *in vacuo*. Sodium bicarbonate (200 mM in deionized water; 25 µl) was added to each tube, followed by an equal volume of dansyl

chloride (2.5 mg/ml in acetone), to give a final reaction pH of about 9.5. The tubes were sealed with silicone rubber stoppers and incubated at 45°C for 1 h. Once dansylated, the mixture was evaporated to dryness *in vacuo* and the residue dissolved in aqueous pyridine (1:1 v/v; 20 µl). Samples (4 µl) were spotted on to 15 cm^2 polyamide sheets and chromatographed for about 45 min in each of the following solvent systems, in the order given: (i) 1st dimension, H_2O:formic acid (98.5:1.5, v/v); (ii) 2nd dimension, acetic acid:toluene (10:90, v/v); (iii) 2nd dimension, methanol:butyl acetate:acetic acid (40:60:2, v/v); finally chromatograms were rerun in the 1st dimension in solvent (i). Chromatograms were viewed under UV light and the derivatives identified and quantified by comparing the fluorescence intensity of each spot with that of known standards of the same compound. A permanent record of each plate was made by photographing under UV light using Ilfodata HS 23 film (Ilford) and a Wratten No. 3 filter (Kodak). Plates were reused after washing for 3 h in acetone:water: 880 ammonia (50:46:4, v/v).

2.3.2.2. Fluorescamine Procedure

At appropriate time intervals throughout incubation, samples (usually 25 µl, containing 0-50 nm of peptide) were withdrawn from the medium and added to 2.5 ml of sodium

tetraborate (0.1 M in deionized water; adjusted to pH 6.2 with HCl). Fluorescamine (0.5 ml; 0.15 mg/ml acetone) was added, while mixing rapidly on a vortex mixer, and the fluorescence yield determined using excitation and emission wavelengths of 390 nm and 480 nm respectively. The concentration of peptide in each sample could be calculated from the fluorescence yield.

2.3.2.3 Embryo Extraction

After incubation with the appropriate peptide, embryos were washed rapidly with 50 ml distilled water, blotted dry, weighed and extracted at 100°C for 20 min with acetic acid (5 M; 1 ml) in a stoppered tube to prevent evaporation. Prior to boiling, ornithine (20 µl; 2 mM) was added to each tube as an internal standard to check the efficiency of extraction and dansylation. The extract was cooled, centrifuged (1500 g; 5 min) and samples of the supernatant solution (usually 20-50 µl) dansylated and chromatographed as described above (section 2.3.2.1).

2.4 Discussion

2.4.1 Embryo Culture

Sterile Procedures. Although barley grains were germinated under sterile conditions, special precautions to maintain sterility during incubation were shown to be unnecessary; interference from contaminating microorganisms was negligible.

Thus, the rate of peptide uptake was identical whether embryo excision and incubation were performed in the open laboratory or in a UV sterilized room using presterilized incubation media. Moreover, in the absence of barley embryos, peptide uptake from incubation media exposed to the atmosphere was indetectable during standard incubation periods (8 h).

Embryo Excision. Electron microscopy was used to confirm that no visible damage is caused to the scutellar epethelial cells during excision (see figure 4.5, section 4.3.5).

Oxygenation of the Medium. Preliminary experiments showed that reproducibility was greatly improved by saturating the imbibition and incubation media with oxygen immediately prior to use. However, a continuous supply of oxygen throughout incubation was found to be unnecessary; it did not affect the rate of peptide uptake.

Incubation Conditions. Sodium phosphate:citric acid buffer (50 mM, pH 3.8) was found to be optimal for transport (section 3.1.3.9) and therefore employed unless otherwise stated.

Preliminary experiments showed that for embryos dissected from grains germinated for 6-72 h, uptake of Gly-Sar over a 5 h incubation period (in terms of μm/g fresh wt) remains relatively constant. Thus, for convenience, 24 h embryos were routinely employed; younger embryos were

difficult to dissect from the endosperm, older ones had root and shoot systems which were both cumbersome and easily damaged.

Embryo extraction. Washing, prior to extraction, seemed to be efficient. Peptides which are not transported by the barley embryo (e.g. peptides containing D-amino acid residues; section 3.2.5) were not detected in embryo extracts. Moreover, preliminary experiments showed that the rapid washing procedure employed does not result in any significant loss of accumulated peptide or amino acid from the embryo.

Extraction was initially performed at 100°C with 10% TCA. Extraction was efficient; after 10 min the recovered amino acid pool was identical with the pool obtained after 60 min when the embryo had completely disintegrated. However, TCA was difficult to remove in vacuo prior to dansylation. In addition, debris from the embryo, which disintegrated during extraction, often interfered with subsequent dansylation and chromatography of the extracts. Acetic acid was found to be an equally efficient extractant but with two advantages over TCA; it was more readily removed prior to dansylation and it caused little or no disintegration of the embryo. Several diverse peptides were tested for their solubility in 5 M acetic acid at the maximum levels that might be

accumulated by barley embryos; all were soluble. In addition, the extraction procedure caused no detectable hydrolysis of any peptide tested.

Extraction results in considerable deamidation of both glutamine and asparagine. This must be borne in mind when considering the endogenous amino acid pool or the uptake of peptides containing these two amides.

2.4.2 Dansyl Chloride Procedure

Dansyl chloride (1-dimethylamino-naphthalene-5-sulphonyl chloride) reacts with primary and secondary amino groups to form fluorescent sulphonamide derivatives. This reaction is now routinely employed in protein sequencing and a number of chromatographic methods for the separation and identification of dansyl-amino acids have been developed (see Seiler, 1970, for a general review).

Dansyl chloride also reacts with the α-amino groups of small peptides. It was found that these derivatives could also be successfully separated and identified by two-dimensional thin-layer chromatography, and accurately quantified from the intensities of the fluorescent spots. Thus, the dansyl chloride reaction can be used to determine the concentration of any particular peptide in solution, and therefore provides a suitable means of monitoring peptide

disappearance from the incubation medium. In addition, the excellent separation of dansyl-amino acids and peptides that can be achieved allows cell extracts to be examined, not only to monitor the accumulation of a peptide (or amino acids derived from it), but also to study the size, composition and changes in the free amino acid pool.

A number of photographs of typical chromatograms are presented throughout this thesis. Thus, figures 3.11 and 3.23 each show a series of chromatograms illustrating the disappearance of peptide from the incubation medium. Similarly figure 3.15 shows chromatograms of dansylated barley extracts, illustrating both the endogenous amino acid pool, and the intact uptake of certain peptides. However, it should be emphasized that these black and white photographs are rather poor representations of the real chromatograms.

2.4.2.1 Dansylation Reaction

The methods employed (section 2.3.2.1) were adapted from Hartley (1970). In order to obtain suitable fluorescence intensities for subsequent quantification, between 0.5 and 5 nm of any single amino acid or peptide were dansylated. The method is therefore extremely sensitive, at least two orders of magnitude better than ninhydrin.

A large excess of dansyl chloride must be employed in order to obtain a quantitative reaction with peptides. Thus,

in certain situations (e.g. cell extracts) where large amounts of dansyl-reactive compounds were known to be present, the amount of dansyl chloride was increased accordingly.

The pH at which the dansylation reaction is performed is important if quantitative yields are to be obtained (Hartley, 1970). The reaction pH was checked routinely, although only during the dansylation of cell extracts was a problem ever incurred. Care had to be taken to remove all traces of extractant (TCA or acetic acid) prior to dansylation.

2.4.2.2 Chromatographic Separation and Identification

Dansyl derivatives were separated by two-dimensional thin-layer chromatography on 15 cm^2 polyamide sheets (Woods & Wang, 1967) using three separate solvent systems (Ramshaw et al., 1970). Chromatography in the first dimension often gave poor resolution due to retardation by debris present at the origin. Plates were therefore routinely rerun in the first solvent, resulting in more regular, and consequently more easily quantifiable spots. In samples where considerable debris (e.g. insoluble protein and salts) was present, the insoluble material was removed by centrifugation (10,000 g for 2 min) immediately prior to spotting.

In certain circumstances, where accurate quantification was not essential, 5 cm^2 polyamide sheets were employed; only 1-2 minutes were then required in each solvent, allowing the rapid analysis of many samples.

Identification of dansylated compounds can be facilitated by comparing their mobility with a range of standard dansyl-amino acids run on the reverse of the polyamide sheet. The colour of spots is also an aid to identification. While most dansyl-amino acids fluoresce green, the histidine derivative is orange, tyrosine yellow, and dansyl-NH_2 and -OH blue. Certain dansyl-peptides also fluoresce at unusual wavelengths.

Figure 2.1 shows a typical separation of dansyl amino acids (from a barley embryo extract) after chromatography in all three solvents. However, certain groups of amino acids are more easily resolved after separation in the first two solvents only. These amino acids (proline/valine/ γ-aminobutyric acid; leucine/isoleucine) were routinely estimated prior to running in the third solvent.

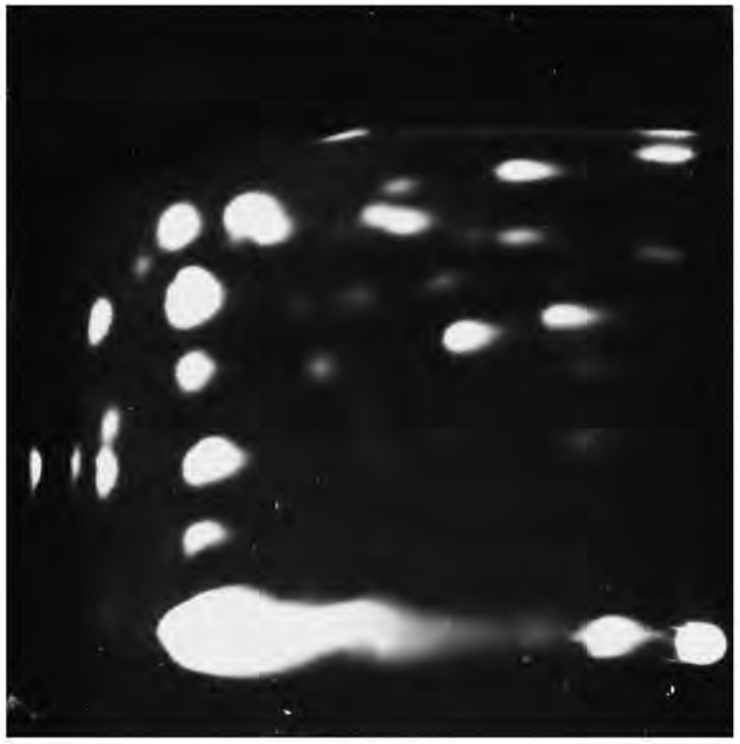
The chromatographic locations of various dansyl amino acids after separation in the first and second solvents only, and after all three solvents, are shown diagrammatically in figures 2.2 and 2.3 respectively. The positions to which dansyl derivatives of the peptides used in this study

Figure 2.1: Chromatographic Separation of Dansyl-Amino Acids

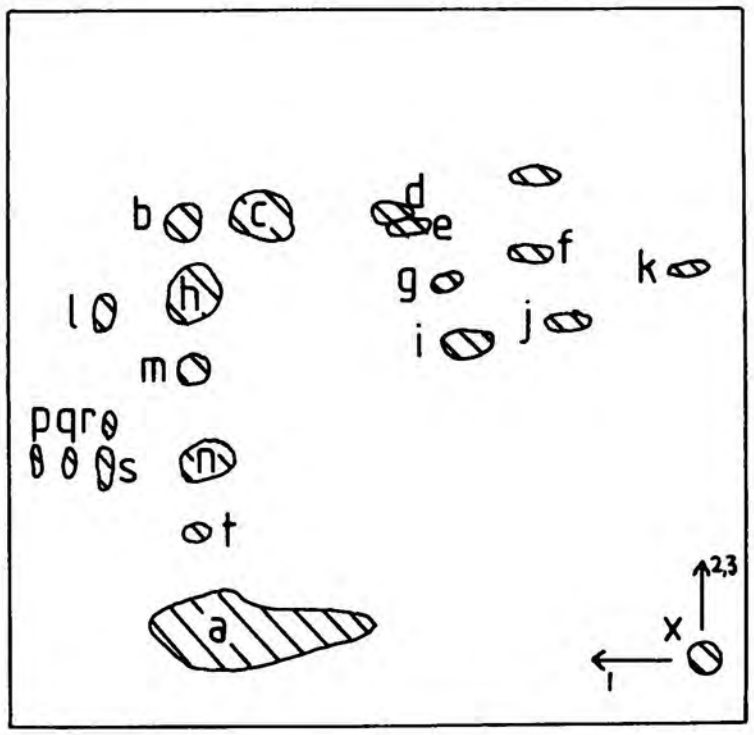
(I) Photograph of a polyamide plate after chromatographic separation of a dansylated extract of barley embryos. Embryos were incubated for 6h in 2mM Gly-Sar prior to extraction.

(II) Diagram showing the identity of each dansylated derivative: a) dansyl-hydroxide, b) dansyl-ammonia, c) proline/valine/GAB, d) isoleucine, e) leucine, f) histidine, g) phenylalanine, h) alanine, i) ornithine (internal standard), j) lysine, k) tyrosine, l) glycylsarcosine, m) glycine, n) glutamic acid, p) arginine, q) asparagine, r) threonine, s) serine, t) aspartic acid, x) origin.

I



II



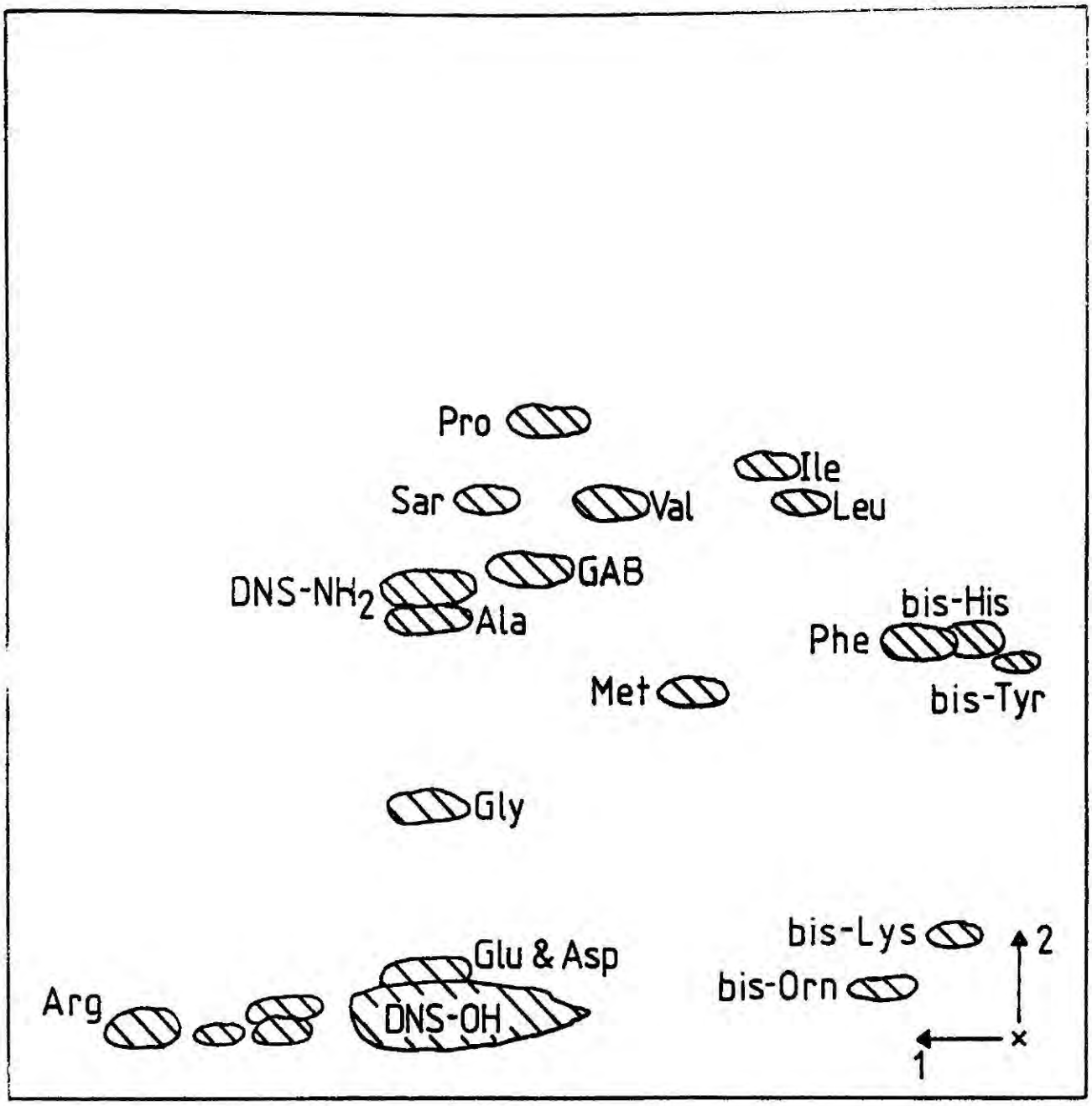


Figure 2.2: Diagram Illustrating the Chromatographic Separation of Dansyl-Amino Acids: After Two Solvents

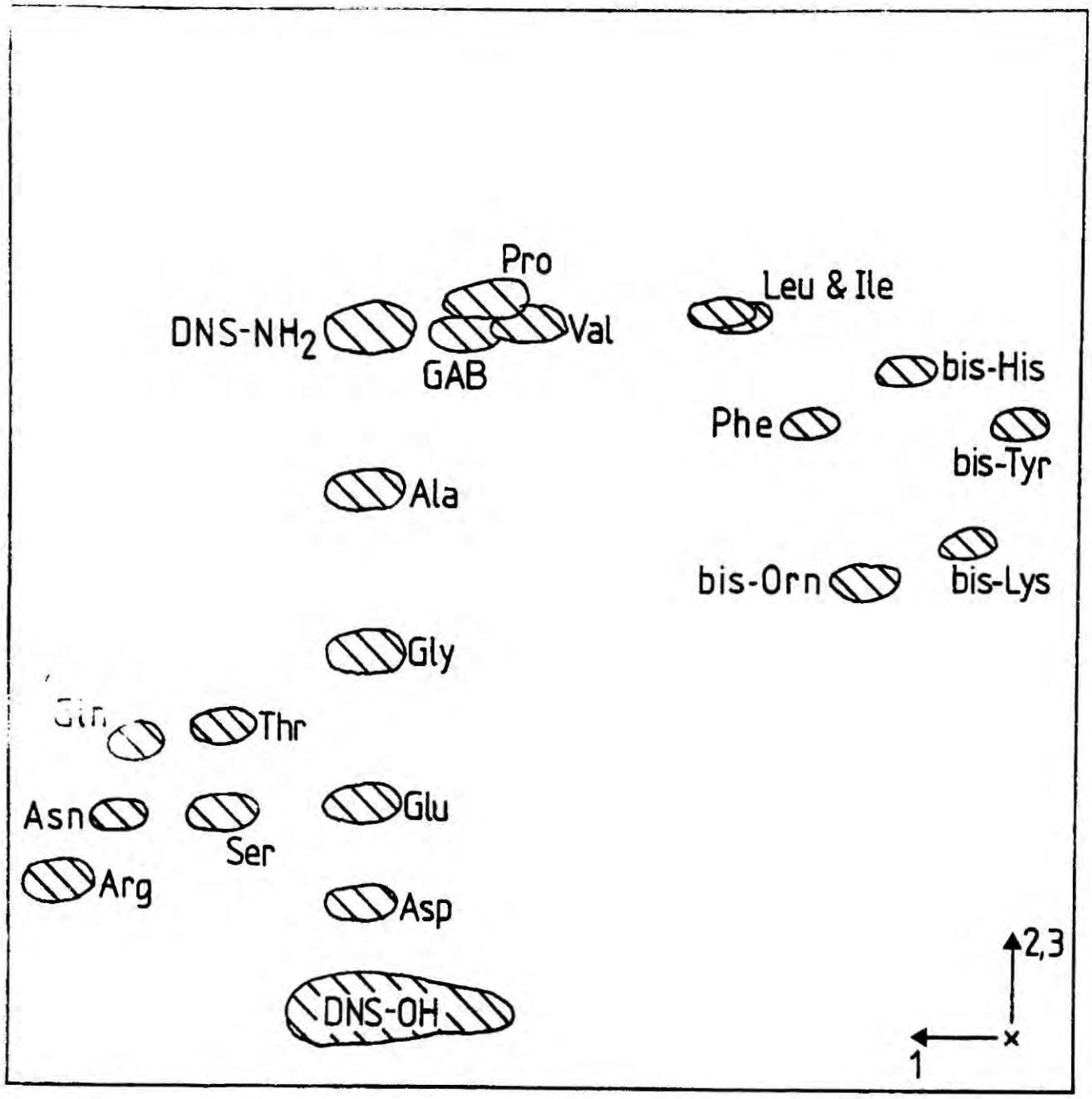


Figure 2.3: Diagram Illustrating the Chromatographic Separation of Dansyl-Amino Acids: After Three Solvents

chromatograph, after all three solvents, are shown in appendix 2.

2.4.2.3 By-Products

Two main by-products are produced during the dansylation reaction. The reaction of excess reagent with water to form dansyl hydroxide is unavoidable but fortunately presents no problems; this derivative is readily separated from all dansyl-amino acids and peptides during chromatography. However, the dansyl-NH_2 derivative, formed by reaction with ammonia, chromatographs in the region of proline and valine and, if present in large quantities, can interfere both with the estimation of these amino acids and certain dansyl-peptides. This problem can generally be avoided by ensuring the removal of all ammonia *in vacuo* prior to dansylation, and using freshly deionized water for the bicarbonate buffer.

Certain amino acids have side chains which form derivatives with dansyl chloride (e.g. Lys, His, Tyr); thus, the bis-dansyl derivatives will normally be detected. However, if these amino acids are present in a peptide (except at the N-terminus) the mono-derivatives will form (e.g. ε-dansyl-lysine). These may be detected after acid hydrolysis of the peptide (see appendix 2).

Glutamine and asparagine are detected intact after dansylation. Deamidation to the corresponding acid does not

occur (G. Bell, personal communication).

2.4.2.4 Quantification

The fluorescence intensity of spots resulting from the chromatographic separation of dansyl derivatives has been used previously for quantitative purposes (Seiler, 1970). However, such separations have generally been performed on silica gel which has certain inherent problems. Drying is critical as it can cause the fluorescence to fade rapidly. Considerable fading is also observed on storage. The present use of polyamide sheets overcomes these problems. Little or no fading occurs either during drying or subsequent storage (for at least a month, in the dark).

Visual estimation of fluorescence intensity, by comparison with known standards, was used routinely and found to be accurate to within ± 5-10% (under most circumstances). The validity of visual estimation was confirmed using an automatic thin-layer scanner attached to a recording spectrofluorimeter (using excitation and emission wavelengths of 340 nm and 485 nm respectively). For certain experiments where greater accuracy was required, the thin-layer scanner was used exclusively. Further confirmation that visual estimation is meaningful has been obtained using tritiated dansyl chloride. Estimation of the radioactivity present in each spot (using a Panax thin-layer scanner RTLS-1A)

corresponded accurately with the fluorescence determined visually (Payne & Bell, 1979).

Ornithine was selected as a suitable internal standard for checking the efficiency of extraction and dansylation, as it is absent from barley extracts. In addition, dansyl-ornithine is readily separated from most dansyl-peptides, giving a uniform, and therefore easily quantifiable spot.

Most amino acids give a similar quantum yield with dansyl chloride (Hartley, 1970). However, the quantum yield achieved with any given peptide depends upon its structure. Thus, quantification was always performed with reference to known standards of the same peptide. Most amino acids and peptides were readily estimated by these means. However, dansyl-arginine and certain larger peptide derivatives (e.g. dansyl-pentaglycine) chromatograph close to the solvent front and often give uneven spots or are obscured by debris carried with the solvent front. Similarly certain peptides (e.g. Gly-Asp) may be obscured by the dansyl-hydroxide spot. If necessary this difficulty could be overcome by employing different solvent systems.

The standard dansyl chloride procedure is often unsuitable for studying the uptake of peptides with substituted α-amino or α-carboxyl groups. However, the assay can be adapted to overcome some of these problems; this is

dealt with elsewhere (section 3.2.1.1).

2.4.2.5 Conclusions

The dansyl chloride procedure provides a rapid and extremely sensitive method for monitoring peptide transport. It does not require labelled substrates and its high sensitivity entails the use of only very small amounts of otherwise expensive peptides.

However, the principal advantage of this method is the extra information available (in addition to the straightforward rate of peptide uptake), which cannot easily be obtained by other current procedures:

(i) The degree to which a peptide is hydrolysed and the site of hydrolysis.

(ii) The metabolic fate of peptide-derived amino acids. In barley (sections 3.1.3.1; 3.1.3.4) and bacteria (Payne & Bell, 1977b) rapid and specific metabolism of certain amino acids is evident.

(iii) Amino acid exodus. It is now clear that in barley (section 3.1.3.5) and bacteria (Payne & Bell, 1977b, 1979) rapid exodus of amino acids, derived from absorbed peptides, can occur. This has a number of implications for the regulation of peptide transport (Payne & Bell, 1977c) and the use of radiotracers to monitor peptide uptake. The dansyl chloride procedure allows uptake and exodus to be

monitored simultaneously.

The dansyl chloride method would seem to be suitable for studies of peptide transport in most, if not all, other organisms. Indeed, the method has already been successfully adapted for use in both bacteria (Payne & Bell, 1979) and yeast (Nisbet & Payne, 1979a).

2.4.3 Fluorescamine Procedure

During incubation of barley (and other organisms) with a peptide, free amino acids may appear in the incubation medium, either as a result of peptide uptake, hydrolysis and exodus, or the general leakage of endogenous amino acids (section 3.1.3.5). As most reagents for the assay of peptides react equally well with free amino acids (e.g. ninhydrin, dansyl chloride), a time-consuming separation step must be included in order to study the uptake of a peptide by monitoring its disappearance from the incubation medium. However, under certain conditions fluorescamine, a fluorogenic reagent for primary amines, will react with peptides yet give negligible fluorescence with free amino acids. This property was exploited in the development of a rapid and sensitive assay for peptide transport.

Fluorescamine (4-phenylspiro-(furan-2(3H),1'-phthalan)-3,3'-dione) was first introduced as a reagent for the fluorometric assay of amines (Weigele et al., 1972;

Udenfriend et al., 1972). It reacts rapidly and specifically with primary amino groups to form a fluorescent derivative; excess reagent is hydrolysed to yield non-fluorescent, water-soluble products. Ammonia gives no significant fluorescence yield.

The mechanism of the fluorescamine reaction in aqueous solution is not yet fully understood although it has been investigated in some detail (de Bernardo et al., 1974; Stein et al., 1974; Chen et al., 1978). The reaction conditions can have a considerable influence on the fluorescence yield; reaction is especially dependent upon pH (Udenfriend et al., 1972). This is due, at least in part, to the degree of protonation of the primary amino group; fluorescamine only seems to react with the non-protonated moiety. As peptide α-amino groups generally have a considerably lower pK than those of free amino acids, it is apparent that at a suitably low pH only peptides will give a significant fluorescence yield. Thus, peptides may be assayed, even in the presence of contaminating amino acids.

The reaction conditions employed (see section 2.3.2.2) were based upon Perrett et al. (1975). Certain precautions were found to be necessary in order to obtain reproducible results:

(i) 'Analar' acetone was used to prepare stock solutions of fluorescamine. Fluorescamine was unstable in 'reagent grade' acetone, presumably because of the relatively high water content. In addition, smaller and more reproducible reagent blanks were obtained.

(ii) Borate buffer was prepared in deionized, distilled water.

(iii) Glassware was thoroughly cleaned (chromic acid) and rinsed in deionized water.

(iv) Rapid mixing was essential during the addition of fluorescamine to the sample to be analysed.

Results are expressed in arbitrary units of fluorescence yield. The fluorescence yield for a given amount of peptide varied somewhat between experiments (although was of course, constant within any single experiment) as a result of variations in the concentration of fluorescamine reagent, made up freshly each day. However, under standard conditions (section 2.3.2.2), 100 nm of diglycine gives a fluorescence yield of about 27,500. For comparison, a 1 µg/ml solution of quinine sulphate in 0.1M H_2SO_4 gives a fluorescence yield of 5820 using a 1 cm path length.

2.4.3.1 Optimum pH for the Assay of Peptide Transport

Fluorescamine reacts optimally with amino acids and peptides at about pH 9.0 and pH 8.0 respectively, depending

upon the particular amines employed. While reduction of the assay pH from pH 8.0 to pH 6.0 increases the ratio of the yield obtained with similar quantities of peptide and amino acid, the overall sensitivity of the assay is decreased (Perrett et al., 1975). Clearly, an appropriate balance must be achieved between these two opposing effects.

Figure 2.4 shows the effect of varying buffer pH on the fluorescence yield with dialanine and alanine. The ratio of yields is optimal (about 40:1) between pH 6.2 and 6.8 inclusive. Although the higher pH gives improved sensitivity, for several reasons pH 6.2 was selected as most appropriate for assaying peptide transport in barley:

(i) The sensitivity at pH 6.2 is still perfectly adequate for the peptide concentrations employed.

(ii) Certain amino acids do give a significant fluorescence yield at low pH (section 2.4.3.5). As the exodus of free amino acids from barley embryos is often quite considerable, the lower pH (pH 6.2) reduces any interference to a minimum.

(iii) Other compounds (probably protein) leaching from barley embryos during incubation also give a significant fluorescence yield with fluorescamine (section 2.4.3.7). Again this interference is minimized at pH 6.2.

It must be noted that, although pH 6.2 was most appropriate for barley, in certain circumstances it might be

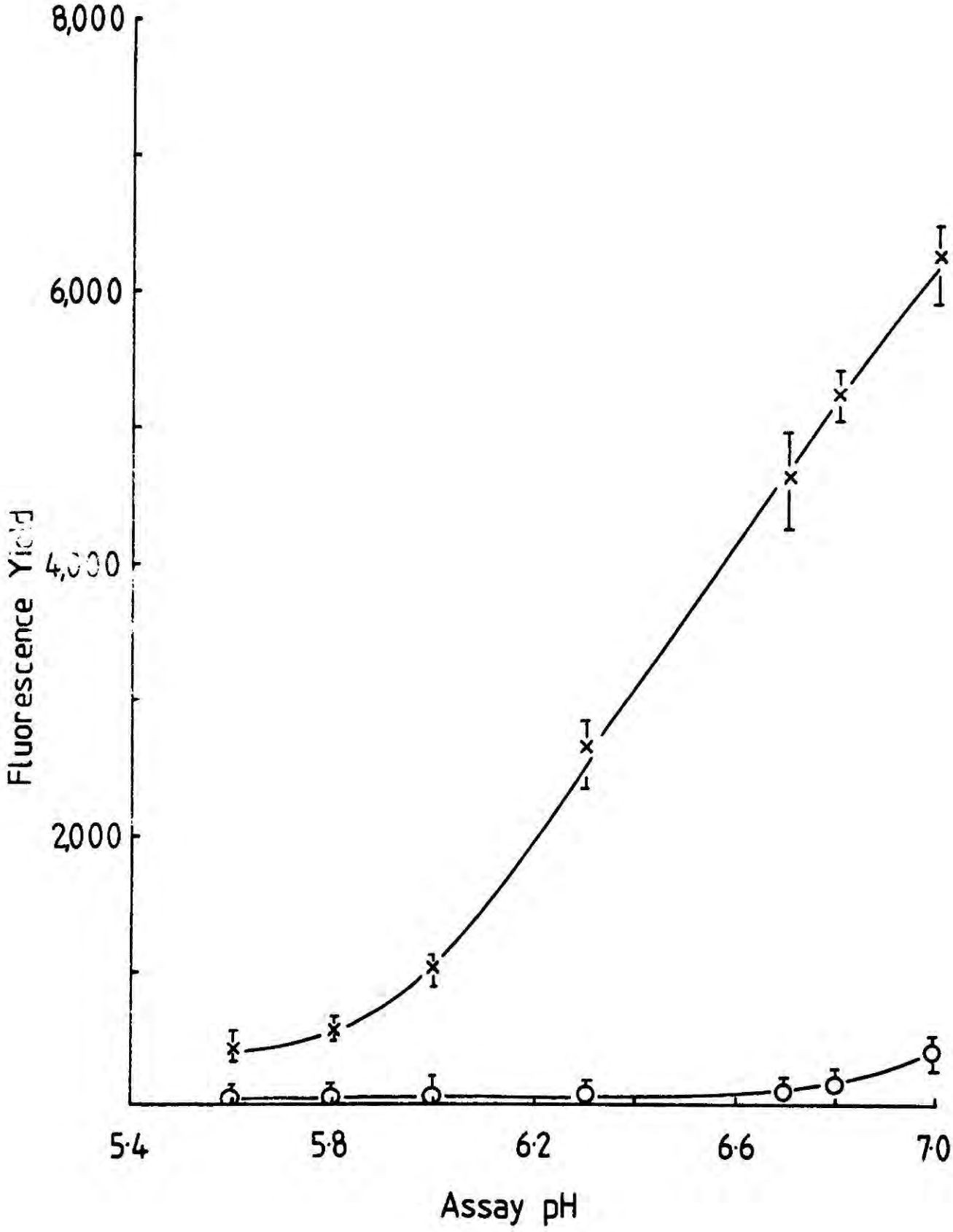


Figure 2.4: Effect of Assay pH on the Reaction of Fluorescamine with Alanine and Dialanine

10nm of alanine (o) or dialanine (x) were assayed under standard conditions (section 2.3.2.2) with the pH of the borate buffer adjusted appropriately, using conc. HCl. Each value is the average of three separate determinations. Bars show the range of values obtained.

advantageous to employ a higher assay pH:

(i) If greater sensitivity is required.

(ii) In studies on organisms where amino acid exodus is minimal (e.g. yeast: T.M. Nisbet, personal communication).

(iii) To assay the uptake of those peptides whose constituent amino acids, if they undergo exodus, do not react well with fluorescamine, even at pH 6.8.

2.4.3.2 Buffer Composition

Although borate is most commonly employed as the buffer in the fluorescamine reaction, under certain conditions phosphate is known to give an improved reaction rate with primary amines, as well as reduce the rate of reagent hydrolysis (Stein et al., 1974). In addition, the concentration of buffer ions can affect the fluorescence yield.

Figure 2.5 shows the effects of varying concentrations of both borate and phosphate buffer on the fluorescence yield obtained with dialanine. Clearly, the greatest sensitivity at pH 6.2 is achieved with 0.1 M tetraborate. In no case was a significant reaction with amino acids obtained. Interestingly, unlike borate, the fluorescence yield in phosphate increases as the buffer concentration is decreased, although the sensitivity never reaches that of borate; lower phosphate concentrations are unsuitable as the buffering capacity is insufficient.

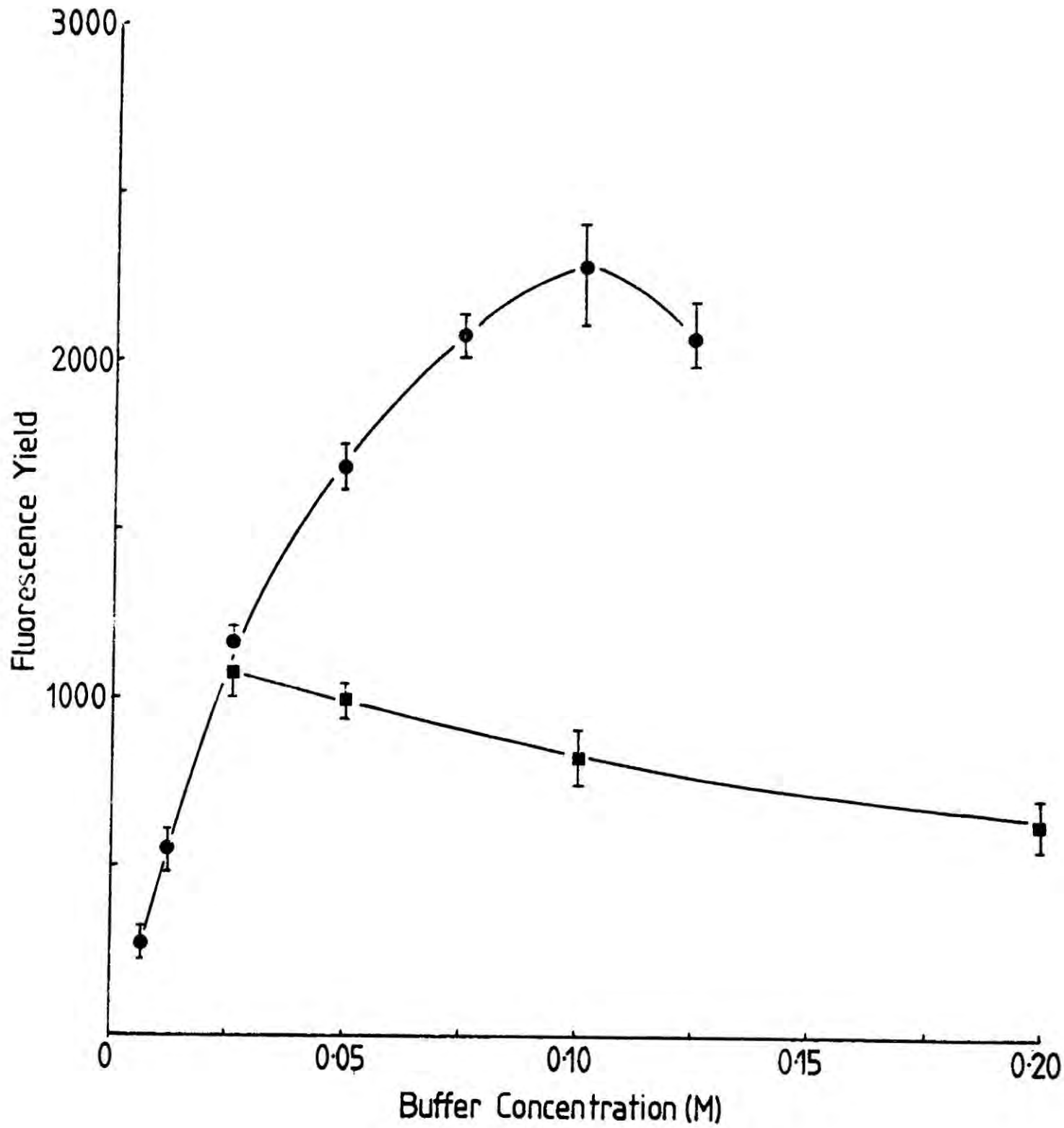


Figure 2.5: Effect of Buffer Composition and Concentration on the Fluorescamine Reaction

10nm of dialanine was assayed by the fluorescamine method using either phosphate (■) or tetraborate (●) buffers of varying concentration, each adjusted to pH 6.2. Each value is the average of three separate determinations. Bars show the range of values obtained.

2.4.3.3 Fluorescamine Concentration

The fluorescence yield obtained with dialanine increases as the concentration of the added fluorescamine reagent is increased (figure 2.6). Although at first sight this might suggest that under standard assay conditions (0.15 mg fluorescamine/ml) fluorescamine becomes limiting, this cannot be the case; increasing the dialanine concentration also gives an increased fluorescence yield (section 2.4.3.4). It seems that for a given amount of fluorescamine only a very small, though constant, proportion of peptide (or other amino compound) reacts to form a fluorescent derivative. Any increase in fluorescamine concentration affects the fluorescence yield simply by increasing the proportion of peptide which reacts. The reasons for this are still unclear (Chen et al., 1978).

Thus, the sensitivity of the fluorescamine reaction can be increased by increasing the reagent concentration. However, for present purposes, a relatively low reagent concentration was selected as suitable:

(i) Sensitivity was still adequate; a linear response was obtained over the required peptide concentration range (section 2.4.3.4).

(ii) Fluorescamine is expensive.

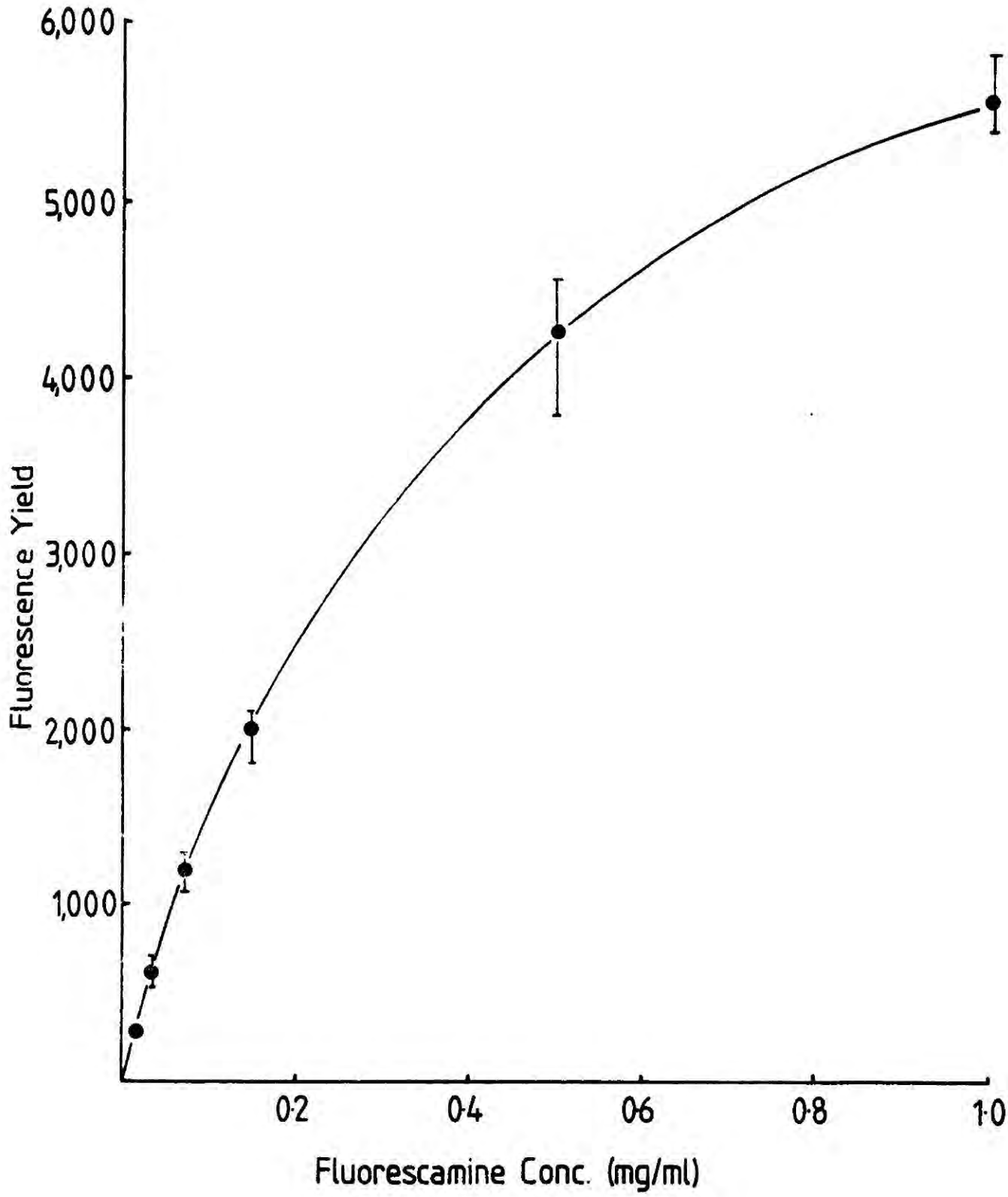


Figure 2.6: Effect of Fluorescamine Concentration on the Fluorescence Yield with Dialanine

Assays were performed under standard conditions (section 2.3.2.2), using 10nm dialanine. Each value is the average of three separate determinations. Bars show the range of values obtained.

(iii) Quenching can occur in the presence of excess reagent, probably due to the presence of hydrolysis products (de Bernardo et al., 1974).

2.4.3.4 Peptide and Amino Acid Concentration

A linear relationship is observed between peptide concentration and fluorescence yield, at least up to 100 nm of peptide; approximate linearity is maintained with up to 250 nm (figure 2.7). Thus, the amounts of peptide assayed during transport studies (generally less than 50 nm) are well within the linear range.

Under the same conditions (pH 6.2), most free amino acids give no significant reaction. In addition, an identical fluorescence yield with diglycine or trialanine is obtained when the assay is performed in the presence of an equal amount of free alanine. Thus, not only do amino acids fail to react, they also do not interfere with the reaction between fluorescamine and peptides (although see below).

2.4.3.5 Interference from Free Amino Acids

Most free amino acids were found to give less than 3% of the fluorescence yield of an equivalent amount of peptide under standard conditions. Thus, even if a dipeptide is absorbed, hydrolysed, and the resultant amino acids are all released to the medium, the error in the estimated rate of peptide uptake due to the released amino acids will still

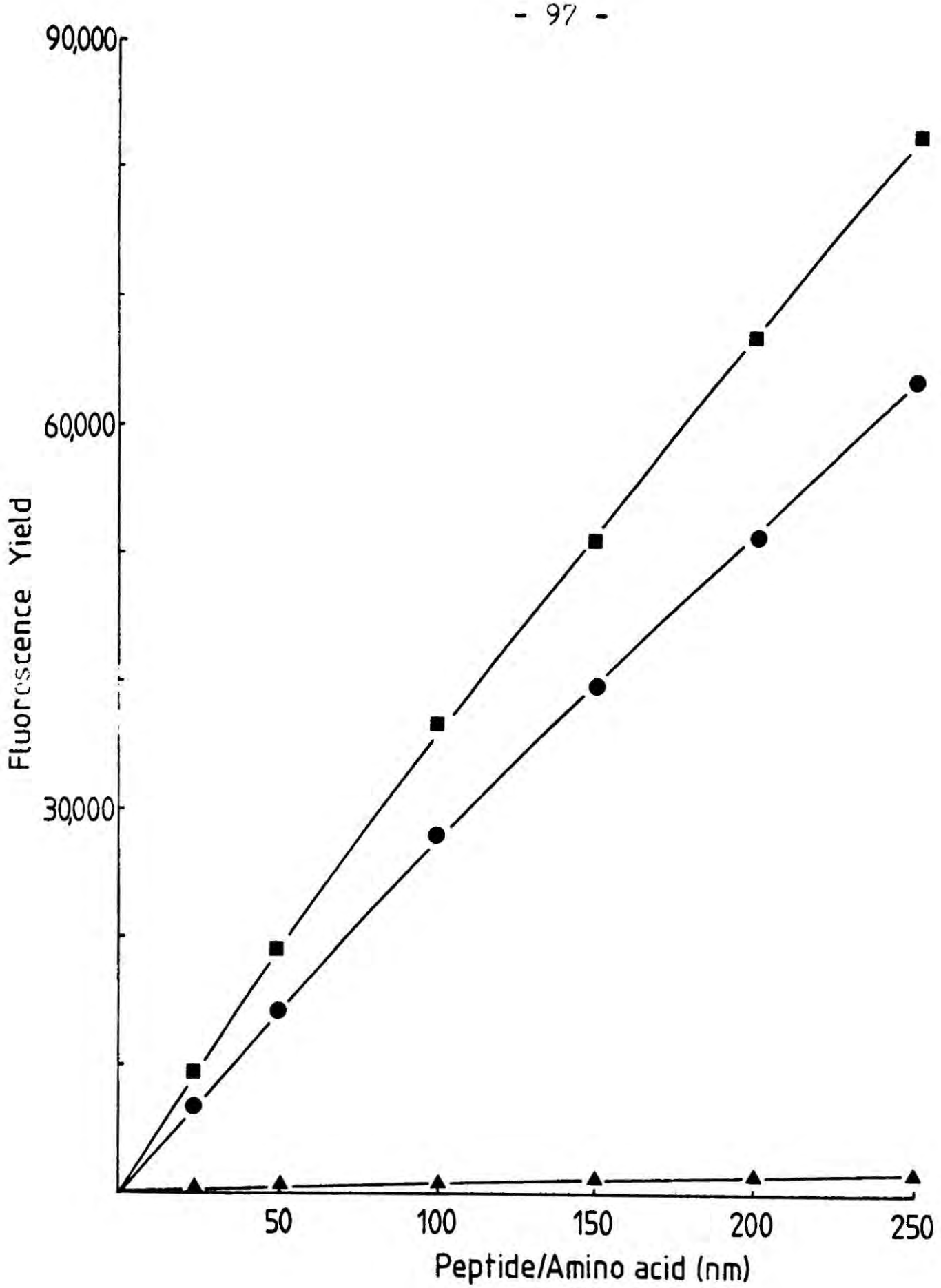


Figure 2.7: Effect of Peptide and Amino Acid Concentration on Fluorescence Yield

Fluorescamine assays were performed under standard conditions (section 2.3.2.2) with varying concentrations of alanine (▲), diglycine (●) or trialanine (■). Each value is an average of three separate determinations.

be less than 6%.

However, two amino acids, phenylalanine and histidine, do give a significant fluorescence yield. Unlike most primary amines the yield increases for several minutes after mixing (reaction is normally complete within a few seconds). No explanation can be offered for this behaviour, although it is clear that care must be taken in interpreting the results of uptake studies involving peptides containing these two amino acids.

2.4.3.6 Secondary Amines

Secondary amines, including proline, prolyl-peptides and N-methylated peptides, do not form a fluorescent derivative with fluorescamine (although they may be assayed after chemical conversion to primary amines; Weigele *et al.*, 1973). Thus, it is not possible to monitor the uptake of peptides with a secondary amino group using the standard fluorescamine procedure.

However, not only do secondary amines fail to form a fluorescent derivative with fluorescamine, they also interfere with the reaction between fluorescamine and primary amines (table 2.1). It was shown that this interference was not the result of quenching, either by the secondary amines themselves or by any non-fluorescent derivative they might form with fluorescamine. It therefore seemed likely that secondary

Table 2.1: Effect of Secondary Amines on the Fluorescamine Reaction

Peptide(s)	Fluorescence Yield
Ala-Ala-Ala (50nm)	15,800
Sar-Gly-Gly (500nm)	-20
Pro-Gly (500nm)	-11
Ala-Ala-Ala (50nm) + Sar-Gly-Gly (500nm)	6,050
Ala-Ala-Ala (50nm) + Pro-Gly (500nm)	4,200
Gly-Gly (50nm)	14,500
Pro (50nm)	-30
Pro-Gly-Gly (50nm)	-10
Gly-Gly (50nm) + Pro (50nm)	12,900
Gly-Gly (50nm) + Pro-Gly-Gly (50nm)	12,100

Assays were performed under standard conditions (section 2.3.2.2). Each value is an average of three separate determinations.

amines interfere with the reaction between fluorescamine and primary amines by successfully competing for reagent. Results obtained from studies on the effect of varying Sar-Gly-Gly concentrations on the fluorescence yield achieved with trialanine (fig. 2.8) are also compatible with this idea, and further support is provided by the known ability of proline to react extremely rapidly with fluorescamine to form non-fluorescent derivatives (Toome & Manhart, 1975; Chen et al., 1978).

Thus, not only is it impossible to assay the transport of prolyl- or N-methylated peptides using the fluorescamine procedure, but care must also be taken when employing fluorescamine in conditions where proline or other secondary amines might be present (see also section 3.2.1.1).

2.4.3.7 Exodus from Barley Embryos

During incubation a certain amount of fluorescamine-reactive material leaches from the barley embryo. The free amino acids appearing in the medium are insufficient to account for all this reactivity; it is possible that proteins are also important. Under most circumstances interference from this material is negligible. Only when monitoring uptake at low peptide concentrations, or of peptides which give a poor fluorescence yield (e.g. acidic peptides), does

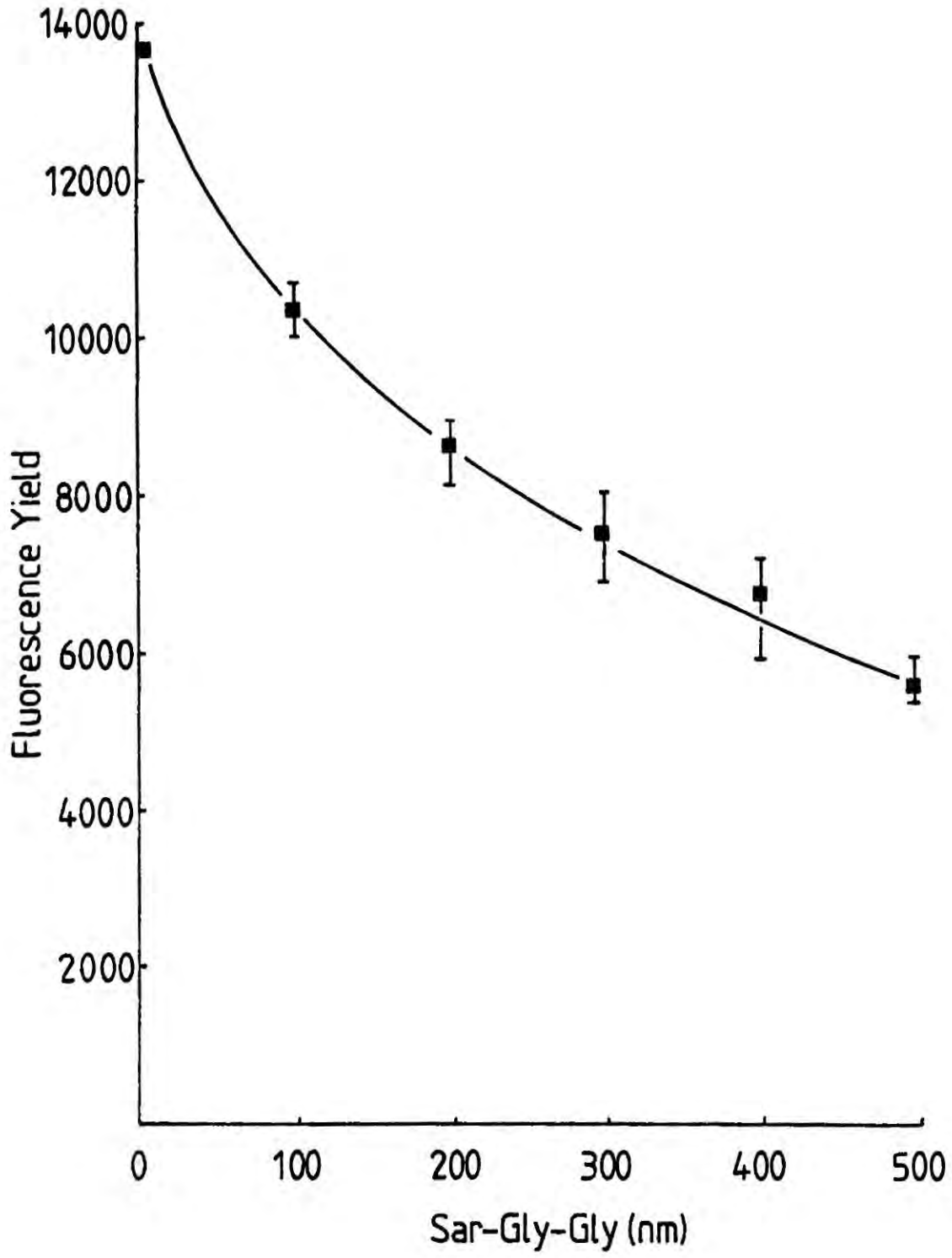


Figure 2.8: Effect of Sar-Gly-Gly Concentration on the Reaction of Fluorescamine with Trialanine

The fluorescamine reaction was performed under standard conditions (section 2.3.2.2) with 50nm of trialanine. Each value is the average of three separate determinations. Bars represent the range of values obtained.

it become important. However, this background fluorescence is reproducible and, when necessary, can be accounted for in calculating the rate of peptide uptake.

2.4.3.8 Conclusions

The fluorescamine method provides an extremely rapid and sensitive method for studying peptide transport. Although it clearly provides less information on the mechanism of uptake (e.g. the metabolic fate of the peptide) than the dansyl chloride procedure, it is very much more rapid and extremely useful as a general scanning procedure. It is envisaged that the assay will, to a large extent, be used in conjunction with the dansyl chloride method.

The fluorescamine assay is also applicable to other organisms. It has been successfully adapted to monitor peptide transport in both bacteria (Payne & Bell, unpublished results) and yeast (Nisbet & Payne, 1979a), and has recently been automated.

2.5 Expression of Results

Throughout this study, peptide uptake is expressed in terms of embryo fresh weight. Two possible sources of confusion arise. Firstly, when studying uptake by embryos of different ages, embryo weights will vary while the size of the absorptive surface (the scutellum) may remain more

or less constant. Thus, results are not strictly comparable. Secondly, when expressing concentrations of peptide or amino acid within the embryo, 1 g fresh weight is assumed to be equivalent to 1 ml intracellular fluid. While adequate for present purposes, this is clearly only an approximation. The actual concentrations of peptide within the embryo will be rather greater than the values presented, as the 24 h embryo is only about 80% water (table 5.1, section 5.3.1). In addition, it is assumed that the peptide is evenly distributed throughout the embryo; again this may not be the case. Thus, the values presented represent the minimum possible concentrations of peptide in the intracellular fluid. The actual concentrations may be considerably higher.

In certain experiments peptide uptake was not detected. However, it must be remembered that this really represents the limitations of the detection methods employed. For example, peptide uptake could be as rapid as 0.2 μm/g fresh wt./h from a 1 mM solution (about 8% of the rate of uptake of most peptides), yet be indetectable using dansyl chloride to monitor peptide disappearance from the medium. Examination of embryo extracts for intact peptide (or an increase in the peptide's constituent amino acids) is rather more sensitive. Except for alanine, where the large endogenous pool makes small increases hard to detect, rates

of uptake as low as 0.025 μm/g/h may be measured, providing of course that intracellular amino acid metabolism is minimal (see section 3.1.3.1). However, the sensitivity of both these methods will depend upon many factors, including the incubation period, peptide concentration, and the reactivity of the peptide being studied with the fluorescent labels. Thus, the above limits are only approximate; sensitivity can be much better or much worse under different circumstances. Zero rates of uptake are therefore expressed as not detectable (ND).

CHAPTER 3

PEPTIDE TRANSPORT BY THE BARLEY SCUTELLUM

3.1 Characterization of Peptide Transport

3.1.1 Introduction

Preliminary experiments showed that, after incubation of barley embryos with any one of a variety of physiological peptides (containing only the L-isomer of 'protein' amino acids), a considerable increase in the peptide's constituent amino acids was detectable in embryo extracts. However, the high level of peptidase activity associated with the embryo precluded the demonstration of intact uptake of any of these peptides. Thus, peptides containing the non-physiological amino acid sarcosine (see section 1.1.1) were employed. Barley embryos were found to take up these peptides intact, with a minimum of hydrolysis. Peptide transport was therefore characterized using these peptides. Subsequently, the uptake of physiological peptides was also demonstrated. Although the intact uptake of physiological peptides could not be demonstrated directly, transport was shown to be mediated by the same system(s) as the sarcosine-containing peptides.

3.1.2 Methods

Peptide uptake was assayed using the dansyl chloride procedure unless otherwise indicated. Uptake of sarcosine-containing peptides was monitored by examination of embryo extracts, while the uptake of amino acids and physiological

peptides, both on their own and in the presence of potential competitive inhibitors, was followed by the disappearance of peptide from the incubation medium.

Unless stated otherwise, standard incubation conditions were employed throughout (section 2.3.1).

3.1.2.1 Separation of Embryo Tissues

In certain experiments embryos were separated into their three main tissues, the shoot, root and scutellum, either before or after incubation (section 3.1.3.3). In order to facilitate separation and to provide approximately similar amounts of each tissue, embryos from barley grains germinated for 48 h (in a spray room; section 2.3.1) were employed.

3.1.2.2 Determination of pH Optimum

All buffers used were at 50 mM unless otherwise stated. pH was shown not to vary by more than 0.15 units during incubation, using a glass electrode pH meter.

3.1.2.3 Resolution of Amino Acids

In early experiments DNS-proline and DNS-γ-amino-butyric acid were not resolved chromatographically; the combined pool of these two amino acids is therefore referred to as proline/γ-aminobutyric acid. Only in subsequent experiments was this 'single' spot resolved and routine separation of these dansyl-amino acids achieved (section 2.4.2.2).

3.1.2.4 Metabolic Inhibitors

CCCP, the only inhibitor employed which was poorly soluble in water, was made up as a stock solution in 100% ethanol. Equivalent amounts of ethanol alone, added to the incubation medium, did not affect uptake.

Anoxia was achieved by bubbling nitrogen through the medium for 20 min prior to adding the embryos and throughout further incubation.

All inhibitors, including sodium acetate, were employed in media buffered with sodium phosphate:citric acid. This must be distinguished from certain experiments (sections 3.3.2.5; 3.3.2.6) in which acetate buffer completely replaced the sodium phosphate:citric acid.

3.1.2.5 Kinetic Analysis of Peptide Transport

The effect of substrate concentration on the rate of peptide uptake was studied using the fluorescamine method. A number of limitations became apparent:

(i) In order to obtain initial rates of uptake only short incubation periods could be employed, during which uptake approximated to linearity. This reduced sensitivity.

(ii) At high substrate concentrations (>10 mM) such a small proportion of the total peptide present in the medium was absorbed, that it became impossible to obtain meaningful results.

(iii) At low substrate concentrations, compounds released to the medium by barley embryos interfered significantly with the assay.

Thus, it was not possible to monitor peptide uptake over the whole concentration range required to obtain accurate kinetic constants using standard transformations of the Michaelis-Menten equation. Instead, uptake was measured over a rather restricted concentration range and the kinetic constants determined from the data by statistical analysis. This was performed using a computer programme devised by Dr. J.T. Gleaves (Botany Dept., Durham University), based on Colquhoun (1971).

3.1.3 Results

3.1.3.1 Amino Acid Uptake

The uptake of an amino acid can be monitored in one of two ways; by observing its disappearance from the incubation medium (assuming extracellular deamination is unimportant) or the increase in its concentration within the embryo. Both these methods have been employed for several amino acids, amongst which glycine and isoleucine serve as typical examples (figure 3.1). For glycine (and two other amino acids, serine and glutamic acid) a discrepancy was noted between the rates of uptake obtained by each method; measuring an increase in the amino acid pool within the

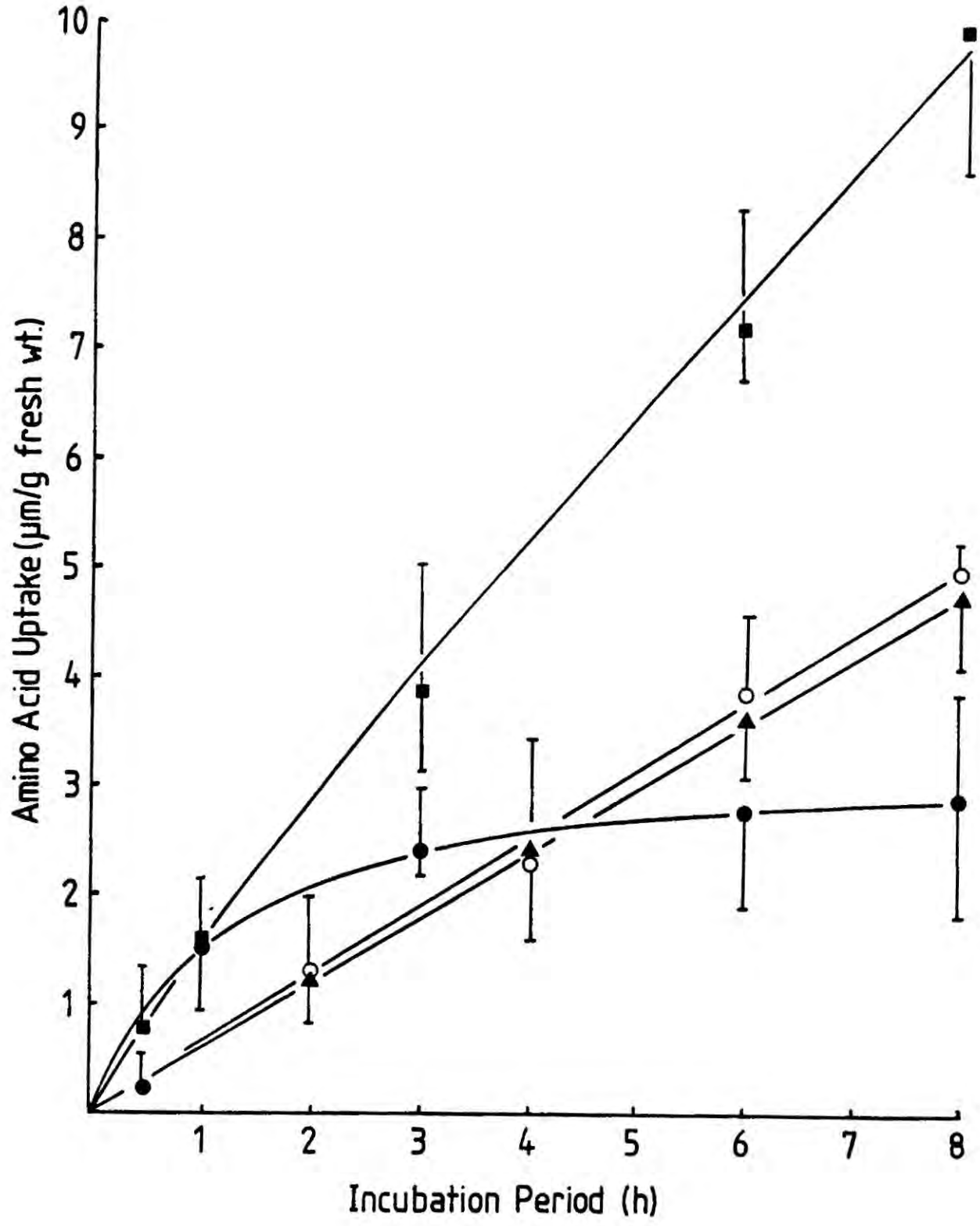


Figure 3.1: Uptake of Glycine and Isoleucine by Barley Embryos

Incubations were carried out in 1mM amino acid, under standard conditions (section 2.3.1). Dansyl chloride was used to follow the disappearance of glycine (■) and isoleucine (o) from the medium. In addition, increases in the pools of glycine (●) and isoleucine (▲) in the embryo were monitored, also using dansyl chloride. Each value is the average of four separate determinations. Bars represent the range of values obtained.

Table 3.1: Accumulation of Amino Acids by Germinating Barley Embryos

	Amino acid accumulation (μm/g fresh wt.)		
Amino acid concentration in medium	0	2mM	20mM
Histidine	1.0	8.2	70
Valine	1.8	9.6	58
Leucine	1.0	8.0	60
Proline	4.0	10.2	70
Phenylalanine	0.6	8.6	58

Embryos were incubated for 8h in 0, 2 or 20mM solutions of each amino acid, under standard conditions (section 2.3.1). Dansyl chloride was used to determine the level in the embryos of the particular amino acid with which they were incubated.

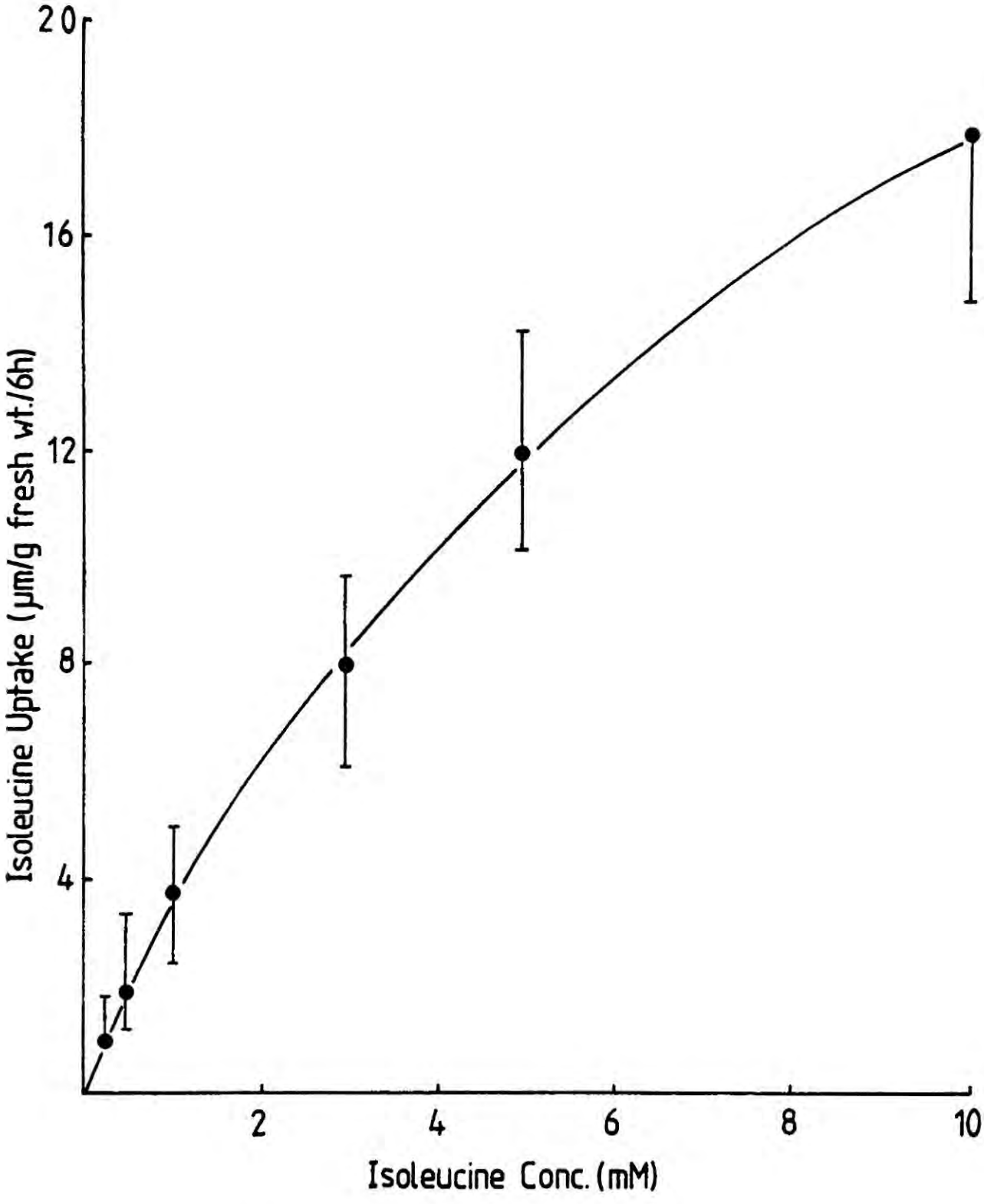


Figure 3.2: Effect of Medium Concentration on the Uptake of Isoleucine by Barley Embryos

Standard incubation conditions were employed (section 2.3.1). Uptake was determined by monitoring increases in the free isoleucine pool of the embryos after 6h incubation. Each value is the average of four separate determinations. Bars represent the range of values obtained.

embryo leads to an underestimate of the actual rate of uptake. This is due to the rapid metabolism of a proportion of the absorbed amino acid. However, for isoleucine (and the amino acids listed in table 3.1) the two methods give comparable results, implying the lack of any significant metabolic degradation by barley embryos. Thus, the uptake of peptides containing these amino acids could be estimated by measuring the increase in the amino acid pool of the embryo. Indeed, this method gives comparable results with the rates of uptake estimated by other means (section 3.1.3.4) and, under certain circumstances, can be the most suitable procedure.

The linearity of uptake over an 8 h period, together with the absence of any intracellular metabolism, shows that the accumulation of isoleucine within the embryo over similar periods is a valid estimate of the rate of uptake. A similar assumption holds for several other amino acids. Thus, many amino acids are absorbed by barley embryos at similar rates (table 3.1). Uptake is against the concentration gradient and does not seem to be limited by the degree to which an amino acid can accumulate; an increased external concentration of amino acid results in an equivalent increase in the amount accumulated by the embryo. This indicates that, within the concentration range studied, amino acid

uptake is not saturated. Certainly, isoleucine uptake does not saturate within this range (figure 3.2).

D-amino acids do not seem to be taken up by barley embryos. No uptake of D-alanine, D-leucine or D-valine could be detected on monitoring the incubation medium. Furthermore, examination of embryo extracts after incubation also failed to show any uptake . However, under similar conditions the corresponding L-amino acids are taken up quite rapidly.

3.1.3.2 Uptake of Sarcosine-Containing Peptides

The three sarcosine-containing peptides, Gly-Sar, Gly-Sar-Sar and Gly-Sar-Sar-Sar, are all taken up intact by barley embryos (figure 3.3). During a 6 h incubation period very little hydrolysis occurs. Free sarcosine appearing in the embryo always represents less than 10% cleavage of the absorbed peptide. Both Gly-Sar and Gly-Sar-Sar are accumulated in the embryo to a higher concentration than they are supplied in the external medium; Gly-Sar-Sar-Sar is not.

It seems that intracellular metabolism of free sarcosine is unimportant. Thus, if long incubation periods are employed, such that the disappearance of Gly-Sar from the incubation medium is detectable, the loss is exactly equivalent to the sum of Gly-Sar plus sarcosine present in the embryo.

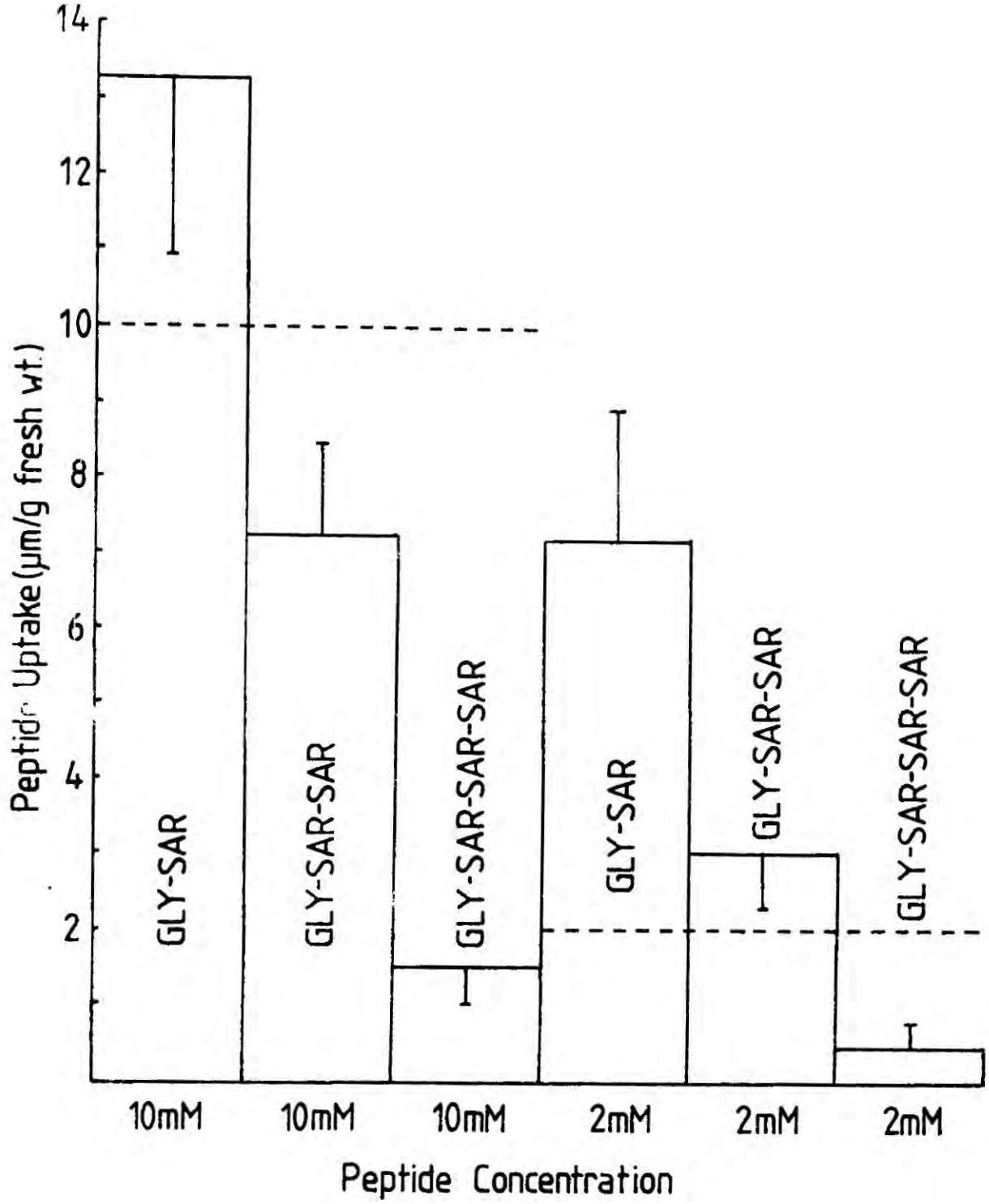


Figure 3.3: Intact Uptake of Sarcosine-containing Peptides by Barley Embryos

Incubations were carried out in 2 or 10mM peptide solutions under standard conditions (section 2.3.1). Values were obtained by embryo extraction after a 6h incubation period. Dotted lines represent the concentration of peptide in the medium. Each value is an average of at least four separate determinations. Bars represent the range of values obtained.

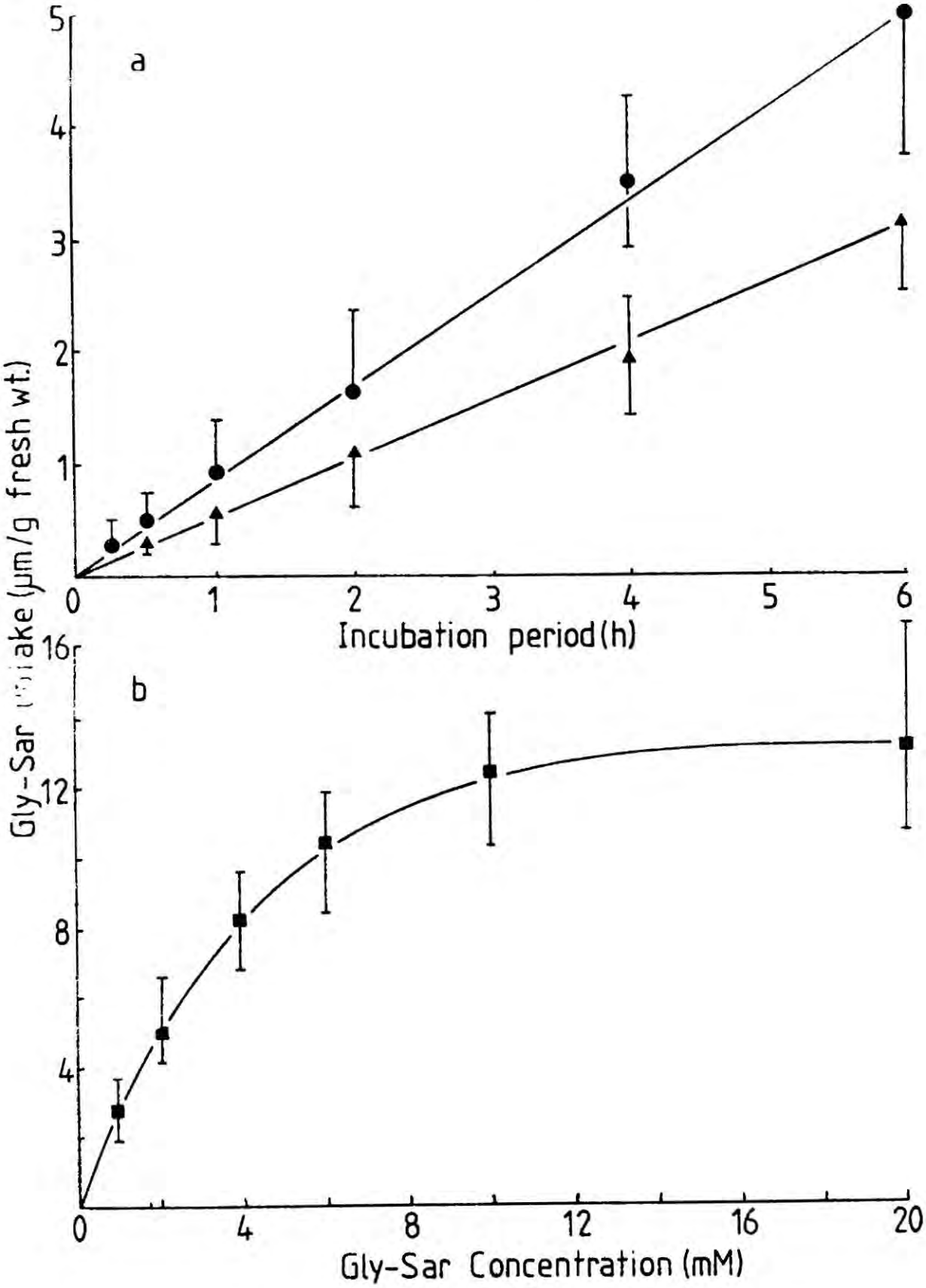


Figure 3.4a & b: Characteristics of Intact Gly-Sar Uptake

(a) Effect of varying incubation period upon the uptake of Gly-Sar (●) and Gly-Sar-Sar (▲) from a 2 mM solution.

(b) Effect of medium concentration on the uptake of Gly-Sar during a 6h incubation period.

All incubations were carried out in 50mM sodium phosphate:citric acid buffer, pH 4.2. Each value, obtained by embryo extraction, is the average of at least three separate determinations. Bars represent the range of values obtained.

Similarly, although removal of the external supply of peptide allows a certain proportion of the accumulated sarcosine and Gly-Sar to leak from the embryo, the total amount of each compound (in the embryo plus the medium) remains constant throughout further incubation (section 4.3.4). It therefore seems clear that the amount of intact peptide plus free sarcosine detected in the embryo will be an accurate reflection of the total amount of Gly-Sar absorbed.

Accumulation of Gly-Sar and Gly-Sar-Sar increases linearly with time for at least 6 h (figure 3.4a); preliminary studies indicated that uptake may show a steady increase for as long as 48 h. Gly-Sar uptake also exhibits saturation kinetics (figure 3.4b).

3.1.3.3 Site of Peptide Absorption

Table 3.2 shows the accumulation of Gly-Sar by each of the separated tissues of 48 h barley embryos. It is clear that the scutellum absorbs Gly-Sar to a far greater extent than the roots and shoots, despite the scutellum having a lower surface area:volume ratio than the other two tissues (estimated visually). From these data, and the relative weights of each tissue, it can be calculated that if the Gly-Sar absorbed by each organ separately were evenly distributed throughout the embryo, the concentration would

Table 3.2: Uptake of Glycylsarcosine by Various Tissues of the Barley Embryo

	Separated tissues[a]	Intact embryos[b]
Shoots	0.71	6.2
Roots	0.26	4.5
Scutellum	10.30	9.7
Whole embryo	4.50[c]	7.3

Incubations were for 6h under standard conditions (section 2.3.1), in 2mM Gly-Sar. Tissues from 48h embryos were employed. Values are expressed as μm/g fresh wt. of tissue; each is the average of two separate determinations.

(a) Uptake by each of the separated tissues.

(b) Gly-Sar present in each tissue after incubation of intact embryos.

(c) Peptide present in the whole embryo was calculated from the amount of peptide absorbed by each of the separated tissues, taking the weight of each tissue into consideration.

reach 4-5 µm/g fresh wt. However, the actual concentration of Gly-Sar achieved by intact embryos over the same period was 7.3 µm/g fresh weight. Thus, it seems that excision reduces net peptide uptake by the scutellar epithelium. This may not be a reduction in the rate of uptake, but a result of the increased leakage of absorbed peptide through the cut surface. About 30% more amino acids leak from the separated tissues than the intact embryo (results not presented).

Although separated roots and shoots only take up Gly-Sar rather poorly, high concentrations may be found in these tissues after incubating intact embryos with the peptide (table 3.2); once absorbed by the scutellum, peptide seems to become more or less evenly distributed throughout the embryo. This was also illustrated by incubating excised embryos on damp filter paper, such that only the scutellar surface came into contact with the medium containing Gly-Sar. After incubation, embryos were dissected into their three main tissues and each extracted separately. Gly-Sar was shown to accumulate to about the same extent in each organ, including the root and shoot which had never come into contact with the peptide solution.

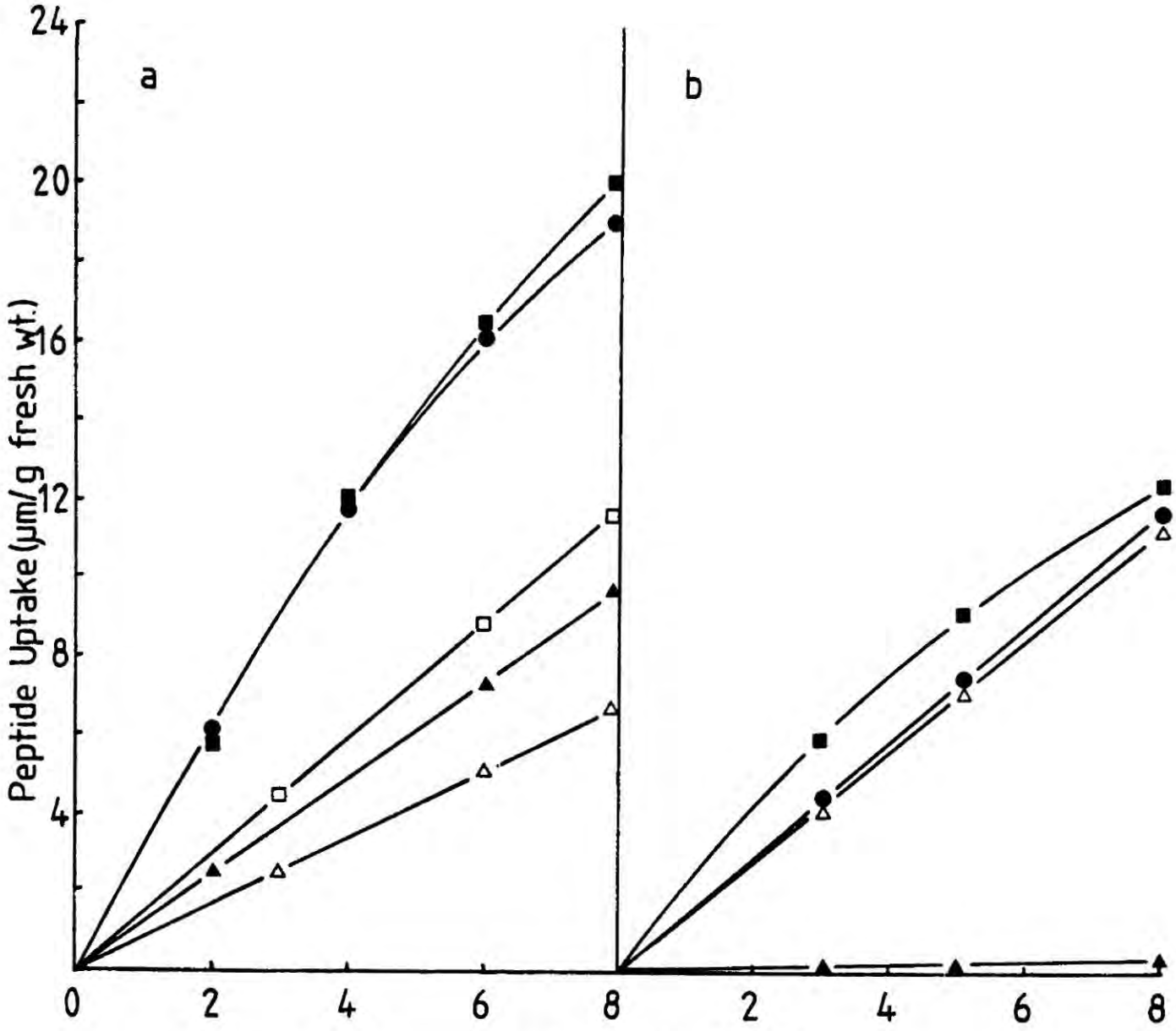
3.1.3.4 Uptake of Physiological Peptides

The uptake of alanine and a number of alanyl peptides

from a 1 mM solution, obtained by monitoring their disappearance from the incubation medium, is illustrated in figure 3.5a. Figure 3.5b shows similar results for a series of glycine peptides. In addition, many other structurally diverse peptides, containing for example lysine, proline, and glutamic acid residues, show comparable rates of uptake (sections 3.2.2; 3.2.3). The apparent 'tailing off' in the rate of uptake after several hours incubation is caused by substrate limitation. If incubations are performed under conditions in which substrate does not become limiting, uptake remains linear for at least 8 h.

Thus, it is clear that barley embryos are able to remove not only amino acids, but also both dipeptides and small oligopeptides from the incubation medium. Table 3.3 shows the rates of uptake of alanine and glycine from equimolar solutions of their homopeptides. Clearly, amino acids can be taken up more rapidly when presented as a peptide than as the free amino acid. This is also illustrated for a mixed dipeptide, glycylisoleucine (figure 3.6), isoleucine being absorbed more rapidly from the peptide than as the free amino acid.

Examination of embryo extracts, after 8 h incubation in a 1 mM solution of a physiological peptide, always



Figures 3.5a & b: Uptake of Alanyl and Glycyl Peptides

Uptake of alanyl (a) or glycyl (b) peptides from 1mM solutions was monitored by following their disappearance from the incubation medium, using dansyl chloride. Standard incubation procedures were employed (section 2.3.1). (a) alanine (▲), dialanine (■), trialanine (●), tetraalanine (□), pentaalanine (△). (b) glycine (△), diglycine (●), triglycine (■), tetra-, penta- and hexaglycine (▲). Each value is an average of at least two separate determinations.

Table 3.3: Rates of Uptake of Alanine and Glycine Residues from Equimolar Solutions of the free Amino Acids and their Homopeptides

	Rate of amino acid uptake (μm/g fresh wt./h)	
	Alanine	Glycine
Alanine	1.3	-
Dialanine	5.8	-
Trialanine	8.7	-
Tetraalanine	6.0	-
Pentaalanine	4.5	-
Glycine	-	1.4
Diglycine	-	2.8
Triglycine	-	5.7
Tetraglycine	-	0
Pentaglycine	-	0

Incubations were in 1mM solutions of each amino acid or peptide, under standard conditions (section 2.3.1). Uptake was monitored by following the disappearance of amino acid/peptide from the medium throughout 5h incubation, during which period rates of uptake remained constant.

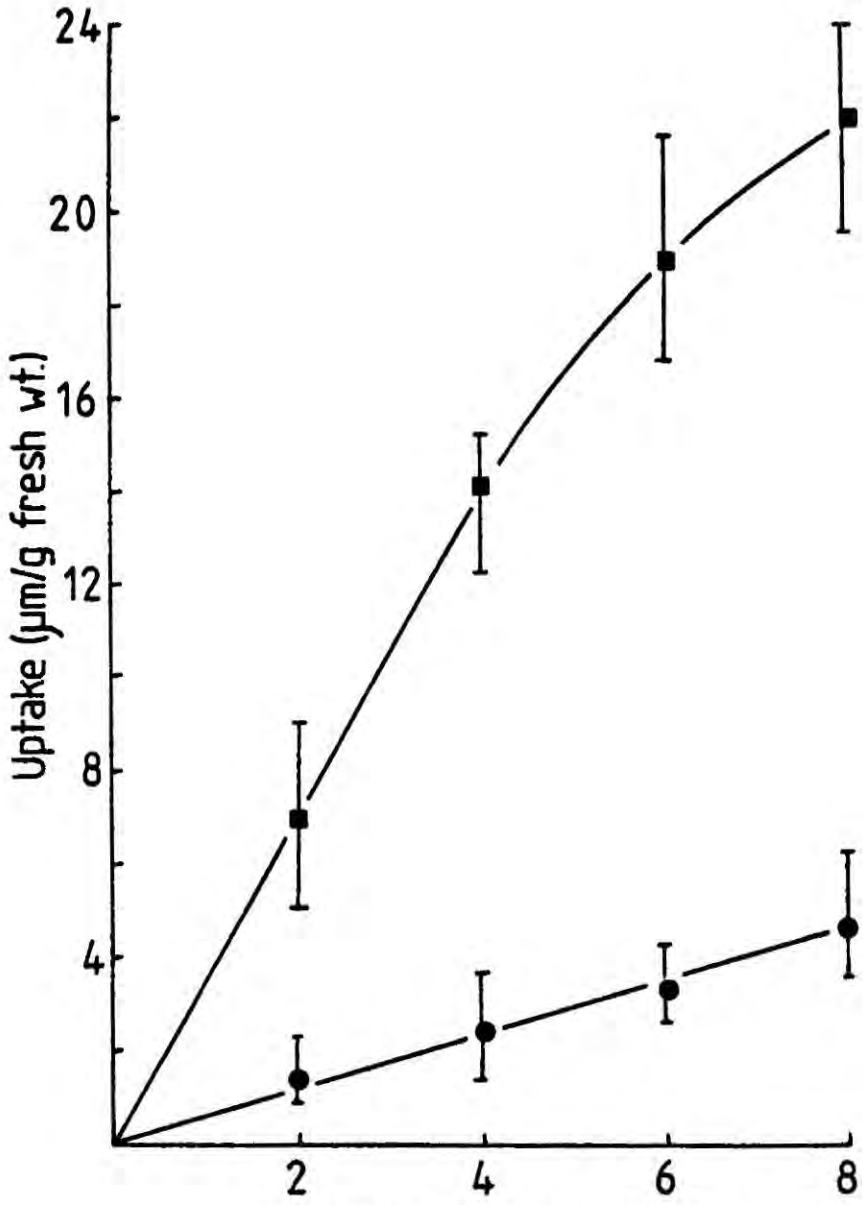


Figure 3.6

Uptake of glycylisoleucine (■) and isoleucine (●) from 1mM solutions was monitored by following their disappearance from the incubation medium, using dansyl chloride. Standard incubation conditions were employed (section 2.3.1). Each value is an average of four separate determinations. Bars represent the range of values obtained.

failed to show any intact peptide uptake. However, if as little as 0.5% of the total peptide lost from the medium had remained intact within the embryo it would have been detected.

3.1.3.5 Amino Acid Exodus

During incubation of barley embryos, free amino acids appear in the medium (see section 3.3.2.2). While most of these amino acids appear as a result of exodus from the embryo, and occur whether or not any peptide is present, others are clearly derived from the peptide added to the medium (table 3.4). In the latter case, these amino acids might arise as a consequence of peptide uptake, followed by hydrolysis and exodus of the resultant amino acids. Alternatively, the amino acids may be produced as a direct result of extracellular hydrolysis, raising the possibility that peptide uptake might actually occur by the sequential process of hydrolysis and amino acid uptake. However, the relative rates of peptide and amino acid uptake argue against this; glycylisoleucine provides a typical example (figure 3.6 and table 3.4). The rate of isoleucine uptake from a 1 mM solution of glycylisoleucine is about 3.0 μm/g fresh wt./h, yet it is only 0.6 μm/g fresh wt/h from a 1 mM solution of free isoleucine. As the maximum level of free isoleucine in the medium never exceeds 0.15 mM during incubation, any explanation for the disappearance of peptide

Table 3.4: Appearance of free Amino Acids in the Medium during Incubation of Barley Embryos

Peptide	None				1mM Glycylisoleucine				1mM Dialanine				1mM Trialanine			
Incubation period (h)	0	3	6	8	0	3	6	8	0	3	6	8	0	3	6	8
Gly-Ile	-	-	-	-	500	270	60	15	-	-	-	-	-	-	-	-
Ala-Ala	-	-	-	-	-	-	-	-	500	350	120	15	-	-	-	-
Ala-Ala-Ala	-	-	-	-	-	-	-	-	-	-	-	-	500	350	125	50
Ala	0	20	40	60	0	30	60	80	0	150	280	360	0	250	350	500
Ile	0	0	0	0	0	30	60	80	0	0	0	0	0	0	0	0
Gly	0	0	10	20	0	0	20	40	0	0	10	20	0	0	10	20
Pro/GAB	0	20	40	80	0	20	40	80	0	20	40	80	0	20	40	80
Others	0	30	30	30	0	30	30	30	0	30	30	30	0	30	30	30

Incubations were carried out with three embryos in a total volume of 500 µl of medium under standard conditions (section 2.3.1). Values are expressed as nm of compound present in the incubation medium.

from the medium based on extracellular hydrolysis appears untenable.

In E.coli, amino acids derived from the uptake of individual peptides generally undergo exodus (Payne & Bell, 1977b, 1979). However, in the eukaryotic yeast, S. cerevisiae (Nisbet & Payne, 1979a), and in barley embryos, only a small proportion leave the cell. This can almost certainly be attributed to the high capacity of the eukaryotic vacuole to store amino acids, regulating their concentration in the cytoplasm, as opposed to the prokaryotic means of achieving such regulation by allowing excess amino acids to leave the cell.

3.1.3.6 Kinetic Analysis of Peptide Uptake

Two peptides, diglycine and trialanine, with widely different competitive abilities (section 3.1.3.7), and therefore presumably different affinities for a binding site (K_t), were selected for a detailed kinetic analysis of transport (for methods see section 3.1.2.5). The initial rates of peptide uptake were monitored over the concentration range 0.2 - 10 mM; a total of 98 separate readings for diglycine and 76 for trialanine were obtained (figures 3.7 & 3.8).

The following three models were fitted to the data, using least-squares analysis to obtain the best fit:

(i) linear uptake
(ii) Michaelis-Menten kinetics
(iii) Michaelis-Menten kinetics plus a linear diffusion component

For both diglycine and trialanine, analysis of variance shows that the Michaelis-Menten model fits the data very much better than do simple linear kinetics; the probability of obtaining such a high variance ratio between the two models if linear uptake were the underlying process is negligible ($P \ll 0.001$). Addition of a linear diffusion component to the Michaelis-Menten model does not significantly improve the fit with the available data.

For diglycine, the Michaelis-Menten model will satisfactorily account for all the variability in the data ($P \gg 0.05$). However, for trialanine, the probability of obtaining the observed results, if the only process operating obeys simple Michaelis-Menten kinetics, is rather small ($0.025 > P > 0.01$). Thus, while the Michaelis-Menten model fits the bulk of the data, there remains the possibility (at least for trialanine) that an additional process is also affecting uptake to some extent. It seems unlikely that this is simply a superimposed linear diffusion component, although it is possible that an additional mediated process might be operating. However, it seems equally likely that the extra variability may

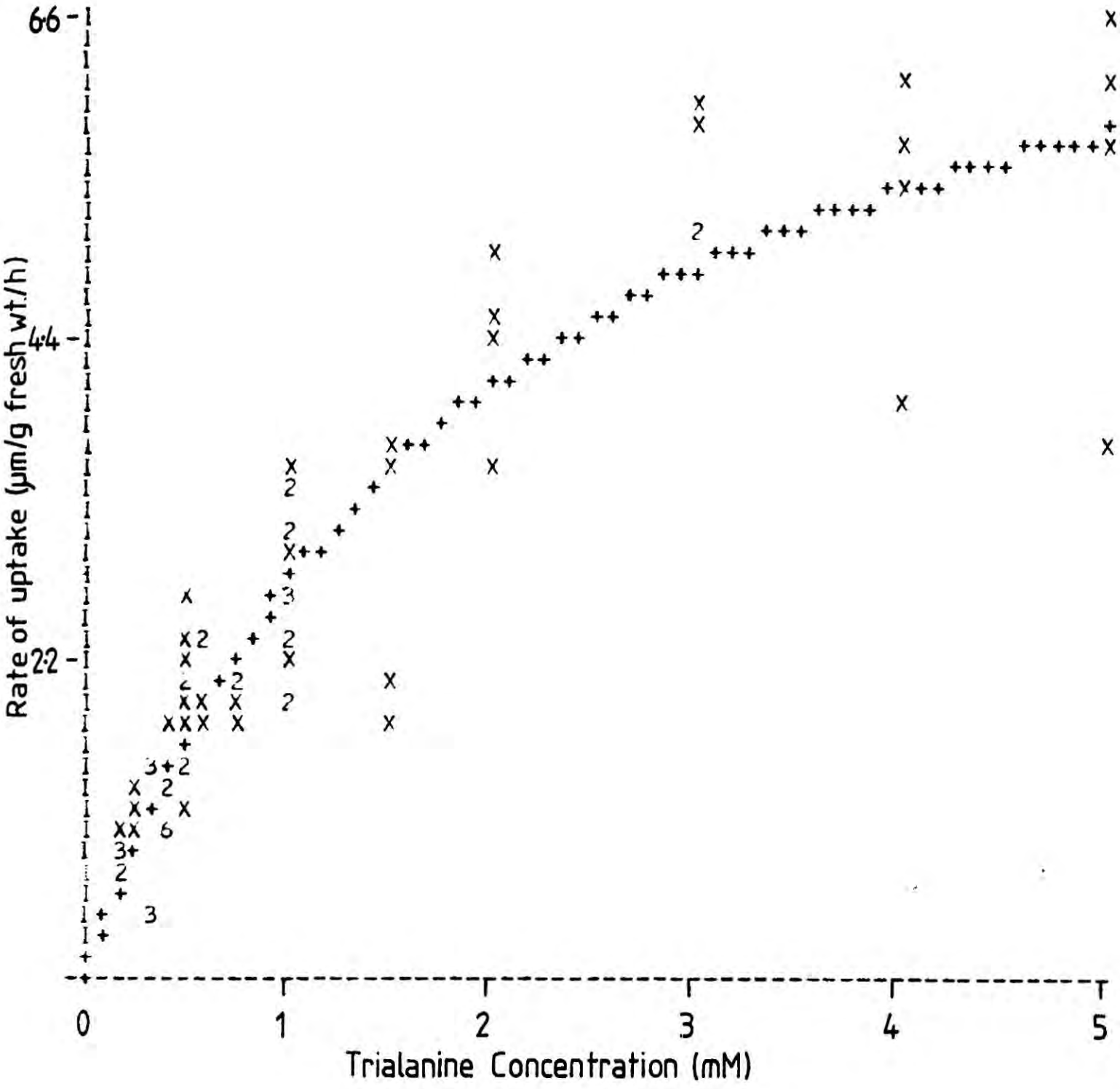


Figure 3.7: Effect of Substrate Concentration on Trialanine Uptake

Uptake of trialanine was monitored using fluorescamine. Incubations were carried out under standard conditions. The curve is the best-fitting Michaelis-Menten hyperbola, fitted by least-squares analysis (section 3.1.2.5). Each point (x) is the result of a separate determination. Where a number of separate determinations gave identical rates of uptake, this number is presented.

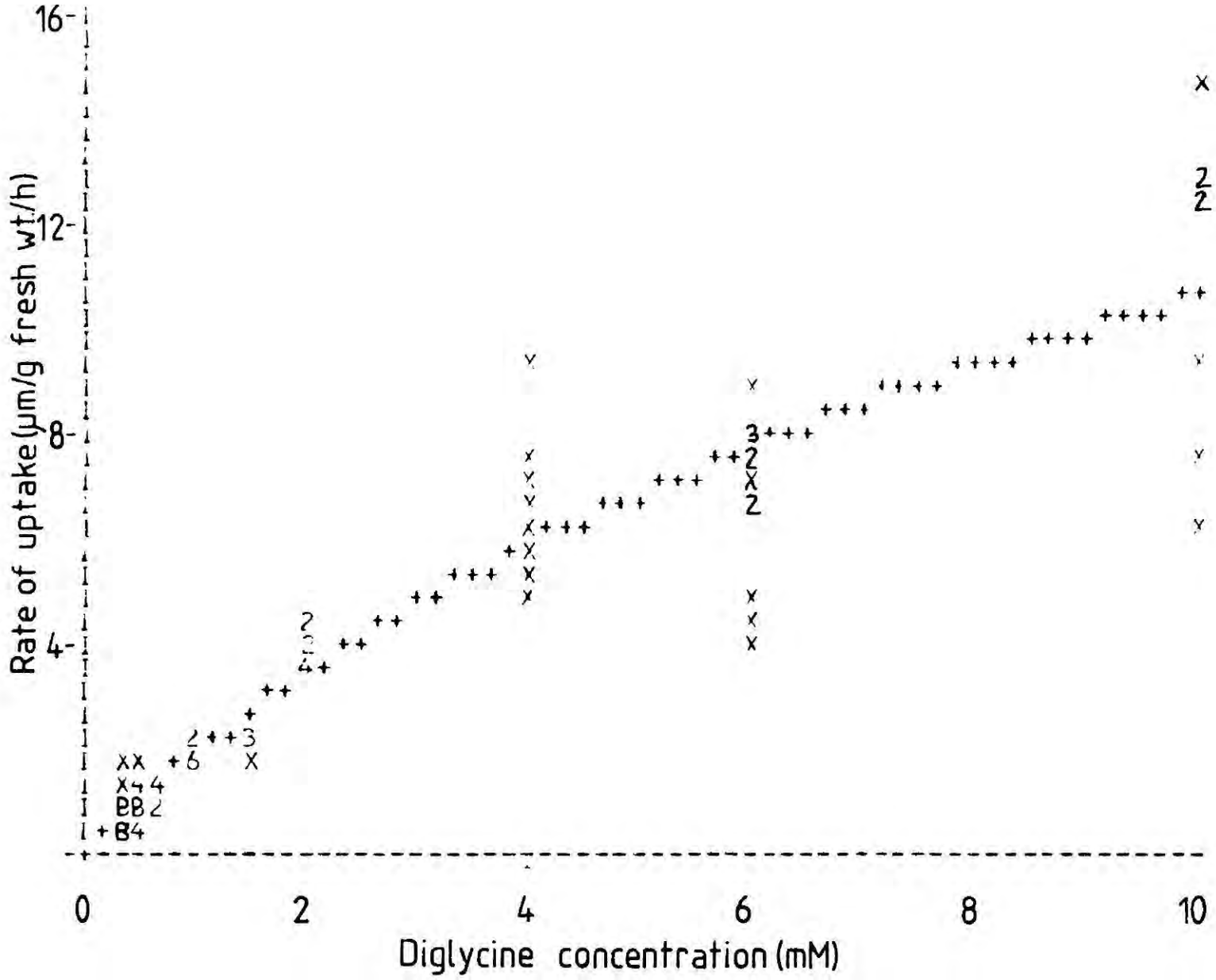


Figure 3.8: Effect of Substrate Concentration on Diglycine Uptake

Uptake of diglycine was monitored using fluorescamine. Incubations were carried out under standard conditions. The curve is the best-fitting Michaelis-Menten hyperbola, fitted by least-squares analysis (section 3.1.2.5). Each point (x) is the result of a separate determination. Where a number of separate determinations gave identical rates of uptake, this number is presented. B indicates 11 identical determinations.

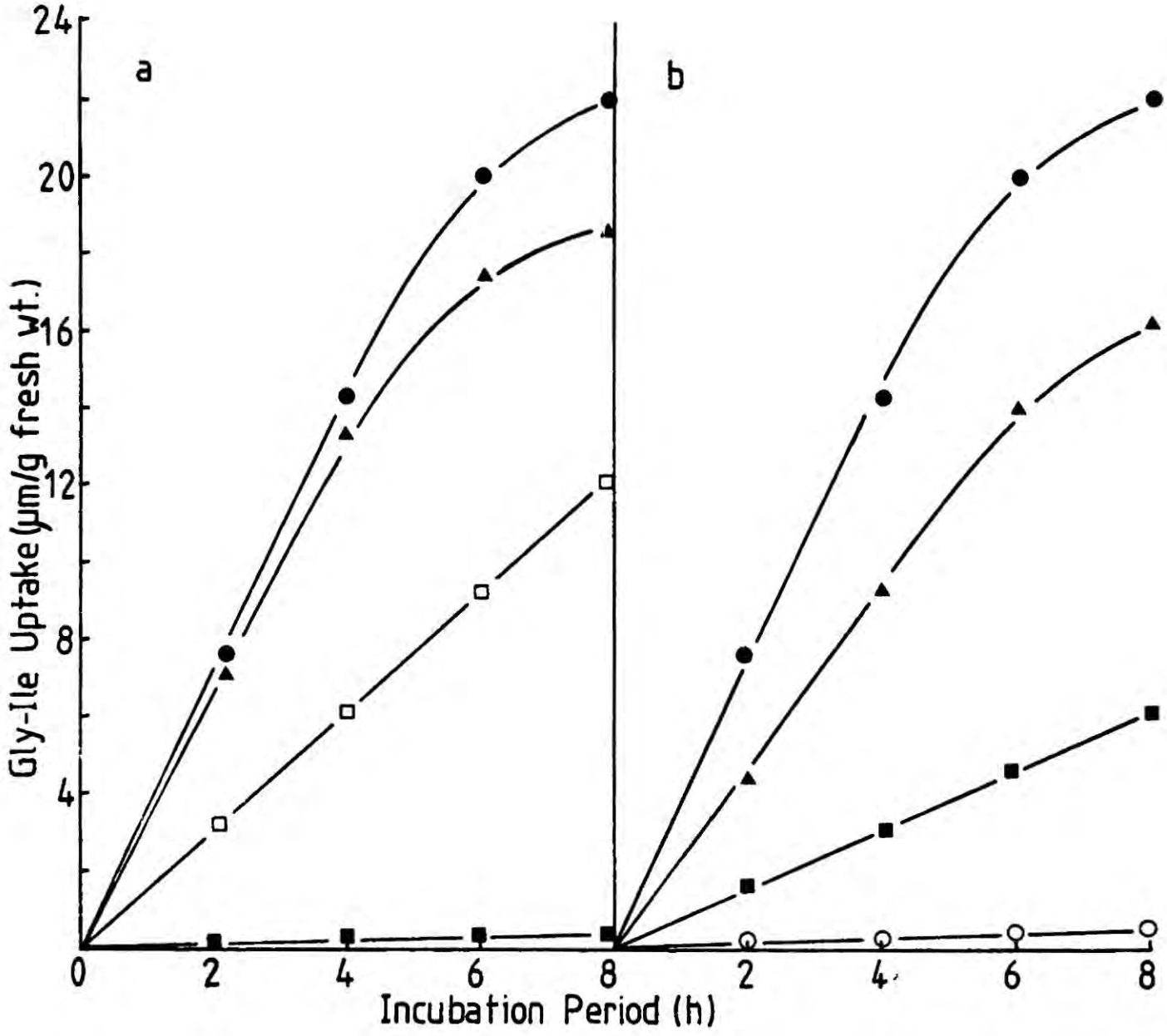
arise as a result of such circumstances as 'unstirred layers' at the scutellar surface or the non-uniform nature of barley embryo cells. Even if a second transport system is present, at the peptide concentrations employed here, it appears relatively unimportant.

Kinetic parameters obtained from the best fitting Michaelis-Menten curves are as follows:

	Vmax (μm/g fresh wt/h)	K_t (mM)
Diglycine	20.5	10.3
Trialanine	7.8	1.9

3.1.3.7 Competitive Inhibition of Peptide Uptake

If two substrates are handled by the same transport system, then, at appropriate concentrations (i.e. saturation), there will be competition and consequent mutual inhibition of uptake. Figure 3.9a shows the effects of various dipeptides on the uptake of glycylisoleucine. Clearly, transport is inhibited. Inhibition varies from almost 100% by dialanine to 5% by diglycine, while mixed dipeptides inhibit by an intermediate amount. The results of similar experiments with a variety of amino acids and oligopeptides as competitors are shown in figure 3.9b. Both tri- and tetra-alanine inhibit uptake of the dipeptide glycylisoleucine. None of the amino acids tested showed any inhibitory effects.



Figures 3.9a & b: Competitive Inhibition of Glycylisoleucine Uptake

Uptake of Gly-Ile from a 1mM solution, in the presence of competing dipeptides (a) or amino acids and oligopeptides (b), was monitored using dansyl chloride. Standard incubation conditions were employed (section 2.3.1). All competitors were at 9mM. Each value is an average of at least two separate determinations.

(a) Gly-Ile alone (●) and in the presence of Gly-Gly (▲), Ala-Ala (■), Gly-Ala (□) or Ala-Gly (□).

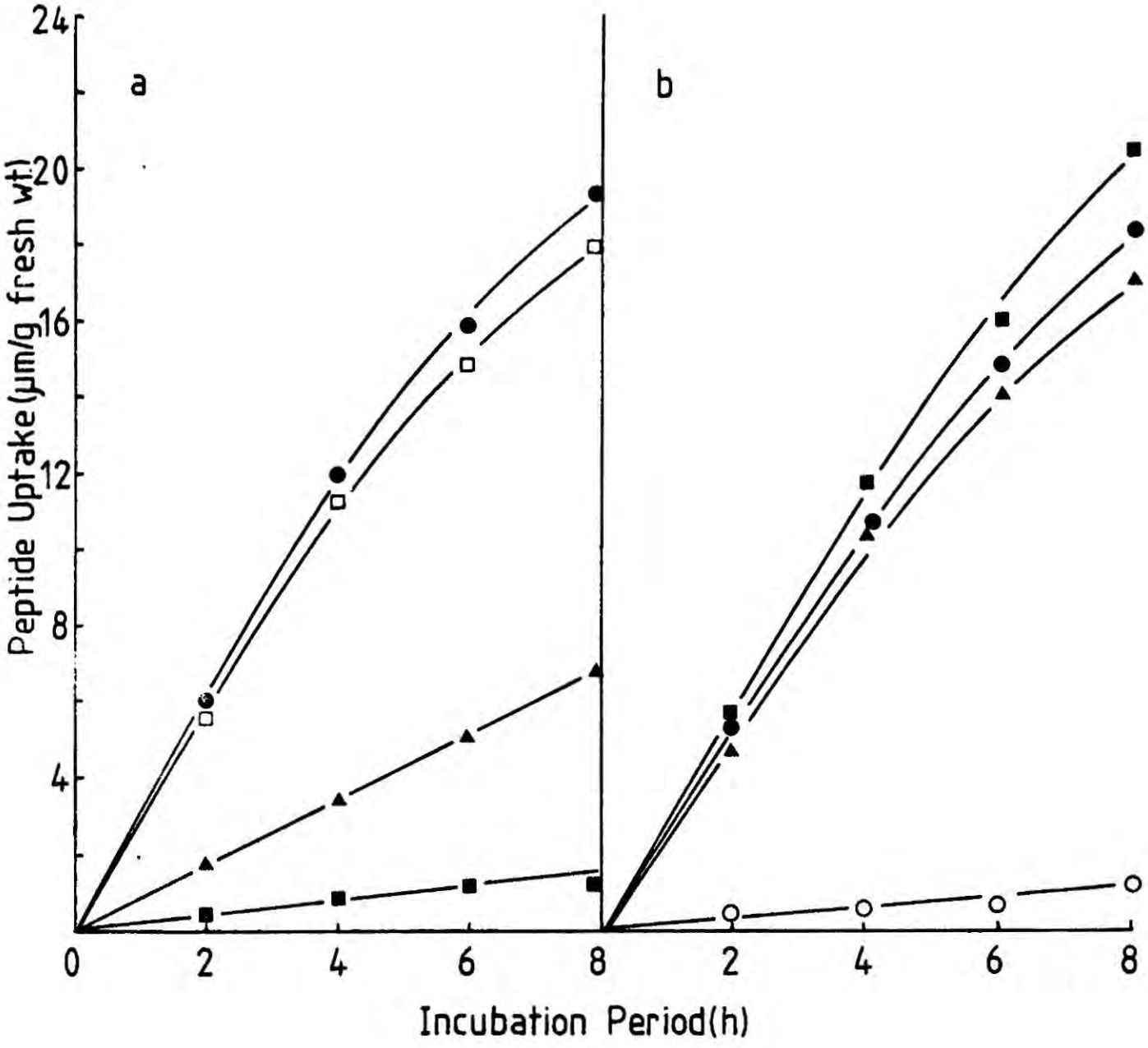
(b) Gly-Ile alone (●) and in the presence of Ala-Ala-Ala (○), Ala (●), Gly (●), Gly-Gly-Gly (▲) or Ala-Ala-Ala-Ala (■).

The uptake of both di- and trialanine is also inhibited by a range of di- and tripeptides (fig. 3.10a & b). Again, amino acids do not act as competitors, while dipeptides inhibit tripeptide uptake and vice versa.

Figure 3.11 illustrates one such competition experiment, in which the disappearance of Gly-Ile from the incubation medium is monitored. The ornithine standard remains constant on each plate, indicating an equal efficiency of dansylation. In the absence of any competitor, the level of Gly-Ile decreases rapidly during 8 h incubation (3.11 a-d). However, in the presence of 9 mM trialanine, the rate of Gly-Ile uptake is considerably reduced (3.11 e-h). The plates also show the small amount of free isoleucine appearing in the medium, in contrast with the large amount of peptide utilized. In addition, some exodus of the endogenous embryo amino acids is seen.

Similar competition experiments between a variety of peptides, containing many different amino acid residues, are reported elsewhere (section 3.2). Significantly, peptides which are transported slowly, if at all (e.g. peptides containing D-amino acid residues; section 3.2.5), show no inhibitory effects.

In all these experiments the high concentrations of competitor employed ensure that it remains in considerable



Figures 3.10a & b: Competitive Inhibition of Dialanine and Trialanine Uptake

Uptake of trialanine (a) and dialanine (b) from a 1mM solution, in the presence of competing amino acids and peptides, was monitored using dansyl chloride. Standard incubation conditions were employed (section 2.3.1). All competitors were at 9mM. Each value is an average of at least two separate determinations.

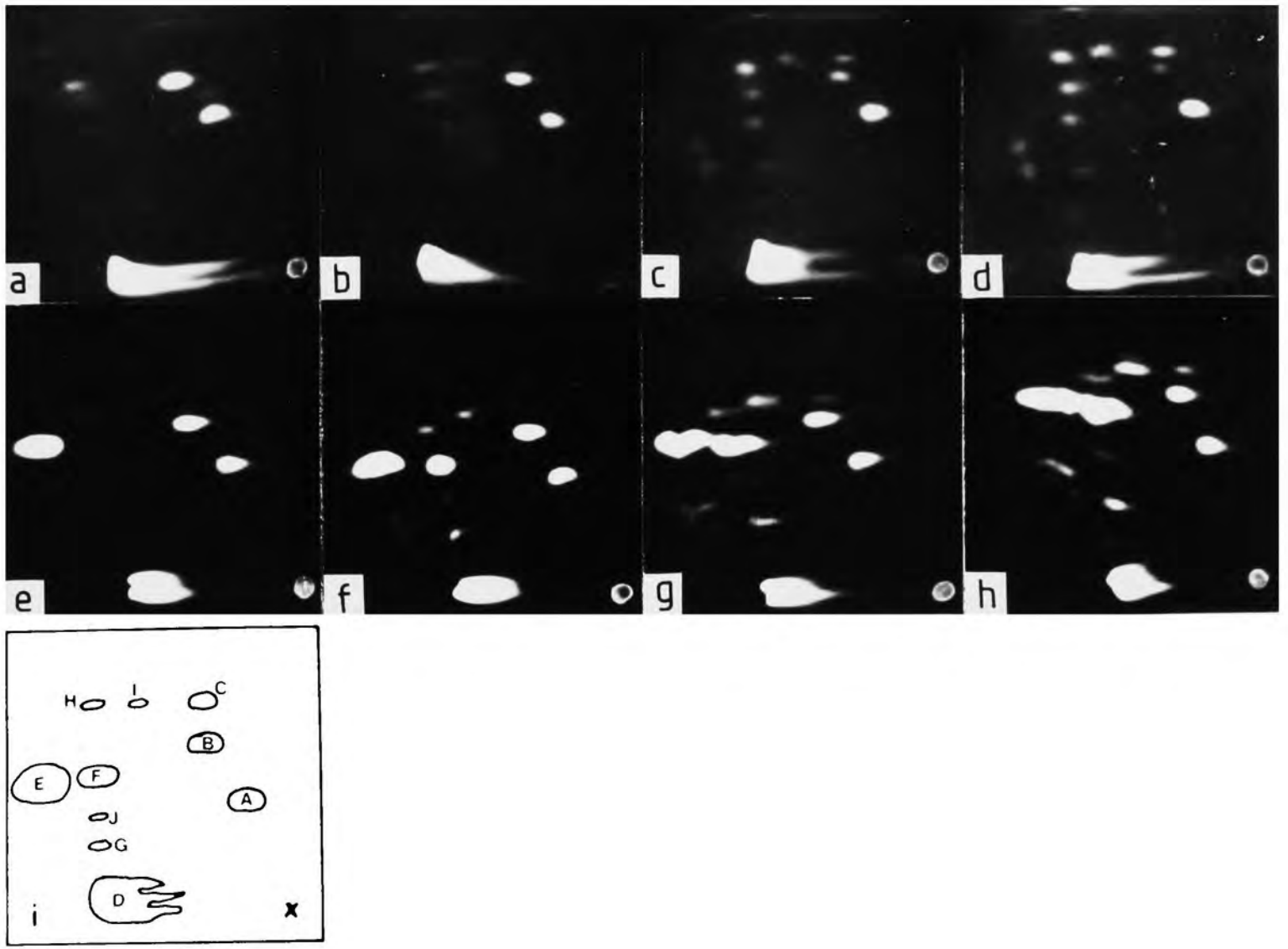
(a) Trialanine alone (●) and in the presence of Ala (●), Gly-Gly (●), Gly-Gly-Gly (□), Ala-Gly (▲), Ala-Ala (■), Gly-Ile (■), Val-Val (■) or Phe-Glu (■).

(b) Dialanine alone (■) and in the presence of Gly-Gly (●), Gly-Gly-Gly (▲), Ala-Ala (○), Gly-Ile (○) or Phe-Glu (○).

Figures 3.11a-i: Uptake of Glycylisoleucine in the Presence and Absence of Trialanine

Developed chromatograms of dansylated samples of the incubation medium photographed under UV light. Incubations were carried out under standard conditions (section 2.3.1) in 1mM glycylisoleucine (a-d) or 1mM glycylisoleucine plus 9mM trialanine (e-h). Samples were removed and dansylated after 0h (a and e), 3h (b and f), 6h (c and g) and 8h (d and h) of incubation.

(i) Diagram indicating locations of some of the dansylated derivatives: A) ornithine (internal standard), B) glycylisoleucine, C) isoleucine, D) dansyl-hydroxide, E) trialanine, F) alanine, G) glutamic acid, H) dansyl-ammonia, I) proline/valine/GAB, J) glycine, X) origin.


a
b
c
d
e
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g
h
i
H
I
C
B
E
F
A
J
G
D
x

excess throughout incubation.

3.1.3.8 Competition Against Sarcosine-Containing Peptides

Gly-Sar uptake saturates at about 10 mM (section 3.1.3.2). Table 3.5 shows the effects of a variety of potential competitive inhibitors on the accumulation of Gly-Sar and Gly-Sar-Sar from 10 mM solutions. It is clear that there is inhibition by all the peptides tested, yet free amino acids show no detectable inhibitory effect.

The above results, together with those in the preceding section (section 3.1.3.7), indicate that di- and tripeptides compete for a common transport system (although other interpretations are possible; see section 3.1.4.4). However, the possibility that an additional system(s) exists, specific for, say, dipeptides, cannot be excluded. If such a system were to be present, then it should not be possible to achieve complete inhibition of dipeptide transport by a tripeptide. However, if a separate system does not exist then, at a high enough concentration of competitor, complete inhibition should be observed. Thus, the uptake of Gly-Sar was measured in the presence of a range of concentrations of di- and trialanine. Plots of

$$\frac{1}{I} \quad \text{vs} \quad \frac{1}{1 - \frac{V_i}{V_o}}$$

(where I is the inhibitor concentration and V_i and V_o are

Table 3.5: Uptake of Sarcosine-containing Peptides in the presence of various Amino Acids and Peptides

Inhibitor	Inhibition of uptake (%)	
	Gly-Sar	Gly-Sar-Sar
Glycine	0	0
Alanine	0	0
Diglycine	53	55
Dialanine	80	60
Glycylisoleucine	72	65
Divaline	72	60
Phenylalanylglutamate	72	60
Triglycine	46	55
Trialanine	82	73
Tetraalanine	67	-
Pentaalanine	30	-

Data are presented as percentage inhibition of Gly-Sar or Gly-Sar-Sar uptake. 100% uptake of these two peptides was 12.8μm/g fresh wt. and 7.8μm/g fresh wt., respectively. Incubations were for 6h under standard conditions (section 2.3.1) with the sarcosine-containing peptides at 10mM and all competitors at 8mM (except pentaalanine at 0.8mM). All figures are the average of four or six separate determinations, obtained by embryo extraction.

- = not determined.

the rate of uptake in the presence and absence of an inhibitor, respectively; Inui & Christensen, 1966) intersect the ordinate of a value of approximately 1.0 (figure 3.12). This indicates that at infinite concentrations of either di- or trialanine, the uptake of Gly-Sar will be very considerably, if not totally, inhibited. If an additional, specific transport system is present, it is clearly of little importance at the peptide concentrations employed. Figure 3.13 is a similar plot for the inhibition of Gly-Sar-Sar uptake by di- and trialanine. Again the intersection of the ordinate is at approximately 1.0.

3.1.3.9 Effect of pH on Peptide Transport

Figure 3.14a shows the effect of pH on the uptake of Gly-Sar. Uptake shows a pronounced optimum at pH 3.8, at which there is considerable accumulation of peptide against a concentration gradient. This optimum is also illustrated by figures 3.15 a-d where, as the pH increases from 3.8 (fig. 3.15a) to 5.8 (fig. 3.15d), the amount of Gly-Sar (and sarcosine and substance A) decreases while the endogenous pool of amino acids remains at a more or less constant level. The amount of sarcosine on each plate is low, representing less than 10% hydrolysis of the absorbed peptide. Substance A is an unknown, produced from Gly-Sar during extraction with TCA.

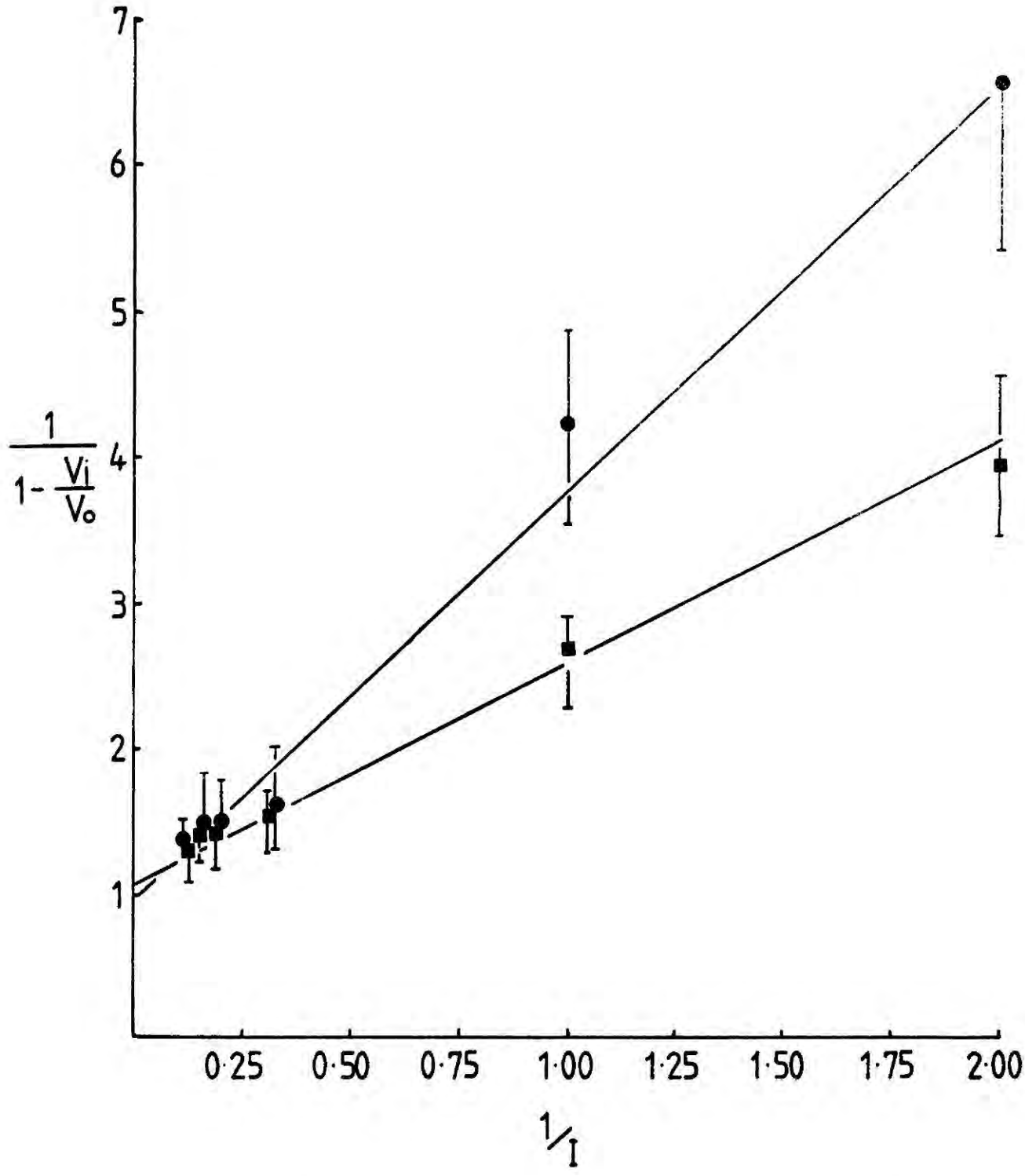


Figure 3.12: Inhibition of Gly-Sar Uptake by Di- and Trialanine

Plots of $^{1}/_{I}$ against $\frac{1}{1-\frac{Vi}{Vo}}$ (where I = inhibitor concentration and V_i and V_o the rate of uptake in the presence and absence of competitor, respectively). Uptake of Gly-Sar from a 10mM solution, in the presence of various concentrations of dialanine (●) or trialanine (■), was obtained by embryo extraction after 6h incubation. Each value is the average of at least three separate determinations. Bars represent the range of values obtained.

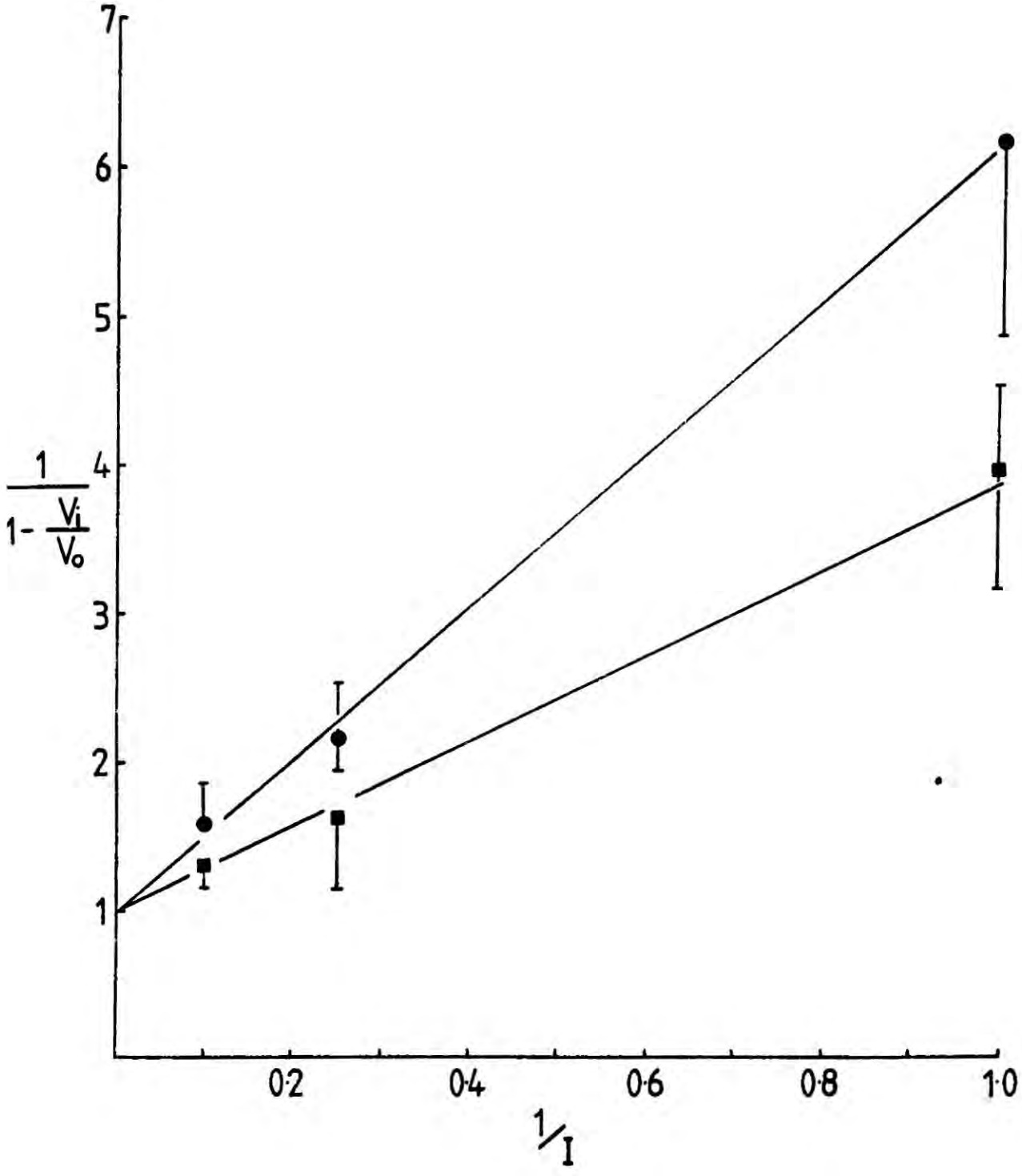
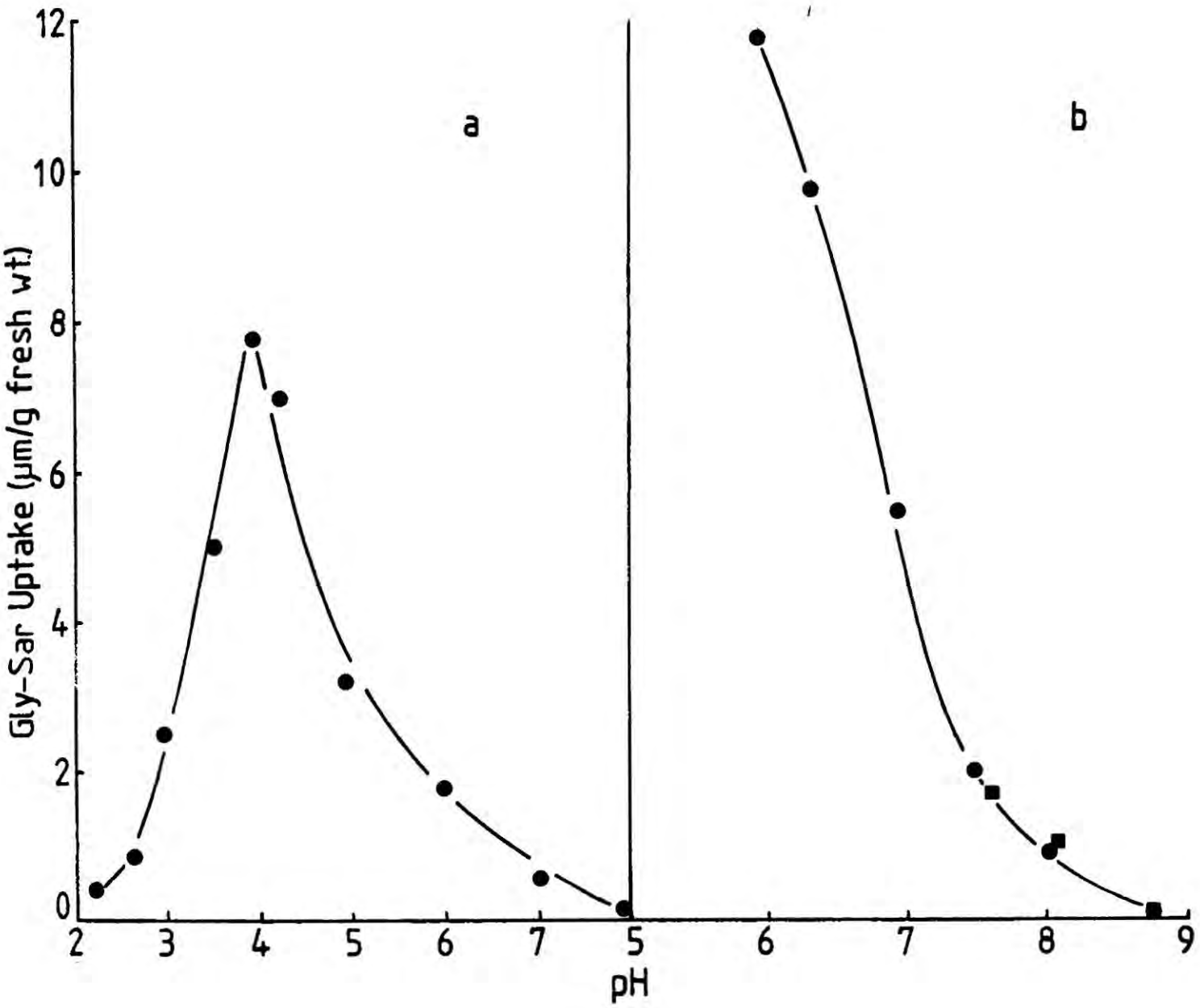


Figure 3.13: Inhibition of Gly-Sar-Sar Uptake by Di- and Trialanine

Plots of $1/I$ against $\frac{1}{1-\frac{Vi}{Vo}}$ (where I = inhibitor concentration and V_i and V_o the rate of uptake in the presence and absence of competitor, respectively). Uptake of Gly-Sar-Sar from a 10mM solution, in the presence of various concentrations of dialanine (●) or trialanine (■), was obtained by embryo extraction after 6h incubation. Each value is the average of at least three separate determinations. Bars represent the range of values obtained.

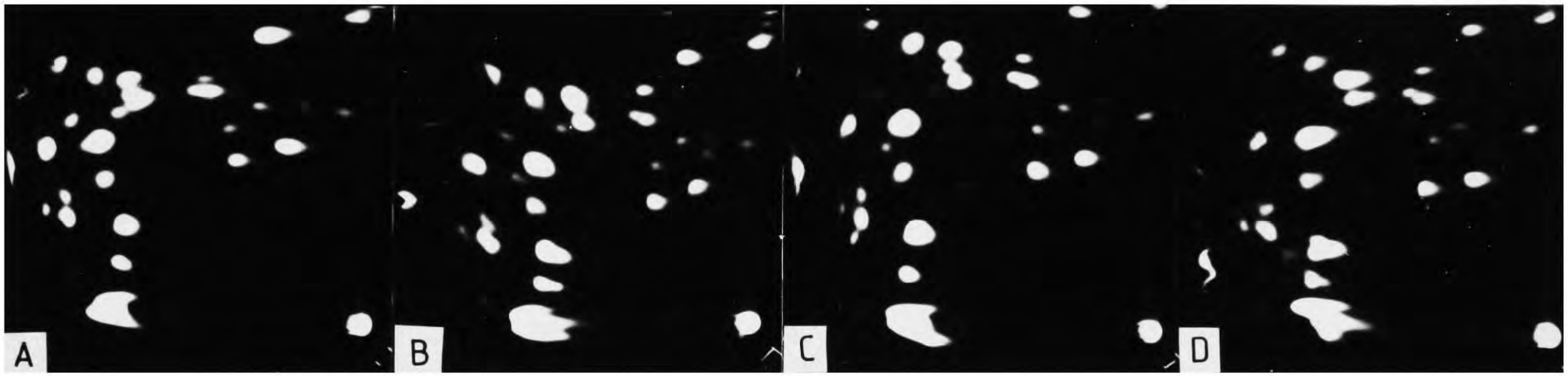


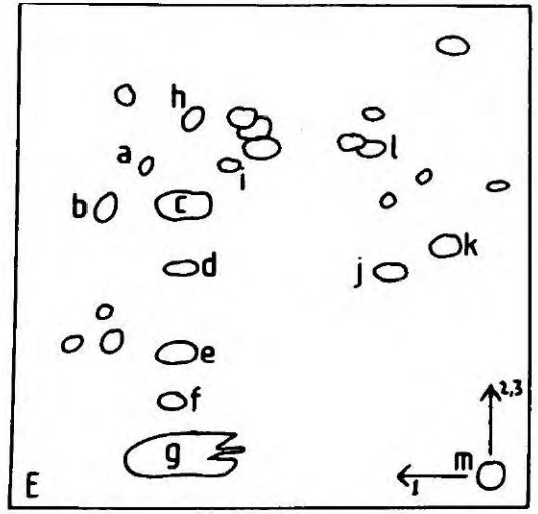
Figures 3.14 a & b: Effect of pH on Gly-Sar Uptake

Uptake of Gly-Sar from a 2mM(a) or 20mM(b) solution was monitored by embryo extraction. Incubations were for 6h in 50mM buffer: (a) sodium phosphate:citric acid; (b) tris (■), sodium phosphate (●). Each value is an average of at least two separate determinations.

Figures 3.15a-e: Effect of pH on Gly-Sar Uptake

Developed chromatograms of dansylated barley embryo extracts photographed under UV light. Embryos were incubated with 2mM Gly-Sar for 6h in 50mM sodium phosphate:citric acid buffer at (A) pH 3.8, (B) pH 4.2, (C) pH 5.2 or (D) pH 5.8. (E) Diagram indicating the locations of some of the dansylated derivatives: a) unknown A, b) Gly-Sar, c) alanine, d) glycine, e) glutamic acid, f) aspartic acid, g) dansyl-hydroxide, h) dansyl-ammonia, i) sarcosine, j) ornithine, k) lysine, l) leucine, m) origin.


A
B
C
D


a
b
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f
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h
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1
2,3
E

Figure 3.16 shows the effect of pH on the uptake of two physiological peptides, Gly-Ile and trialanine. Again a clear optimum is seen at about pH 4.0.

A number of other buffers were tested to confirm that pH, and not the ionic composition of the buffer, was responsible for the optimum shown in figure 3.14a. Thus, citric acid:sodium citrate (pH 3.6-5.8) and sodium phosphate (pH 5.8-8.0) buffers gave results identical to those obtained with the mixed sodium phosphate:citric acid buffer (results not shown). The effects of phosphate and tris buffers on Gly-Sar uptake, over the range pH 5.8-9.0, are shown in figure 3.14b. Again, the results are similar to those obtained with the phosphate:citric acid buffer. Neither changes in the buffer concentrations, nor exchanging Na^+ for K^+ had any significant effect on Gly-Sar uptake (fig. 3.17).

3.1.3.10 Effect of Metabolic Inhibitors on Peptide Transport

The effects of a variety of typical metabolic inhibitors on Gly-Sar uptake are shown in table 3.6. Although the low levels of Gly-Sar made accurate estimation rather difficult, all the inhibitors tested reduced uptake by at least 90%. As embryos were not pre-incubated with the inhibitors it is perhaps not surprising that complete

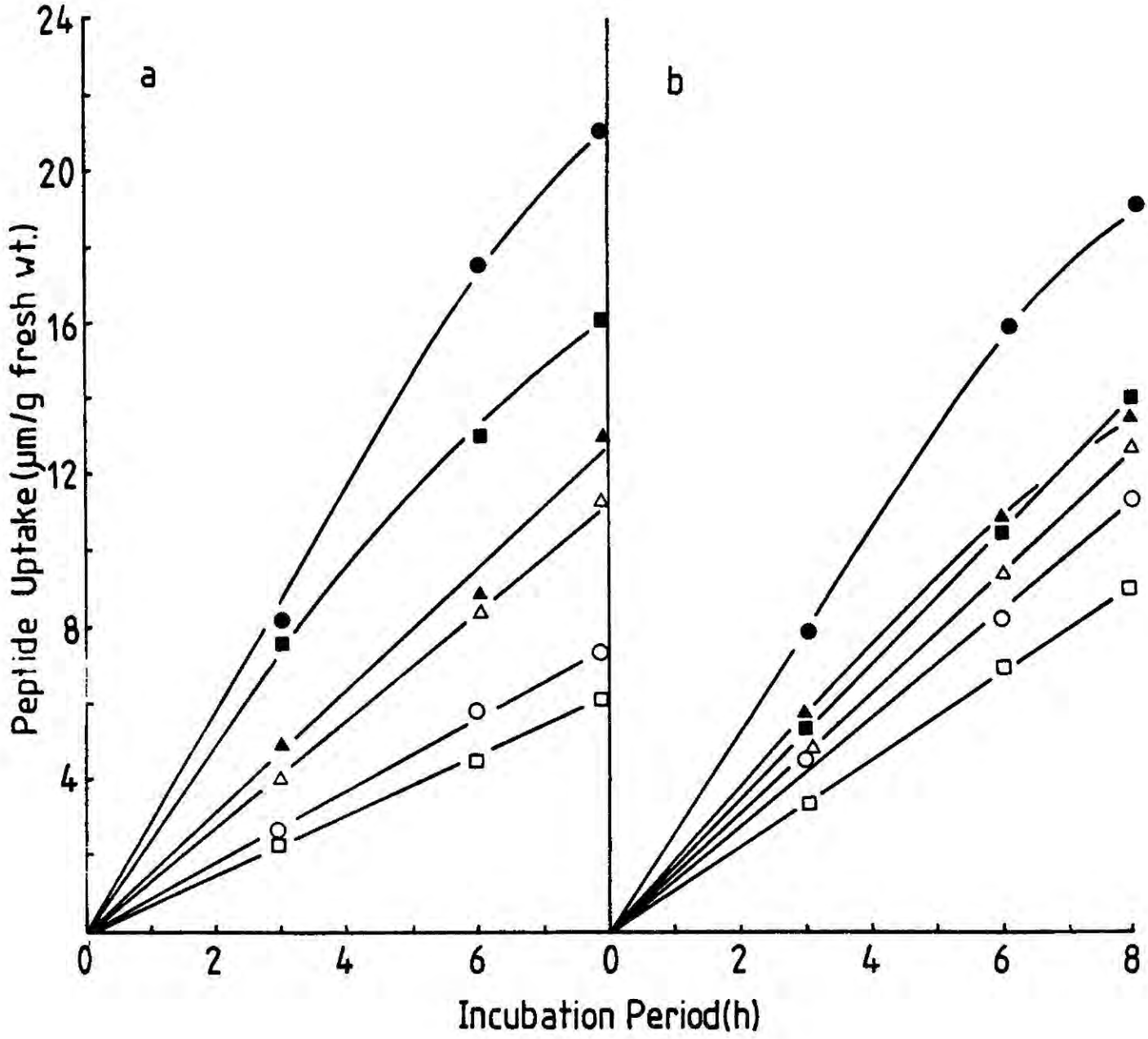


Figure 3.16a & b: Effect of pH on the Uptake of Physiological Peptides

Uptake of trialanine (a) or glycylisoleucine (b), from 1mM solutions, was monitored using dansyl chloride to follow their disappearance from the medium. Incubations were in 50mM sodium phosphate: citric acid buffer at pH 3.0 (▲), pH 4.0 (●), pH 5.0 (■), pH 6.0 (△), pH 7.0 (○) or pH 8.0 (□). Each value is the average of two separate determinations.

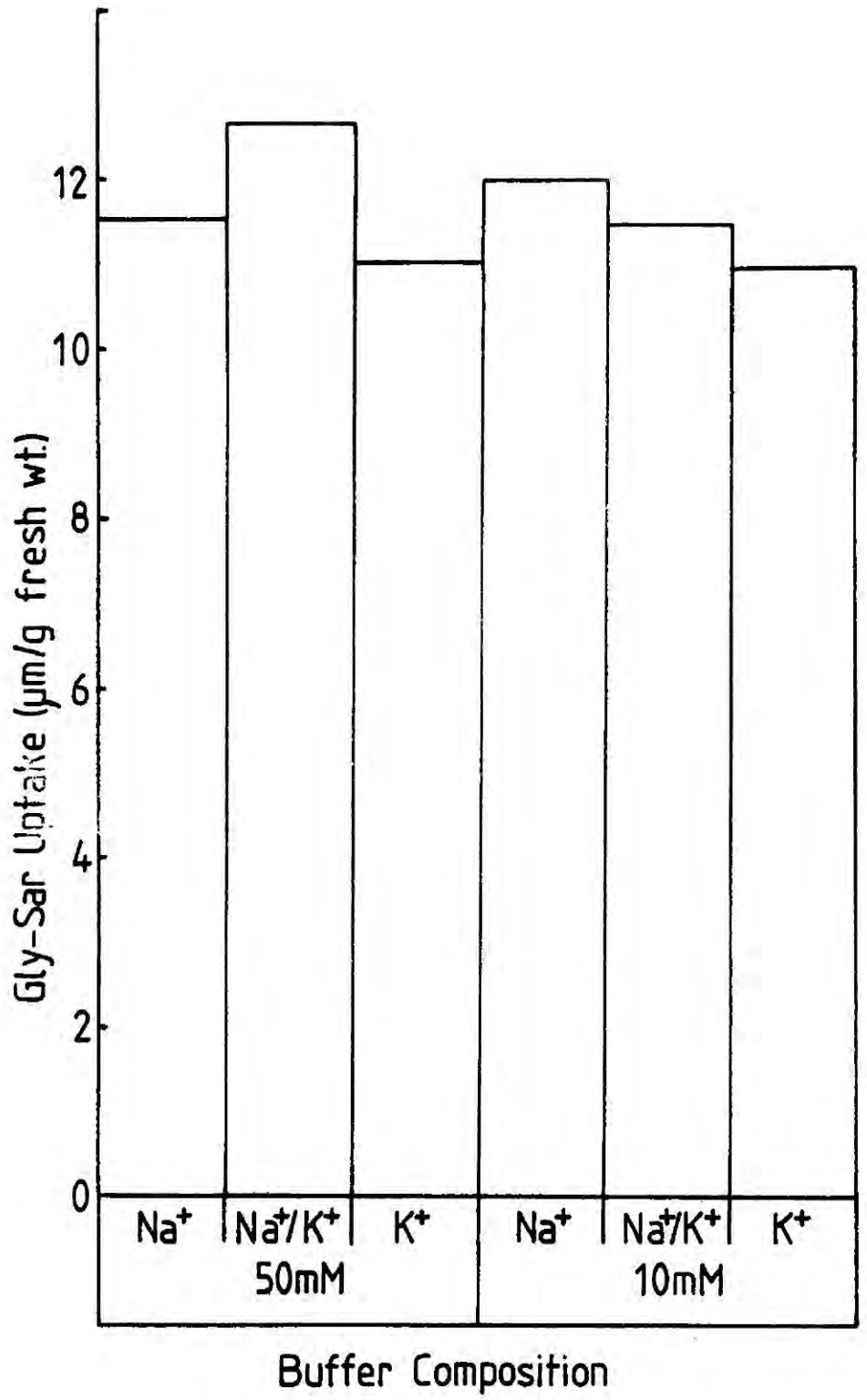


Figure 3.17: Effect of Buffer Composition on Gly-Sar Uptake

Uptake of Gly-Sar, from a 20mM solution, was monitored by embryo extraction. Incubations were for 6h in 50mM phosphate buffer at pH 5.8. Each value is the average of two separate determinations.

Table 3.6: The Effect of Metabolic Inhibitors on Gly-Sar Uptake

Buffer	Phosphate:citric acid (50mM)[a]				Phosphate (50mM)	
pH	2.2	3.4	4.6	5.8	5.8[a]	6.2[b]
No inhibitor	0.3	4.9	4.0	1.8	1.8	11.0
DNP (0.3mM)	1.4	0.5	0.1	0	0	0.5
Na Azide (1.5mM)	0.3	0.2	0.2	0	0	0.8
KCN (4mM)	-	0.6	0.8	0.9	0.9	-
Anoxia	-	-	-	-	-	1.0

Values are given as μmole of Gly-Sar per g fresh wt., obtained by embryo extraction after 6h incubation.
Incubation media contained Gly-Sar at (a) 2mM or (b) 20mM.

inhibition was not achieved. At pH 2.2 the inhibitors had little or no effect on the low levels of accumulation observed, implying that the residual uptake at this low pH is non-active. Metabolic inhibitors reduced Gly-Sar-Sar uptake in a similar fashion (results not shown). However, no effect on the low levels of Gly-Sar-Sar-Sar uptake was observed. In the presence of 0.3 mM DNP, or 1.5 mM azide, uptake of the following physiological peptides from the incubation medium was indetectable: Gly-Gly, Gly-Gly-Gly, Ala-Ala, Ala-Ala-Ala, Gly-Ile, Phe-Glu, Leu-Leu, Gly-Gly-Leu, Leu-Gly-Gly.

Although the specificities of these inhibitors in plant tissue are not well known, the similar effects exerted by each, and the analogy with their mode of action in microorganisms, makes it likely that they generally inhibit energy supply, rather than specifically interfere with a component of the peptide transport system.

3.1.4 Discussion

3.1.4.1 Peptide Transport is an Active, Mediated Process

The uptake of Gly-Sar by barley embryos satisfies many of the generally accepted criteria for an active transport process; saturation kinetics, a requirement for metabolic energy and intact accumulation against a concentration gradient. At the pH employed, Gly-Sar will carry no net

charge. Thus, the possibility that it enters the cell passively, down an electrochemical gradient, seems remote. Although it has not been confirmed that Gly-Sar inside the embryo is present in an osmotically active form, the concentrations achieved (10-20 mM) seem too high for the peptide to be present in a 'bound' form. Besides, the exodus of Gly-Sar from the cell, observed under certain conditions (section 4.3.4), also tends to indicate that the peptide is present in free solution.

The uptake of physiological di- and tripeptides shows similar characteristics. Uptake is again dependent upon a supply of metabolic energy and exhibits typical Michaelis-Menten kinetics. However, direct evidence that these peptides are actively transported cannot be obtained as their rapid hydrolysis precludes the demonstration of intact accumulation. Nevertheless, evidence that uptake is independent of hydrolysis (section 3.1.4.3) and mediated by the same system as Gly-Sar (section 3.1.4.4) implies that the transport of physiological peptides is also an energy-dependent process. Possible mechanisms for coupling metabolic energy to peptide transport are discussed below (section 3.3.3.5).

The possibility that peptide absorption by barley embryos is the result of energy-dependent binding to sites

within the cell wall, rather than uptake across the plasmalemma, may be discounted. Detached roots and shoots are unable to absorb peptides from the medium, and therefore cannot possess such binding sites. However, these tissues may accumulate considerable quantities of peptide transferred to them from the scutellum (section 3.1.3.2). As the roots and shoots lack extracellular binding capacity, this accumulated peptide must be within the cell. Similarly, peptide may be detected in roots and shoots of embryos incubated such that only the scutellar surface comes into contact with the peptide; again, it seems most probable that transport to these tissues is intracellular.

3.1.4.2 Amino Acid Uptake

All the amino acids tested were absorbed by barley embryos against a concentration gradient; uptake was stereo-specific (section 3.1.3.1). Thus, it seems likely that active transport system(s) operate in the scutellum. This is not surprising considering the large amounts of free amino acids produced in the endosperm during germination (section 5.4.2).

However, at equivalent substrate concentrations, free amino acids are accumulated much more slowly than peptides. As the concentrations at which uptake was assayed correspond well with the levels of peptide and amino acid in the

barley endosperm (section 5.4.2), it seems likely that peptides will play at least as important a role as amino acids in the absorption of nitrogen by the scutellum (see also section 6.1).

3.1.4.3 Distinction Between Peptide and Amino Acid Transport

Physiological peptides are hydrolysed so rapidly that, under present circumstances, intact uptake into the embryo cannot be demonstrated. The possibility must therefore be considered that peptide uptake actually occurs as a result of extracellular hydrolysis followed by amino acid uptake (for possible models see section 1.1.1). However, for a number of reasons, such a scheme is of little or no significance here (although see section 3.1.4.5):

(i) The rate of uptake of an amino acid residue from a peptide is far more rapid than uptake of the free amino acid itself (section 3.1.3.4).

(ii) There is no evidence for extracellular peptidase activity. Although amino acids appear in the medium during incubation with a peptide, this is almost certainly the result of exodus from the embryo after intracellular hydrolysis (section 3.1.3.5). This view is supported by the fact that DNP and other metabolic inhibitors which prevent peptide absorption also prevent the appearance of hydrolysis products in the medium. Except in the unlikely case that

peptidases themselves require a direct input of energy (section 4.4.5), this indicates that peptide hydrolysis is dependent upon prior transport into the cell.

In addition, dipeptides are not detected in the medium during incubation with oligopeptides. This is clearly illustrated by results obtained with peptides containing D-amino acids (section 3.2.5). Barley embryos are unable to absorb Gly-D-Leu, Gly-D-Ala and Ala-D-Ala. These dipeptides would therefore be expected to accumulate in the medium if produced from tripeptides by extracellular hydrolysis. However, during incubation with Gly-Gly-D-Leu, Gly-Gly-D-Ala and Ala-Ala-D-Ala no trace of the respective dipeptides could be detected.

(iii) While mutual inhibition of uptake between most peptides is observed, in no case was any inhibition of peptide uptake by an amino acid detected. This suggests the existence of quite distinct transport systems for amino acids and peptides.

(iv) During incubation, only low concentrations of free amino acids appear in the medium, eliminating the possibility that extensive extracellular hydrolysis could account for peptide disappearance, unless accompanied by rapid uptake of the liberated amino acid residues. Direct measurements show that the rate of amino acid uptake is far too slow to

make this a real possibility (section 3.1.3.5). This is particularly well illustrated if the stereospecific requirements for transport are considered (section 3.2.5). While D-alanine and D-leucine may be rapidly absorbed when presented as certain peptides, barley embryos are unable to absorb the free amino acids.

(v) Certain peptides (Gly-Sar, Gly-Sar-Sar, L-Leu-D-Leu, Val-β-Ala, β-Ala-Ala and β-Asp-Ala; sections 3.2.4, 3.2.5) may be accumulated intact against a concentration gradient. Evidence from competition experiments, that these peptides are absorbed by the same system that handles all physio-peptides (sections 3.1.4.4, 3.2.4, 3.2.5), shows that all peptides are handled by a system which at least has the potential of operating independently of hydrolysis.

Thus, in barley embryos the peptide transport system(s) is quite distinct from amino acid uptake. This is similar to the situation in all other organisms in which peptide transport has been characterized (section 1.1.1). In isolated barley embryos, as in E.coli and S. cerevisiae, the utilization of any peptide seems to require the operation of a peptide transport system(s); extracellular hydrolysis is unimportant. This is in contrast with the situation in the mammalian gut, where it is becoming increasingly

evident that a considerable proportion of peptides may be hydrolysed by membrane-bound peptidases and actually absorbed as amino acids.

In barley, as in all other organisms examined, the use of peptidase-resistant substrates shows that, at least in certain circumstances, peptide transport may operate in the absence of hydrolysis (see (v) above). However, the possibility of an obligatory coupling between hydrolysis and transport cannot be eliminated for most physiological peptides. Only in S. typhimurium has it yet been possible to show that the transport of all peptides is independent of hydrolysis (using peptidase-deficient strains; J.W. Payne, unpublished results).

3.1.4.4 Number of Peptide Transport Systems

There is often considerable mutual inhibition of uptake between peptides. The most probable explanation for this effect is competition for a common transport site, although other possibilities (e.g. competition for an energy supply; feedback regulation of transport) should at least be borne in mind. Using present techniques it is both difficult and time-consuming to study transport kinetics in detail (sections 3.1.2.5, 3.1.3.6), and impossible to demonstrate conclusively that inhibition is indeed competitive. However, other possible explanations appear unlikely:

(i) Non-specific inhibition is ruled out because peptides (e.g. certain peptides containing D-amino acids; section 3.2.5) which are not transported, show no competitive ability. In general, the competitive ability of a peptide seems to be related to its rate of uptake.

(ii) Trans-inhibition by a mechanism of feedback regulation, whether by absorbed peptides or amino acids derived from them, also appears improbable. Individual peptides are absorbed linearly for considerable periods of time with no evidence for regulation, and free amino acids do not affect peptide uptake, even though they are accumulated to a considerable extent by the embryos. Although small amounts of physiological peptide may remain intact within the embryo (although too little to detect by present means), the amounts are insignificant compared with the amounts of intact Gly-Sar or peptide-derived amino acids which can be accumulated without affecting peptide transport, and therefore seem unlikely to regulate uptake.

(iii) The observed slopes of the Inui-Christensen plots (figures 3.12, 3.13; section 3.1.3.8) cannot be explained on the basis of non-competitive inhibition (appendix 3).

A variety of dipeptides can inhibit tripeptide uptake and vice versa. In several cases inhibition is virtually complete, implying that only a single transport system

exists, capable of handling both di- and tripeptides. The inhibition of Gly-Sar and Gly-Sar-Sar uptake by various physiological di- and tripeptides shows that they too are handled by this same transport system. Additional evidence that a range of structurally diverse peptides also compete for the same transport system is presented elsewhere (section 3.2).

Reciprocal plots for the inhibition of Gly-Sar and Gly-Sar-Sar uptake show that the transport of these peptides may be completely inhibited by both dialanine and tri-alanine. Thus, it seems that there is no transport system unique to either di- or tripeptides. Although the possibility remains that an additional system(s) with restricted specificity may exist, or that there are several systems, each capable of handling both di- and tripeptides with different specificities, there is no evidence for this; all peptides tested, whatever their structure, seem to be handled by the same transport system (if they are transported at all).

One apparent anomaly in the data is the poor competitive abilities of di- and triglycine compared with their rates of uptake. Although a direct relationship between these two parameters would not necessarily be expected, it is a general observation that peptides which

are transported more rapidly show greater competitive abilities. Glycine peptides are the only peptides which deviate considerably from this relationship; this is considered elsewhere (section 3.2.3.2).

Thus, on the available evidence, barley seems to be similar to other eukaryotic organisms studied to date, in having a single peptide transport system with broad specificity for di- and tripeptides (section 1.1.3). However, unlike barley and other eukaryotes, many bacteria have, in addition to the oligopeptide permease which handles both di- and tripeptides, a second permease specific for dipeptides. It would be interesting to know why bacteria possess this additional system. It may be that the improved ability of the bacterial oligopeptide system to handle large peptides (four to six residues), relative to the corresponding eukaryotic systems (section 3.1.4.5), has been at the expense of a high affinity for dipeptides. Certainly in E. coli, the oligopeptide permease handles dipeptides rather poorly compared with tripeptides (Alves & Payne, personal communication), while in barley and other eukaryotes di- and tripeptides are transported at similar rates.

3.1.4.5 Size Restriction for Peptide Uptake

Di- and tripeptides are handled by the same transport

system in barley. However, the situation with regard to larger peptides is slightly ambiguous. Small amounts of Gly-Sar-Sar-Sar are taken up by barley embryos. However, uptake is not affected by metabolic inhibitors and is therefore unlikely to represent active uptake via the peptide transport system. Furthermore, the uptake of tetra-, penta- and hexaglycine is indetectable, although tetra- and pentaalanine are absorbed by barley embryos. The rate at which alanine is absorbed by the embryo from these peptides is greater than from an equivalent concentration of the free amino acid. In addition, these peptides competitively inhibit the uptake of Gly-Sar. Thus, it seems clear that at least a portion of their uptake is mediated by the peptide transport system. However, considerable quantities of free alanine appear in the medium during incubation with these larger peptides. It is not clear whether this is the result of exodus from the embryo or a result of extracellular hydrolysis. The possibility that hydrolysis followed by amino acid uptake plays a role in the utilization of these larger peptides cannot, therefore, be excluded. Furthermore, traces of di-/trialanine appear in the incubation medium, raising the possibility that tetra- and pentaalanine may be hydrolysed extracellularly to di- and tripeptides prior to absorption.

Thus, although it seems likely that the barley peptide transport system is able to handle certain tetra- and pentapeptides, other possible explanations for the results cannot yet be discounted. Again, there is a clear similarity between the barley peptide transport system and similar systems in other eukaryotic organisms (section 1.1.4.1); while di- and tripeptides are absorbed efficiently, larger peptides are handled less well, if at all.

3.1.4.6 pH Optimum for Peptide Transport

The uptake of Gly-Sar and physiological peptides shows an optimum at about pH 3.8. Although this may seem low, it is in good agreement with the pH reported for the barley endosperm *in vivo*, and the pH optima of the endosperm proteases (section 1.3.4.1). A similar acidic pH optimum was found for glutamine transport by the maize scutellum (Stewart, 1971).

The increase in peptide uptake as the pH is lowered from pH 8.0 to 3.8 cannot be attributed to a general increase in membrane permeability, or to non-specific adsorption, as uptake at pH 3.8 is against a concentration gradient and inhibited by a variety of metabolic inhibitors. Only at pH 2.2 may the small amount of residual uptake be non-active; inhibitors do not affect uptake at this low pH.

It remains to be established whether the influence of pH is exerted primarily through changes in protonation of the peptide substrate, of a protein carrier, or an effect on energy supply (the possibility that a proton gradient may be involved in energizing peptide transport is considered elsewhere; section 3.3.3.5).

3.1.4.7 Kinetic Studies of Peptide Transport

The kinetics of transport were only studied in detail for two peptides, diglycine and trialanine. The uptake of both these peptides conforms to a saturable, Michaelis-Menten-type mechanism. There is no evidence for a significant diffusional (non-mediated) component to uptake. This is in agreement with competition experiments which show that 100% inhibition of transport can be achieved.

The transport parameters, K_t and V_{max}, differ slightly for the two peptides examined. Any attempt at an explanation of these differences would be premature and must await a clearer understanding of the mechanism of transport. However, the K_t for transport (1.9 mM for trialanine) is very similar to the concentrations of peptide present in the barley endosperm (section 5.4.2). Interestingly, the K_m of the embryo dipeptidases varies between 0.3 and 15.8 mM, depending upon the substrate (Sopanen, 1976).

No evidence for bi- or multiphasic kinetics (Epstein, 1976) has been obtained, although this has been claimed for amino acid transport in many plant tissues (Shtarkshall & Reinhold, 1974; Hancock, 1975; King, 1976; Borstlap, 1977; Harrington & Smith, 1977; Lien & Rognes, 1977; Soldal & Nissen, 1978). However, the limited peptide concentration range employed here may not have revealed the full situation.

It is also worth noting that peptide uptake is linear (except where substrate becomes limiting) for at least 8 h, and possibly very much longer. This is similar to other transport processes in plants (see Higgins & Payne, 1979) yet markedly different from peptide transport in the mammalian gut, which is rarely linear for more than about 30 min. This is presumably due to the ability of plant cells to deposit unwanted metabolites in the vacuole, eliminating the need to regulate transport per se. The considerable quantities of free amino acids which can be accumulated by barley embryos (section 3.1.3.1) provide a good illustration of their storage capacity.

3.2 Structural Requirements for Peptide Transport

3.2.1 Requirement for the Terminal Amino and Carboxyl Groups

The N-terminal α-amino group and C-terminal α-carboxyl group are clearly important structural features of any peptide; at physiological pH's they will be predominantly positively and negatively charged, respectively. It might be anticipated that these groups will play a crucial role in determining the binding of a peptide to its transport site.

3.2.1.1 Methods

Standard fluorescamine and dansyl chloride procedures were employed throughout, as indicated, although certain modifications were introduced for particular peptides.

Peptide esters: Methyl and ethyl ester linkages were found to be labile at pH 9.6, during the dansylation reaction. The reaction time was therefore restricted to 20 min, during which period hydrolysis was insignificant although peptide labelling still reached completion (at least for the low concentrations of peptide employed).

Peptides with a modified N-terminal amino group: Alkyl derivatives of di- and triglycine were synthesised by Dr. J.W. Payne (Payne, 1974).

A number of difficulties are associated with the

application of fluorescence techniques to the transport of peptides with modified α-amino groups:

(i) Substitution of the α-amino group of a peptide interferes with its reaction with dansyl chloride. Acetylated peptides show no reaction, while alkylated peptides react only poorly. Thus, for those peptides whose reaction with dansyl chloride is insufficient, uptake of peptide from the medium was monitored by following the level of the peptides' constituent amino acids, after acid hydrolysis. Although somewhat less sensitive than following the uptake of intact peptide directly, rates of uptake greater than 5 μm/g/8 h, from a 1 mM solution, can be detected.

(ii) Both alkylated and acetylated peptides give a negligible fluorescence yield with fluorescamine. Their uptake cannot therefore be monitored directly. However, it is possible to study the competitive effects these peptides exert on the uptake of unsubstituted peptides. The failure of peptides with acyl or large alkyl substituents to give a fluorescent product with fluorescamine makes this method ideal for such studies. Peptides with smaller alkyl substituents (especially N-methylated peptides) are less useful as they interfere with the reaction between other peptides and fluorescamine (section 2.4.3.6). However, in the presence of a constant amount

of a competing N-methylated peptide, the fluorescence yield obtained with varying concentrations of an unsubstituted peptide is still linear, although considerably lower than in the absence of the secondary amine (section 2.4.3.6). Thus, the competitive effects of peptides with small alkyl substituents can still be studied if their concentration remains constant throughout incubation. This is a reasonable assumption if high initial concentrations are employed and the peptide is only transported relatively poorly.

(iii) The available peptides are restricted, mainly to derivatives of di- and triglycine which, even when unsubstituted, are known to be poor substrates for the barley peptide transport system (section 3.2.3.2).

3.2.1.2 Results

3.2.1.2.1 C-Terminal Carboxyl Group

Peptides lacking a C-terminal carboxyl group.

β-alanine can be considered as an aspartic acid residue with the α-carboxyl group replaced by a hydrogen atom. GAB bears a similar relationship to glutamic acid. Table 3.7 shows the rates of uptake of a number of peptides with glutamic acid or aspartic acid at the C-terminus, together with the corresponding GAB or β-alanine derivatives. Clearly, replacement of the carboxyl group with a hydrogen

Table 3.7: Rates of Uptake of various Peptides and their Analogues lacking a C-terminal Carboxyl Group

Peptide	Rate of uptake (μm/g fresh wt./h)
Gly-Asp	2.6
Val-Asp	2.4
Gly-Glu	2.2
Val-β-Ala	1.3
Gly-GAB	ND
Gly-β-Ala	ND
Gly-Gly-β-Ala	ND

Disappearance of peptide from a 1mM solution was monitored using dansyl chloride. Incubations were carried out for 8h under standard conditions (section 2.3.1). Each value is the average of two separate determinations.

ND = not detectable.

Table 3.8: Rates of Uptake of various Peptides and their Analogues lacking a C-terminal Carboxyl Group

Peptide	Rate of uptake (μm/g fresh wt./h)
Gly-Asp	3.2
Val-Asp	3.3
Gly-Glu	4.0
Val-β-Ala	2.4
Gly-GAB	1.0
Gly-β-Ala	0.6
Gly-Gly-β-Ala	0.4

Uptake from a 2mM solution of each peptide was monitored using fluorescamine. Incubations were carried out for 4.5h under standard conditions (section 2.3.1). Each value is the average of two separate determinations.

atom considerably reduces peptide uptake.

Table 3.8 gives the rates of uptake of the same peptides, monitored using fluorescamine. The greater sensitivity of this method, together with the higher peptide concentrations employed, show that all those peptides whose uptake was indetectable using dansyl chloride are in fact taken up by barley embryos, albeit slowly. Again it is clear that the lack of a C-terminal carboxyl group impairs peptide transport.

The only one of these peptides detected intact in embryo extracts was Val-β-Ala; 3.9 μm/g fresh wt. accumulated over an 8 h incubation period. This is rather less than the amount of peptide lost from the medium (10.9 μm/g fresh wt/8 h); the difference is accounted for by the increase in free valine in the embryo, showing that a proportion of the absorbed peptide is hydrolysed.

Neither Gly-GAB or Gly-β-Ala competitively inhibit dialanine uptake, although some inhibition by Val-β-Ala is detectable (table 3.9).

Peptides with substituted C-terminal carboxyl groups. Table 3.10 shows the rates of uptake of a number of peptides and their ester or amide derivatives, determined using the fluorescamine method. Clearly, substitution of the carboxyl group reduces the rate of uptake considerably.

Table 3.9: Inhibition of Dialanine Uptake by various Peptides with Modified C-termini

Competing peptide	Concentration (mM)	Inhibition of uptake (%)
Gly-Gly-OEt	9	0
Gly-GAB	18	0
Gly-Tyr-NH_2	9	12
Gly-β-Ala	9	0
Gly-Leu-NH_2	18	0
Val-β-Ala	9	40

Uptake of dialanine from a 1mM solution was monitored over a 6h incubation period, using dansyl chloride. 100% uptake of dialanine was 16μm/g fresh wt. Incubations were carried out under standard conditions (section 2.3.1). Each value is the average of two separate determinations.

Table 3.10: Rates of Uptake of various Peptides and their Derivatives with Modified Carboxyl Groups

Peptide	Rate of uptake (μm/g fresh wt./h)
Ala-Ala-Ala	4.0
Ala-Ala-Ala-OMe	0.8
Asp-Phe	3.5
Asp-Phe-OMe	0.6
Gly-Gly	2.6
Gly-Gly-OEt	0.4
Gly-Gly-NH_2	0.3
Gly-Leu	4.2
Gly-Leu-NH_2	0.5
Gly-Tyr	3.4
Gly-Tyr-NH_2	1.2

Peptide uptake from a 2mM solution was monitored using fluorescamine. Incubations were carried out for 4.5h under standard conditions (section 2.3.1). Each value is the average of two separate determinations.

The dansyl chloride procedure gave similar rates of uptake for the unsubstituted peptides, although it was not sensitive enough to detect uptake of their esters or amides.

None of the peptides tested inhibited the uptake of dialanine (table 3.9). The apparent inhibition by Gly-Tyr-NH_2 is almost certainly the result of acetate inhibition (section 3.3.2.5). The peptide is only supplied as its acetate salt, and equivalent concentrations of acetate alone affect dialanine uptake in a similar manner.

None of the peptides was detected intact in the embryo after 8 h incubation in a 1 mM solution. However, tyrosine increased in the embryo after incubation with Gly-Tyr-NH_2, corresponding to a rate of uptake of 0.2 μm/g fresh wt/h). Traces of tyrosine and Gly-Tyr also appeared in the incubation medium. While the uptake of Gly-Tyr-NH_2 (as judged by the appearance of tyrosine in the embryo) was totally inhibited by 9 mM dialanine, the appearance of Gly-Tyr in the medium was unaffected. Thus, it seems that the observed uptake of Gly-Tyr-NH_2 is the result of peptide uptake after extracellular deamidation. Although this interpretation requires that the unsubstituted Gly-Tyr in the medium should actually increase in the presence of dialanine, such an increase, if it occurred, would not have been detectable.

3.2.1.2.2 N-Terminal Amino Group

Acetylated peptides. Table 3.11 shows the effect of three acetylated peptides, acetyl-Gly-Gly, acetyl-Gly-Leu and acetyl-Ala-Ala, on the uptake of dialanine. Although acetyl-Ala-Ala and acetyl-Gly-Leu inhibit dialanine uptake, the effect is very much less than that produced by the unsubstituted peptides (judging from previous results, free dialanine would be expected to compete at least as well as Gly-Leu). The lack of an inhibitory effect with acetyl-Gly-Gly is perhaps not surprising if the poor competitive ability of diglycine itself is considered (section 3.1.3.7).

Intact uptake of these peptides cannot be monitored as they fail to react with dansyl chloride. However, during 8 h incubation with 2 mM acetyl-Gly-Leu, a small increase in free leucine is detected in the embryo, showing it can be absorbed to some extent.

A further indication that acetylated peptides may be taken up was obtained by monitoring changes in the levels of their constituent amino acid in the medium, after acid hydrolysis. The exodus of amino acids from the embryo made accurate quantification difficult, and the particularly high levels of alanine precluded the use of this method for acetyl-Ala-Ala. However, the lower levels of glycine and leucine undergoing exodus allowed the uptake of acetyl-Gly-Leu and

Table 3.11: Inhibition of Dialanine Uptake by N-substituted Peptides

Competing Peptide	Inhibition of uptake (%)
Sar-Ala	ND
Sar-Ser	ND
Sar-Gly	ND
Sar-Gly-Gly	ND
Propyl-Gly-Gly	24
Isopropyl-Gly-Gly	ND
Isobutyl-Gly-Gly	8
Dimethyl-Gly-Gly	ND
Acetyl-Gly-Gly	ND
Gly-Leu	100
Acetyl-Gly-Leu	36
Acetyl-Ala-Ala	17
Butyl-Gly-Gly-Gly	ND
Isobutyl-Gly-Gly-Gly	ND

Uptake of dialanine from a 1mM solution was monitored over a 4.5h incubation period, using fluorescamine (except in the presence of Gly-Leu where dansyl chloride was employed). All results were confirmed using dansyl chloride. Incubations were carried out under standard conditions (section 2.3.1) with the competing peptides at 18mM. Each value is the average of two separate determinations. 100% uptake of dialanine was 2.9 µm/g fresh wt./h.

ND = no inhibition detectable.

acetyl-Gly-Gly to be compared with the corresponding unsubstituted peptides using this method; acetylation led to a considerable reduction in the rate of peptide uptake.

Alkylated peptides. Table 3.11 also shows the effects of several alkylated peptides on dialanine uptake. Although propyl-Gly-Gly and isobutyl-Gly-Gly appear to inhibit uptake to some degree, most N-alkylated peptides had no detectable effect.

The dansyl chloride method was not sensitive enough to detect the uptake of any alkylated peptide from the incubation medium (dimethyl-diglycine, which does not react with dansyl chloride, was monitored by changes in glycine levels after acid hydrolysis), although unsubstituted di- and triglycine are both absorbed at a rate of about 12 µm/g/8 h from solutions of an equivalent concentration (1 mM).

Examination of embryo extracts showed that N-methylated (sarcosyl) peptides are taken up intact, to a small extent (about 1 µm/g/fresh wt/8 h, from a 2 mM solution). Free sarcosine appears in the embryo, indicating that a proportion of the absorbed peptide is hydrolysed (table 4.1, section 4.3.1). Propyl-diglycine is also taken up intact (1.2 µm/g fresh wt/8 h, from a 2 mM solution), again undergoing partial hydrolysis (propyl-glycine appears in the embryo at

3 µm/g fresh wt/8 h). No other alkylated peptides were taken up intact, although during incubation with isobutyl-diglycine, isobutyl-glycine appears in embryo extracts (0.9 µm/g fresh wt/8 h, from a 2 mM solution).

3.2.1.3 Discussion

In considering these results, the possibility that certain of the substituted peptides may undergo extracellular modification (e.g. deacylation, deamidation), and that the active species is actually the unsubstituted peptide, should be borne in mind. However, this possibility seems unlikely as in no case (except Gly-Tyr-NH_2) was the unsubstituted peptide detected in the medium during incubation.

It seems that both the N- and the C-termini of a peptide are important in conferring affinity for the transport system, although neither group is essential.

Peptides with substituted C-termini (esters and amides) show reduced rates of uptake and no detectable competitive ability. This is not simply a steric affect, as peptides lacking the carboxyl group (GAB and β-alanine derivatives) are also very poor substrates. One anomaly is Val-β-Ala. This peptide is absorbed fairly rapidly and shows a corresponding competitive ability. In addition, it can be detected intact within the embryo, although a certain proportion of the absorbed peptide is hydrolysed. No

explanation for the unexpectedly high rate of transport of this peptide can be advanced at present.

Peptides with a modified α-amino group are also absorbed relatively slowly, compared with their unsubstituted counterparts. However, despite the restricted substrates available it does seem that substitution of the N-terminus has less of an effect on uptake than substitution of the C-terminus. Thus, for example, acetyl-Gly-Leu inhibits dialanine uptake quite strongly, while at similar concentrations Gly-Leu-amide has no detectable effect. Although most N-terminal substituted derivatives show no competitive ability this is probably a consequence of employing derivatives of di- and triglycine. However, it is noticeable that isobutyl- and propyl-diglycine are still able to compete with dialanine to a certain extent, as well as being absorbed by the embryos. Further evidence suggesting that the C-terminus is more important than the N-terminus is presented elsewhere (section 3.2.4; 3.2.5.3).

N-methylated (sarcosyl) peptides do not show any detectable competitive ability yet may be detected intact within the embryos; the presence of free sarcosine also shows they may be absorbed at quite a rapid rate. This apparent anomaly seems to be the result of the uptake of

N-methylated peptides by an alternative mode of entry, unavailable to other peptides (see chapter 4).

The requirement for the N- and C-terminal groups of a peptide is also a feature of peptide transport in all other organisms which have been studied. In S. cerevisiae, certain N-acetyl and N-benzyloxycarbonyl derivatives of both di- and tripeptides may be used as growth substrates, although many other growth-supporting peptides are not utilized after acetylation (Naider et al., 1974; Becker & Naider, 1977; Jones, 1977; Marder et al., 1977). However, substitution of the C-terminal group seems to have a less drastic effect on peptide utilization; the methyl esters of all peptides tested are able to serve as growth substrates, albeit after an increased lag phase (Naider et al., 1974; Becker & Naider, 1977). Although it must be emphasized that the growth tests employed with yeast are indirect and very insensitive, it nevertheless appears that in S. cerevisiae, the N-terminus is more important than the C-terminus in conferring affinity for the peptide transport system. This is in contrast with the situation in barley, where the C-terminus seems to be most important. However, in a second yeast, Candida albicans, the requirements seem to be similar to barley; peptide methyl esters cannot be used as growth substrates (Lichliter et al., 1976).

Both peptide termini seem to be important for peptide transport by the mammalian gut, although the requirements have not been studied systematically. Amidation or methylation of the C-terminal α-carboxyl group reduces affinity considerably (Addison et al., 1974, 1975b). Similarly, peptides with a substituted α-amino group are poor substrates for transport (Rubino et al., 1971; Burston et al., 1972; Addison et al., 1974, 1975b; Das & Radhakrishnan, 1974).

The situation in bacteria seems to be somewhat different, although it must be remembered that results based on growth tests do not distinguish between slow uptake and no uptake at all. In E.coli, N-substituted di- and oligopeptides may be utilized as long as the positive charge is retained. Thus, N-alkylated derivatives are utilized while N-acetylated or dialkylated derivatives are unable to serve as growth substrates (Payne, 1971a, 1974; Becker & Naider, 1974). A similar requirement for the N-terminus is shown by several other bacterial species (S. typhimurium: Jackson et al., 1976. Pseudomonas: Cascieri & Mallette, 1976; Miller & Becker, 1978. Streptococcus: Law, 1978).

The requirement for the C-terminal carboxyl group differs for the di- and oligopeptide systems. Dipeptides apparently require an unsubstituted carboxyl group in order

to enter through the dipeptide permease, although dipeptides lacking a carboxyl group can still enter via the oligopeptide permease (Payne & Gilvarg, 1968a; Fickel & Gilvarg, 1973; Payne, 1973; Hirshfield & Price, 1975). Oligopeptides, on the other hand, remain good growth substrates, even if the carboxyl group is removed or substituted (Payne & Gilvarg, 1968a; Fickel & Gilvarg, 1973; Payne, 1973; Allen et al., 1978). However, recent results using fluorescence procedures show that, despite their seemingly normal nutritional availability, C-substituted oligopeptides are actually transported at decreased rates (J.W. Payne, unpublished results). Hence the C-terminus is important, even in this system.

It is interesting to consider how a transport system which will accommodate both di- and oligopeptides can have requirements for both the C-terminal carboxyl and the N-terminal amino group; binding sites aligned for the two charged groups of a dipeptide cannot also be aligned for the same group in a tripeptide, and vice versa. A further relevant feature is the degree of protonation of the terminal groups. This will vary according to their pK_a/pK_b and the pH of the medium. If the transport system only handles, for example, peptides with a protonated amino group (as it seems to in E.coli; Payne, 1971a),

certain structural modifications to a peptide may affect transport, simply by altering the pK_b of the peptide.

Protonation of the α-amino group seems unlikely to be important in barley; at pH 3.8 only a negligible proportion of the peptide will be in the unprotonated form. However, at this pH, the degree of protonation of the carboxyl group would seem to be more relevant. Amino acid transport by Ehrlich's cells is terminated by protonation of the carboxyl group (Garcia-Sancho et al., 1977b).

3.2.2 Transport of Proline-Containing Peptides

Proline is of particular importance in barley, in that it is one of the most abundant amino acids of the endosperm storage protein. It has also been suggested that, as a result of the apparent inability of barley proteases and peptidases to cleave bonds involving proline (section 5.4.4.2), a large proportion of peptides produced in the endosperm will have terminal proline residues, although work presented below (chapter 5) shows this view to be unfounded.

Proline is also unique amongst the protein amino acids in that it has an imino group rather than an α-amino group. In view of the importance of the N-terminal group for peptide transport (section 3.2.1), it is of interest to ascertain whether peptides with an N-terminal proline

residue are handled by the general peptide transport system. In addition, proteases and peptidases of broad specificity are normally unable to cleave bonds involving proline (including barley peptidases; section 5.4.4.2); specific enzymes are generally required. It therefore seems possible that a specific peptide transport system(s) might exist to handle peptides with terminal proline residues.

3.2.2.1 Results

Figure 3.18 shows the uptake of a number of peptides containing proline residues at either the C- or N-terminus. During incubation, only small amounts of proline appeared in the medium, indicating extracellular hydrolysis is unimportant (see section 3.1.3.5). Clearly these peptides are absorbed by barley embryos at rates comparable with most other physiological peptides.

For the following representative peptides, no intact uptake could be detected in the embryo after 8 h incubation in a 1 mM solution, although large increases in free proline were apparent; Ala-Pro, Gly-Pro, Pro-Gly-Gly and Gly-Gly-Pro.

In the presence of 9 mM Gly-Ile, no uptake of the following peptides was detectable over an 8 h period, from a 1 mM solution; Pro-Gly-Gly, Ala-Pro, Pro-Tyr and Gly-Pro. Pro-Gly was taken up slowly (2.5 µm/g/8 h) compared with

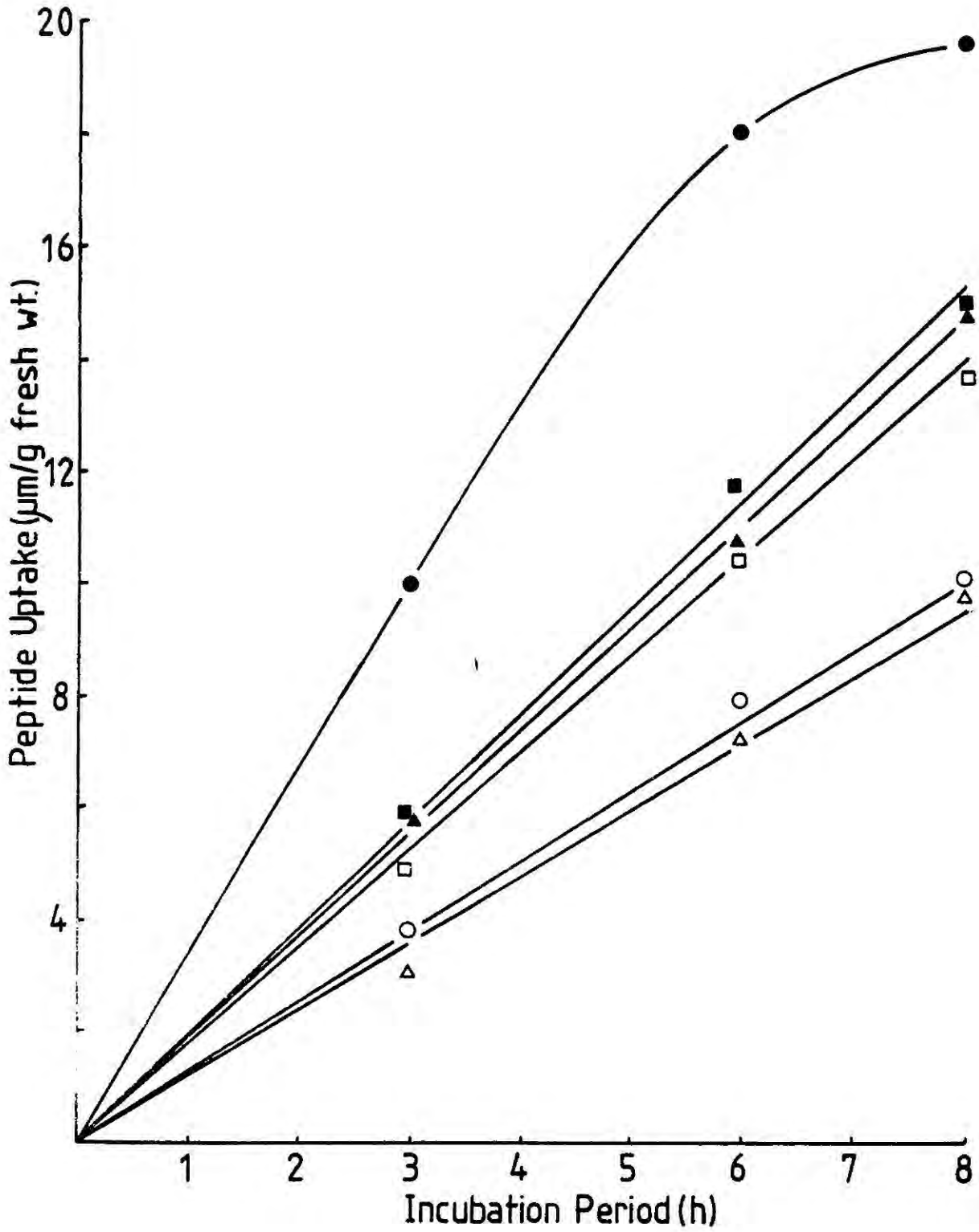


Figure 3.18: Uptake of Proline-containing Peptides

Uptake of peptides from a 1mM solution was monitored by following their disappearance from the medium, using dansyl chloride: Ala-Pro (●), Gly-Pro (■), Phe-Pro (▲), Gly-Gly-Pro (●), Pro-Val (□), Pro-Gly (○), Pro-Tyr (△), Pro-Gly-Gly (○). Standard incubation conditions were employed (section 2.3.1). Each value is an average of at least two separate determinations.

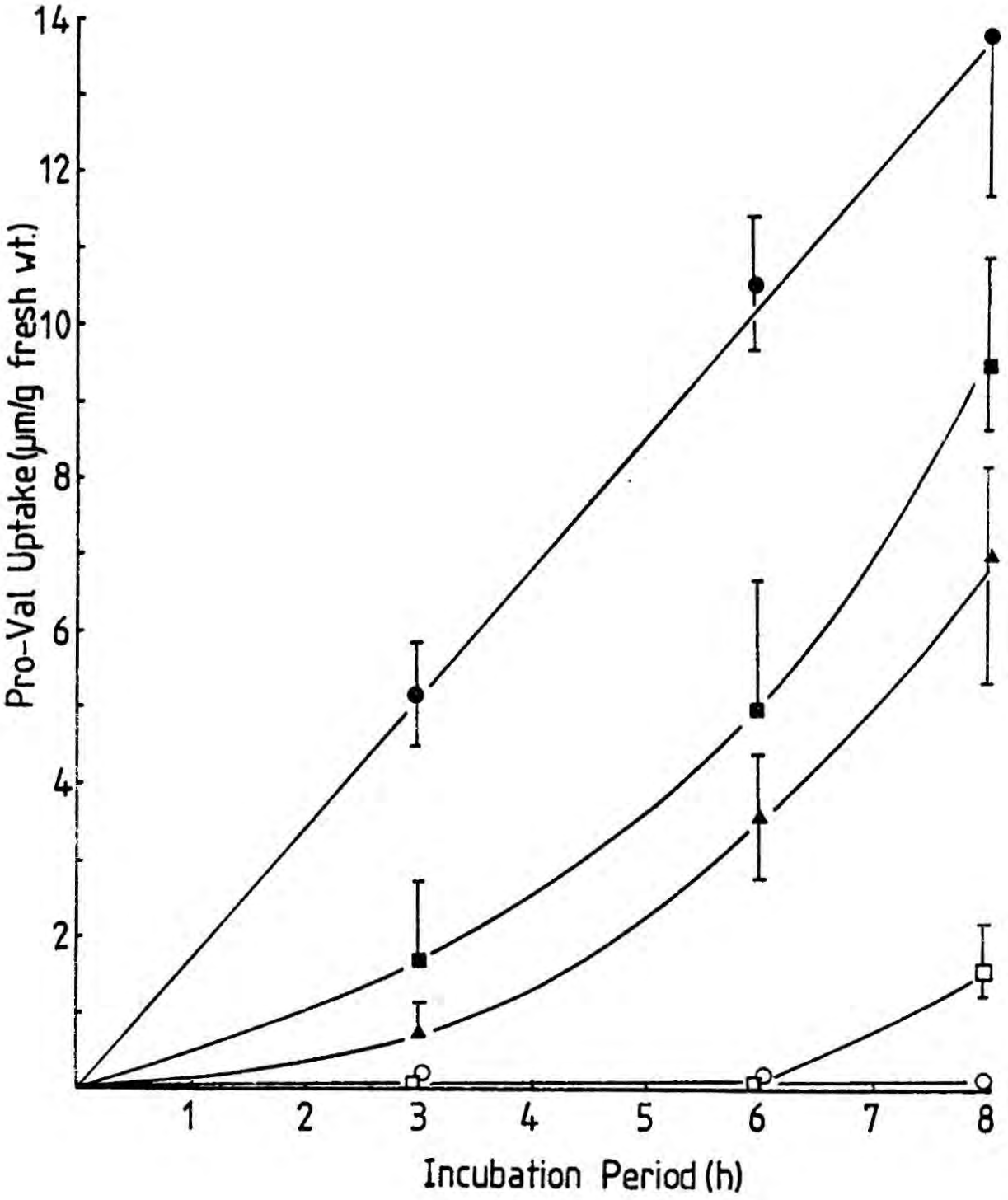


Figure 3.19: Inhibition of Pro-Val Uptake by Dialanine

Uptake of Pro-Val from a 1mM solution, alone (●), and in the presence of 1mM (■), 2mM (▲), 5mM (□), or 9mM (○) dialanine, was monitored using dansyl chloride. Standard incubation procedures were employed (section 2.3.1). Each value is an average of three separate determinations. Bars represent the range of values obtained.

its rate of uptake in the absence of Gly-Ile. Figure 3.19 shows the effects of varying concentrations of dialanine on the uptake of Pro-Val. Again considerable inhibition is apparent. The increase in the rate of uptake towards the end of incubation reflects the decrease in the concentration of competing dialanine, following its uptake by the embryo.

3.2.2.2 Discussion

Despite the apparent requirement for a primary α-amino group for peptide transport (section 3.2.1), peptides with an N-terminal proline residue (a secondary amino group) are transported by barley embryos at rates comparable with most other peptides. Competition for transport shows that uptake is mediated by the same transport system. Indeed, the ability of Gly-Ile to inhibit totally the uptake of several di- and tripeptides containing proline indicates that, if a specific proline-peptide transport system is present, it is of little importance under the conditions employed. As the substrate concentrations used here are similar to the physiological concentrations in the barley endosperm (section 5.4.2), it seems unlikely that a specific transport system for proline-peptides will exist.

It seems therefore, that the peptide transport system is able to handle prolyl-peptides, while discriminating

against most other secondary amines. A similar situation holds for dipeptides with a C-terminal proline residue. While an unsubstituted peptide bond is normally a requirement for peptide transport (see below, section 3.2.4), dipeptides with a C-terminal proline residue are still excellent substrates. It is interesting to consider how a binding site can make such distinctions.

The ability to handle proline-containing peptides further emphasises the similarity between the barley peptide transport system and analogous systems in other organisms. In the mammalian gut, what little evidence is available indicates that proline-containing peptides may be handled by the same system as other peptides (Das & Radhakrishnan, 1974; Addison et al., 1975c). Similarly, in E.coli (Payne, 1971b) and S. typhimurium (Jackson et al., 1976; Yang et al., 1977) it is clear that both the di- and oligopeptide systems are capable of handling proline-containing peptides.

The ability of barley embryos to hydrolyse prolyl-peptides as fast as they are absorbed, such that intact peptide never accumulates in the embryo, is also worthy of note. Despite suggestions to the contrary (section 5.4.4.2), barley does contain peptidases capable of cleaving peptide bonds involving proline.

3.2.3 Specificity Towards Amino Acid Side Chains

It seems unlikely that the amino acid side chains of a peptide will be important in conferring specificity for transport (section 1.1.3). It has already been observed that a range of di- and tripeptides containing aliphatic side chains (alanine, glycine, isoleucine) all compete for the same transport system (section 3.1.3.7). These observations are extended here to cover peptides of widely differing structures.

3.2.3.1 Results

At pH 3.8, most peptides will have no overall charge. However, peptides containing aspartic acid or glutamic acid will tend to bear a negative charge and peptides containing lysine, histidine or arginine residues will be positive. Table 3.12 shows that a number of these 'charged' peptides are absorbed by barley embryos at similar rates to all other peptides. Furthermore, trialanine inhibits the uptake of a number of charged peptides (table 3.13). Analysis of the medium, using dansyl chloride, endorsed these conclusions and showed that the disappearance of peptide measured with fluorescamine did not simply arise from extensive extracellular hydrolysis.

Table 3.12: Uptake of Peptides Bearing a Positive or Negative Charge

Peptide	Rate of uptake (μm/g fresh wt./h)
Glu-Ala	2.8
Phe-Glu	3.7
Glu-Lys	3.0
Gly-Asp	3.3
Glu-Glu	3.2
His-Ala	3.5
Arg-Arg	1.0
Lys-Lys	2.3
Ala-His	2.7
Lys-Ala	3.0

Uptake of peptide from a 2mM solution was monitored using fluorescamine. Incubations were carried out under standard conditions (section 2.3.1). Each value is the average of three separate determinations.

Table 3.13: Inhibition of the Uptake of 'Charged' Peptides by Trialanine

Peptide	Trialanine	Rate of uptake (µm/g fresh wt./h)
Glu-Glu	-	2.6
Glu-Glu	+	ND
Glu-Ala	-	2.3
Glu-Ala	+	0.4
Ala-His	-	2.2
Ala-His	+	0.3
Glu-Lys	-	2.2
Glu-Lys	+	ND
Lys-Lys	-	1.7
Lys-Lys	+	ND
His-Ala	-	2.5
His-Ala	+	ND
Lys-Ala	-	2.3
Lys-Ala	+	0.3

Uptake of peptides from a 1mM solution, in the presence (+) or absence (-) of 9mM trialanine, was monitored using dansyl chloride. Incubations were carried out under standard conditions (section 2.3.1). Each value is the average of two separate determinations.

ND = not detectable

3.2.3.2 Discussion

The amino acid side chains of peptides seem to have little influence on their ability to be transported by barley embryos. In addition to peptides containing aliphatic and proline residues (sections 3.1.3.7; 3.2.2; 3.2.5), peptides containing aromatic (phenylalanine; section 3.1.3.7), imidazole (histidine), basic (arginine, histidine, lysine) and acidic residues (glutamic and aspartic acids) are all absorbed at broadly similar rates. The ability of trialanine to inhibit the uptake of representatives of all types of peptide, indicates that they all enter via a common system.

Thus, it appears that the barley peptide transport system has little or no specificity for the amino acid side chains of a peptide. No evidence has been obtained to suggest that additional system(s) may exist with restricted specificity. This is again similar to the situation in bacteria, yeasts and the mammalian intestine (section 1.1.4.2).

Despite the ability to handle most, if not all, physiological peptides, differences in their rate of uptake and/or competitive ability are apparent. It will not be possible to rationalize these differences until detailed kinetic constants have been obtained. However, glycine homopeptides seem to have a particularly poor affinity for transport in

barley (section 3.1.3.7; 3.1.4.5). Indeed, this seems to be a general observation for other transport systems; glycine homopeptides are poor substrates in E.coli (Payne & Bell, 1979), S. typhimurium (Yang et al., 1977), and possibly the mammalian gut (see discussion to Payne, 1977). The reason for this is not clear, but it does serve as a warning against basing too general a conclusion on results obtained using glycine peptides to study peptide transport.

3.2.4 Requirement for an α-Peptide Bond

If a transport system is to differentiate between peptides and amino acids, one of the most obvious structural differences on which discrimination might be based is the peptide bond.

Substitution of the peptide bond, as in Gly-Sar and Gly-Sar-Sar, does reduce the rate of uptake by barley embryos, although a considerable amount of absorption still takes place. Thus, from a 2 mM solution, Gly-Sar and Gly-Sar-Sar are absorbed at rates of 7 μm/g/6 h and 3 μm/g/6 h respectively, compared with rates of about 15 μm/g/6 h for both di- and triglycine (sections 3.1.3.2; 3.1.3.4). This is similar to bacteria, yeast and the mammalian gut, in which methylation of the peptide bond leads to reduced rates of uptake and resistance to peptidase activity (section 1.1.1).

Dipeptide transport in E.coli is apparently specific for an α-peptide bond; several peptides containing β-, γ- and ε-linkages show no affinity for the transport system (Payne, 1972c). Similar observations have been made in Pseudomonas (Cascieri & Mallette, 1976); S. typhimurium (Yang et al., 1977) and Streptococci (Law, 1978), albeit with a limited number of peptides. In the mammalian gut however, peptides containing β-linkages may be absorbed, though rather slowly. Such peptides are normally resistant to hydrolysis (Matthews, 1975). Similarly, peptide transport in barley does not seem to have an absolute requirement for the α-peptide bond. Table 3.14 shows the rate of uptake of a number of peptides containing unusual peptide linkages. Although the rate of uptake is rather lower than that achieved by most α-linked peptides (17-23 μm/g/6 h), it is clear that there is still considerable uptake of some of these peptides. Despite the limited number of such peptides investigated, it is interesting to note that the only peptide which shows no detectable uptake is Ala-ε-Lys. In this peptide the backbone is elongated between the peptide bond and the C-terminus. The other three peptides, on the other hand, are altered in the spatial relationship between the bond and the N-terminal amino group. This accords with data presented elsewhere which indicates that the C-terminus is more important in determining transport capabilities than the N-terminus (sections 3.2.1.3;

Table 3.14: Uptake of Peptides with Unusual Peptide Linkages

Peptide	Rate of uptake[a] (µm/g fresh wt./6h)	Intact peptide[b] in the embryo after 6h incubation (µm/g fresh wt.)
β-Asp-Ala	8	8
β-Ala-Ala	12	12
Ala-ε-Lys	ND	ND
γ-Glu-Ala	14	ND

(a) Uptake of peptides from a 2mM solution was monitored using fluorescamine. (b) Intact peptide in the embryo was detected with dansyl chloride. Standard incubation conditions were employed (section 2.3.1). Each value is the average of two separate determinations.

ND = not detectable

3.2.5.3).

It is also of interest to note that peptides with a β- or γ- peptide linkage have an increased molecular distance between the terminal amino and carboxyl groups, yet they are still absorbed quite rapidly. Thus, an exact spatial relationship between the termini seems unimportant, as might be anticipated from the ability of the peptide transport system to handle both di- and oligo-peptides.

Both β-Ala-Ala and β-Asp-Ala are absorbed intact against a concentration gradient. No evidence for significant hydrolysis was obtained. However, although γ-Glu-Ala is rapidly absorbed from the incubation medium, it does not remain intact within the embryo. Similarly, in E.coli, γ-glutamyl peptides are absorbed and hydrolysed, although apparently independently of the peptide permeases (Payne, 1972c). It is possible that these peptides are being transported as a consequence of the vectorial operation of the γ-glutamyl cycle (section 1.2.2.1). The rapid hydrolysis of γ-Glu-Ala at least shows that barley contains a γ-glutamyl peptidase. This possibility certainly merits further investigation.

Finally, when considering the peptide bond, the chemical configuration may be relevant. While most peptides exist

predominantly in the trans form (Marsh & Donohue, 1967), peptide bonds in which the amino group is provided by proline or sarcosine will consist of a mixture of both isomers; up to 50% may be in the cis form (Evans & Rabenstein, 1974). In view of recent evidence showing that certain peptidases may be specific for the trans form of the peptide bond (Lin & Brandts, 1979), the possibility that the rate of cis-trans transition may limit transport is worthy of consideration.

3.2.5 Stereochemical Requirements for Transport

The virtual absence of D-amino acids in protein-derived peptides makes it highly improbable that a specific system for D-peptides will exist, at least in the barley scutellum. In addition, the considerable effect which a D-amino acid will have on the overall 'shape' of a peptide might be expected to exclude such peptides from the general peptide transport system. This feature has been investigated in some detail in barley embryos. In addition, studies using D-peptides, the results of which are presented below, have provided substantial evidence that all peptides are absorbed intact by an active, mediated process (section 3.1.4.1).

3.2.5.1 Methods

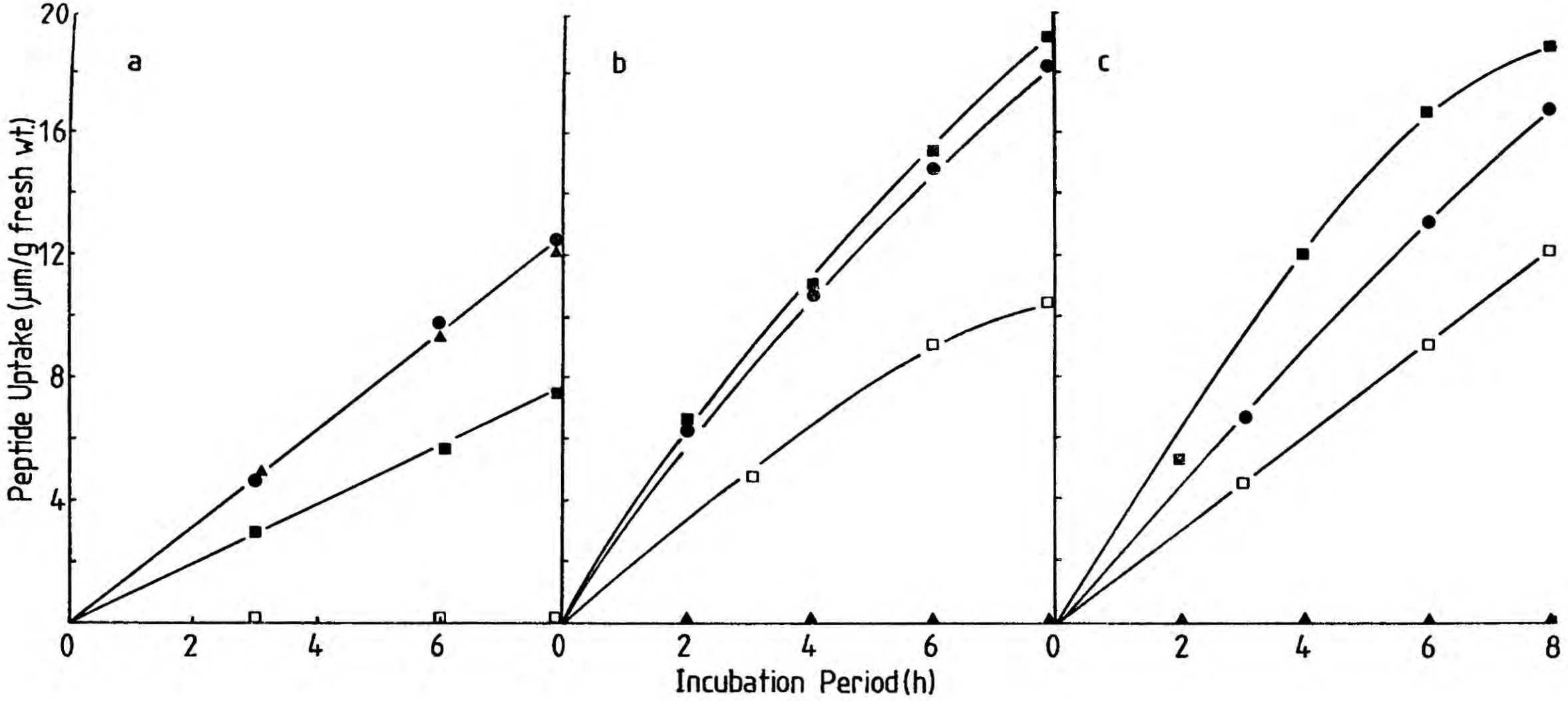
Standard dansyl chloride procedures were employed to

monitor peptide disappearance from the incubation medium and to analyse embryo extracts.

3.2.5.2 Results

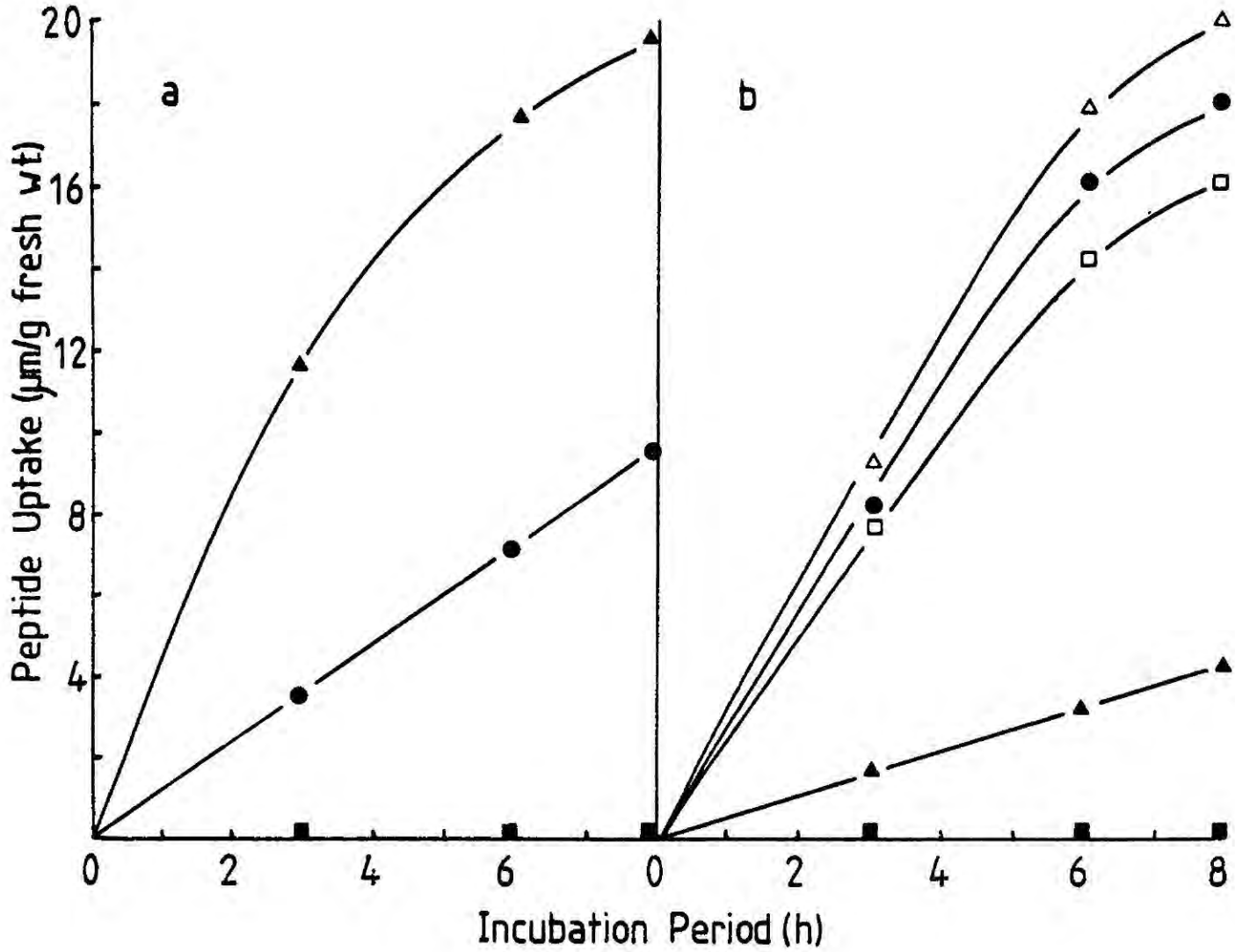
The rates of uptake of a variety of leucine-containing di- and tripeptides are illustrated in figures 3.20 a-c. It is clear that the presence of a D-amino acid in a peptide can have a pronounced effect on its rate of uptake. A similar effect is observed for analogous series of peptides containing valine or alanine (figs. 3.21 & 3.22). The influence of a D-amino acid is illustrated in figure 3.23. While Gly-Gly-L-Leu disappears from the incubation medium over an 8 h incubation period (3.23 a-d), no change in the level of Gly-Gly-D-Leu is detectable over a corresponding period (3.23 e-h)

Uptake of the following peptides could not be detected by monitoring their disappearance from the incubation medium (1 mM peptide, for 8 h) and neither was there any increase in the levels of their constituent amino acids in the embryo: Gly-D-Ala, Gly-D-Leu, L-Ala-D-Ala, D-Ala-D-Ala, D-Leu-D-Leu, Gly-Gly-D-Leu L-Ala-L-Ala-D-Ala and D-Ala-D-Ala-D-Ala. However, an increase in free valine was detected in the embryo on incubation with L-Val-L-Val-D-Val, corresponding to a rate of uptake of 0.2 µm of the tripeptide/g fresh wt/h .



Figures 3.20a-c: Uptake of Peptides Containing L- and D-Leucine

Uptake was followed by monitoring peptide disappearance from the incubation medium, using dansyl chloride. Standard incubation conditions were employed (section 2.3.1). Each point represents the average of at least four independent experiments. Uptake from a 1mM solution of each of the following peptides was studied: (a) L-Leu-L-Leu (●), L-Leu-D-Leu (■), D-Leu-L-Leu (▲), D-Leu-D-Leu (□); (b) Gly-L-Leu (●), Gly-D-Leu (▲), L-Leu-Gly (■), D-Leu-Gly (□); (c) Gly-Gly-L-Leu (●), Gly-Gly-D-Leu (▲), L-Leu-Gly-Gly (■), or D-Leu-Gly-Gly (□).



Figures 3.21a & b: Uptake of Stereoisomers of Peptides containing Alanine and Valine

Uptake was followed by monitoring peptide disappearance from the medium using dansyl chloride. Each value is the average of at least four independent experiments. Uptake from a 1mM solution of the following peptides was studied:

(a) Stereoisomers of dialanine; LL (▲), LD (■), DL (●) and DD (■).

(b) Tripeptides of alanine and valine; L-Val-L-Val-L-Val (□), L-Val-L-Val-D-Val (■), L-Ala-L-Ala-L-Ala (△), L-Ala-D-Ala-L-Ala (■), D-Ala-D-Ala-D-Ala (■), L-Ala-L-Ala-D-Ala (■), L-Ala-Gly-Gly (●) or D-Ala-Gly-Gly (▲).

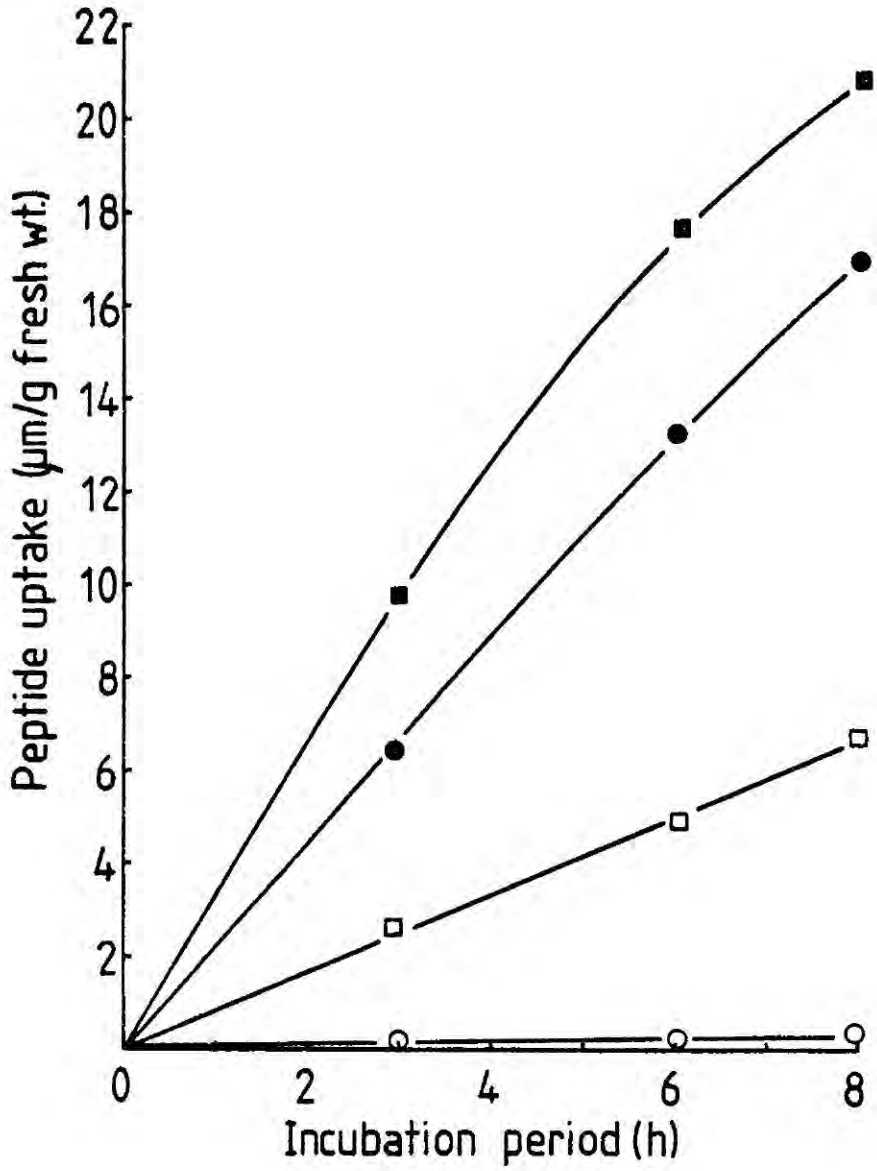


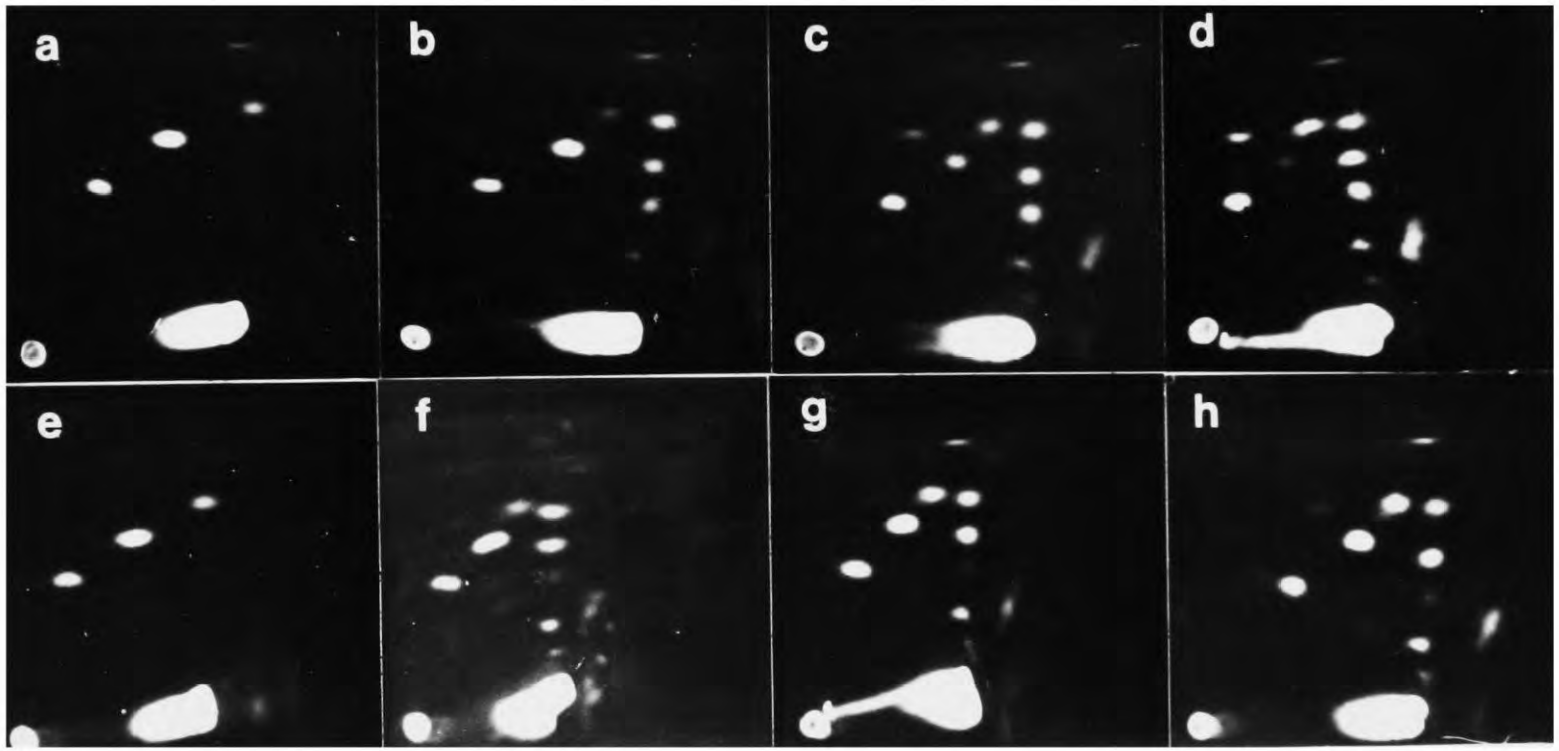
Figure 3.22: Uptake of the Stereoisomers of Dipeptides Containing Alanine and Glycine

Incubations were carried out under standard conditions (section 2.3.1) using 1mM peptide: Gly-L-Ala (●), Gly-D-Ala (o), L-Ala-Gly (■), D-Ala-Gly (□). Uptake from the medium was monitored using dansyl chloride. Values are the average of at least two separate determinations.

Figures 3.23a-i: Uptake of Gly-Gly-L-Leu and Gly-Gly-D-Leu by Barley Embryos

Developed chromatograms of dansylated samples of the incubation medium photographed under UV light. Incubations were carried out under standard conditions (section 2.3.1) in 1mM Gly-Gly-L-Leu (a-d) or Gly-Gly-D-Leu (e-h). Samples of the medium were removed and dansylated after 0h (a and e), 3h (b and f), 6h (c and g) and 8h (d and h).

(i) Diagram showing the identities of some of the dansylated derivatives: a) origin, b) dansyl-hydroxide, c) ornithine (internal standard), d) leucine, e) Gly-Gly-D-Leu or Gly-Gly-L-Leu, f) proline/valine/GAB, g) dansyl-ammonia, h) alanine, i) glycine, j) glutamic acid, k) aspartic acid.


a
b
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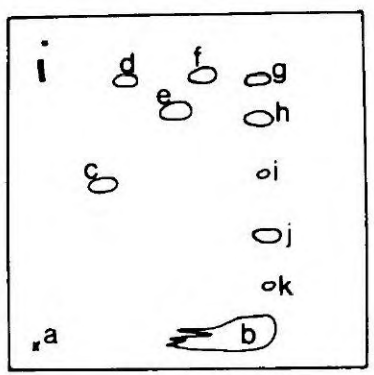

i
d
f
g
e
h
c
i
j
k
a
b

Intact Peptide Uptake. Of all the peptides examined here, only L-Leu-D-Leu could be detected intact in embryo extracts, reaching a concentration of 2.85 µm/g fresh wt. after 8 h incubation. Although disappearance of the peptide from the medium indicated a rate of uptake of 8 µm/g fresh wt/8 h, this apparent discrepancy is accounted for by the increase in free leucine levels in the embryo, indicating partial hydrolysis of the peptide.

During incubation with tripeptides containing D-residues, no intact dipeptides were detected in the incubation medium or in the embryo, which might have arisen as a result of partial hydrolysis of the tripeptide (any more than 4% hydrolysis of the original peptide would have been detected).

Competition for Transport. Dialanine uptake was reduced by a nine-fold excess of a number of di- and tri-peptides containing both L- and D-amino acids (figure 3.24). Certain peptides failed to exert any competitive effect at a 9:1 molar excess and were therefore studied at higher competitor:substrate ratios (figure 3.25). Similarly, a number of leucine- and alanine- containing peptides were shown to inhibit Gly-Ile uptake (fig. 3.26 a & b). The intact uptake of Gly-Sar was also inhibited by several peptides containing both L- and D-amino acid residues (table 3.15).

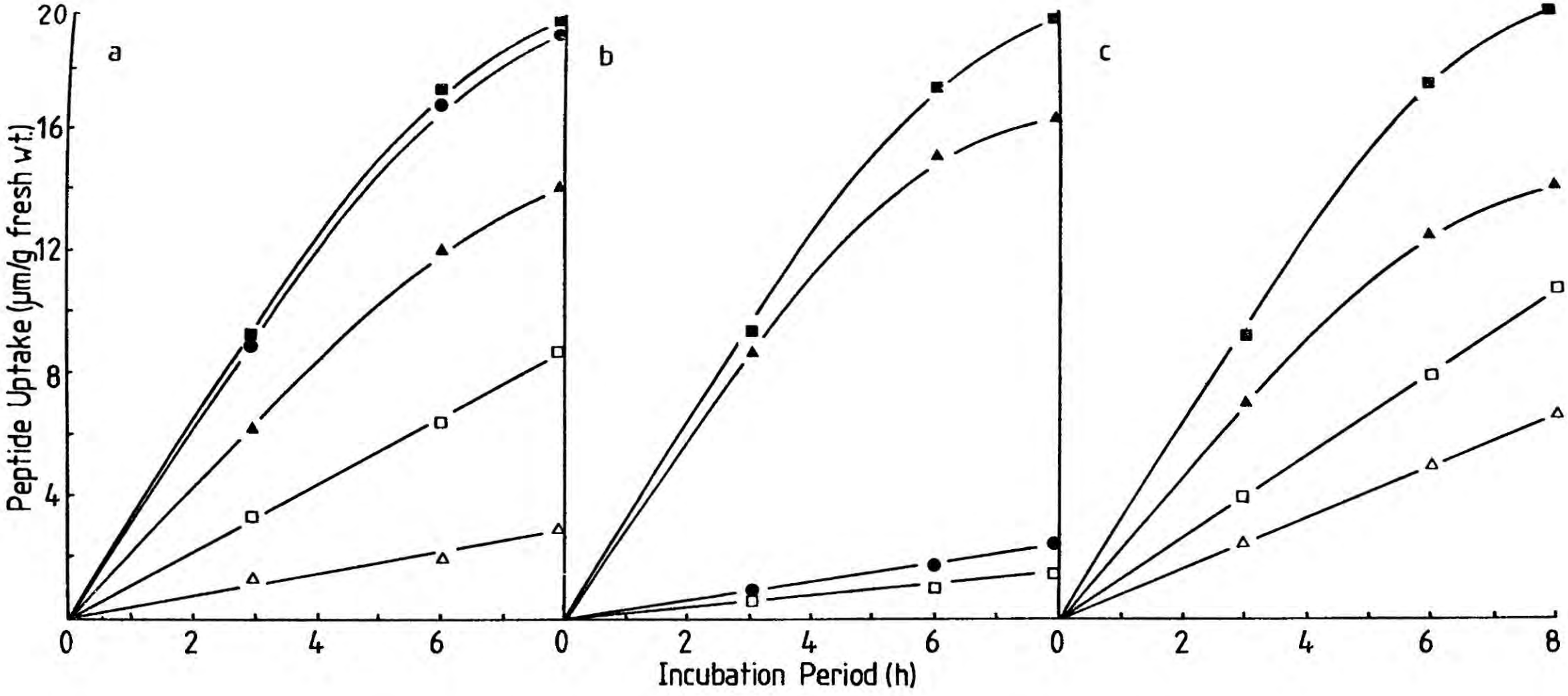


Figure 3.24a-c: Inhibition of Dialanine Uptake by the Stereoisomers of various Di- and Tripeptides

Embryos were incubated under standard conditions (section 2.3.1) with 1mM dialanine and the appropriate competing peptide at 9mM. Dialanine uptake was followed by monitoring its disappearance from the medium. Each value is the average of at least four separate determinations. Each figure shows the uptake of dialanine in the absence of any competitor (■), and in the presence of:

(a) L-Leu-Leu (△), L-Leu-D-Leu (▲), D-Leu-L-Leu (□) or D-Leu-D-Leu (●);
(b) Gly-L-Leu (●), Gly-D-Leu (■), L-Leu-Gly (□) or D-Leu-Gly (▲);
(c) Gly-Gly-L-Leu (□), Gly-Gly-D-Leu (■), L-Leu-Gly-Gly (△) or D-Leu-Gly-Gly (▲)

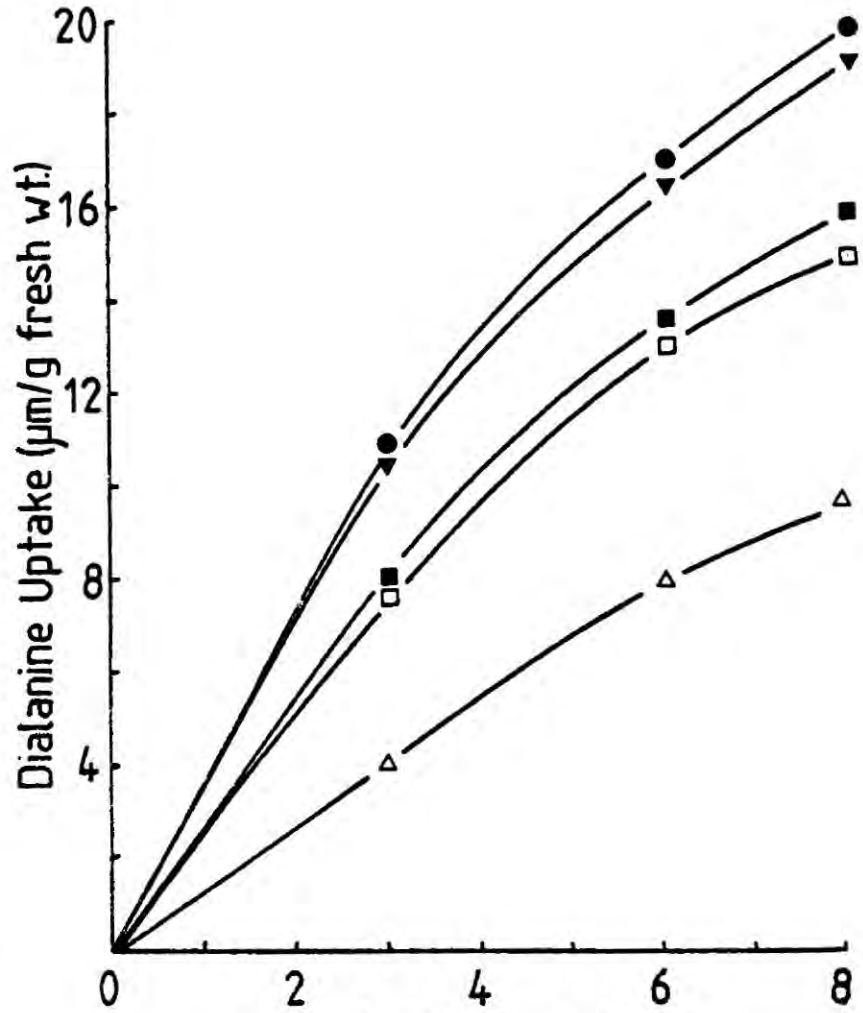
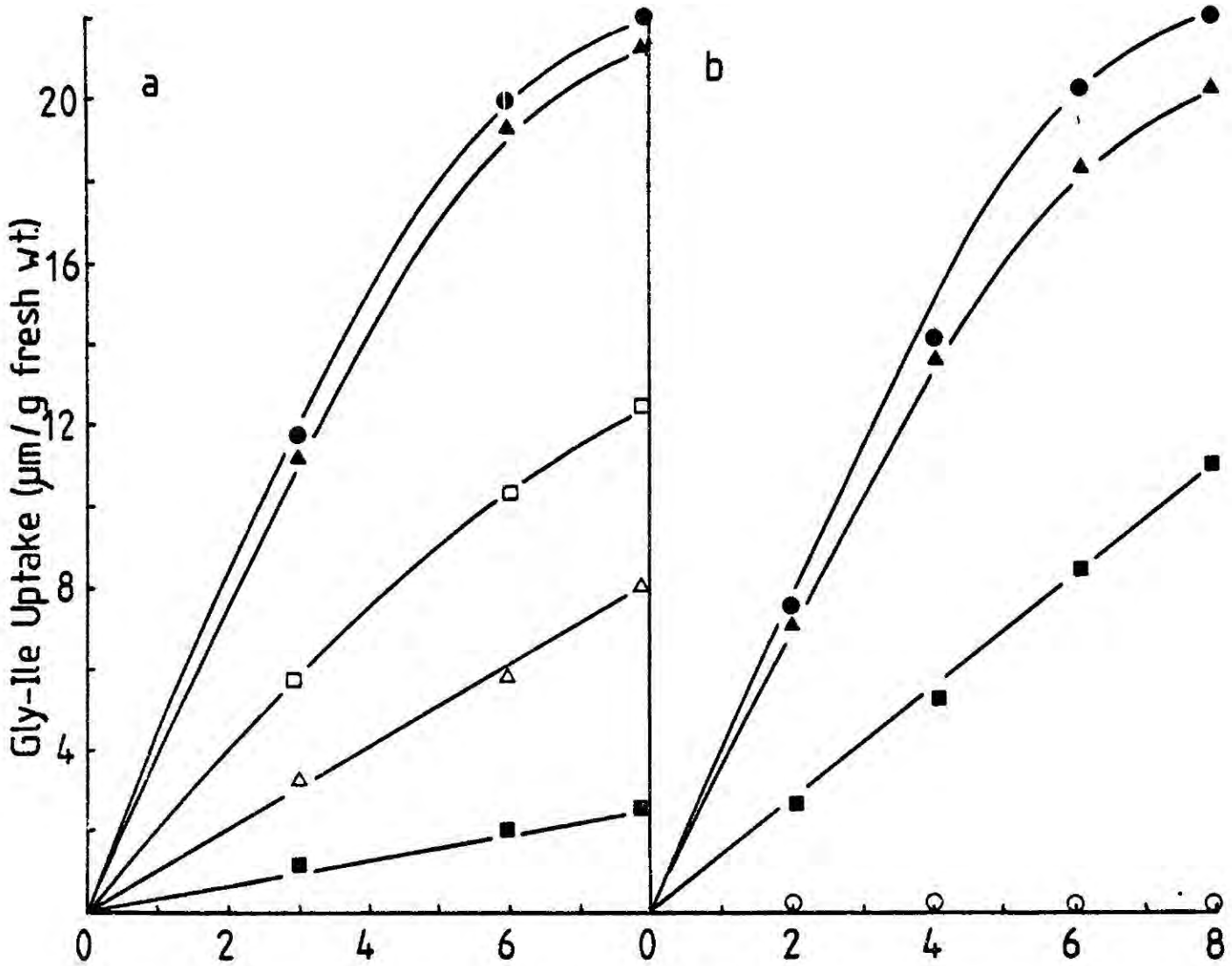


Figure 3.25: Inhibition of Dialanine Uptake by High Concentrations of Competing Di- and Tripeptides

Uptake of dialanine from a 1mM solution was followed by monitoring its disappearance from the medium, using dansyl chloride. Incubations were carried out under standard conditions (section 2.3.1) in the absence of any competitor (●), and in the presence of 45mM Gly-D-Leu (▼), 45mM Gly-Gly-D-Leu (■), 22.5mM L-Leu-D-Leu (△) or 22.5mM D-Leu-D-Leu (□). Each value is the average of at least two separate determinations.



Figures 3.26a & b: Inhibition of Gly-Ile Uptake by Stereoisomers of various Peptides

Uptake of Gly-Ile from a 1mM solution was monitored using dansyl chloride. Incubations were carried out under standard conditions (section 2.3.1). All competitors were at 9mM. Each value is the average of at least two separate determinations. Uptake was studied in the absence of a competitor (●) and in the presence of:

(a) Gly-Gly-D-Leu (▲), Gly-Gly-L-Leu (△), D-Leu-Gly-Gly (□) or L-Leu-Gly-Gly (■).

(b) D-Ala-D-Ala-D-Ala (●), L-Ala-L-Ala-L-Ala (○), Gly-D-Ala (●), Gly-L-Ala (■), D-Ala-Gly (▲) or L-Ala-Gly (■).

Table 3.15: Inhibition of Gly-Sar Uptake by various Peptides Containing L- and D-residues

Competing Peptide	Inhibition of Gly-Sar Uptake (%)
L-Leu-L-Leu	94
D-Leu-L-Leu	93
L-Leu-D-Leu	84
Gly-L-Asn	77
Gly-Gly-L-Leu	74
D-Leu-D-Leu	35
Gly-Gly-D-Leu	2

Incubations were carried out under standard conditions (section 2.3.1) for 6h in 2mM Gly-Sar, with the competing peptide at 8mM. Gly-Sar uptake was determined by its intact accumulation in the embryo. 100% uptake of Gly-Sar was 6.6 μm/g fresh wt./6h. Each value is the average of at least two separate determinations.

3.2.5.3 Discussion

It is apparent from the above results that peptides containing a D-amino acid are handled very poorly by the barley peptide transport system, compared with peptides composed solely of L-amino acid residues. A similar conclusion has been arrived at for peptide transport in various bacteria (E.coli: Levine & Simmonds, 1962; Becker & Naider, 1974; Allen et al., 1978; Payne, unpublished results. S. typhimurium: Yang et al., 1977. Pseudomonads: Shankman et al., 1962; Cascieri & Mallette, 1976. Streptococci: Kihara et al., 1961; Law, 1978. Lactobacilli: Yoder et al., 1965), yeasts (Lichliter et al., 1976; Becker & Naider, 1977), and the mammalian gut (Asatoor et al., 1973; Matthews, 1975). Many early reports for both bacteria and mammalian tissues indicated that the requirement for L-isomers was absolute, although the advent of more sensitive fluorescence techniques has led to the realization that certain peptides containing D-amino acids can enter various cells (J.W. Payne, unpublished results). Similarly, application of the sensitive dansyl-detection technique shows that in barley few, if any, peptides are actually excluded.

Good agreement is found between the rate of peptide uptake by barley embryos and their relative abilities to

compete for transport. As competitive ability is normally considered as reflecting affinity for a transport site, it appears that the reduced rates of uptake of D-peptides are a result of their decreased affinity for a transport site, rather than a lower rate of translocation across the membrane. Furthermore, this agreement implies that there is not only competition for binding but competition for transport itself, making it likely that any peptide containing a D-amino acid which can bind at the transport site will also undergo transport. A similar argument also applies to other studies on the structural requirements for peptide transport.

The position of a D-amino acid in the peptide chain is important in determining the efficiency with which it is taken up by barley; this presumably reflects the substrate binding specificity of the transport site. Thus, for both di- and tripeptides, substitution of a D- for an L-residue at the C-terminus reduces peptide transport to a greater extent than a similar change at the N-terminus. The presence of more than one D-amino acid residue reduces uptake still further. This feature is well illustrated by the dileucine series: LL and DL have comparable rates of uptake (although the latter shows a poorer competitive ability), uptake of LD is about 50% lower and uptake of DD

is indetectable. Similar results are obtained for analogous series of di- and tripeptides containing glycine, leucine, alanine and valine.

The effect of positional substitution of a D-residue has only rarely been studied systematically in other organisms. Similar positional effects to those described here for barley have been reported for dialanine accumulation by Streptococcus faecalis (Kihara et al., 1961) and dialanine uptake by rat jejunum (Asatoor et al., 1973). For tripeptides, the positional effect of a D-amino acid is variable, depending upon the organism involved. In the bacterium, E.coli (Becker & Naider, 1974), and the yeast, S. cerevisiae (Becker & Naider, 1977), the LLD form of a tripeptide is apparently taken up more readily than corresponding isomers with the D-residue nearer the N-terminus. In contrast, Candida albicans exhibits similar stereospecific requirements to barley; DLL-trimethionine may be utilized, while LLD is excluded (Lichliter et al., 1976). These differing tripeptide specificities may be explained on the basis of other structural requirements for transport (Payne, 1972c). Thus, the LLL and LLD isomers of a tripeptide may be superimposed, except for the terminal carboxyl group which is not essential for transport in either E.coli (Payne & Gilvarg, 1968a) or S. cerevisiae (Marder et al., 1977). Similarly, comparison of the LLL and DLL isomers

shows that here the α-amino group is out of alignment. This group is essential for peptide transport in E.coli (Payne, 1971a) and also seems to assume some importance in S. cerevisiae (Marder et al., 1977; Becker & Naider, 1979). Thus, the DLL isomer might be expected to show a lower affinity for the peptide binding site. In Candida, which apparently exhibits opposite requirements in relation to positional stereospecificity, an analogous explanation may be invoked; the structural requirements for peptide transport by this yeast are correspondingly different, with the N-terminal α-amino group being unimportant and the C-terminal carboxyl group being essential. Thus, by analogy, it might be expected that in barley the terminal carboxyl group of a peptide will be more important than the terminal amino group in determining transport capabilities. This prediction is at least partly borne out experimentally (sections 3.2.1; 3.2.4).

Assuming that inhibition of transport reflects competition for a transport site (section 3.1.4.4), the present results provide further support for the view that only a single transport system operates in barley, capable of handling both di- and tripeptides. In addition, it is clear that those peptides containing D-amino acids which are absorbed by barley embryos enter through the general

peptide transport system and not a system specific for D-peptides.

3.3 Energy Supply and Membrane Permeability

Barley embryos contain a considerable pool of free amino acids. During incubation, a certain proportion of this pool leaks out into the external medium (section 3.1.3.5). Both the composition of the pool, and the proportion which undergoes exodus, are dependent upon the conditions of germination and incubation. This seems to be the result of changes in membrane permeability which may have an important bearing on the mechanism by which peptide transport is energized.

3.3.1 Methods

Dansyl chloride was used to assay the free amino acids in the incubation medium and in barley embryo extracts. The use of inhibitors has been described previously (section 3.1.2.4).

3.3.2 Results

3.3.2.1 The Free Amino Acid Pool of Barley Embryos

Table 3.16 shows the free amino acid pool of barley embryos germinated for 24 h under two sets of conditions, in water (the standard procedure employed throughout this study), or in a spray room, exposed to the atmosphere. Although the overall composition of the amino acid pool is similar in both cases, the levels of two amino acids, alanine and γ-aminobutyric acid, are considerably

Table 3.16: Free Amino Acid Pool of Barley Embryos Germinated Under Different Conditions

	Amino acids (µm/g fresh wt.)	
	Water[a]	Steam Room[b]
Gly	1.2	1.4
Ala	30.0	3.0
Glu/Gln	8.9	8.4
Asp/Asn	1.6	1.2
Ser	2.2	1.1
Thr	0.7	0.6
Leu	1.7	1.6
Ile	1.1	1.0
Pro	5.7	6.1
Val	3.0	2.6
GAB	4.8	1.1
Lys	1.2	1.5
Phe	0.8	0.8
His	1.3	1.1
Tyr	1.0	0.8
Met	0.4	0.2
Arg	1.5	1.7
E	0.9	1.1
F	3.0	3.3
G	1.9	1.8
Others	<0.2	<0.2
Weight of 20 embryos	162 mg	205 mg

Seeds were germinated for 24h (a) in water at 25°C or (b) on polythene granules in a steam room at 28°C (section 2.3.1). Embryos were extracted and the amino acid pool analysed using dansyl chloride. Each set of data is the average of four separate extractions of six embryos each. Substances E, F, and G are unknown dansyl-reactive compounds (see appendix 2).

higher in the embryos of grains germinated in water. Embryos from the grains germinated in water also weigh rather less. It seems almost certain that both these effects are the result of oxygen limitation in the water-germinated seeds. A continual supply of oxygen throughout the germination of seeds in water substantially reduced the amounts of alanine and GAB and increased the embryo weights. Besides, similar changes in the amino acid pools of many plant tissues, under anaerobic conditions, have often been reported (see Discussion).

The composition of the amino acid pool was fairly similar in the various organs of the embryo (shoot, root and scutellum), although the concentrations (in terms of µm/g fresh wt.) were generally a little greater in the scutellum (table 3.17). However, two amino acids did vary in their distribution within the embryo. Proline was primarily located in the scutellum, while GAB was abundant in the scutellum and particularly the roots, yet only present in small amounts in the shoots (a similar distribution of GAB in barley embryos has been reported elsewhere; Inatomi & Slaughter, 1971).

3.3.2.2 Amino Acid Exodus

Over an 8 h incubation period, 20 - 30% of the embryo amino acids are lost to the medium (table 3.18). However,

Table 3.17: Free Amino Acid Pools of Embryo Tissues

	Amino Acids (µm/g tissue fresh wt.)			
	Whole Embryos	Roots	Shoots	Scutellum
Gly	1.0	0.8	0.6	1.8
Glu	4.7	3.6	6.0	5.6
Ser	1.4	1.2	1.4	2.0
Leu	0.9	0.8	0.8	1.4
Ile	0.8	0.6	0.7	0.8
Pro	3.8	1.8	1.9	6.8
Val	2.1	2.0	2.0	2.4
GAB	5.9	7.8	1.1	6.0
Phe	0.5	0.4	0.3	0.7
His	0.9	0.5	0.6	0.9
Others	not determined			

Embryos, from grains germinated for 24h, were incubated for 8h under standard conditions (section 2.3.1) and the amino acids in whole embryos or the separated tissues determined using dansyl chloride. Each value is the average of two separate determinations.

Table 3.18: Amino Acid Exodus from Barley Embryos

	Amino Acids (μm/g fresh wt.)					
	0h			8h		
	Medium	Embryo	Total	Medium	Embryo	Total
Gly	0	1.2	1.2	0.4	0.9	1.3
Ala	0	30.0	30.0	8.0	19.0	27.0
Glu/Gln	0	8.9	8.9	1.8	4.8	6.6
Asp/Asn	0	1.6	1.6	0.3	0.7	1.0
Ser	0	2.2	2.2	0.9	1.7	2.6
Leu	0	1.7	1.7	0.5	1.0	1.5
Ile	0	1.1	1.1	0.4	0.8	1.2
Pro	0	5.7	5.7	2.4	2.9	5.3
Val	0	3.0	3.0	0.6	3.1	3.7
GAB	0	4.8	4.8	5.8	5.4	11.2
Lys	0	1.2	1.2	0.2	0.9	1.1
Phe	0	0.8	0.8	0.2	0.6	0.8
His	0	1.3	1.3	0.2	1.2	1.4
Others	not determined			not determined		

24h barley embryos were incubated for 0h or 8h in 1 ml of 50mM sodium phosphate:citric acid buffer, pH 3.8 (six embryos per ml of medium). The amino acids in embryo extracts and in the medium were determined using dansyl chloride. Each set of data is the average of four separate determinations.

as the relative volumes of the medium and embryo are about 20:1, a gradient of 50:1 in favour of the embryo is still maintained. There seems to be little or no selectivity in the release of amino acids to the medium. However, while the total amount of most amino acids remains fairly constant during incubation (embryo plus medium), there is a marked increase in the overall level of GAB.

3.3.2.3 Effect of pH on Exodus

The pH of the incubation medium has little effect on amino acid exodus (except in acetate buffer; section 3.3.2.6), although an effect on the composition of the amino acid pool is observed (table 3.19). While most amino acids remain at a relatively constant level (in the embryo plus the medium), regardless of the pH at which the embryos are incubated, the total amount of GAB increases markedly as the medium pH is lowered from pH 8 to pH 3. Over the same pH range there is a corresponding decrease in the amount of both glutamic acid and aspartic acid. Most of the additional GAB present at low pH, and glutamic acid and aspartic acid at high pH, is found in the medium; the pool size within the embryo remains relatively constant.

3.3.2.4 Effect of Metabolic Inhibitors on Exodus

Metabolic inhibitors affect both the composition of the embryo amino acid pool and exodus into the incubation medium

Table 3.19: Effect of Medium pH on Amino Acid Exodus from Barley Embryos

	Amino acids released to the medium (µm/g fresh wt. embryo)						Amino acids remaining in the embryo (µm/g fresh wt. embryo)					
Medium pH	3	4	5	6	7	8	3	4	5	6	7	8
Gly	1.0	0.4	0.6	0.8	0.4	0.5	1.0	1.3	1.5	0.9	1.3	0.6
Ala	8.8	8.8	8.1	8.8	9.8	7.9	14.2	17.3	15.0	14.2	17.5	14.4
Glu	2.3	2.7	4.2	7.3	9.8	14.4	3.5	3.8	3.9	4.2	4.2	4.6
Asp	0.9	0.9	1.3	1.7	2.5	3.5	1.7	1.7	1.6	1.8	1.9	2.0
Leu	0.7	1.0	1.0	0.6	0.8	0.8	1.3	1.4	1.0	1.5	1.3	1.0
Ile	0.4	0.5	0.8	0.5	0.5	0.5	0.7	0.8	0.8	0.6	1.0	0.7
Pro	3.5	3.5	3.1	2.9	3.3	3.5	2.1	2.9	3.3	3.5	3.5	3.1
Val	1.3	1.6	2.2	1.6	1.8	1.9	3.3	2.9	2.5	2.8	2.9	3.3
GAB	14.2	5.1	3.1	2.9	2.3	1.3	4.2	3.9	4.0	2.9	2.5	1.4

Embryos were incubated for 8h in 50mM sodium phosphate:citric acid buffer at the appropriate pH(six embryos/ml medium). Amino acids in the embryo and medium were determined using dansyl chloride.

(table 3.20). Thus, CCCP, azide and DNP all increase amino acid exodus relative to the buffer control. In 0.3 mM DNP, exodus is such that over an 8 h incubation period, equilibration across the membrane occurs (table 3.21; see also section 4.3.3). Further evidence that DNP allows equilibration across the membrane is provided by its effects on the intact uptake of physiological peptides. In the absence of DNP, physiological peptides do not accumulate intact within the embryo. However, in the presence of 0.3 mM DNP, intact peptides are accumulated within the embryo to the same concentration as they are supplied extracellularly (section 4.3.3; see also section 4.4.3). Interestingly, DNP does not promote the intact accumulation of D-peptides.

The total amount of most amino acids (in the embryo plus medium) remains unaffected by the presence of these inhibitors. However, each inhibitor causes a large increase in the level of GAB and a corresponding decrease in both glutamic and aspartic acid.

3.3.2.5 Effect of Acetate on Peptide Transport

Acetate ions seem to have a similar effect on barley embryos as anaerobiosis and the various metabolic inhibitors employed above (DNP, azide, etc.). Thus, the uptake of physiological peptides cannot be detected if the sodium

Table 3.20: Effect of Metabolic Inhibitors on Amino Acid Exodus from Barley Embryos

	Embryos							Medium							Total						
	No Inhibitor	10^{-4}M CCCP	5mM Acetate	1.5mM Azide	0.15mM DNP	0.3mM DNP	Anoxia	No Inhibitor	10^{-4}M CCCP	5mM Acetate	1.5mM Azide	0.15mM DNP	0.3mM DNP	Anoxia	No Inhibitor	10^{-4}M CCCP	5mM Acetate	1.5mM Azide	0.15mM DNP	0.3mM DNP	Anoxia
Gly	0.4	0.9	0.6	1.0	1.1	1.4	0.7	0.9	0.5	0.8	0.2	0.6	0.10	0.7	1.3	1.4	1.4	1.2	1.7	1.5	1.4
Ala	8.0	16.8	12.4	24.0	20.0	33.0	24.2	19.0	12.0	19.6	12.0	12.8	2.10	11.6	27.0	28.8	32.0	36.0	32.8	35.1	35.8
Glu	4.6	1.7	1.7	1.1	2.0	2.6	1.6	5.8	1.7	2.3	1.3	0.7	0.13	2.0	10.4	3.4	4.0	2.4	2.7	2.7	3.6
Asp	1.1	0.5	0.5	0.8	0.7	1.4	0.8	1.9	0.5	0.5	0.4	0.3	0.05	0.6	3.0	1.0	1.0	1.2	1.0	1.5	1.4
Ser	0.9	-	-	-	-	-	-	1.7	-	-	-	-	-	-	2.6	-	-	-	-	-	-
Leu	0.5	1.3	0.6	1.8	1.8	1.9	1.0	1.0	0.7	1.2	0.6	0.5	0.10	1.0	1.5	2.0	1.8	2.4	2.3	2.0	2.0
Ile	0.4	0.7	0.2	1.1	1.2	1.5	0.6	0.8	0.3	1.0	0.3	0.4	0.05	0.8	1.2	1.0	1.2	1.4	1.6	1.6	1.4
Pro	2.4	3.2	2.4	4.3	5.8	6.8	3.2	2.9	1.1	3.0	0.7	0.9	0.45	2.0	5.3	4.3	5.4	5.0	6.7	7.3	5.2
Val	0.6	1.9	1.5	2.2	2.1	3.0	1.4	3.1	1.1	2.2	0.7	0.8	0.25	1.8	3.7	3.0	3.7	2.9	2.9	3.3	3.2
GAB	5.8	14.4	13.6	19.2	13.8	18.8	12.2	5.4	2.3	4.7	1.7	2.0	0.63	4.9	11.2	16.7	18.3	20.9	15.8	19.4	17.1
Lys	0.2	0.7	0.3	0.8	0.8	1.8	0.6	0.9	0.9	1.1	0.4	0.4	0.10	0.6	1.1	1.6	1.4	1.2	1.2	1.9	1.2
Phe	0.2	0.5	0.2	0.3	0.4	0.7	0.3	0.6	0.1	0.6	0.5	0.2	0.05	0.4	0.8	0.6	0.8	0.8	0.6	0.8	0.7
His	0.2	0.9	0.4	0.7	0.9	1.3	0.7	1.2	0.4	1.4	0.3	0.5	0.05	0.7	1.4	1.3	1.8	1.0	1.4	1.4	1.4

24h embryos were incubated for 8h in 50mM sodium phosphate:citric acid buffer, pH 3.8, containing the appropriate 'inhibitor' (six embryos/ml of medium). Amino acids in the medium and embryo extracts, determined using dansyl chloride, are expressed as μm/g fresh wt. Each value is the average of at least two separate determinations.

Table 3.21: Concentration of Amino Acids in The Embryo and Medium after Incubation with DNP

	Buffer alone		Buffer + 0.3mM DNP	
	Medium	Embryo	Medium	Embryo
Gly	0.02	0.88	0.05	0.10
Ala	0.40	19.00	1.32	2.10
Glu	0.23	5.80	0.11	0.10
Asp	0.06	1.90	0.06	0.05
Leu	0.02	1.00	0.08	0.10
Ile	0.02	0.80	0.06	0.05
Pro	0.12	2.80	0.31	0.45
Val	0.32	3.10	0.16	0.20
GAB	0.29	5.40	0.75	0.63
Lys	0.01	0.90	0.07	0.10
Phe	0.01	0.60	0.03	0.05
His	0.01	1.20	0.05	0.05
Others	not determined		not determined	

Amino acid concentrations in the medium are expressed as µm/ml, and in the embryo as µm/g fresh wt. Embryos were incubated for 8h under standard conditions (6 embryos/ml medium) (section 2.3.1) and the levels of amino acids in the embryos and medium determined using dansyl chloride.

phosphate:citric acid buffer is replaced by acetate buffer at the same pH (metabolic inhibitors also prevent peptide uptake, section 3.1.3.10). Similarly, acetate buffer considerably reduces the amount of Gly-Sar uptake by barley embryos (fig. 3.27; table 3.22; see also tables 3.23 and 3.24). This inhibitory effect is dependent upon the acetate concentration, but not the counterion (fig. 3.27).

3.3.2.6 Effect of Acetate on Amino Acid Exodus

5 mM acetate has little effect on amino acid exodus (table 3.20). However, at higher external concentrations, acetate causes a considerable loss of amino acids from the embryo. This effect is strongly influenced by pH (table 3.23).

Figure 3.28 also illustrates the effect of acetate on the amino acid pool. Thus, although the fluorescence intensity of the ornithine standard is similar in figures 3.28a&b (indicating uniform extraction and dansylation), the presence of acetate in the incubation medium (fig. 3.28b) reduces the level of all other amino acids. For most amino acids this loss is the result of exodus; amounts recovered in the incubation medium complement those lost from the embryo (table 3.24). However, tables 3.23 and 3.24 both show that, as for recognised metabolic inhibitors (table 3.20), acetate causes an overall reduction in the

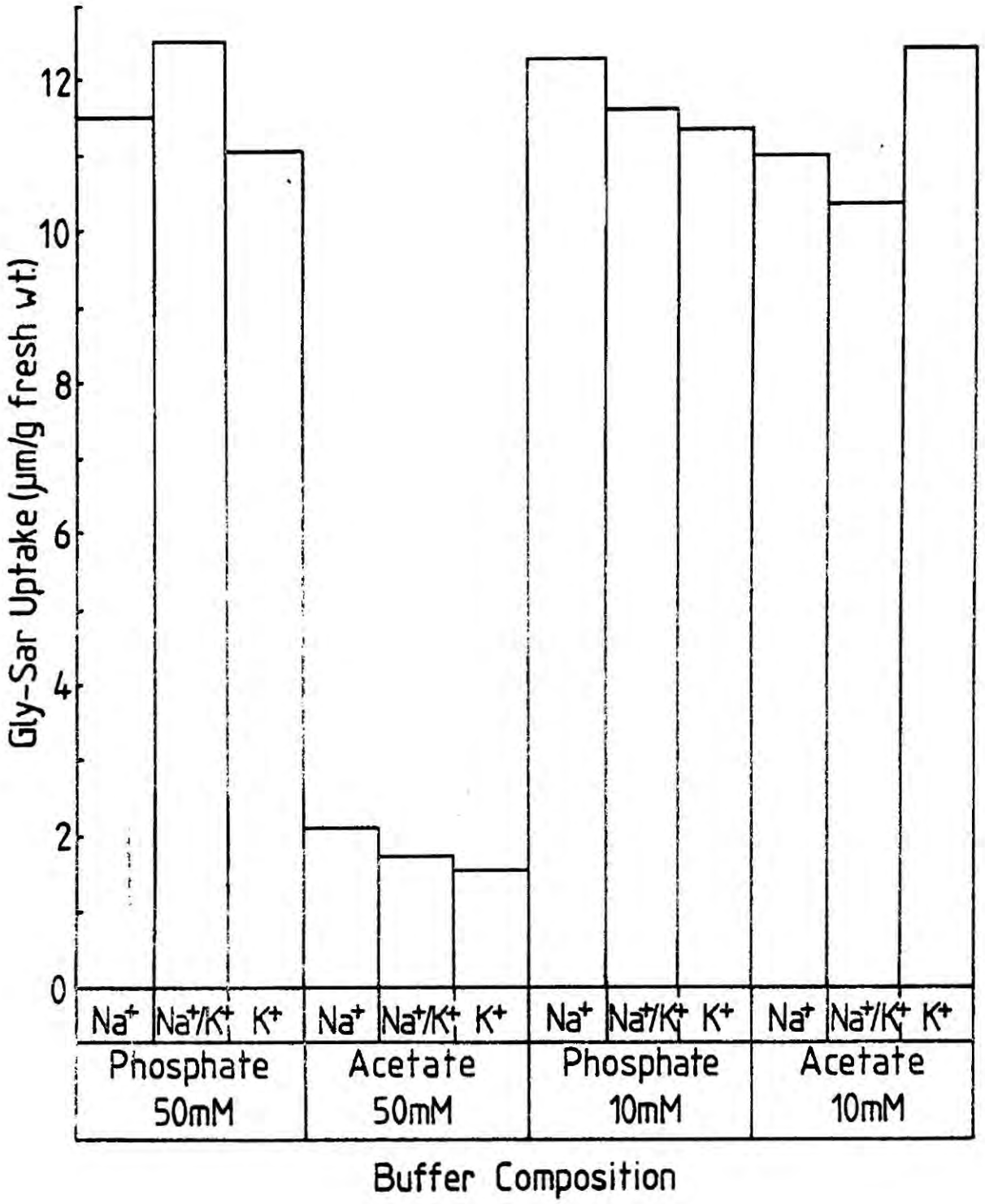


Figure 3.27: Effect of Acetate on Gly-Sar Uptake

Gly-Sar uptake from a 20mM solution was monitored by embryo extraction. Incubations were carried out at pH 5.8 for 6h. Each value is the average of two separate determinations.

Table 3.22: Effect of Acetate Buffer on Gly-Sar Uptake

	Gly-Sar uptake (μm/g fresh wt./6h)	
Buffer pH	Acetate buffer (50mM)	Phosphate:citric acid buffer (50mM)
3.8	0.8	7.0
4.4	0.5	4.0
5.2	0.3	2.4
5.8	0.1	1.8

Intact uptake of Gly-Sar from a 2mM solution was monitored by embryo extraction. Incubations were for 6h in the appropriate buffer. Each value is the average of at least two separate determinations.

Table 3.23: Effect of Acetate on the Amino Acid Pool of Barley Embryos at Various pH Values

Buffer ions	Acetate					Phosphate
pH of incubation medium	3.6	4.0	4.6	5.2	5.8	5.8
Gly-Sar	5.0	3.8	2.6	1.8	1.8	13.0
Gly	0	0	0.3	0.8	2.0	2.0
Ala	0	0.2	1.3	9.2	11.5	14.1
Glu	0	0	0	0.8	2.9	4.5
Asp	0	0	0	0.5	0.8	0.5
Leu/Ile	0	0	0	0.5	1.3	1.0
Pro/GAB	0	0	0.3	4.7	6.3	2.0
Val	0	0	0	1.2	1.5	1.5
Lys	0	0	0	1.2	1.5	1.5
Others	not determined					

Values are expressed as μmole of amino acid/peptide per g of embryo (fresh wt.). Embryos, from grains germinated for 24h, were incubated for 6h in 50mM buffer containing 20mM Gly-Sar. The composition of the amino acid pool was determined by embryo extraction. Each value is the average of at least two separate determinations.

Table 3.24: Effect of Acetate on the Exodus of Amino Acids from Barley Embryos

	Embryo extract			Incubation medium		
Buffer	Phosphate:Citric acid	Acetate		Phosphate:Citric Acid	Acetate	
pH	4.5	3.6	5.8	4.5	3.6	5.8
Gly-Sar	4.3	0.3	0.2	-	-	-
Gly	1.8	0	2.2	0	2.4	0.2
Ala	12.8	0.5	13.1	2.6	14.4	2.9
Glu	7.5	0	3.0	0	2.4	1.2
Leu/Ile	1.4	0	1.4	0	1.6	0
Pro/GAB	2.9	0.2	3.7	0.8	8.5	1.7
Val	2.2	0	2.2	0	2.5	0
Lys	2.2	0	2.2	0	2.5	0
Others	not determined					

Embryos, from grains germinated for 24h, were incubated for 6h in 50mM buffer containing 2mM Gly-Sar. Amounts of each amino acid/peptide in embryo extracts, and released into the incubation media, are expressed as μm/g fresh wt. Each value is the average of at least two separate determinations.

Figures 3.28a-c: Exodus of Amino Acids from Barley Embryos in Acetate

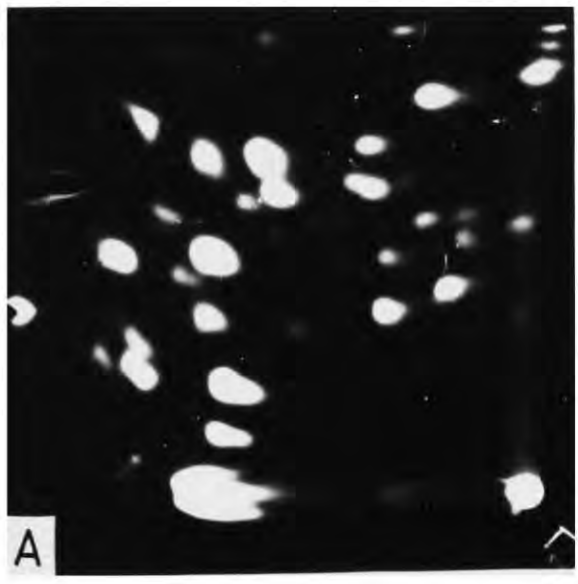
Developed chromatograms of dansylated barley extracts photographed under UV light. Embryos were incubated for 6h in 2mM Gly-Sar as follows:

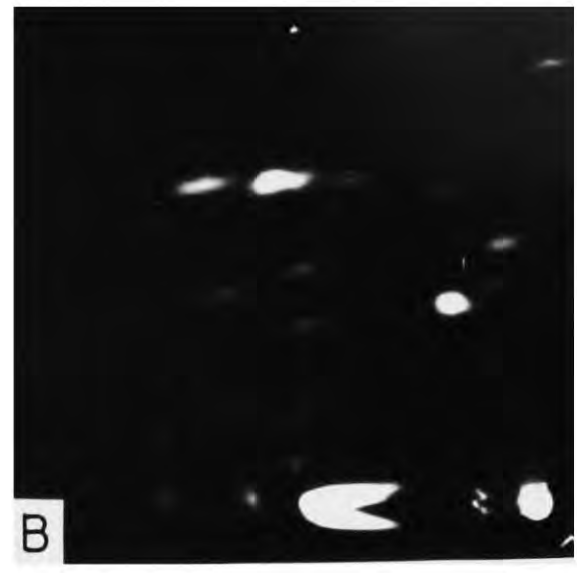
(A) 50mM sodium phosphate:citric acid buffer, pH 3.8.

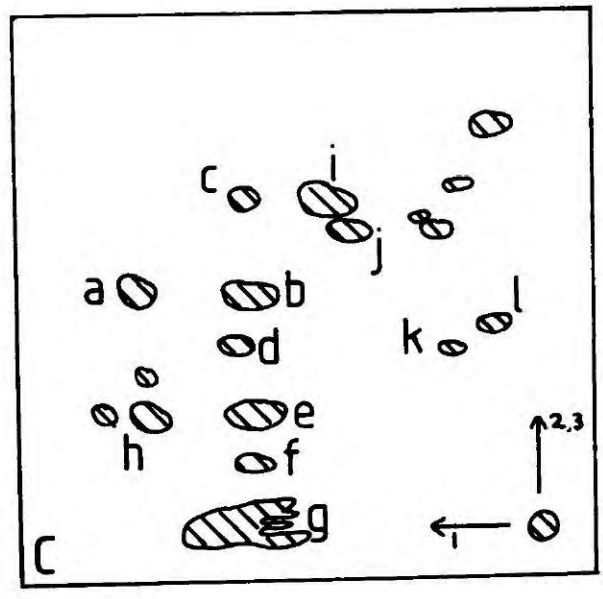
(B) 50mM sodium acetate buffer, pH 3.8.

(C) Diagram showing the locations of some of the dansylated derivatives:

a) Gly-Sar, b) alanine, c) dansyl-ammonia, d) glycine, e) glutamic acid, f) aspartic acid, g) dansyl-hydroxide, h) serine, i) proline/GAB, j) valine, k) ornithine, l)lysine.


A


B


c
i
j
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f
g
2,3
1
C

levels of glutamic and aspartic acids and a corresponding increase in GAB.

3.3.2.7 Effect of Metabolic Inhibitors on Embryo Weight

Incubation of barley embryos with metabolic inhibitors causes a reduction in embryo weight, compared with incubation in buffer alone (table 3.25). The greatest reduction in weight is seen with those inhibitors which produce the greatest amino acid exodus. For example, acetate only causes a reduction in embryo weight at low pH; likewise, amino acid exodus is only promoted by acetate at low pH. However, these two effects do not seem to be directly related. The proportion of amino acids lost to the medium is often very much greater than the percentage decrease in embryo weight.

3.3.3 Discussion

The results presented above indicate that anaerobiosis, acetate, and a range of metabolic inhibitors all exert a number of similar effects on barley embryos: a reduction in embryo weight, the inhibition of peptide uptake, an increase in amino acid exodus, and the synthesis of GAB (with a corresponding decrease in the pool of glutamic acid). It seems probable that all these effects are the secondary results of interference with a single, primary process. The most likely candidate for this primary process seems to

Table 3.25: Effect of Metabolic Inhibitors on Embryo Weight

Buffer (50mM)	Inhibitor	pH	Weight of twelve embryos (mg)
Phosphate:citric acid	-	3.8	96.6
Phosphate:citric acid	5mM acetate	3.8	99.2
Phosphate:citric acid	1.5mM azide	3.8	77.8
Phosphate:citric acid	0.15mM DNP	3.8	82.4
Phosphate:citric acid	0.3mM DNP	3.8	79.8
Phosphate:citric acid	Nitrogen (anoxia)	3.8	83.8
Acetate	-	3.6	72.6
Acetate	-	4.0	76.8
Acetate	-	4.6	85.4
Acetate	-	5.2	95.3
Acetate	-	5.8	103.4

Embryos, from grains germinated for 24h, were incubated for 8h in the appropriate buffer and blotted dry prior to weighing.

be the production/maintenance of a proton gradient across the plasmalemma.

For several years it has been known that substantial quantities of H^+ ions may be absorbed or excreted across plant cell membranes. Many roles for such fluxes have been postulated, including regulation of intracellular pH, morphogenetic responses to hormones, stomatal and other plant movements, photosynthetic phosphorylation and metabolite transport. Proton movement is dependent upon a supply of metabolic energy. Thus, the production of a proton gradient across the plasmalemma can be inhibited by anaerobiosis and metabolic inhibitors (see Smith & Raven, 1974, 1976; Raven & Smith, 1977, for general reviews). Indeed, it seems that some of the inhibitors employed here (the uncouplers DNP and CCCP) actually exert their inhibitory effects by increasing the permeability of cell membranes to protons, at least in bacterial and animal systems (Hopfer et al., 1968; Green, 1977; Kessler et al., 1977). There is also a certain amount of evidence that in plant cells too, the primary effect of both DNP and CCCP is to increase the permeability of the plasmalemma to protons (Humphreys, 1975; Felle & Bentrup, 1977; Dejaegere & Neirinckx, 1978).

3.3.3.1 Inhibitory Properties of Acetate

While acetate is not generally recognised as a metabolic

inhibitor, it clearly has a similar effect on barley embryos to that achieved with anaerobiosis or inhibitors of electron transport and oxidative phosphorylation.

Acetate has long been known to interfere with bacterial and fungal growth (Hobson, 1969). Inhibitory effects on transport processes have also been reported; for example, the uptake of sugars in S. cerevisiae (Okada & Halvorson, 1964), amino acids in Ehrlich cells (Garcia-Sancho et al., 1977a) and K^+ in Chlorella (Tromballa, 1978). These inhibitory properties (and similar properties of other weak acids) are apparent only at low pH values, below the pK of the carboxyl group, implying that it is the undissociated species that is important (Simon & Beevers, 1952; Hentges, 1967). It has been proposed that inhibition arises from an 'uncoupling' effect, the undissociated acetic acid molecule (CH_3-COO^-H^+) conducting protons across the cytoplasmic membrane (Hueting & Tempest, 1977a,b).

In barley, acetate appears to act in a similar manner. Thus, the acetate-promoted release of pool amino acids is pH-dependent, significant release only occurring below pH 4.6 and being barely detectable above pH 5.0. As the pK of acetic acid is 4.8, it appears that amino acid exodus is promoted by the undissociated form of the molecule. At higher pH values, little or no amino acid exodus is apparent,

although peptide transport is still inhibited. However, this inhibition is only observed at high acetate concentrations at which the amount of the undissociated species is correspondingly high.

Thus, it seems highly likely that the primary effect of acetate on barley embryos is the disruption of a proton/electrochemical gradient. This view is substantiated by two additional lines of evidence:

(i) Acetate affects barley embryos in a similar manner to anaerobiosis and various metabolic inhibitors. It is generally accepted that one of the main effects of inhibiting aerobic respiration in plant cells is the breakdown of proton gradients.

(ii) The observed effects of acetate on barley embryos (e.g. amino acid exodus, GAB synthesis) could all arise as the secondary results of the disruption of a proton gradient (see below).

3.3.3.2 Amino Acid Exodus

During incubation of barley embryos, amino acids leak into the medium. This leakage is increased by metabolic inhibitors until in some cases over 95% of the amino acids are lost from the embryo and equilibration occurs across the cell membrane. Further evidence that equilibration occurs is provided by the intact accumulation of physiological

peptides in the presence of DNP (section 3.3.2.4) and the effects of DNP on the uptake of N-methylated peptides (see section 4.4.3).

It has been known for several years that aerobic respiration is essential for the maintenance of plant cell membrane integrity (Handley & Overstreet, 1963). Anaerobiosis and metabolic inhibitors rapidly cause a general increase in the leakage of ions, amino acids and sugars from plant cells (Robertson et al., 1951; Marschner & Mengel, 1966; Marschner et al., 1966; Hiatt & Lowe, 1967; Smirnova et al., 1968; Humphreys, 1977). This leakage can be prevented by Ca^{2+} ions (Rains et al., 1964; Marschner & Mengel, 1966; Smith, 1978).

As yet, the precise nature of this increase in permeability is not known. It is possible that the general disruption of membrane structure allows leakage through 'holes' in the membrane (Marschner et al., 1966). The reduction of leakage by Ca^{2+} ions has been used in support of this idea; Ca^{2+} ions may stabilize membrane structure. Alternatively, removal of an energy supply may permit specific transport systems to operate passively, allowing substances to leave the cell down an electrochemical gradient. Evidence obtained in barley supports this latter view. Leakage promoted by DNP is stereospecific,

indicating that a carrier-mediated process is involved, rather than exodus occurring as a result of a non-specific increase in membrane permeability (section 4.3.3).

It therefore seems feasible that the amino acid leakage observed could be passive, mediated exodus occurring as a result of the disruption of a proton gradient. External pH changes are known to affect leakage from plant cells (Rains et al., 1964; Marschner et al., 1966; Hiatt & Lowe, 1967). In addition, there has recently been a proliferation of reports indicating that the transport of organic solutes by plant cells may be energized by a H^+-symport mechanism, according to the chemiosmotic theory of Mitchell (1976) (section 3.3.3.5). These systems may operate passively in the absence of a proton gradient, allowing their substrates to leave the cell down an electrochemical gradient. The ability of Ca^{2+} ions to prevent exodus may be explained by the stimulatory effects these ions are known to have on plant membrane-bound ATPases (Hodges, 1976). These ATPases are believed to be responsible for the active extrusion of H^+ ions. Thus, Ca^{2+} ions may maintain membrane integrity by stimulating the production of a proton gradient across the plasmalemma.

3.3.3.3 Amino Acid Metabolism: GAB Synthesis

The composition of the endogenous amino acid pool of

barley embryos is relatively constant, regardless of the conditions to which the embryos are subjected. However, under certain conditions, rather large fluctuations in the relative amounts of one or two amino acids become apparent.

Embryos from grains germinated under conditions of oxygen limitation (under water) have greatly increased levels of two amino acids, GAB and alanine. Similarly, during incubation of embryos with a variety of metabolic inhibitors, the level of GAB increases while glutamic acid and aspartic acid both decrease. Any increase in alanine that may be caused by the metabolic inhibitors will be masked by the high levels of alanine already present in the embryo.

It has often been reported that anaerobic conditions lead to an increase in alanine and GAB in plant tissues, at the expense of aspartic acid and glutamic acid respectively (Naylor & Tolbert, 1956; Dixon & Fowden, 1961; Thompson et al., 1966; Effer & Ranson, 1967; Guinn & Brinkerhoff, 1970; Streeter & Thompson, 1972a). However, it seems possible that the reported increases in alanine were actually β-alanine. Certainly this may be the case in the present study; alanine and β-alanine are inseparable by the chromatographic procedures employed here. It is difficult to explain how α-alanine is synthesised at the

expense of aspartic acid (see for example Streeter & Thompson, 1972a). However, β-alanine can arise from aspartic acid by simple α-decarboxylation in an exactly analogous fashion to the production of GAB from glutamic acid. Aspartate decarboxylase is found in many bacteria and animal tissues and is generally active under the same conditions as glutamate decarboxylase (Najjar, 1955; Utter, 1961). In addition, it has been reported that β-alanine does accumulate in plant tissues under anaerobic conditions (Miflin & Lea, 1977).

Evidence obtained in barley indicates that proton movements may also be responsible for decarboxylations. Thus, acetate promotes GAB synthesis (particularly at low pH); a similar effect has been noted for a number of other weak acids in Chlorella (Lane & Stiller, 1970). In addition, GAB synthesis is increased simply by lowering the pH of the external medium.

Two possible mechanisms may be proposed to try and explain the increase in GAB synthesis resulting from the inhibition of aerobic respiration.

(i) the permeability of intracellular membranes may be increased, exposing glutamate, normally present in the vacuole, to glutamate decarboxylase which is located in the cytoplasm (Dixon & Fowden, 1961). This view is

supported by evidence indicating that GAB is synthesised from a normally non-metabolizable 'sub-fund' of glutamate (Zemlyanukhin & Ivanov, 1977).

(ii) The increase in GAB synthesis may be the result of a decrease in cytoplasmic pH. This could be a positive response in an attempt to maintain the cytoplasmic pH by decarboxylations. Alternatively, GAB synthesis could simply be due to the increased activity of glutamate decarboxylase at low pH. The pH optimum for the enzyme in plants is pH 5.9 (Streeter & Thompson, 1972a,b).

Both these models fit the hypothesis that the primary effect of inhibiting anaerobic respiration is to disrupt proton gradients. Evidence presented here tends to support the latter view. GAB synthesis is promoted by conditions under which significant general exodus does not occur (e.g. low pH; acetate at high pH). Thus, GAB synthesis does not seem to be directly related to a generalized increase in membrane permeability. However, the possibility that, under these conditions, intracellular permeability barriers break down more extensively than the plasmalemma, does not allow (i), above, to be completely discounted.

A further indication that the breakdown of intracellular compartmentation is not the main cause of the anaerobic synthesis of GAB comes from observations on

proline synthesis. Proline does not accumulate in barley embryos under any of the adverse conditions employed here, yet it is well known that proline accumulates in barley tissues under specific stress (e.g. low temperature: Shiomi & Hori, 1978. Water stress: Singh et al., 1973; Stewart, 1978; Hanson & Tully, 1979. Nutrient deficiency: Göring & Thien, 1979). It therefore seems likely that GAB and proline synthesis are regulated by entirely separate processes. However, like GAB, proline is also apparently synthesised in the cytoplasm from glutamic acid (Boggess et al., 1976; Stewart et al., 1977). Thus, under aerobic conditions sufficient glutamate is available in the cytoplasm for the synthesis of large amounts of proline. This indicates that glutamate is also available for conversion to GAB. A factor other than glutamate availability must therefore be important in regulating GAB synthesis.

Finally, preliminary observations on E.coli indicate that anaerobiosis and inhibitors of electron transport specifically promote the synthesis of GAB, β-alanine and putrescine (the decarboxylation product of ornithine) (J.W. Payne, personal communication). Clearly, in a prokaryote, disruption of intracellular permeability barriers cannot be important. In addition, these bacterial decarboxylases are only active at low pH (about pH 5.0; Najjar, 1955; Utter, 1961), again implying that the primary

effect of all the inhibitors employed is to reduce cytoplasmic pH.

The lowering of cytoplasmic pH by acetate and uncouplers can readily be explained by their ability to facilitate the movement of protons across cell membranes (sections 3.3.3 and 3.3.3.1). However, it is less easy to explain the apparent ability of anaerobiosis and inhibitors of electron transport to lower the cytoplasmic pH. Possibly, normal cellular metabolism leads to the production of hydrogen ions which, under aerobic conditions, are expelled from the cell, maintaining a constant intracellular pH. If aerobic respiration is inhibited, the active extrusion of H^+ ions may be inhibited leading to an accumulation of protons within the cell. This implies that the primary function of the proton pump is to maintain cellular pH at a constant level.

The activation of decarboxylases by the inhibition of aerobic respiration would be expected to remove protons from the cytoplasm. This possibility should be borne in mind when studying proton movements under such conditions. In addition, the apparent reduction in the cytoplasmic pH of both plant and bacterial cells, as a result of the inhibition of aerobic respiration, has considerable implications for the measurement of proton fluxes;

cytoplasmic pH has generally been considered to remain constant when interpreting the results of such studies.

3.3.3.4 Reduction in Embryo Weight

The reduction of embryo weight in the presence of metabolic inhibitors is only observed in conditions in which considerable exodus of amino acids (and presumably other substrates) from the embryo is apparent. Thus, osmotic effects seem likely to be important. The exodus of metabolites from the embryo will lead to a reduction in osmotic pressure. Consequently, water will leave the cells, resulting in an overall decrease in embryo weight.

3.3.3.5 Energy Supply to Peptide Transport

Peptide transport by barley embryos is clearly an active process, requiring an input of metabolic energy (section 3.1.4.1). It is not possible to exclude hydrolysis of the peptide bond as a driving force for peptide uptake (section 1.1.5), although such a possibility seems unlikely as active transport can occur in the absence of hydrolysis, at least for certain peptides. In addition, the ability of such a variety of metabolic inhibitors to prevent transport indicates that a less specific method of energization is operative.

Na^+ or K^+ ions are not required for peptide transport by barley embryos, although they seem to be necessary for peptide

transport in animal cells (Matthews, 1975). This is similar to other plant transport processes where Na^+-cotransport is normally unimportant. However, Sopanen et al. (1978) have reported that high concentrations of these monovalent cations actually inhibit peptide transport in barley. Similarly, high concentrations of Na^+ or K^+ ions inhibit amino acid uptake by maize scutella (Stewart, 1971). It seems likely that these inhibitory effects are achieved by the disruption of a proton gradient; such an explanation has often been invoked to explain the inhibitory effects of these ions in other plant tissues. In addition, Ca^{2+}/Mg^{2+} ions reverse the Na^+/K^+ inhibition of peptide transport. It seems likely that these divalent ions prevent the disruption of proton gradients (section 3.3.3.2).

More direct evidence for the involvement of an H^+ ion gradient in energizing peptide transport comes from studies with metabolic inhibitors. In addition to their effect on the general exodus of metabolites from plants cells (section 3.3.3.2), it seems that anaerobiosis and metabolic inhibitors can also inhibit transport more specifically. Thus, in several plant systems, low concentrations of DNP do not promote general exodus, yet may still inhibit the uptake of various compounds into the cell (Robertson et al., 1951; Schoolar

& Edelman, 1970; Jensén, 1978). Low concentrations of DNP also inhibit amino acid uptake by plant cells (e.g. Harrington & Smith, 1977; Lien & Rognes, 1977; Soldal & Nissen, 1978).

It seems that in barley too, the inhibition of peptide transport by metabolic inhibitors is not simply a result of increased membrane leakiness. For example, under certain conditions (e.g. acetate at high pH) little or no amino acid exodus is apparent, indicating that the membrane has not become generally leaky, yet peptide transport is still strongly inhibited. This is particularly well illustrated by the effects of acetate on the uptake of Gly-Sar from a 20 mM solution (table 3.23; section 3.3.2.5). After 6 h incubation in the absence of acetate, Gly-Sar does not attain a concentration in the embryo equivalent to that in the extracellular medium. Thus, if acetate acts simply by increasing membrane leakiness, it would not be expected to inhibit Gly-Sar uptake. Indeed, it might even be expected to increase uptake, allowing peptide to enter the cell down a concentration gradient. However, acetate does inhibit Gly-Sar uptake under these conditions, indicating a rather more specific effect on transport than simply increasing the leakiness of the cell membrane.

If analogies with microbial and animal systems are permitted, two possible modes of energization remain; direct energization by phosphate bond energy, or transport dependent upon H^+/electropotential gradients. Several lines of evidence point towards the latter possibility.

(i) Anaerobiosis and metabolic inhibitors, which inhibit peptide transport, all seem to cause the breakdown of proton gradients in barley embryos (see above).

(ii) The undissociated acetic acid molecule, which it would seem can only act by disrupting a H^+ ion gradient (section 3.3.3.1), is a powerful inhibitor of peptide transport.

(iii) The inhibitory effects of K^+/Na^+ ions can be explained on a basis of energization by proton gradients.

(iv) It seems unlikely that, even if inhibitors of oxidative phosphorylation were 100% efficient, they would totally inhibit an ATP-dependent transport system. The high levels of metabolizable sugars in the scutellum should allow significant quantities of ATP to be produced anaerobically, by substrate-level phosphorylation.

(v) Even if uptake is ATP-dependent, retention of peptide within the embryo requires an H^+-ion gradient. Gly-Sar exodus is promoted by DNP and acetate (section 4.3.4) both of which seem to cause exodus by interfering with the proton gradient. As exodus is apparently stereospecific (section 4.3.3), uptake and exodus are probably mediated

by the same transport system. It therefore seems likely that peptide uptake is also H^+-dependent.

(vi) There has recently been a proliferation of reports indicating that many transport processes in plants are dependent upon a proton gradient. In particular, transient depolarization of the membrane and rapid pH changes have been observed during the transport of several metabolites; sucrose in Ricinus cotyledons (Komor, 1977; Komor et al., 1977; Hutchings, 1978a,b), maize scutella (Humphreys, 1978); Samanea pulvini (Racusen & Galston, 1977), Chlorella (Komor & Tanner, 1976; Schwab & Komor, 1978), Lemna fronds (Novacky et al., 1978b), and phloem loading in Ricinus (Malek & Baker, 1977; Baker, 1978); glucose in maize roots (Kennedy, 1977) and Impatiens roots (Jones et al., 1975); amino acids in Lemna fronds (Novacky et al., 1978a), oat coleoptiles (Etherton & Nuovo, 1974; Etherton & Rubinstein, 1978) and tomato internodes (van Bel & van Erven, 1976, 1979). Thus, plant cells seem to be able to couple transport to H^+-ion movements.

It therefore seems likely that peptide transport in barley is energized according to the chemiosmotic theory (see Mitchell, 1976). This is unlike similar systems in bacteria where peptide transport is apparently ATP-dependent

and animal tissues in which a Na^+-peptide cotransport system may operate.

Unlike most transport systems, where all substrates bear the same charge, peptides handled by a single system can be neutral, cationic or anionic, depending upon their amino acid sequence. Certain peptides (e.g. trilysine, triglutamic acid) may be multiply charged. Unless a variable H^+:peptide stoichiometry is invoked, certain peptide:H^+ carrier complexes will be neutral. Transport will therefore be non-electrogenic, dependent only upon a pH gradient, normally considered unable to support a high accumulation ratio in the absence of an electropotential gradient. In certain extreme cases, although uptake may be down a pH gradient, it may be against an electropotential gradient. Alternatively, a high H^+:peptide stoichiometry would have to be invoked; this would make peptide transport energetically inefficient. Thus, if H^+-peptide cotransport is confirmed, this system will be of particular interest in studying the mechanism of proton-dependent transport in plant cells.

3.4 Final Discussion

The existence of a peptide transport system in the scutellum of germinating barley embryos seems firmly established. The system is independent of amino acid uptake, yet will handle di-, tri- and possibly even larger peptides. It is highly probable that all peptides are transported intact; hydrolysis is rapid, yet subsequent to transport. No evidence has been obtained (although see chapter 4) for the existence of any further peptide transport systems with restricted specificity. All peptides which are absorbed seem to be handled by this one system.

3.4.1 Structural Requirements for Transport

The structural requirements for peptide transport by barley embryos have been examined in some detail, and are very similar to all other peptide transport systems (table 3.26). The only major difference observed between those organisms in which peptide transport has been characterized is the existence of two separate systems in E.coli and other bacteria. A possible reason for this has been discussed elsewhere (section 3.1.4.4). However, despite the additional dipeptide transport system in many bacteria, the structural requirements for transport are still similar to those o other organisms. In fact, considering the very different environments in which the barley, bacterial, yeast and

Table 3.26: Comparison of the Peptide Transport Systems of Various Organisms

	Plants (Barley Scutellum)	Animals (Mammalian Gut)	Fungi (S. cerevisiae)	Bacteria (E. coli)
Number of systems	Probably only one main system	Probably only one main system	Probably only one main system	Separate di- and oligopeptide systems
Relation to amino acid transport	Independent	Independent	Independent	Independent
Role of membrane-bound peptidases in peptide utilization	Probably none	Of importance for most peptides	Probably none	None
Active, intact transport	Yes	Yes	Yes	Yes
Energy supply	Possibly H^+-linked	Na^+-dependent	ND	Probably dependent on phosphate bond energy
Specificity for peptide structure:				
Terminal amino group	Medium	High	High	High
Terminal carboxyl group	High	High	Medium/Low	High (dipeptides) Low (oligopeptides)
Amino acid side chains	Low	Low	Low	Low
∝- Peptide bonds	Medium	High	ND	High
L- Amino acid residues	High	High	High	High
Maximum chain length	Probably 5(a)	Probably 3, although may be up to 5(a)	Probably 5(a)	5-6 (for oligopeptide system

(a) Peptides of more than 5 residues not studied
ND = not determined

mammalian systems operate, it is, at first sight, perhaps surprising how similar they appear to be. A great deal of this similarity is doubtless due to the need to specifically recognize a peptide and distinguish it from other metabolites (the structural requirements for transport will present a 'mirror-image' of the shape of the peptide binding site). A number of features of peptide structure seem to be important in conferring affinity for the transport system; no single component predominates. This is perhaps not surprising if non-peptide material is to be excluded. For example, if the amino group were the one single requirement for uptake, amino acids, amino sugars and other metabolites would also bind to the transport site and reduce peptide transport, either competitively or non-competitively. Thus, all the characteristic structural features of a peptide, the peptide bond and the N- and C-terminal groups, are almost invariably important for the binding process. Any structural modifications which alter the spatial relationships between these groups (e.g. D-amino acid residues) might reasonably be expected to reduce binding. Similarly, the variable nature of the amino acid side chains makes them an uncharacteristic component of peptide structure and consequently unimportant with regard to peptide recognition. Thus, it is perhaps not surprising (at least

for systems which handle peptides of the same size) that, although affinities and maximum rates may vary, the structural requirements for peptide transport in all species are very similar.

Certain structural requirements for peptide transport do not seem easily compatible with each other. For example, it is difficult to envisage a binding site requiring both the N- and C-terminal groups of a peptide yet being able to accommodate peptides of different lengths. Similarly, it is surprising that the transport system is only able to handle N-substituted peptides very poorly, yet will handle N-terminal prolyl peptides extremely efficiently. It is interesting to speculate that such peptides may have distinct binding sites, possibly on separate 'binding proteins'. If this were so, competition for transport may then represent competition of the loaded binding proteins for a single membrane-bound transport system. Evidence for 'binding proteins' in plant transport processes has been presented, although it is by no means conclusive (Amar & Reinhold, 1973; Wahlström & Eriksson, 1976; Rubinstein et al., 1977).

3.4.2 Relationship Between Peptide Transport and Peptidase Activity

It has been shown that the transport of peptides by the barley scutellum can operate independently of peptidase

activity (section 3.1.4.3). However, these results should be considered in the light of recent evidence indicating that, at least in bacteria, peptide transport is indirectly affected by peptidase activity (J.W. Payne, unpublished results). Thus, peptides normally enter bacteria and are rapidly cleaved so that a significant peptide pool never accumulates. However, in peptidase-deficient strains, peptides accumulate intact until a given concentration is attained, at which point exodus from the cell equals the rate of uptake and no further net transport can be detected. In wild-type Salmonella, at least, the level of intact peptide that can be accumulated before exodus is significant is such that no uptake can be detected at all under most conditions. A similar situation might therefore be envisaged in wild-type cells, in which a structurally modified peptide is a good substrate for transport, yet peptidase-resistant; net uptake will be indetectable despite its adequacy as a transport substrate.

It seems less likely that exodus will be important in the barley embryo than it will in bacteria. Once a peptide has entered the cytoplasm of an embryo cell, it may be rapidly transported to a vacuole or to neighbouring cells, preventing the accumulation of a sufficient concentration in the cytoplasm for exodus to become significant. At present, techniques are not available to test this

possibility in barley. However, the possibility that the rates of transport measured in barley may reflect net movements of peptide, rather than the actual rate of entry into the cell, should be borne in mind.

It is also interesting to note that the model proposed for bacteria is very similar to the model proposed to explain evidence for subcellular compartmentation in the barley embryo (model II, section 4.4.7). The only difference is that inhibition of the entry of peptides into the hydrolytic compartment (vacuole?), rather than a lack of peptidase activity *per se*, allows intact peptide to accumulate in the cell and this limits net uptake as the rate of exodus increases.

3.4.3 Comparison With Other Studies

During the course of this work an independent study of peptide transport by germinating barley embryos was initiated elsewhere (Sopanen *et al.*, 1977, 1978). It is encouraging to note the substantial agreement between their results and those presented here, especially considering the entirely different methods employed. Sopanen and his co-workers employed radioactive peptides to monitor uptake; the lack of suitable substrates restricted their studies to sarcosine-containing peptides and the physiological peptide diglycine. This choice of diglycine proved in

some ways fortunate. Most peptides are hydrolysed and a high proportion of the resultant amino acids undergo exodus, a circumstance which would lead to a severe underestimate of peptide uptake using radioactive methods. However, glycine released from diglycine is rapidly metabolised by barley embryos (section 3.1.3.1) and consequently radioactivity remains in the embryo. In other ways the use of diglycine was less than ideal. Glycine-peptides are clearly anomalous with respect to peptide transport (section 3.2.3.2).

From their studies they have concluded that the barley scutellum possesses a single peptide transport system which handles di- and tripeptides, yet is independent of amino acid uptake. Uptake is inhibited by anaerobiosis and metabolic inhibitors and is independent of Na^+ ions. Peptide transport is optimal between pH 4 and 5. In agreement with present results, Gly-Sar and Gly-Sar-Sar were found to be transported intact, and diglycine to be a relatively poor substrate for transport.

Thus, the broad similarity between their conclusions and the data presented here is striking. However, two obvious, although by no means fundamental, differences are apparent. Firstly, Sopanen *et al.* claim a detectable non-mediated component to uptake. This may well be the result

of the techniques employed. They used isolated scutella, dissected from the bulk of the embryo. Thus, uptake by damaged tissue at the cut surface may well account for any non-mediated absorption. However, statistical analysis of their data, using the same methods as were applied to data obtained in the present study, has shown that simple Michaelis-Menten kinetics fit their data at least as well as Michaelis-Menten kinetics plus the proposed diffusion component (results not presented).

The second difference is in the reported rates of peptide uptake. Sopanen et al., consistently quote rates 5-10 times greater than those given here. These differences are not a function of the variety of barley employed. A sample of Himalaya (the variety studied by Sopanen et al.) was tested here, using fluorescence methods to monitor uptake, and it behaved identically to Maris Otter, Winter (results not presented).

A good deal of the apparent difference in the rate of peptide uptake is almost certainly a result of the terms in which it is expressed. Sopanen et al. employed excised scutella as opposed to the intact embryos used in this study. However, as uptake is almost entirely through the scutellar epithelium, rather than the rest of the embryo surface (section 3.1.3.3), the amount of absorptive

surface will be the same for excised scutella as for the intact embryos, while the overall weight of the scutellar tissue alone is considerably less. This will clearly lead to a discrepancy in rates of uptake when expressed in terms of μm/g tissue employed.

In addition, however, there is probably a real difference in rates. Sopanen et al. employed three-day seedlings which they found gave optimal rates of uptake (D. Burston, personal communication). Preliminary investigations, here, have indicated that the rate of peptide uptake, in terms of μm/g embryo, remains approximately constant during the first few days of germination (results not presented). Given that the scutellar epithelium is the only significant absorptive surface, the amount of transport per scutellum must increase several-fold in the first 3 days of germination, in parallel with the increase in embryo weight. Thus, it seems probable that three-day scutella do indeed absorb peptides rather more rapidly than scutella from 24 h embryos.

In conclusion, the characteristics ascribed to the barley peptide transport system by Sopanen et al. independently confirm some of the results presented here and endorse the suitability of the new methods employed; any differences in the results may be readily accounted for.

CHAPTER 4

SUBCELLULAR COMPARTMENTATION

4.1 Introduction

In order to simplify discussion, barley embryo cells have, until now, been assumed to be uniform 'black boxes', surrounded by a single membrane. Although such a view may be justified, the possibility of transport into various intracellular organelles certainly merits careful consideration. This has a number of implications for peptide transport:

(i) Peptides may be excluded from certain organelles. Where these occupy a relatively large proportion of the cell volume this can lead to a serious underestimate of the concentration of intact peptide within the cell fluid, and thus, an underestimate of the degree of accumulation.

(ii) An additional peptide transport system(s) may be present in the membrane surrounding a particular organelle. This raises the possibility that movement into the organelle, rather than across the plasmalemma, is the rate-limiting step in transport. Observed concentration gradients may represent gradients across the intracellular membrane, the cytoplasm remaining more or less at equilibrium with the extracellular space.

(iii) Peptidases may be unevenly distributed throughout the cell. Thus, peptides may be hydrolysed within the cytoplasm immediately they enter the cell. Alternatively,

it is possible that all peptides can exist intact in the cytoplasm; they must enter an organelle (e.g. a lysosome) before hydrolysis will occur.

There is now considerable indirect evidence for the compartmentation of metabolites and metabolic reactions within the plant cell (see Oaks & Bidwell, 1970, for a general review), yet little direct information is available concerning the mechanism or the organelles involved. Few transport studies have been performed on isolated plant organelles, and in studies of transport by intact plant cells (and those of other eukaryotes) the possibility that the plasmalemma may not be the only membrane limiting uptake has rarely been considered. However, there is a considerable amount of evidence to suggest that hydrolases are compartmentalized within the plant cell (section 4.4.8). Thus, subcellular compartmentation seems likely to be of particular importance in the utilization of peptides. Studies on the uptake of certain N-methylated peptides, described in this chapter, indicate that this is indeed the case; peptides are apparently transported across an intracellular membrane prior to hydrolysis.

4.2 Methods

4.2.1 Peptide Uptake

Peptide uptake was monitored using the standard dansyl

chloride procedures described previously (section 2.3.2). Sar-Gly uptake was always determined by examining embryo extracts for accumulated peptide.

In experiments where the incubation pH was varied, media were maintained at the appropriate pH with 50 mM sodium phosphate:citric acid buffer.

4.2.2 Peptide and Amino Acid Exodus

Again, standard incubation conditions were employed. Six embryos were pre-loaded during a 4 h preincubation with 2 mM peptide, rapidly but thoroughly washed, blotted dry, and placed in 1 ml of fresh incubation medium lacking any peptide (although containing the appropriate additives as described in results). After a further 4 h incubation, the distribution of peptide (or amino acids) between the embryo and the medium was monitored using dansyl-chloride.

General exodus of the amino acid pool was similarly followed, although here, preincubation was carried out in buffer alone, in the absence of any added peptides.

4.2.3 Electron Microscopy

24 h barley embryos were fixed at 4°C with glutaraldehyde (2.5%) and formaldehyde (1.0%) in sodium cacodylate buffer (0.1 M; pH 7.0). After 24 h the embryos were washed in buffer (two changes, 30 min each) and placed in osmium tetroxide (1% in buffer) for a further 6 h. Embryos were

dehydrated serially into 100% ethanol followed by propylene oxide, embedded in Spurr's resin (Spurr, 1969) and sectioned. Sections were stained with uranyl acetate (Watson, 1958) and alkaline lead citrate (Reynolds, 1963) and photographed using an AEI EM 6b or a Phillips EM 400 electron microscope at 60 kV.

4.3 Results

4.3.1 Characterization of Sarcosylglycine Uptake

Three N-methylated peptides, Sar-Gly, Sar-Gly-Gly and Sar-Ser, are taken up intact by barley embryos, although peptidase activity results in the release of some free sarcosine within the embryo (table 4.1). Increases in free glycine or serine, corresponding to the increase in free sarcosine, are not detected as these amino acids are rapidly metabolized by barley embryos (section 3.1.3.1). Free sarcosine is also detected in the incubation medium; the low concentration (< 0.05 mM) shows that most sarcosine is retained within the embryo against a large concentration gradient (40:1).

The sarcosine appearing in the embryo during incubation with Sar-Gly is not a result of free sarcosine uptake following extracellular peptide cleavage. Although free sarcosine is absorbed from a 2 mM solution at a rate of 1.9 µm/g fresh weight/8 h (a rate sufficient to explain its uptake from Sar-Gly on the basis of hydrolysis followed by

Table 4.1: Uptake of Sarcosyl Peptides Alone, and in the Presence of Various Amino Acids and Peptides

Added amino acid/peptide		None	Gly-Ile (8mM)	Ala-Ala-Ala (8mM)	Gly-Gly (8mM)	Sar (8mM)	Ala (8mM)	Gly (16mM)
Sar-Gly	Intact peptide	1.1	0.9	1.1	1.1	1.2	0.9	1.1
	Free sarcosine	2.1	0.3	0.3	0.9	9.7	1.9	2.2
Sar-Gly-Gly	Intact peptide	1.2	0.9	1.1	-	-	-	-
	Free sarcosine	1.4	0.4	0.3	-	-	-	-
Sar-Ser	Intact peptide	0.4	0.4	0.6	-	-	-	-
	Free sarcosine	6.7	0.4	0.4	-	-	-	-
Gly-Sar	Intact peptide	10.3	3.2	-	-	-	-	-
	Free sarcosine	0.8	0	-	-	-	-	-

The amounts of intact peptide and free sarcosine detected in barley embryos after 8h incubation in a 2mM solution of the appropriate sarcosyl peptide (in the presence or absence of additional amino acids/peptides, as indicated) are expressed as μm/g fresh wt. Each value is the average of at least four separate determinations.
- denotes not determined.

sarcosine uptake), the fact that the external concentration of free sarcosine never exceeds 0.05 mM during incubation with Sar-Gly, while the rate of sarcosine uptake is proportional to its external concentration (at least up to 2 mM; data not presented), makes this possibility untenable. Thus, Sar-Gly is absorbed intact. A certain proportion is hydrolysed intracellularly to give a pool of free sarcosine, most of which remains within the embryo.

Figures 4.1 and 4.2 show the kinetics of Sar-Gly uptake from a 2 mM solution. Intact peptide increases to a level of about 1.1 µm/g fresh weight within 4 h; this level then remains constant throughout any further incubation. However, the production of free sarcosine from Sar-Gly increases linearly for at least 24 h, after an initial lag of an hour or so. Thus, if the total Sar-Gly uptake is assumed to be the sum of intact peptide plus any free sarcosine produced, uptake remains approximately linear for at least 24 h.

The amount of intact Sar-Gly accumulated by barley embryos over a 4 h incubation period is proportional to its external concentration (fig. 4.3). As no further Sar-Gly is accumulated if the incubation period is extended to 6 h or 8 h, it seems that the peptide present in the embryo after 4 h represents the maximum amount which may be accumulated at any given external concentration.

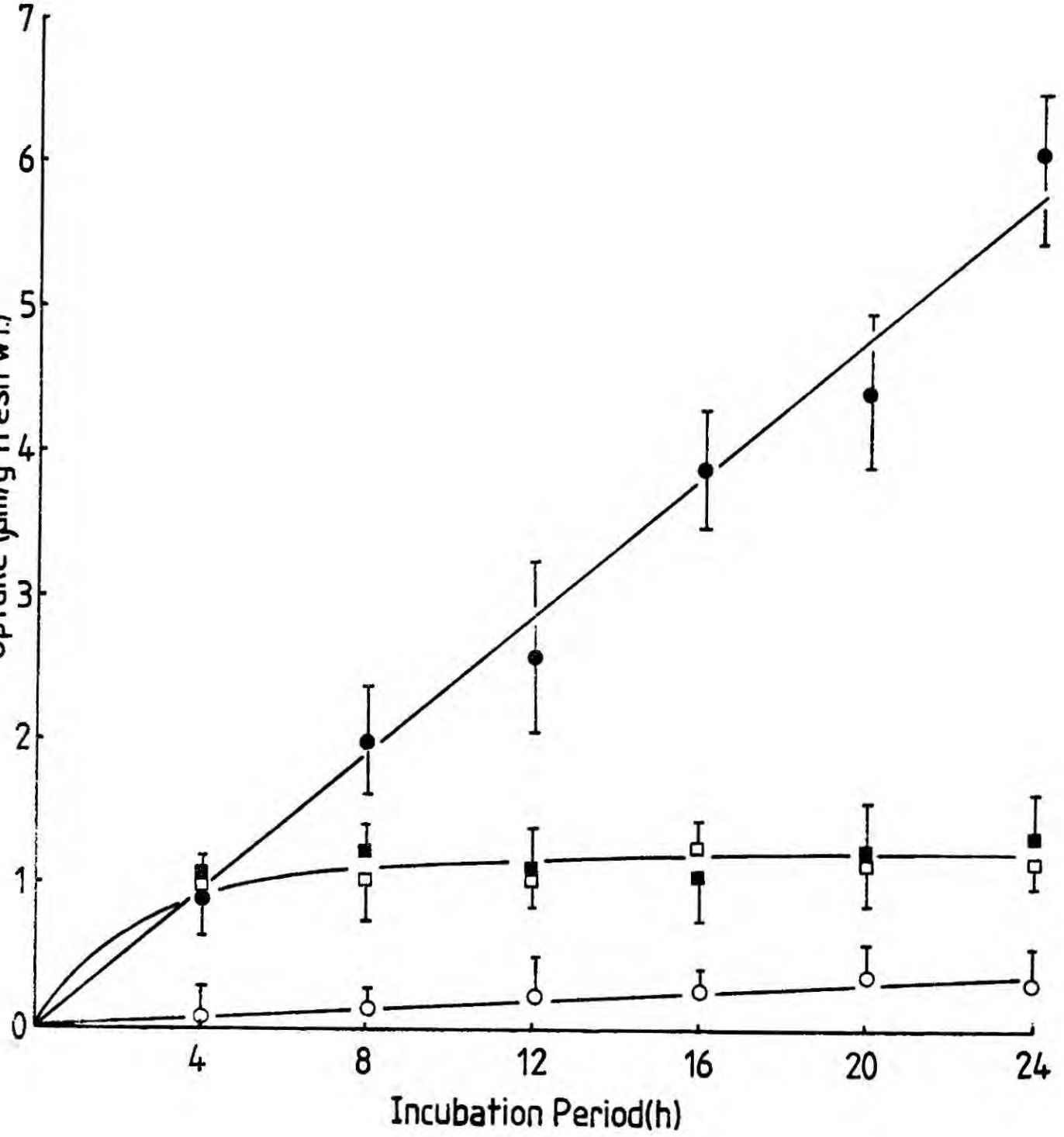


Figure 4.1: Uptake of Sar-Gly in the Presence and Absence of Gly-Ile: Long Incubation Periods

Sarcosine (●,○) and Sar-Gly (■,□), accumulated from 2mM Sar-Gly alone, (solid symbols) and in the presence of 8mM Gly-Ile (open symbols), were determined by embryo extraction. Incubations were carried out under standard conditions (section 2.3.1). Each value is the average of four separate determinations. Bars represent the range of values obtained.

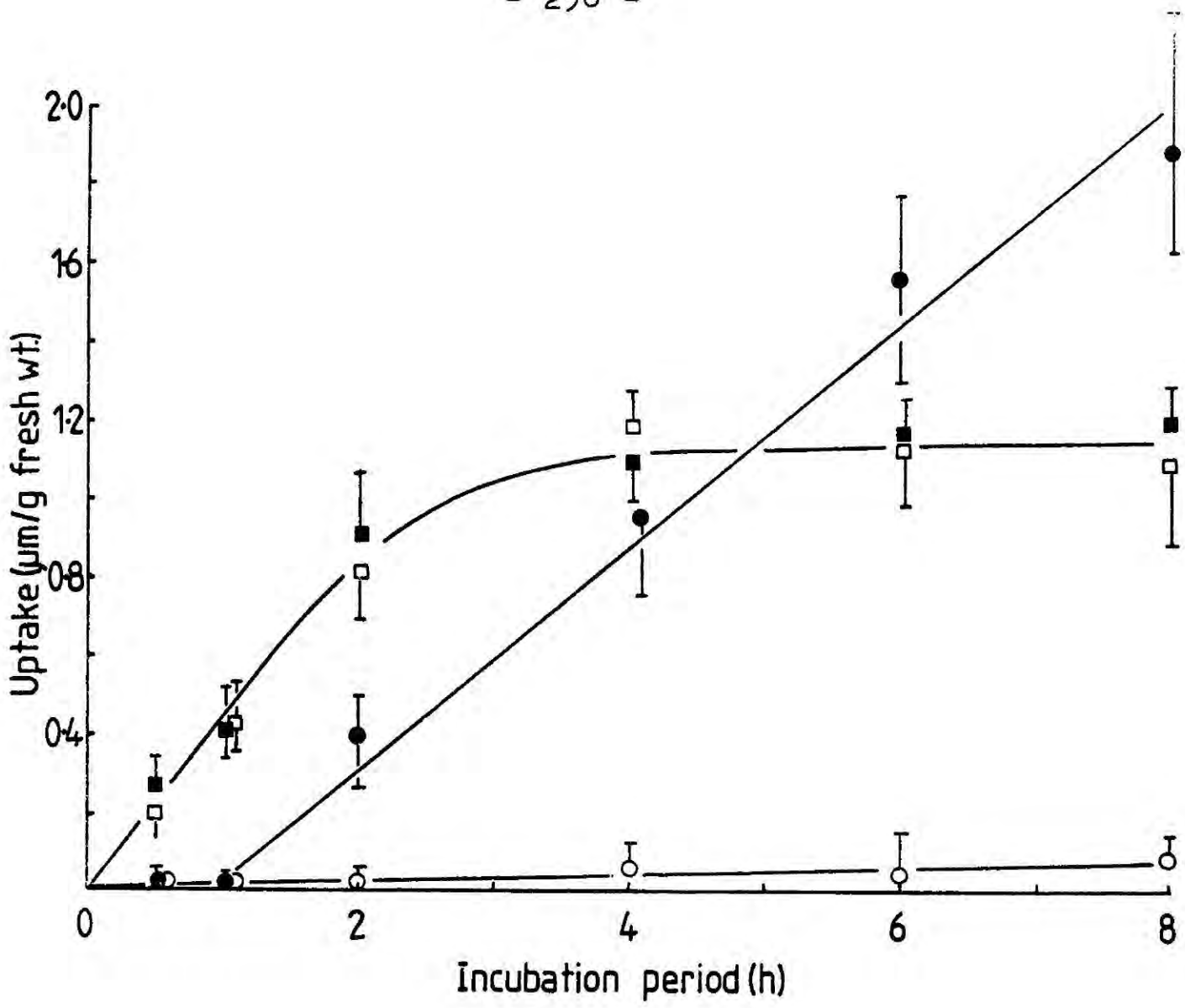


Figure 4.2: Uptake of Sar-Gly in the Presence and Absence of Gly-Ile: Short Incubation Periods

Sarcosine (●,○) and Sar-Gly (■,□), accumulated from 2mM Sar-Gly alone (solid symbols), and in the presence of 8mM Gly-Ile (open symbols), were determined by embryo extraction. Incubations were carried out under standard conditions (section 2.3.1). Each value is the average of four separate determinations. Bars represent the range of values obtained.

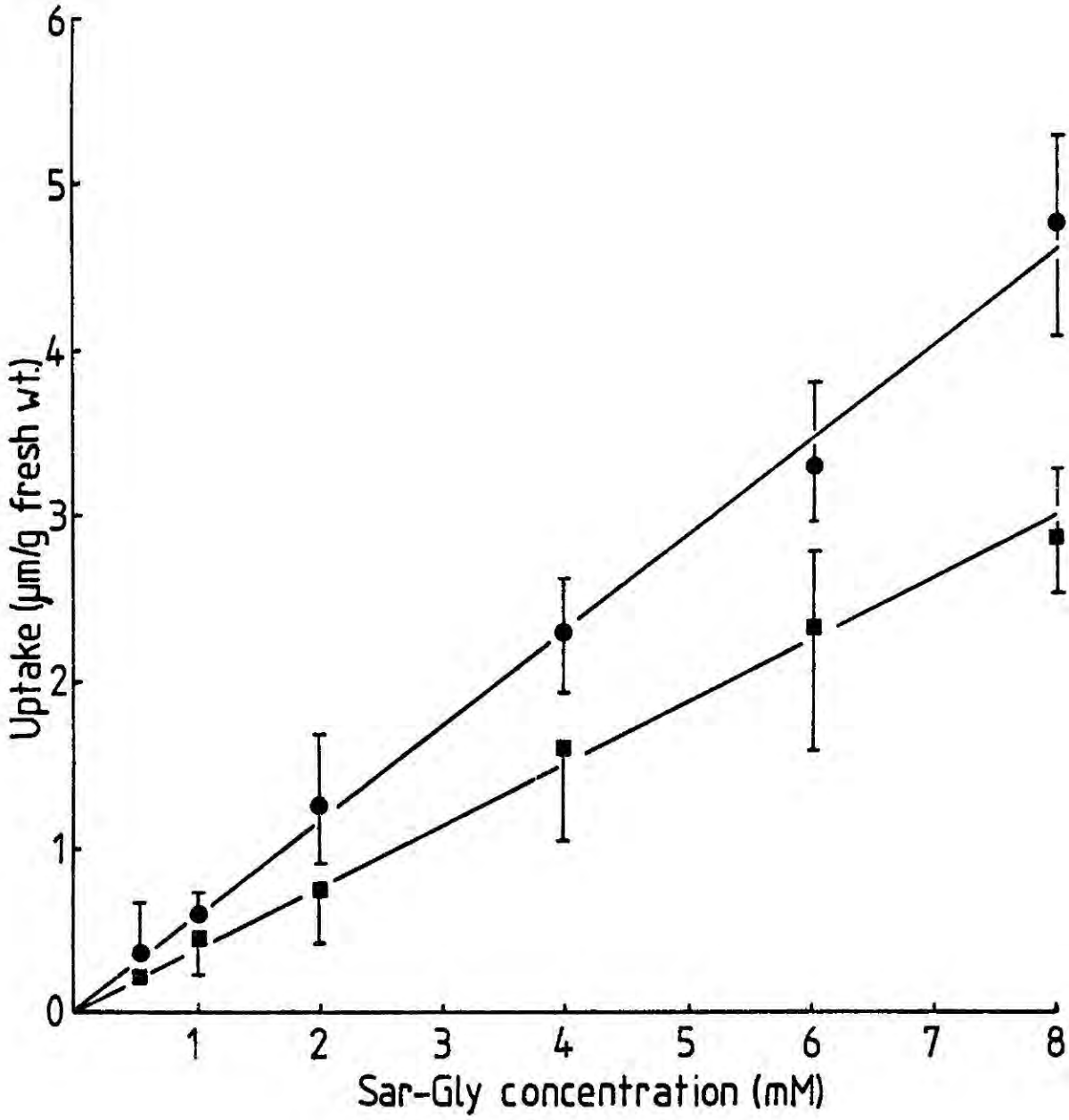


Figure 4.3: Effect of Medium Concentration on Sar-Gly Uptake

The accumulation of sarcosine (■) and Sar-Gly (●) by barley embryos was monitored by embryo extraction. Incubations were for 4h under standard conditions (section 2.3.1) with varying concentrations of Sar-Gly. Each value is the average of at least three separate determinations. Bars represent the range of values obtained.

Uptake of intact Sar-Gly shows an optimum at pH 4.0 (figure 4.4). This is similar to the pH optimum obtained for the uptake of Gly-Sar and a number of physiological peptides (section 3.1.3.9). However, although similar, the pH profile for the hydrolysis of Sar-Gly, as evinced by the appearance of free sarcosine, is clearly different from that for the uptake of intact peptide. This provides additional evidence that peptide uptake and hydrolysis are separate processes (see also section 3.1.4.3).

4.3.2 Inhibition of Sarcosylglycine Uptake

In attempting to demonstrate that accumulation of intact Sar-Gly is mediated by the peptide transport system, a number of unexpected results were obtained. Peptides (e.g. Gly-Ile, Ala-Ala-Ala), which from previous experience would have been expected to competitively inhibit transport, had no significant effect on the accumulation of intact Sar-Gly, Sar-Gly-Gly or Sar-Ser over an 8 h incubation period (table 4.1), although in a similar period they reduced the intact uptake of Gly-Sar several-fold (see also section 3.1.3.8). However, the production of free sarcosine within the embryo, as a result of peptidase activity, was very considerably reduced by these physiological peptides. This reduction was not simply due to increased rates of exodus, as sarcosine was indetectable in the incubation medium.

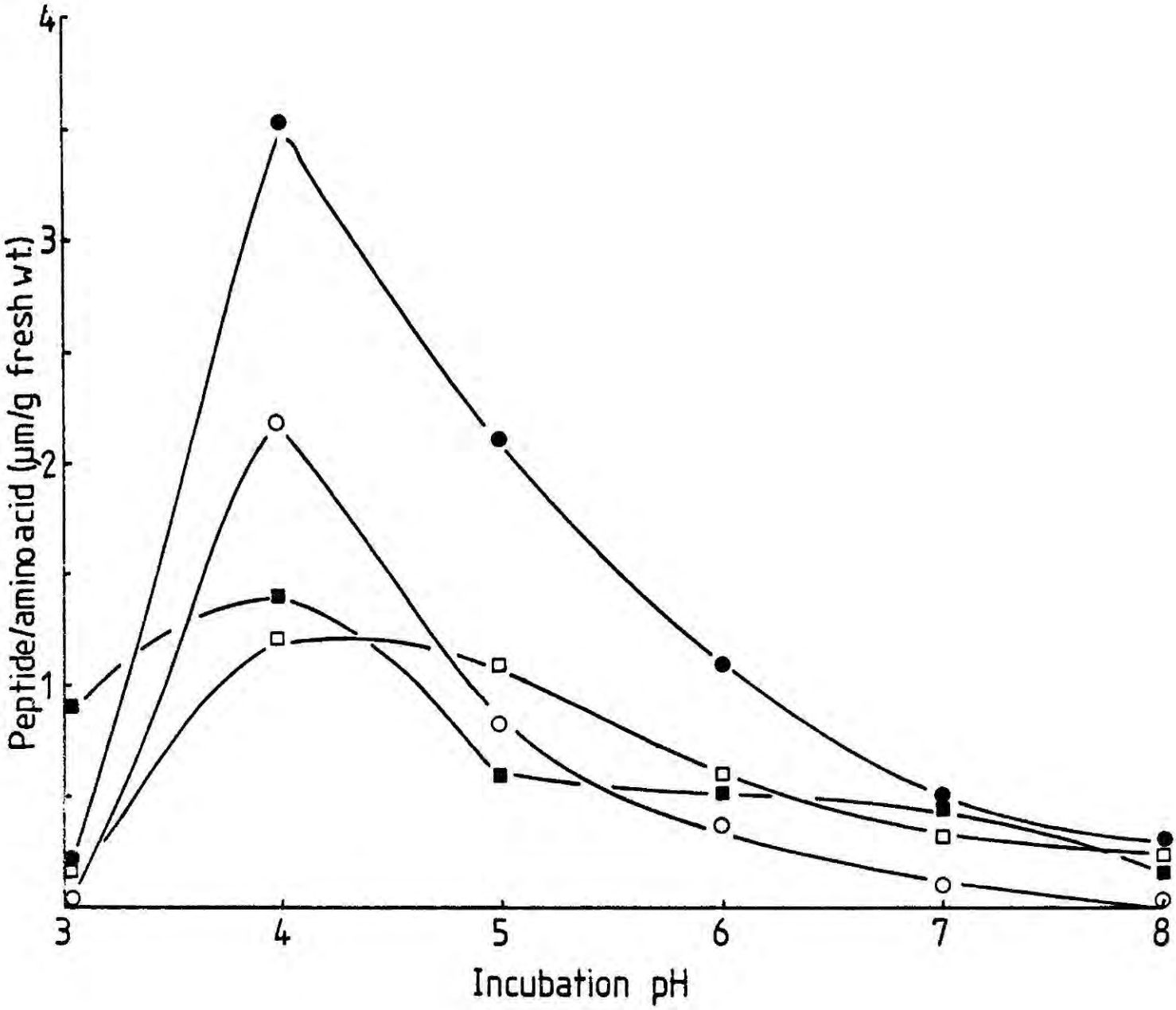


Figure 4.4: Effect of pH on Sar-Gly Uptake

Embryos were incubated for 8h in 2mM Sar-Gly at the appropriate pH and the amounts of Sar-Gly (■) and free sarcosine (○) accumulated, determined by embryo extraction. In addition, the free sarcosine appearing in the medium (□) was monitored using dansyl chloride. The total amount of free sarcosine (embryo plus medium) is also given (●). Each value is the average of two separate determinations.

The effects of Gly-Ile on Sar-Gly uptake were examined after various incubation periods (figs. 4.1 and 4.2). Clearly, Gly-Ile has little or no effect on the accumulation of intact Sar-Gly, leaving even the initial rate of uptake unaffected, yet the production of free sarcosine by hydrolysis is markedly reduced.

Metabolic inhibitors have a similar effect on Sar-Gly uptake as does Gly-Ile (table 4.2). This similarity is also observed if the kinetics of inhibition are examined (results not presented). Again, although the uptake of intact peptide is unaffected by the metabolic inhibitors, the production of free sarcosine is greatly reduced.

Free amino acids, however, unlike peptides, have no detectable effect on the accumulation of intact Sar-Gly or free sarcosine derived from it (table 4.1).

4.3.3 Inhibition of Uptake of Glycylsarcosine and Physiological Peptides

In order to ascertain the unusual nature of the effects of the above 'inhibitors' on the uptake of Sar-Gly, their effects on a number of known substrates of the peptide transport system were also studied.

Gly-Sar is taken up intact by barley embryos, with little hydrolysis (section 3.1.3.2). Both Gly-Ile (table 4.1) and DNP (table 4.2) strongly inhibit this intact

Table 4.2: Effect of Metabolic Inhibitors on the Uptake of Sarcosyl Peptides

Inhibitor		None	Sodium Acetate (5mM)	Sodium Azide (1.5mM)	DNP (0.3mM)	CCCP (0.1mM)
Sar-Gly	Intact peptide	1.1	0.8	1.1	0.9	1.2
	Free sarcosine	2.1	0.3	0	0	0
Gly-Sar	Intact peptide	10.3	-	-	3.0	-
	Free sarcosine	0.8	-	-	0	-

The amounts of intact peptide and free sarcosine detected in the embryo after 8h incubation in a 2mM solution of the appropriate sarcosyl peptide (together with an inhibitor, as indicated) are expressed as μm/g fresh wt. Each value is the average of at least two separate determinations. - denotes not determined.

accumulation (in agreement with results presented previously; sections 3.1.3.8; 3.1.3.10).

Trialanine was found to be almost completely removed from the medium by barley embryos over an 8 h incubation period. However, in the presence of 8 mM Gly-Ile or 0.3 mM DNP, little or no loss from the medium could be detected (results not presented). Table 4.3 shows in detail the effects of DNP and Gly-Ile on trialanine uptake. In the absence of these inhibitors, almost all the trialanine initially present in the medium is absorbed over an 8 h period and hydrolysed to free alanine. No intact peptide can be detected within the embryo. Gly-Ile, however, inhibits trialanine uptake. The lack of any increase in free alanine in the embryo or medium shows that hydrolysis is also prevented. This seems to be due to the inhibition of uptake (preventing trialanine gaining access to the peptidases), rather than to competition for the peptidases per se, as intact trialanine does not accumulate within the embryo.

In the presence of DNP, trialanine can be detected intact within the embryo. An increase in the overall level of free alanine indicates that some hydrolysis has also occurred (unless the increase is actually DNP-induced synthesis of β-alanine; section 3.3.3.3). Nevertheless, DNP still

Table 4.3: Uptake of Trialanine in the Presence of Various Inhibitors

Additives to the incubation medium		None		Trialanine (2mM)		Trialanine (2mM) + DNP (0.3mM)		Trialanine (2mM) + Gly-Ile (8mM)	
		Embryo	Medium	Embryo	Medium	Embryo	Medium	Embryo	Medium
Amino acid/ peptide concentrations in the embryo (μm/g fresh wt.) and medium (μm/ml	Trialanine	0	0	0	0.12	1.3	2.0	0	2.0
	Alanine	29	0.45	97	2.3	2.1	2.6	27	0.30
	Glycine	1.2	0.02	1.3	0.03	0.1	0.08	19	0.32
Total amounts of amino acid/ peptide (embryo plus medium) (nm)	Trialanine	0		120		2065		2000	
	Alanine	1840		6910		2700		1620	
	Glycine	78		92		85		1250	

Embryos were incubated under standard conditions (section 2.3.1) with the appropriate compounds added to the medium as indicated. Peptides/amino acids in the embryo and medium, after 8h incubation, were determined using dansyl chloride. Each value is the average of two separate determinations.

inhibits trialanine uptake by at least 85%.

Evidence has already been presented which shows that DNP can cause equilibration of amino acids across the cell membrane (section 3.3.2.4). This is also illustrated by the results presented in table 4.3. While some alanine and glycine leak into the medium in the absence of DNP, a concentration gradient of at least 20:1 in favour of the embryo is still maintained. However, in the presence of 0.3 mM DNP, similar concentrations of both alanine and glycine are achieved in the embryo and medium. This is the result of exodus, rather than metabolism, as the total amount of each amino acid (in the embryo plus medium) does not vary.

Gly-Ile and DNP have similar effects on diglycine uptake as they do on trialanine uptake. After 8 h incubation in a 2 mM solution, no intact diglycine can be detected in barley embryos. However in the presence of 0.3 mM DNP, intact diglycine accumulates to the level of 0.9 μm/g fresh weight.

Unlike the L-peptides employed above, D-peptides which are not taken up by barley embryos (e.g. D-Leu-D-Leu, D-Ala-D-Ala-D-Ala; section 3.2.5) are not detected intact in embryo extracts after incubation with DNP. Thus, although DNP allows amino acids and L-peptides to equilibrate across the membrane, these D-peptides seem to be totally

excluded from the embryo.

4.3.4 Peptide and Amino Acid Exodus

Sar-Gly also behaves rather anomalously when exodus from the embryo is monitored. Embryos preincubated for 4 h in 2 mM Sar-Gly accumulate similar levels of the intact peptide and free sarcosine. However, during a further 4 h incubation in the absence of an external supply of Sar-Gly, all the accumulated peptide is lost from the embryo and recovered intact in the medium, while only a small proportion of the free sarcosine undergoes exodus (table 4.4). The presence of Gly-Ile in the medium does not seem to affect this exodus.

Gly-Sar behaves in a similar manner to free sarcosine; only a proportion of the accumulated peptide undergoes exodus over 4 h (table 4.5). Free isoleucine, derived from the uptake of Gly-Ile, also shows only partial exodus; 25% is leached in 4 h (table 4.6). However, intact trialanine and diglycine, accumulated during incubation in the presence of DNP, are rapidly lost to the medium, in a manner similar to intact Sar-Gly (table 4.5).

DNP increases exodus; greater proportions of both sarcosine (derived from Sar-Gly) and Gly-Sar are lost from the embryo into DNP than into buffer alone. However, even in DNP some of each compound is retained within the

Table 4.4: Exodus of Sar-Gly and Sarcosine from Barley Embryos

		Sarcosine and Sar-Gly in Embryos and media (nanomoles)			
		After Preloading	After exodus into:		
			Buffer Alone	Buffer + DNP (0.3mM)	Buffer + Gly-Ile (8mM)
Sar-Gly	Embryo	55	0	0	0
	Medium	0	44	54	47
Sarcosine	Embryo	45	33	12	29
	Medium	0	8	36	18

Embryos were preloaded during 4h incubation with 2mM Sar-Gly under standard conditions (section 2.3.1). Exodus was into fresh buffer lacking Sar-Gly but containing certain additives, as indicated (see section 4.2.2 for methods). The amounts of Sar-Gly and sarcosine present in the embryos and media after preloading, and after a further 4h 'exodus period', were determined using dansyl chloride. Each value is the average of at least two separate determinations.

Table 4.5: Exodus of Gly-Sar, Trialanine and Diglycine from Barley Embryos

		Peptide present in embryos and media (nanomoles)			
		After Preloading	After exodus into:		
			Buffer Alone	Buffer + DNP (0.3mM)	Buffer + Azide (1.5mM)
Gly-Sar	Embryo	254	136	99	52
	Medium	0	136	180	197
Trialanine	Embryo	61	0	-	-
	Medium	0	52	-	-
Diglycine	Embryo	42	0	-	-
	Medium	0	47	-	-

Embryos were preloaded during incubation with 2mM peptide for 4h (Gly-Sar) or 8h in the presence of 0.3mM DNP (Diglycine and Trialanine). Exodus was into fresh buffer lacking peptide but containing certain additives, as indicated (see section 4.2.2 for methods). The amounts of intact peptide inthe embryos and media after preloading, and after a further 4h 'exodus period', were determined using dansyl chloride. Each value is the average of at least two separate determinations.

Table 4.6: Exodus of Isoleucine (derived from Gly-Ile) from Barley Embryos

	Isoleucine present in embryos and media (nanomoles)	
	After Preloading	After Exodus
Embryo	1030	800
Medium	0	270

Embryos were preloaded during 4h incubation with 2mM Gly-Ile. The levels of free isoleucine in the embryos and media, after preloading and after a further 4h 'exodus period' in fresh buffer (lacking Gly-Ile), were determined using dansyl chloride (see section 4.2.2 for methods). Each value is the average of two separate determinations.

Table 4.7: Exodus of Endogenous Amino Acids from Barley Embryos

	Buffer alone		Buffer + DNP (0.3mM)	
	Embryo	Medium	Embryo	Medium
Proline	190	56	130	120
Valine	110	46	96	58
GAB	290	300	330	480
Iso-leucine	80	16	44	52
Leucine	100	20	78	60
Others	not determined			

Embryos were preincubated in buffer for 4h (six embryos/ml medium). The levels of each amino acid (nanomoles) in the embryo and medium after a further 4h incubation in fresh buffer (section 4.2.2), in the presence and absence of DNP, were determined using dansyl chloride. Each value is the average of two separate determinations.

embryo (tables 4.4 and 4.5).

The free amino acid pool of barley embryos also undergoes exodus; 25-30% of the amino acids are lost to the medium during 4 h incubation in buffer alone, 50-60% in the presence of DNP (table 4.7). These percentage losses are similar to those achieved for sarcosine and Gly-Sar. Thus, it seems that the loss of sarcosine and Gly-Sar from the embryo, both in the presence and absence of DNP, is part of a general exodus. Only Sar-Gly and intact trialanine and diglycine do not fit this pattern; far greater exodus of these peptides occurs than for any other peptide or amino acid.

The relative volumes of embryo and medium require that 95% of a compound must be lost from the embryo before equilibrium with the medium is attained. Thus, even after 4 h incubation in DNP, complete equilibration of the amino acid pool has not been achieved; compounds remaining in the embryo are still at about 20 times their concentration in the medium. Only Sar-Gly seems to reach equilibrium within the 4 h exodus period. The small amount of Sar-Gly remaining in the embryo at equilibrium would be indetectable by present methods.

4.3.5 Structure of the Scutellum

Figure 4.5 is an electron micrograph of the 24 h barley

Figure 4.5: Electron Micrograph of the 24h Scutellar Epithelium

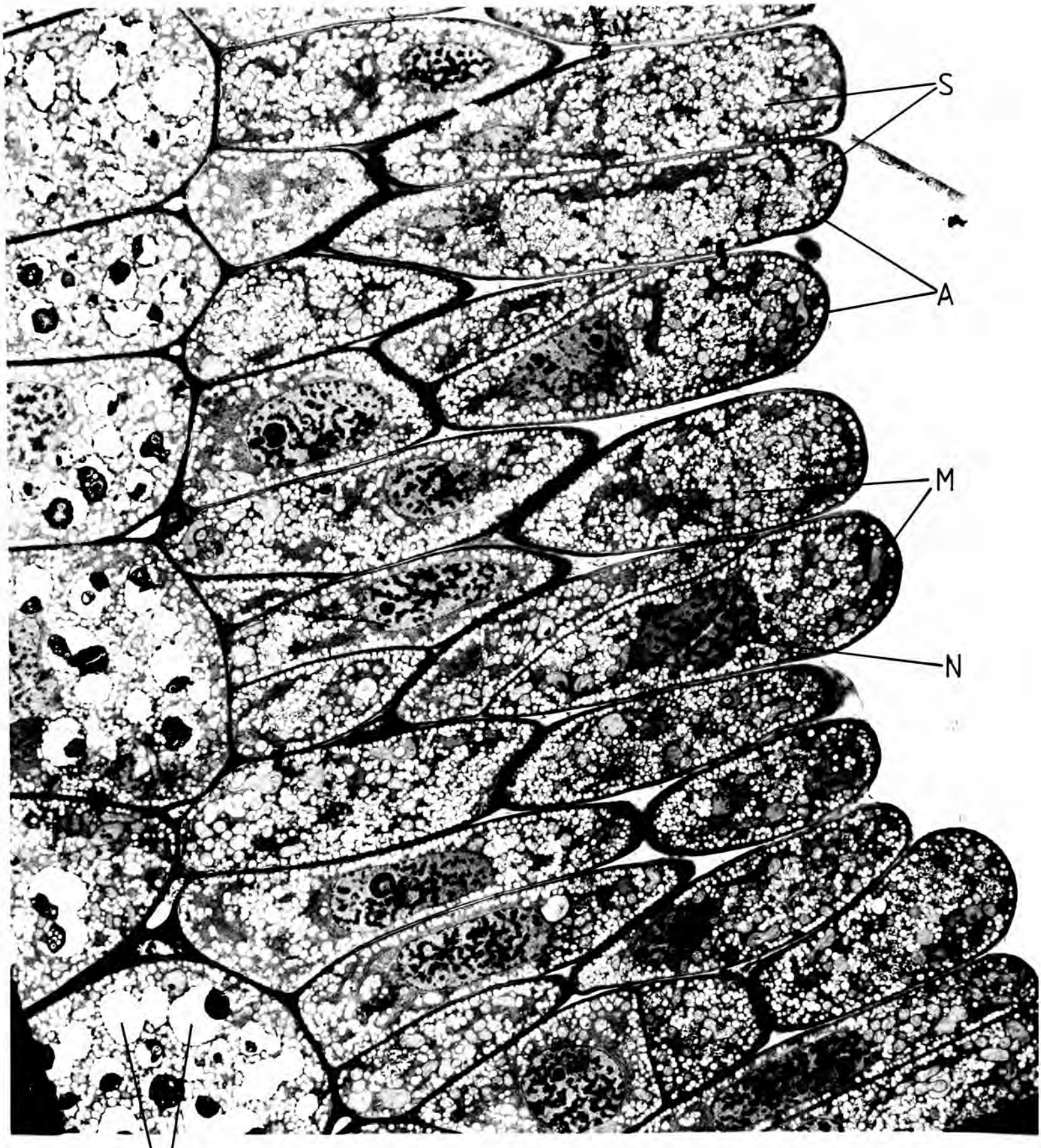
A = Elongated epithelial cells

M = Mitochondria

N = Nuclei

P = Protein bodies (partially empty)

S = Spherosomes (lipid bodies)


S
A
M
N
P
1µm

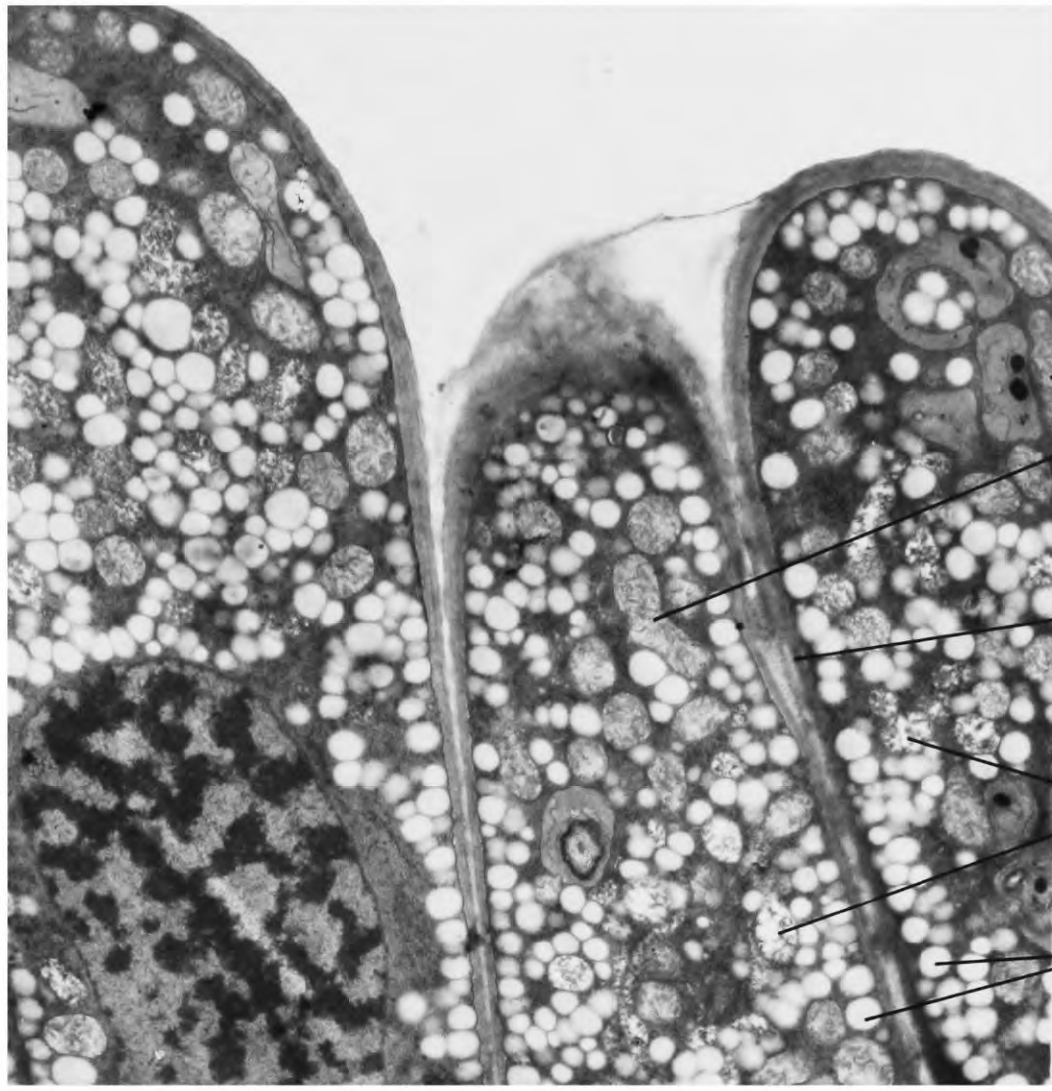
Figures 4.6a & b: Electron Micrographs of the Barley Scutellum

(a) Scutellar epithelial cells

(b) Subscutellar cells, located 2-3 cell layers below the epithelial cells.

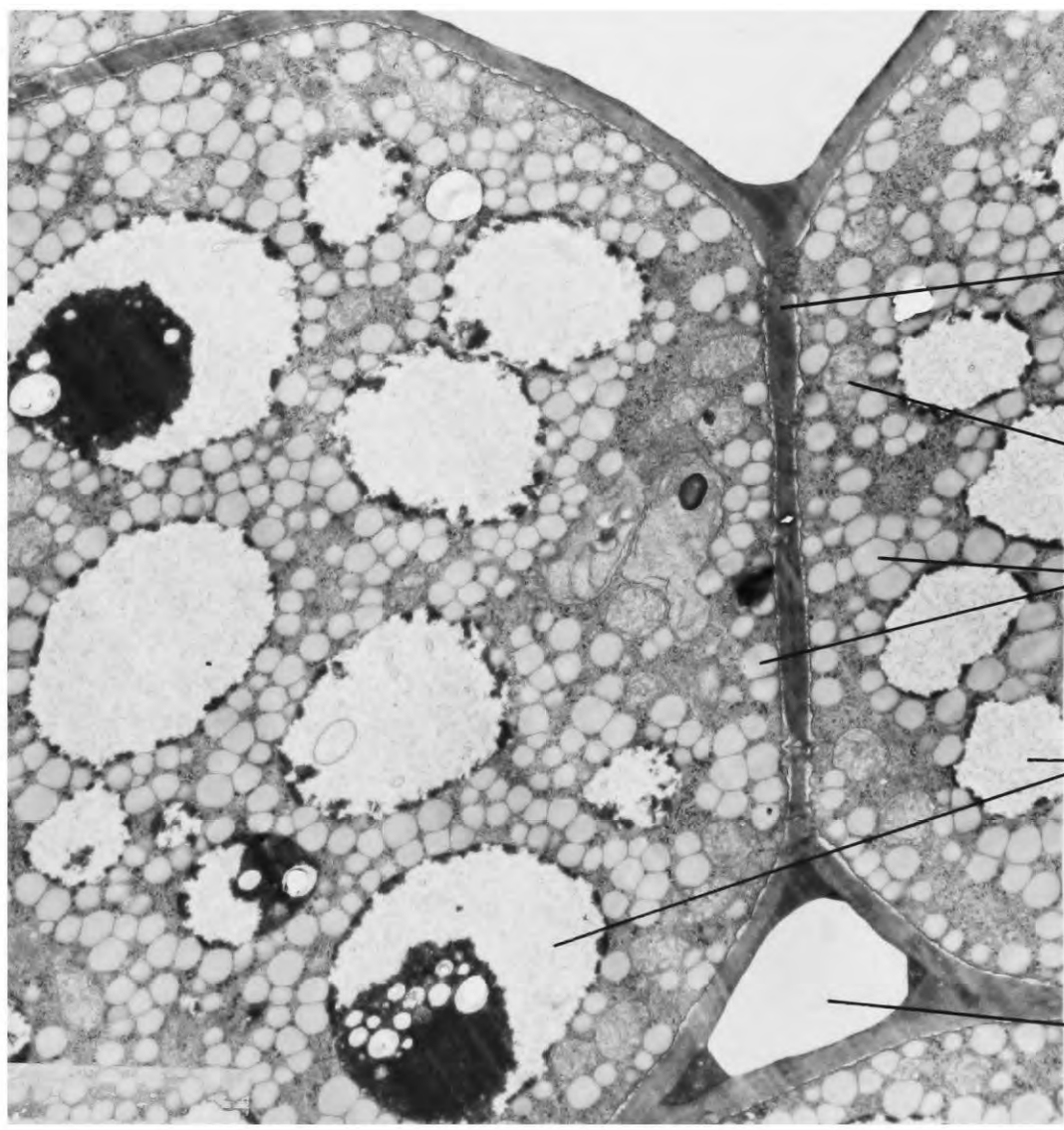
C = Cell walls
I = Intracellular spaces
M = Mitochondria
P = Protein bodies
S = Spherosomes (lipid bodies)

(a)



5µm

(b)



Figures 4.7a & b: Electron Micrographs of the Barley Embryo

(a) Cells from the base of the primordial shoot.

(b) Cells from the base of the primordial root.

C = Cell wall
I = Intracellular space
M = Mitochondria
P = Protein bodies/vacuoles
S = Spherosomes (lipid bodies)

(a)

I

C

M

S

P

5µm

(b)

C

M

P

S

scutellum. Two layers of elongated cells, the scutellar epithelial cells, are present. The cells are in fact rather more elongated than is indicated here, due to the angle at which the section was cut. Beneath the epithelial layers are the larger, isodiametric sub-scutellar cells. The cell walls are relatively thin, as might be expected in an absorptive tissue, and there is little extracellular space within the scutellum or embryo (<10% of the total embryo volume).

The scutellar cells contain large numbers of spherosomes (lipid bodies); the majority of the lipids required for germination are stored in the scutellum (Dure, 1960). However, these inclusions are rather fewer in cells further from the absorptive surface (figure 4.7a), and more or less absent in the shoots and roots. Thus, although the cytoplasm apparently only occupies a small proportion of the total cell volume of the epithelial cells, taking the embryo as a whole at least half the cellular space appears to be free cytoplasm.

The scutellum also contains numerous protein bodies. While the protein bodies in the 24 h scutellar epithelium appear to be rather full of protein (figs. 4.5 and 4.6a), indicating little digestion, the protein bodies of the subscutellar layers are relatively empty (figs. 4.5 and 4.6b).

It is of course possible that the emptiness of these protein bodies is an artefact of preparation, although this seems unlikely to be the case considering the methods of fixation used (N. Harris, personal communication), and the fact that the protein bodies in the epithelial layers remain full, despite being subjected to the same fixation methods. It has been reported elsewhere that the protein bodies of barley scutella can empty within 24 h (Nieuwdorp & Buys, 1964; Zamski, 1973).

Examination of scutellar cells further from the absorptive surface shows that, here, the protein bodies are greatly expanded yet still relatively empty, such that they begin to resemble a typical central vacuole (figs. 4.7a & b). Empty vacuoles are also present in the root and shoot tissues. Thus, even after only 24 h of germination, there seems to be a considerable amount of free space within the protein bodies/vacuoles which would presumably be available for the storage of metabolites.

4.4 Discussion

The amount of Sar-Gly accumulated by barley embryos is relatively small. Consequently estimation by dansyl chloride is only likely to be accurate to ± 20%. However, this lack of accuracy, while making it impossible to perform certain experiments, does not affect any of the general conclusions drawn from the data.

For the purposes of this discussion it is assumed that once absorbed by the scutellum, peptides, and the amino acids derived from them, are evenly distributed throughout the embryo. There is a certain amount of evidence to suggest this is the case (section 3.1.3.3). In addition, the cells of the embryo are assumed to be identical in their ability to absorb and hydrolyse peptides.

Sar-Gly is taken up intact by barley embryos. Within the cell a proportion of the peptide is hydrolysed to its constituent amino acids, although a threshold level of peptide seems to be required before hydrolysis can commence. However, the uptake of this peptide exhibits a number of rather anomalous characteristics. The possibility that its entry into the cell is independent of the peptide transport system must therefore be considered.

4.4.1. Sarcosylglycine is not Bound Extracellularly

A certain proportion of the barley scutellum is occupied by extracellular space, including the cell wall. The possibility that intact uptake of Sar-Gly is the result of binding to specific extracellular sites, rather than uptake across the plasmalemma, should therefore be considered. However, for a number of reasons this seems unlikely:

(i) The extracellular space only occupies a limited proportion (less than 10%) of the total embryo volume

(section 4.3.5). Thus, the observed concentration of Sar-Gly within the embryo as a whole (1.1 µm/g fresh wt.) would represent a concentration of at least 10 µm/ml if totally confined to the extracellular space. This is five times the medium concentration, and therefore represents considerable accumulation against a gradient. However, the accumulated Sar-Gly is readily leached from the embryo. This is difficult to explain if the peptide is indeed bound extracellularly against a concentration gradient.

(ii) During incubation with 2 mM Sar-Gly, the amount of intact peptide which can accumulate is limited (no further uptake occurs after about 4 h incubation). In terms of extracellular binding this would seem to be due to the occupation of all the available binding sites. Why then, do more sites apparently become available to bind Sar-Gly as the medium concentration is increased?

(iii) Physiological peptides do not appear to be bound in the extracellular space; intact accumulation is not observed. This may be due to their rapid removal from the binding sites as a preliminary to transport. However, this explanation seems unlikely as D-peptides, which are not transported, also show no intact accumulation. Thus, if Sar-Gly is bound within the cell wall, the binding site must be very specific. It seems unlikely that such a specific binding site will exist.

Thus, the accumulation of intact Sar-Gly seems unlikely to be the result of extracellular binding. The peptide is almost certainly taken up intact, across the plasmalemma.

4.4.2 Evidence for a Second Transport System

If Sar-Gly were handled by the peptide transport system characterized previously, other peptides might be expected to compete for, and therefore inhibit, uptake. However, although overall uptake (Sar-Gly plus free sarcosine) is indeed reduced by several physiological peptides, the kinetics of inhibition do not suggest direct competition for a single transport system. Only the production of free sarcosine is inhibited; uptake of the intact peptide remains completely unaffected. Thus, the initial rate of Sar-Gly uptake, and the total amount of peptide which can be accumulated, are not affected by the presence of Gly-Ile.

The simplest explanation for this anomalous behaviour is to propose that Sar-Gly (and the other N-methylated peptides tested) enters the cell through a transport system (system B) which is distinct from the general peptide transport system (system A). The possible arrangement of these two systems within the cell is discussed below (section 4.4.7). Although it seems that a considerable proportion of Sar-Gly uptake is mediated by system B, the

possibility that a certain proportion might also enter via system A (however unlikely; section 4.4.7) cannot be eliminated.

4.4.3 Intact Sarcosylglycine Uptake is a Passive Process

It can be calculated that, whatever the extracellular concentration, intact Sar-Gly accumulates to an equivalent concentration intracellularly, assuming that about 60% of the tissue volume is available to the peptide. Judging from electron micrographs this seems to be a reasonable assumption (section 4.3.5). A similar equilibrium is noted for intact Sar-Gly-Gly uptake. The non-equilibrium levels of intact Sar-Ser accumulated may well reflect its particular susceptibility to peptidase action, as evinced by the high levels of free sarcosine produced (table 4.1).

DNP, which causes free amino acids and peptides to equilibrate across the membrane (sections 3.3.3.2; 4.3.4), does not affect intact Sar-Gly uptake, indicating that Sar-Gly achieves equilibrium of its own accord. DNP also permits the intact uptake of trialanine and diglycine, again apparently due to equilibration across the membrane. The levels of these physiological peptides which accumulate within the embryo are similar to those achieved by Sar-Gly in the absence of DNP. This again indicates that, under normal conditions, intact Sar-Gly within the embryo is at equilibrium

with the external medium.

In addition, it seems that the intact uptake of Sar-Gly does not require a supply of metabolic energy. The uptake of peptides via the peptide transport system (system A) is prevented by a range of metabolic inhibitors (sections 3.1.3.10; 4.3.3). However, these same inhibitors have no effect on the intact uptake of Sar-Gly (although they do reduce the amount of sarcosine appearing in the embryo). Thus, the uptake of intact Sar-Gly, via system B, seems to be a passive process, reaching equilibrium (with a 2 mM external solution) within 3-4 h.

4.4.4 The Intact Uptake of Sarcosylglycine is Carrier Mediated

Although the intact uptake of Sar-Gly seems to be the result of passive equilibration across the membrane, it appears that this process is mediated.

Sar-Gly uptake shows a pronounced pH optimum. There is no obvious change in peptide protonation which occurs in this pH range. It therefore seems most likely that this optimum is due to a change in the state of protonation of a membrane carrier protein. Many peptides containing D-amino acids do not accumulate intact in the embryo, and neither do their hydrolysis products appear (section 3.2.5). Thus, the plasmalemma does not seem to be generally permeable to

peptides; transport, if it occurs, must be mediated. Similarly, D-peptides, unlike their L- counterparts, do not equilibrate across the membrane in the presence of DNP. Movement across the membrane, even in the presence of DNP, seems to require the operation of a specific transport system.

Thus, system B, which mediates the intact uptake of Sar-Gly by barley embryos, seems to operate by a process of facilitated diffusion. The system appears to be quite distinct from the peptide transport system previously characterized. The possible locations and roles of these two systems are discussed below (section 4.4.7).

4.4.5 Inhibition of Peptidase Activity

The fact that the production of free sarcosine from Sar-Gly is inhibited by Gly-Ile, while intact Sar-Gly uptake remains unaffected, has a number of important implications concerning the location of peptidases within the cell. Two possible situations may be envisaged:

(i) Sar-Gly transported by system B is inaccessible to peptidase activity. In this case, a certain proportion of Sar-Gly uptake (i.e. that component which is hydrolysed) must be mediated by system A. The inhibitory effects of Gly-Ile on the hydrolysis of Sar-Gly can then be simply explained by its ability to inhibit the uptake of Sar-Gly

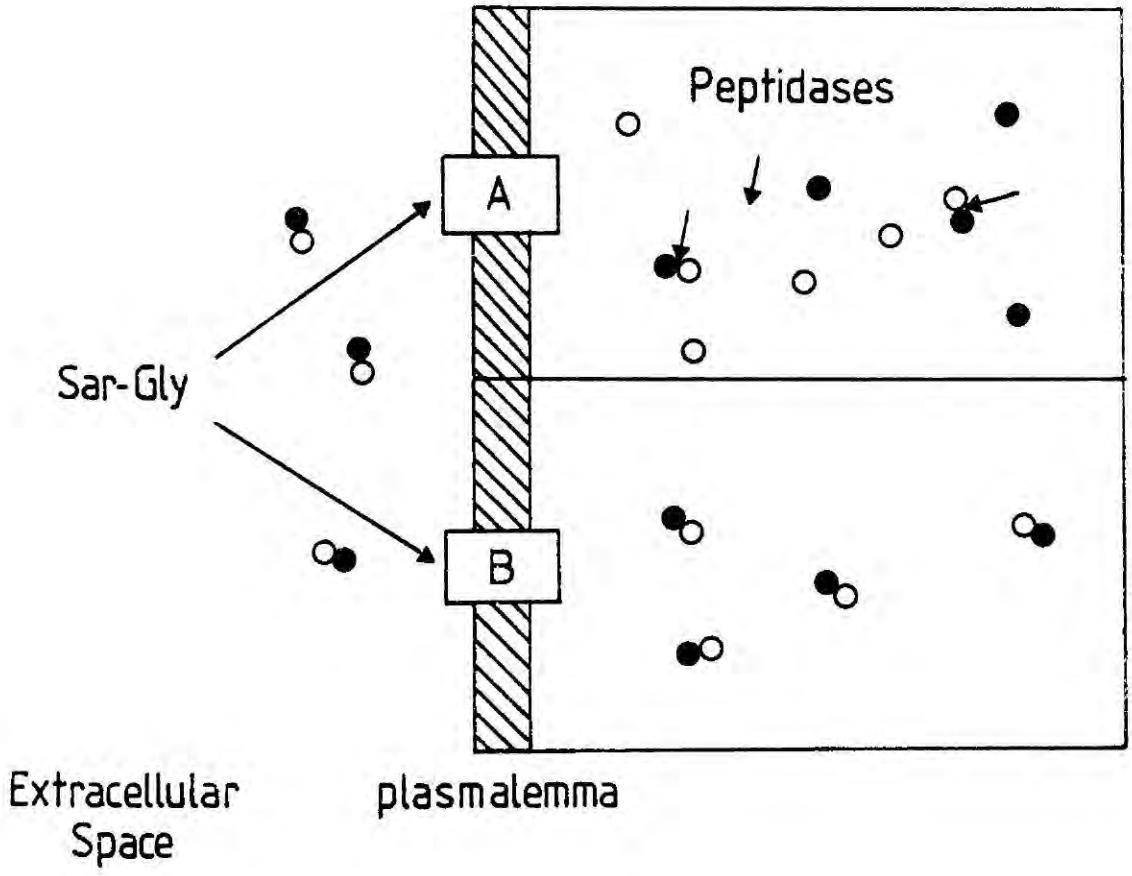
via system A, preventing it gaining access to the peptidases.

Such a model would require that either the peptidases are an integral part of transport system A (the peptide transport system), and consequently inaccessible to peptides entering the cell by other means (previous evidence indicates this is not the case; section 3.1.4.3), or that Sar-Gly entering the cell via system B enters a separate, peptidase-free compartment (see fig. 4.8). A similar model, involving parallel compartments, has been proposed to explain certain anomalies in the transport of sucrose by the maize scutellum (Garrard & Humphreys, 1969). However, there is no cytological evidence for a parallel arrangement of compartments within the plant cell, and evidence presented below (section 4.4.6) indicates that, if compartments exist, they are more likely to be arranged in series. In addition, it seems unlikely that a significant proportion of Sar-Gly uptake is mediated by system A (section 4.4.7). This model is therefore not considered further.

(ii) All Sar-Gly entering the cell has equal access to the peptidases. The inhibition of Gly-Sar hydrolysis can best be explained here, by proposing that Gly-Ile (and other physiological peptides) in some way interferes with peptidase activity. Thus, in the presence of Gly-Ile, the intact uptake of Sar-Gly via system B is unaffected and an equilibrium concentration is reached, as usual, within the

Figure 4.8: Parallel-Compartment Model for Sar-Gly Utilization

Two carriers (plain) are located in the plasmalemma (cross-hatched). Each carrier mediates entry into a separate compartment, only one of which contains peptidases (arrows). See text for discussion.



cell. However, peptidase activity is impaired and the production of free sarcosine, by hydrolysis, drastically reduced.

Gly-Ile could inhibit the hydrolysis of Sar-Gly in one of two ways; by direct competition for the active site(s) of the peptidases themselves, or by preventing Sar-Gly gaining access to the peptidases. Although present information does not permit a distinction to be made between these possibilities, evidence that the peptidases are compartmentalized within the cell (section 4.4.6.1) indicates that the latter is at least a possibility. This idea is indirectly supported by the inhibitory effects of DNP and a number of other metabolic 'inhibitors' on the hydrolysis of Sar-Gly. Like Gly-Ile, these 'inhibitors' do not affect the amount of intact Sar-Gly accumulated by barley embryos, although they drastically reduce the appearance of free sarcosine. Again, hydrolysis seems to be inhibited. It seems probable that these 'inhibitors' exert their effects through a general reduction in energy supply (sections 3.1.4.1; 3.3.3.5). As the hydrolysis of a peptide bond is not believed to require a direct input of energy,[a] it seems most

(a) However, it must be pointed out that there have been a number of recent reports that peptide hydrolysis may require a direct input of energy. These have yet to be explained (Goldberg & St. John, 1976; Etlinger & Goldberg, 1977; Murakami et al., 1977; St. John & Goldberg, 1978).

probable that a reduction in energy supply inhibits peptidase activity by preventing the active transport of peptides into a subcellular compartment containing the peptidases.

4.4.6 Compartmentation Within the Embryo

The different rates at which various peptides and amino acids undergo exodus from the embryo suggest the existence of two distinct compartments within the cell. Intact Sar-Gly leaks rather rapidly from the embryo, while sarcosine, derived from it by hydrolysis, is retained to a much greater extent. It is proposed that they are held in compartments X and Y respectively.

The relatively slow rates of exodus exhibited by the endogenous pool amino acids, accumulated Gly-Sar, and amino acids accumulated as a result of the uptake and hydrolysis of physiological peptides, indicate that these compounds are also present in compartment Y. Sar-Gly alone seems to be retained within compartment X. However, intact trialanine and diglycine, accumulated by the embryo in the presence of DNP, also show the same exodus characteristics as Sar-Gly; they too may be stored in compartment X.

Interestingly, Oaks (1965a) has also obtained evidence for two compartments within the maize scutellum, based on studies of leucine uptake and metabolism.

4.4.6.1 Compartmentation of Peptidases

It seems likely that peptidases are restricted to compartment Y. Amino acids arising from the hydrolysis of absorbed peptides, including sarcosine derived from Sar-Gly, leach rather slowly, suggesting they are both produced and stored in compartment Y. In addition, tri-alanine and diglycine, accumulated intact in the presence of DNP, undergo rapid exodus characteristic of compounds present in compartment X. These peptides can only exist intact in compartment X if it is devoid of peptidase activity. Further credence is lent to this view by the results discussed previously (section 4.4.5) which indicate that Sar-Gly initially enters a peptidase-free compartment and must be transferred to a second compartment before hydrolysis can occur.

4.4.6.2 Organization of Compartments

The fact that compounds present in compartment X are more readily leached than those in Y, indicates that X is most likely to be adjacent to the plasmalemma. This view is supported by the fact that intact Sar-Gly uptake can occur. If compartment Y, containing peptidases, separated X from the plasmalemma, Sar-Gly would be hydrolysed before it could enter the peptidase-free compartment X.

4.4.7 Models for Peptide Utilization

Two simple models (fig. 4.9) can be proposed which will accommodate all the above data, explaining both the evidence for compartmentation and the inhibitory effects of Gly-Ile and metabolic 'inhibitors' on Sar-Gly hydrolysis.

Model I

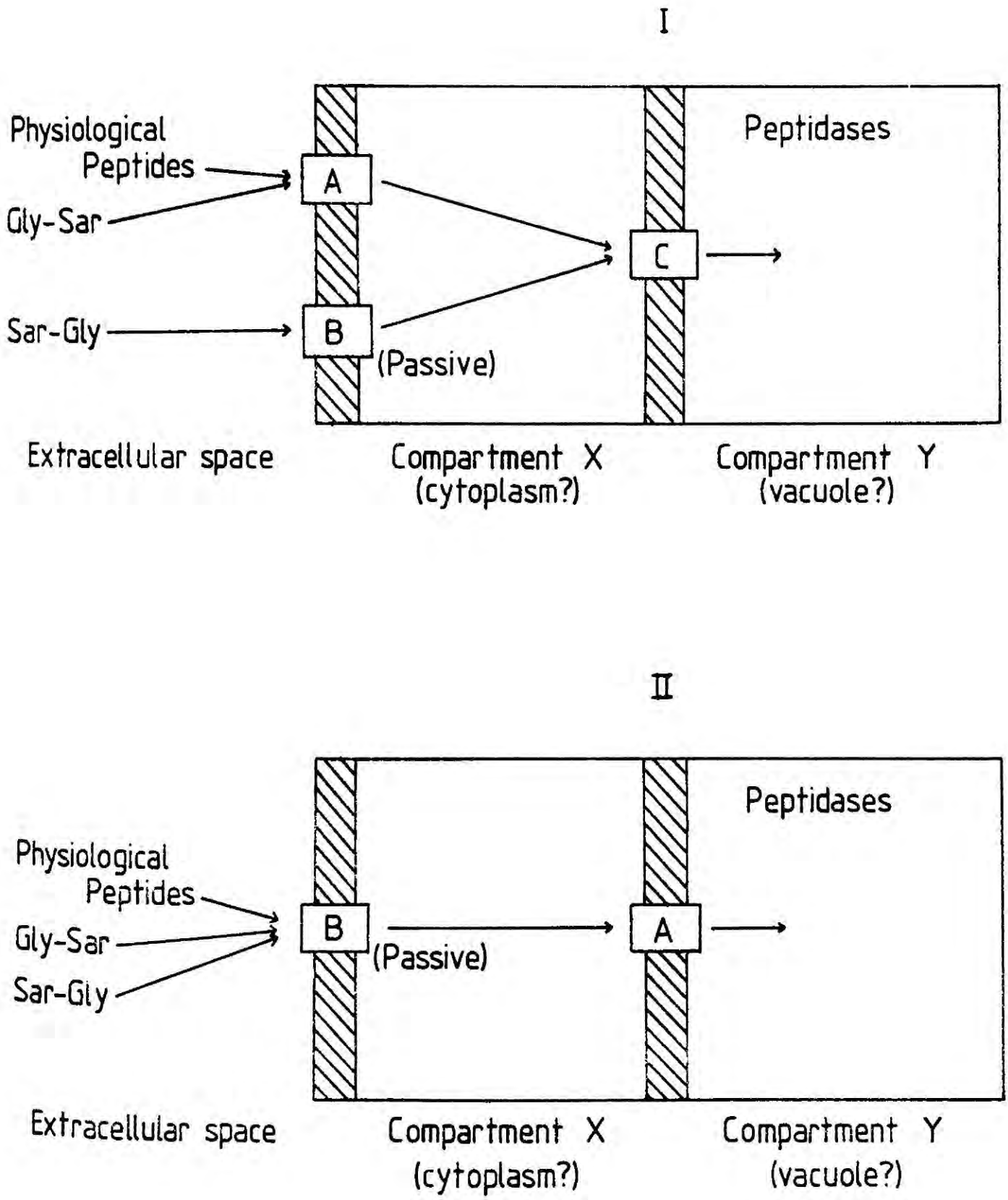
Both transport systems A and B are located in the plasmalemma. Physiological peptides enter the cell via the peptide transport system (system A), initially entering compartment X (peptidase-free). However, these peptides are immediately transferred to compartment Y (the hydrolytic compartment) via transport system C, located in the membrane separating the two compartments. The amino acids produced as a result of peptidase activity remain in compartment Y; the bulk of endogenous amino acids are also apparently stored in this compartment.

Gly-Sar is also handled by both systems A and C, yet is resistant to hydrolase activity; it therefore accumulates intact in compartment Y.

Sar-Gly, like all other peptides, initially enters compartment X intact, although its uptake across the plasmalemma is mediated by a second transport system, system B, which operates by a process of facilitated diffusion. However, Sar-Gly is a poor substrate for

Figure 4.9: Models for Peptide Utilization by Barley Embryos

Various carriers (plain) are located in membranes (cross-hatched). The membrane towards the left represents the plasmalemma, the membrane to the right, an intracellular membrane. See text for discussion.



transport system C, and is therefore only transferred from compartment X to compartment Y at a relatively slow rate. It can be replaced equally rapidly by uptake from the medium, maintaining a constant pool of intact Sar-Gly within the embryo. That proportion of Sar-Gly which is transferred to compartment Y is hydrolysed, the free sarcosine produced remaining within the hydrolytic compartment. The effects of DNP and Gly-Ile on Sar-Gly uptake can be explained as follows: these 'inhibitors' do not affect the uptake of Sar-Gly via system B, but prevent Sar-Gly entering the hydrolytic compartment Y, by inhibiting transport system C. Thus, although hydrolysis is unable to occur, Sar-Gly accumulates intact in compartment X until equilibrium with the external medium is reached.

The possibility that a proportion of Sar-Gly uptake into compartment X is mediated by system A cannot be eliminated. This does not affect the model. It simply requires that Gly-Ile and DNP also inhibit transport system A, which they are known to do (sections 3.1.3.7; 3.1.3.10). However, it seems unlikely that a significant proportion of Sar-Gly uptake is actually mediated by system A as diglycine, which is known to be a very poor competitive inhibitor of the peptide transport system (system A), inhibits the hydrolysis of Sar-Gly to a similar extent as does Gly-Ile.

If this model is correct, what is the physiological function of system B? It does not seem to be a second peptide transport system as it is unable to handle any of the physiological peptides tested. Similarly, the lack of inhibition by amino acids indicates that it is not an amino acid permease (at least for protein amino acids). Its handling of Sar-Gly is probably fortuitous; such peptides are unknown in barley. One possible natural substrate is betaine (N-trimethylglycine) recently reported to be present in germinating wheat in very large quantities (Chittenden et al., 1978). This question clearly requires further investigation.

Model II

In this model it is proposed that transport systems A and B are located on different membranes. System B is located on the membrane separating compartment X from the external environment and is capable of handling physiological peptides as well as Sar-Gly. It operates by a passive diffusion mechanism. Competition for uptake by this system is not observed as its capacity is relatively high compared with the peptide concentrations used. System A, however, is located in the membrane of compartment Y (the peptidase-containing compartment) and the observed competition between peptides for uptake (section 3.1.3.7) is actually competition for entry into this compartment.

Physiological peptides enter compartment X passively, via system B, and are then rapidly removed into compartment Y by system A. Once in compartment Y, hydrolysis takes place. Intact peptides do not accumulate in compartment X as they are transferred extremely rapidly to compartment Y.

Sar-Gly also enters compartment X via system B. In the absence of an inhibitor a proportion is transported into compartment Y, by system A, where it is hydrolysed. Gly-Ile inhibits the hydrolysis of Sar-Gly by competing for transport system A, thus preventing it gaining access to the hydrolases.

4.4.8 Identity of the Subcellular Compartments

Although it is not yet possible to eliminate either of the two models described above, both require the existence of two distinct cellular compartments, X and Y. Peptidase activity is restricted to compartment Y. What is the physiological nature of these compartments? No direct evidence is available to answer this question, although certain speculations can be made, based upon current understanding of the subcellular compartmentation of plant cells.

Compartment X: A number of lines of evidence suggest that compartment X is the cytoplasm. Firstly, compartment X apparently accounts for about 60% of the cell volume

(section 4.4.3). Only the cytoplasm occupies this sort of proportion of the cell (section 4.3.5). Secondly, compartment X separates Y from the external medium (section 4.4.6.2). Again, the cytoplasm, with other organelles embedded within it, fulfils this role. Thirdly, compartment X apparently lacks peptidases (section 4.4.6.1). There is now accumulating evidence to suggest that hydrolases are sequestered in vacuoles, and the cytoplasm may therefore be relatively free of such enzymes (see below).

Compartment Y: It seems likely that compartment Y is one (or more) of the organelles embedded within the cytoplasm of the cell. Although many types of organelle are found in plant cells, most can be given specific functions (e.g. peroxisome, glyoxysome) and occupy such a small proportion of the cell volume that they are unlikely to be significant in relation to peptide uptake. Both plant mitochondria (Day & Wiskich, 1977) and chloroplasts (Nobel & Cheung, 1972; McLaren & Barber, 1977) are known to take up amino acids via specific carriers. However, the absence of any obvious reason why these organelles should absorb or accumulate peptides, together with the relatively small proportion of the total cell volume which they occupy in barley embryos, suggests they will be of little significance here.

The organelles most likely to correspond with compartment Y are the various components of the vacuolar system. Although mature plant cells typically have a single, large central vacuole, recent studies have shown that a whole range of smaller vesicles, most commonly present in immature plant cells, are ontogenetically related to the main cell vacuole. Such vesicles often have rather specialized functions, yet are able to fuse both with each other and the central vacuole, one type of vesicle commonly being derived from another. Together with the central vacuole, these vesicles form the vacuolar system, often considered analogous to the animal lysosomal system (Matile, 1975, 1976, 1978).

Although from a size point of view, vacuoles are clearly an important organelle in many plant tissues, little direct evidence for their involvement in subcellular compartmentation has been obtained. This is mainly due to the labile nature of the tonoplast and the consequent difficulties in isolating intact vacuoles.

It is well known that vacuoles may accumulate substances, including amino acids, against a concentration gradient (Matile, 1976). This implies that metabolites can be selectively and actively transported across the tonoplast, although the suggestion that the amino acids in the vacuole

may not be in an osmotically active form (Matile, 1978) leaves this conclusion in some doubt. However, isolated vacuoles of yeast (Boller et al., 1975) and a higher plant, Hevea brasiliensis (Hanower et al., 1977), do have specific amino acid transport systems, and in the maize scutellum there are suggestions that sucrose is actively transported across the tonoplast (Humphreys, 1973). Animal lysosomes may even be able to absorb peptides (Coffey & de Duve, 1968; Lloyd, 1971; Goldman, 1973; Bouma et al., 1976).

In germinating barley embryos, the most important component of the vacuolar system would seem to be the protein bodies which eventually fuse to give rise to the larger, more typical vacuoles of plant cells.

Although after only 24 h of germination the protein bodies might be expected to be full of protein, with little free space in which to accumulate free amino acids, electron micrographs indicate that within the seedling as a whole there is considerable vacuolar space (section 4.3.5). In addition, the protein bodies of 24 h barley scutella have been reported to be relatively empty (Nieuwdorp & Buys, 1964; Zamski, 1973) and in 4-day maize scutella, the vacuoles apparently occupy 70% of the cell volume (Humphreys, 1973). It is therefore feasible that the bulk of the free amino acids of the barley embryo are stored within the vacuolar system, and that this is synonymous with compartment Y.

Further evidence that compartment Y is part of the vacuolar system is provided by the distribution of peptidases within the cell. Compartment Y contains the scutellar peptidases. Similarly, it seems that the vacuolar system, and in particular the protein bodies, form the hydrolytic compartment of plant cells. Endopeptidase activity is apparently localized within the protein bodies of peas (Matile, 1968), castor beans (Nishimura & Beevers, 1978, 1979), sorghum (Adams & Novellie, 1975b) and barley (Ory & Henningsen, 1969), and in the mature vacuoles from several species (Heftmann, 1971; Matile & Winkenbach, 1971; Parish, 1975). Carboxypeptidase also seems to be a vacuolar enzyme (Nishimura & Beevers, 1978). In certain mature vacuoles proteolytic enzymes have been reported as absent (Butcher et al., 1977), although this may reflect either specialization or a change in function during vacuolar development. Thus, if the vacuolar system is indeed the hydrolytic compartment of the plant cell, it would seem, perhaps naïvely, that compartment Y is also part of this system.

However, although proteases are apparently located within the vacuolar system, nothing is known concerning the sub-cellular location of peptidases within the barley scutellum. Although one might expect them to be sequestered in the

hydrolytic compartment, the pH optima of the peptidases puts this assumption in doubt. The vacuolar fluid is generally considered to be maintained at about pH 4.0 (Kurkdjian & Guern, 1978) and those enzymes known to be present in the vacuoles have acidic pH optima (section 1.3.4.1). However, barley peptidases have pH optima at about pH 8.0 (section 1.3.4.4). Unless further enzymes remain to be characterized, it seems that, if barley peptidases are located within the vacuolar system, the system itself must be compartmentalized to provide a sub-environment at alkaline pH. It is well known that the plant cell does indeed contain a variety of different types of vesicle, probably part of the vacuolar system, which may have specialized internal environments. One such species of vesicle, the spherosome, is particularly abundant in the barley scutellum (section 4.3.5). Spherosomes are small, spherical vesicles characterized by their high lipid content, and believed to be the site of lipid storage in seeds. However, considerable diversity is found amongst this group of organelles and their functions are only poorly understood. They are believed to originate from the endoplasmic reticulum and to be part of the vacuolar system (Buttrose, 1963a,b; Frey-Wyssling et al., 1963) and there have been suggestions that they too are involved in protein hydrolysis. Spherosomes are often found in very close association with

protein bodies (Buttrose, 1963b; Paleg & Hyde, 1964; Jones, 1969a,b,c; Jacobsen et al., 1971; Adams & Novellie, 1975a). It has even been suggested that their membrane may be continuous with that of the protein bodies (Buttrose, 1971). In addition, several reports that they contain proteases have appeared (Matile et al., 1965; Semadeni, 1967; Matile & Spichiger, 1968; Adams & Novellie, 1975b).

It is therefore tempting to speculate that spherosomes, and related vesicles, might be specialized components of the vacuolar system, permitting the operation of enzymes under conditions (e.g. of pH) not prevalent in the major vacuolar cavities. The fact that spherosomes are apparently only surrounded by a half-unit membrane (Schwarzenbach, 1971; Yatsu & Jacks, 1972) has a number of interesting implications if specific transport into and out of the vesicles can be demonstrated.

A further possible identity for compartments X and Y should also be mentioned. There is a certain amount of evidence to suggest that a space exists between plant cell walls and the plasmalemma, analogous to the periplasmic space of Gram-negative bacteria (see Matile, 1975). It is possible that compartments X and Y represent the 'periplasmic space'

and the cytoplasm, respectively. This would entail the existence of 'porin-like' systems (Benz et al., 1978; Lugtenberg et al., 1978; Nakae & Ishii, 1978) for transporting molecules into the 'periplasmic space'. There is no evidence for such systems in plant cells. Indeed, it seems unlikely that there is any restriction of access to the plasmalemma, at least for small molecules. The involvement of such an extracellular compartment therefore seems rather unlikely.

Thus, it seems likely that compartments X and Y represent the cytoplasm and vacuolar system respectively, although it is not yet clear whether Y might represent the whole of the vacuolar system or simply a specialized component of it (e.g. spherosome; protein body). A more definite identification of the subcellular compartments involved in peptide utilization awaits a clearer understanding of the hydrolytic compartment of plant cells, together with more direct evidence (e.g. radioautography, fluorescence microscopy) as to the sites of peptide storage and hydrolysis.

4.4.9 Compartmentation at the Cellular Level

One alternative must also be considered. It has been assumed that the compartments detected represent subcellular compartments. However, the barley embryo comprises a number of tissues, each with various subcellular compartments and

levels of peptidase activity. Although it seems that, once absorbed, peptides are evenly distributed throughout the embryo (section 3.1.3.3), the possibility that the observed compartmentation represents compartmentation between tissues, rather than within the cell, must be borne in mind.

4.5 Conclusions

The utilization of peptides by the barley embryo is clearly rather more complicated than has been assumed hitherto. Peptides seem to enter the cytoplasm intact and must be transported into a subcellular compartment (probably part of the vacuolar system), before hydrolysis can occur. Peptidases do not appear to be free in the cytoplasm but sequestered in a membrane-bound compartment. However, these complications do not significantly affect the conclusions reached previously concerning the barley peptide transport system; they simply put in doubt the original assumption that the transport system is located in the plasmalemma.

Two models have been proposed to explain the presented data. Although the simplicity of model II makes it possibly more attractive, neither model can be discounted on present evidence. A clarification of the situation will require additional approaches (e.g. studies on isolated organelles). However, both models require that a peptide

transport system operates in an intracellular (vacuolar?) membrane, as well as in the plasmalemma. This possibility certainly merits further attention as no convincing demonstration of a transport system in the plant vacuolar membrane has yet been achieved. If, as seems possible, this transport system is actually located in the membrane of protein bodies or spherosomes, the relative ease with which these bodies can be isolated would seem to make it an ideal system for study. The possibility of a peptide transport system in the membrane of protein bodies also has a number of important implications for the digestion of storage proteins in all seeds (section 6.6.2.3).

It must be stressed that the conclusions reached in this chapter are mainly based upon indirect evidence. Although the models proposed are the simplest explanation of the results, they may not be the only possible interpretations and should therefore be regarded as tentative. However, it is clear that the situation in barley is rather more complex than has so far been demonstrated for other peptide transport systems. It should prove instructive to compare the relationship between peptide transport and peptidase activity in such a compartmentalized system, with the situation in 'one-compartment' cells such as E.coli. In addition, the results obtained here have considerable implications for

the study of peptide transport in other eukaryotic systems such as yeast and the mammalian gut. It is hoped that these models will serve as a basis for further studies, not only in barley, but also in other eukaryotes.

CHAPTER 5

THE PEPTIDE POOLS OF GERMINATING BARLEY GRAINS

5.1 Introduction

The existence of a transport system which will accommodate peptides does not prove that this is its role *in vivo*, or that the transport of peptides is of any physiological importance. It is possible, in principle, that the peptide transport system of the barley scutellum functions to transport specialized peptides (e.g. hormones), or even non-peptide material, and that its ability to handle physiological peptides is purely fortuitous. In order to demonstrate the physiological importance of the transport system it is necessary to show that appropriate substrates are available at the right time and in the correct concentrations. Thus, the possibility that a peptide pool is produced in the barley endosperm, during germination, was investigated.

There is a certain amount of indirect evidence for the existence of peptide pools in plant tissues, including the cereal endosperm (section 1.2.3), although few, if any, of these reports can be regarded as conclusive. This is generally a reflection of the rather uncritical techniques which have been employed.

The experimental 'definition' of plant peptides has normally been based on three criteria: (i) they are soluble in various reagents which might be expected to precipitate any proteins (e.g. TCA, 80% ethanol); (ii) they contain organic or α-amino nitrogen; (iii) free amino acids are released during hydrolysis. Clearly, such

criteria could be subject to a number of interpretations.

The non-amino acid, ∝-amino nitrogen fractions, remaining after protein precipitation, have often been termed 'peptide fractions' with little real justification. The size of peptides remaining after precipitation will vary considerably, depending upon the reagents and conditions employed. Large polypeptides and proteins may also remain in solution; hydrolysis of a few such molecules would result in sufficient amino acids to lead to the assumption that a large pool of small peptides is present.

Plant extracts may also contain much soluble, non-peptide, nitrogenous material. The release of free amino acids on hydrolysis may provide 'confirmation' of the presence of peptides, although even this is subject to error. For example, high levels of non-protein, 'bound' amino acids were reported present in ryegrass leaves (Synge, 1951). Although these compounds released free amino acids on hydrolysis, they eventually proved to be N-acylated amino acids rather than peptides (Synge & Wood, 1958).

A further possible source of error in many studies which has not often been considered, is the production (or removal) of peptides during extraction (Synge & Youngson, 1961). Both enzymic cleavage and non-enzymic hydrolysis, by acidic or alkaline extractants, may be important.

Thus, a procedure was developed for the extraction and purification of barley peptides in an attempt to avoid the possible ambiguities discussed above. Three stages were involved; extraction, purification, and finally the identification and characterization of the isolated peptides.

5.2 Materials and Methods

Unless otherwise stated, all materials were as described previously (section 2.2). All other reagents were of analytical grade.

Centrifugations were performed using an M.S.E. Minor (1,500g) or a Gelman Hawksley Haematocrit (10,000g).

5.2.1 Tissue Extraction

Barley grains were surface sterilized and germinated in a spray room as described previously (section 2.3.1). After appropriate periods of germination, sixty seedlings were removed, the embryos and endosperms separated, blotted dry and weighed. Tissues were rapidly ground in a little acetic acid (5M), quantitatively recovered, and the volume made up to 12ml (9ml for embryos) with acetic acid (5M) preheated to 100°C. Extraction was at 100°C for 20 min, in a stoppered tube to prevent evaporation. Extracts were centrifuged for 10 min at 1,500g, the supernatant solutions recovered and evaporated to dryness on a rotary evaporator (Büchi: Rotavapor-R). The residues were redissolved in HCl (0.01M in deionized water; half the original volume) and the pH adjusted to pH 2.0 with 5M HCl. Undissolved material was removed by centrifugation (2 min, 10,000g) and the extracts stored at -20°C until required.

Ethanol and TCA extracts were similarly prepared, replacing 5M acetic acid with 80% (v/v) aqueous ethanol or 10% (w/v) TCA respectively.

5.2.2 Peptide Purification

The high viscosity of embryo and endosperm extracts, due to the high levels of carbohydrate present, were found to affect the subsequent separation of peptides by gel-exclusion chromatography. Thus, the extracts were 'purified' by ion-exchange chromatography prior to separation. This step also removed certain non-peptide material which otherwise interfered with the various assays used to quantify the peptide pool (section 5.3.5).

5.2.2.1 Ion-exchange Chromatography

A column (bed volume 9.5ml) of Dowex 50W-X8 standard H^+ ion-exchange resin (B.D.H. Ltd.) was thoroughly washed with deionized water, equilibrated with HCl (0.01M, in deionized water; pH 2.0) and 1.0ml of extract (in HCl, pH 2) added. Unbound material was eluted with three bed volumes of HCl (0.01M in deionized water), followed by two bed volumes of deionized water, and discarded. Compounds bound to the column (including amino acids and peptides) were eluted with four bed volumes of ammonia (3M in deionized water). Eluate was evaporated to dryness in a rotary evaporator at 40°C, diluting with several volumes of deionized water before complete evaporation was reached, to prevent alkaline hydrolysis of peptide bonds. The residues were redissolved in 1.5ml PBS (phosphate buffered saline: 0.1M NaCl in deionized water, buffered with 0.01M sodium phosphate, pH 7.0), centrifuged for 3 min at 10,000g and the supernatant solutions recovered. These will be referred to as 'purified extracts'.

Resin was regenerated after each run with HCl (0.5M in deionized water).

5.2.2.2 Gel-Exclusion Chromatography

Peptides in the 'purified extracts' were fractionated on a Sephadex G-15 chromatography column (1.5 x 90cm) using PBS as an eluent. A constant flow rate (33.6ml/h) was maintained using a peristaltic pump (Varioperpex 12,000, LKB Ltd.).

1.0ml samples of the 'purified extracts' were loaded on to the column, eluted, and 2.8ml fractions collected. These are referred to below as G-15 fractions.

Column Calibration: A solution containing Blue Dextran 2000 (Pharmacia) and NaBr (200µl; each at 0.5mg/ml) was applied to the column, eluted, and the O.D. of the effluent monitored continuously at 206nm (Uvicord III 2089 dual channel spectrophotometer, LKB Ltd.). The volumes at which the dextran and bromide eluted were taken to be the void volume (V_o) and the total elution volume (V_t) of the column, respectively. The elution volumes (V_p) of a number of known amino acids (200µl, 20mM) and peptides (200µl; 1mM) were similarly determined and the K_d [(a)] for each peptide calculated.

5.2.3 Analysis of G-15 Fractions

5.2.3.1 Absorbance at 206nm

The O.D. of each column fraction was measured at 206nm (1 cm pathlength) using a Unicam SP 500/Gilford 2000

(a) Kd expresses that portion of the column available to peptide but not dextran ($V_p - V_o$), as a fraction of the total intra-particle volume ($V_t - V_o$).

spectrophotometer. Dialanine, dissolved in column eluent (0-680μM), was used as a standard.

5.2.3.2 Fluorescamine Analysis

50μl and 200μl samples of G-15 fractions were assayed for ∝-amino groups using fluorescamine as described previously (section 2.3.2.2). Borate buffer was adjusted to pH 7.0.

5.2.3.3 Dansyl Chloride Analysis

Free amino acids: The free amino acids in each fraction were determined using a modification of the dansyl chloride procedure described previously (section 2.3.2.1). Samples of each fraction (20-400μl, depending upon the levels of amino acid in the fraction) were freeze-dried, together with ornithine (10μl; 0.125mM) as an internal standard, and dansylated using 50μl each of $NaHCO_3$ and DNS-Cl. The labelled amino acids were separated, identified and quantified as described previously. Prior to spotting onto the polyamide sheets, samples (dissolved in pyridine) were centrifuged (2 min, 10,000g) to remove undissolved salts which otherwise interfered with the chromatographic separation.

Peptides: Peptide bonds were acid hydrolysed, and the amino acids released determined by dansylation, as above. Hydrolysis of freeze-dried samples (20-400μl) of each fraction was performed in sealed tubes, using HCl (50μl; 6M) at 105°C for 16h. HCl was removed *in vacuo* over NaOH prior to dansylation.

N-terminal amino acids: N-terminal amino acids were determined as above, except that hydrolysis was performed after dansylation but prior to chromatographic separation.

N-terminal proline residues: Due to the labile nature of the DNS-proline bond (Hartley, 1970), N-terminal proline residues were determined after only 4.5h hydrolysis. The percentage hydrolysis of peptide and DNS-proline bonds achieved within this period, was determined by subjecting samples of proline, Pro-Gly and Pro-Gly-Gly (10, 20, or 50µl; 0.04mM) to similar treatments.

5.2.3.4 TNBS Assay

Standard assay conditions employed were as follows: 1.1ml of amino acid/peptide solution (containing 10-200nm free amino groups) was mixed with 0.9ml of sodium tetraborate (0.13M) and equilibrated at 37°C. A freshly prepared solution of TNBS (250µl; 4mg/ml in water, also at 37°C) was added and thoroughly mixed. After exactly 30 min further incubation at 37°, the absorbance at 420nm was measured (1cm pathlength; Hilger-Watts Uvispek H700 spectrophotometer). For assays in the presence of Cu^{2+} ions, 50µl of $CuSO_4$ (24mM) replaced 50µl of the tetraborate buffer.

In certain experiments (see results) the reaction was terminated after exactly 30 min by the addition of HCl (100µl; 10M) and the absorbance determined at either 340nm or 420nm.

The assay was calibrated using glycine as a standard.

5.2.4 Tissue Water Content

Separated embryos and endosperms from 20 seeds were blotted dry and the fresh weights determined. The tissues were then dried to constant weight, at 75°C under vacuum, and the dry weight recorded. Prior to weighing, tissues were cooled *in vacuo* over silica gel.

Water content was considered to be the difference between the wet and dry weights.

5.2.5 Automatic Amino Acid Analysis

Amino acid compositions of ungerminated barley meal and various column fractions were obtained from acid hydrolysates (6M HCl, 22h, 106°C), using a Locarte amino acid analyser.

5.2.6 G-50 Gel-Exclusion Chromatography

A Sephadex G-50 superfine column (1.6 x 40cm) was equilibrated with PBS at a constant flow rate of 14.1 ml/h. 1ml samples of peptide/protein solution (section 5.3.6) were added to the column and eluted with PBS. The absorbance of the effluent was monitored continuously at 206nm using a Uvicord III dual channel spectrophotometer (LKB Ltd.).

The column was calibrated as before (section 5.2.2.2), using three standard peptides, pentaalanine (200µl, 1mM), insulin α-chain (200µl, 5mg/ml) and insulin β-chain (200µl, 5mg/ml).

5.3 Results

Extracts were made of the endosperm from 1- to 6-day seedlings and the embryos from 1- to 3-day seedlings. In ungerminated grains (day 0) it proved impossible to separate the embryo and endosperm; thus, the complete grain was extracted. In addition, extracts of the proximal (including the embryo) and distal (excluding the embryo) half-grains were made for comparison.

5.3.1 Tissue Water Content

Table 5.1 shows the wet and dry weights and water content of barley tissues at various stages of germination. Clearly, the water content of both the endosperm and embryo increases as germination proceeds.

The endosperm dry weight decreases during germination, while that of the embryo increases (Figure 5.1). As seeds are germinated in the dark, in the absence of any added nutrients, this must reflect the transfer of material from the endosperm to the embryo. However, the total dry weight of the grain (endosperm plus embryo) decreases, presumably due to respiration and/or leaching of material from the grain. Similar changes have frequently been observed (cf. Folkes *et al.*, 1952).

5.3.2 G-15 Column Calibration

It is generally accepted that gel-exclusion chromatography separates substances on the basis of their 'molecular size' (Flodin, 1962; Ackers, 1970). However, this idealized situation is often complicated by the interaction of certain classes of compound with the dextran

Table 5.1: Water Content of Barley Tissues at Different Stages of Germination

Tissue	Age (Days)	Fresh Wt. (g/20 grains)	Dry wt. (g/20 grains)	Water content (%)
Whole Grain	0	0.787	0.719	9.5
Endosperm	1	1.139	0.687	39.9
"	2	1.091	0.546	50.0
"	3	0.998	0.407	59.2
"	4	0.998	0.352	64.7
"	5	0.928	0.261	71.9
"	6	0.771	0.151	80.4
Embryo	1	0.169	0.034	79.9
"	2	1.022	0.090	91.2
"	3	2.068	0.147	92.9
"	4	2.176	0.148	93.2
"	5	3.269	0.219	93.3
"	6	4.820	0.289	94.0

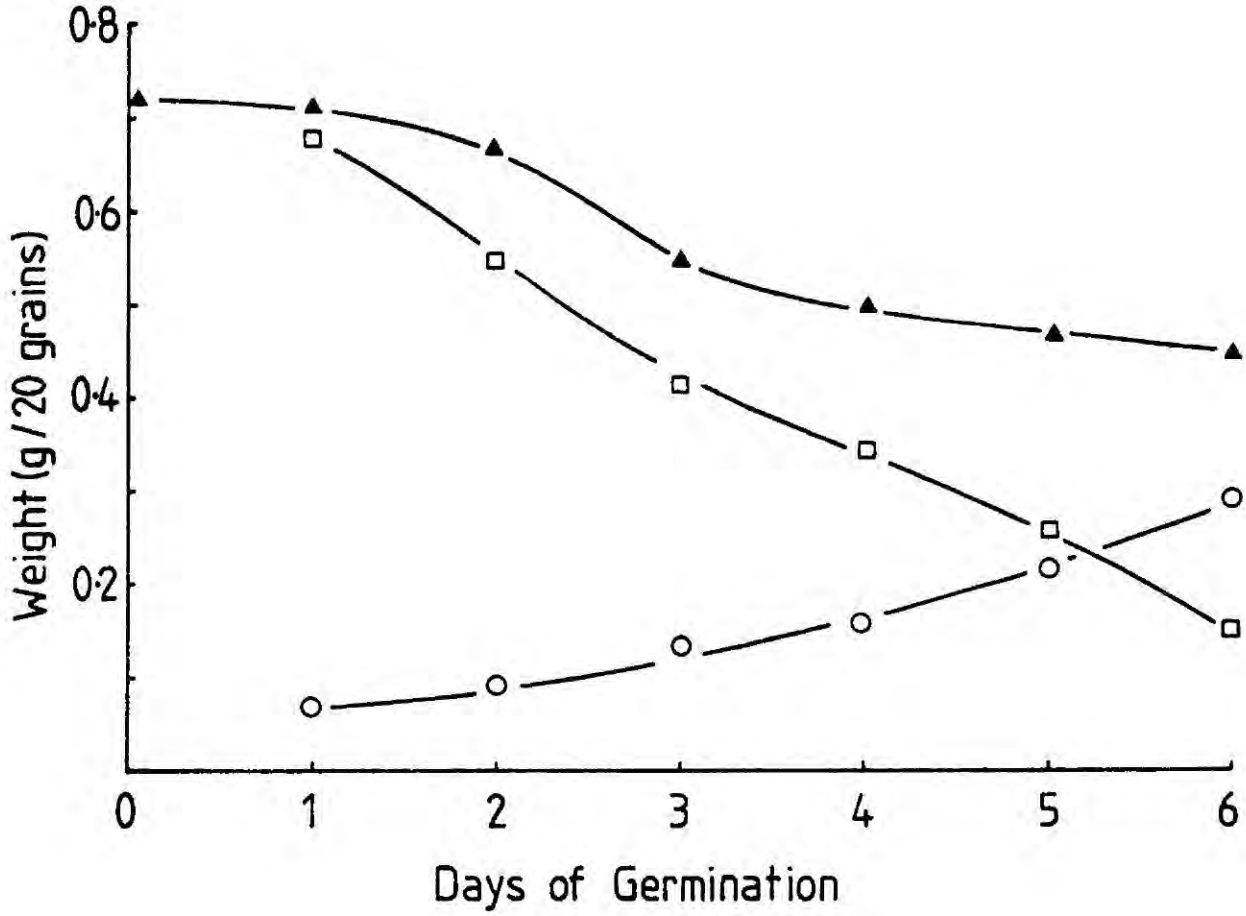


Figure 5.1: Changes in Dry Weight of the Barley Endosperm and Embryo During Germination

Whole grains (▲)
Embryos (○)
Endosperm (□)

gel. Thus, elution was performed with 0.1M NaCl, which has been shown to prevent the interaction of charged peptides with the gel matrix (Payne & Gilvarg, 1968b). Eluent was buffered at pH 7.0 to maintain a constant charge on the peptides.

The 'molecular size' of a peptide depends upon three main factors; its chain length, the amino acid side groups and the degree to which the peptide backbone is folded. For several series of homopeptides, a linear relationship exists between log K_d and the peptide chain length. Thus, for peptides of six or less residues, little or no folding of the peptide chain occurs and separation depends primarily upon the number of amino acid residues (Payne & Gilvarg, 1968b). However, the amino acid side chains will also affect the 'molecular size' of a peptide to a significant extent, and complete separation of a heterogeneous mixture of peptides into di-, tri-, tetrapeptides, etc., will not be achieved by this means.

It is well known that a logarithmic relationship holds between K_d and protein molecular weight (Andrews, 1965); a similar relationship has been reported for peptides of molecular weight 400-2000 (Carnegie, 1965). It seems likely that a similar relationship will also apply to mixtures of small heterogeneous peptides.

Figure 5.2 shows a plot of K_d against log molecular weight, obtained for a number of small peptides. An approximately linear relationship exists, which can be used to calculated the elution volume of any peptide.

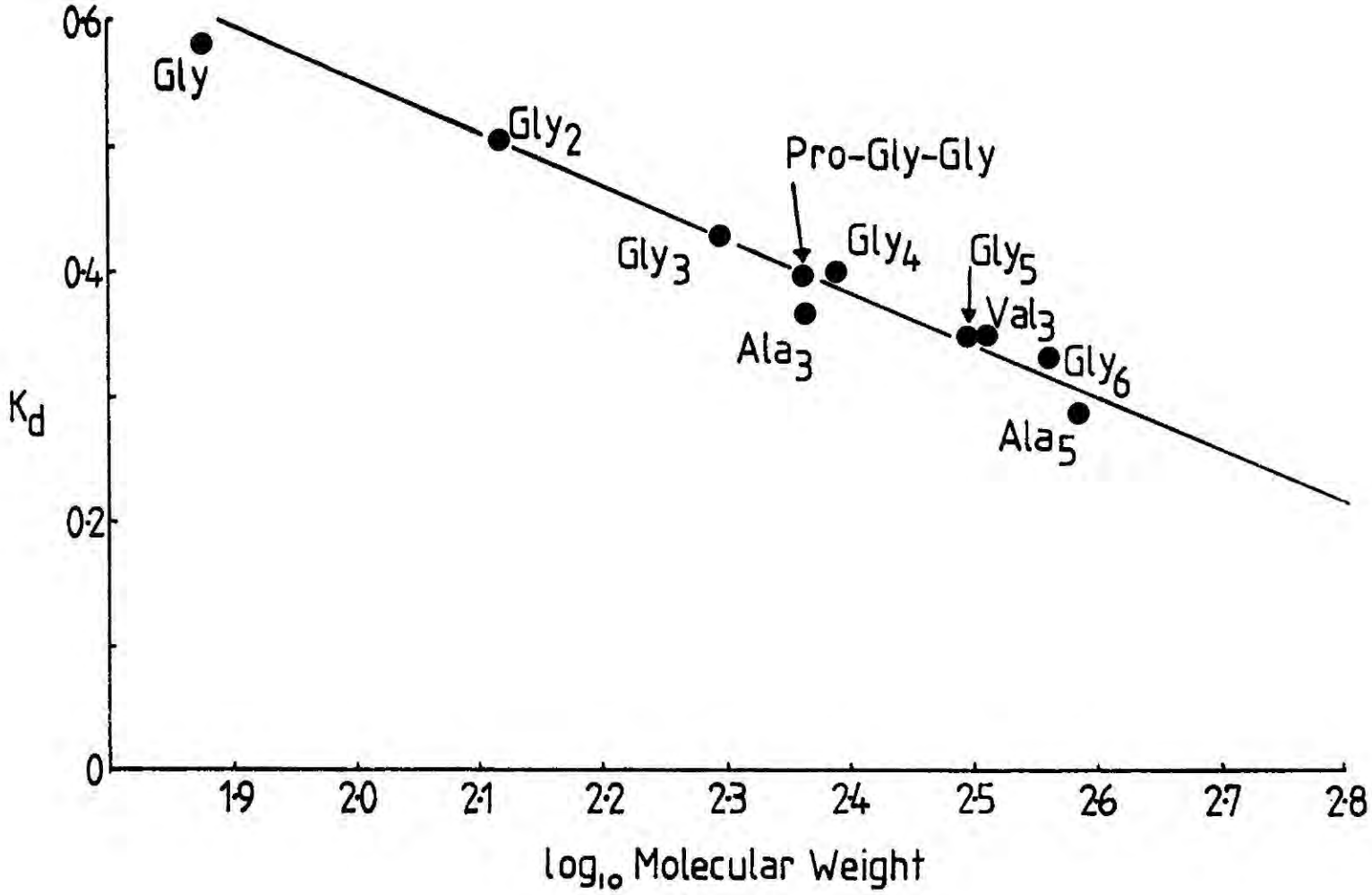


Figure 5.2: K_d as a Function of log_{10} Molecular Weight

Comparison of the K_d for each G-15 column fraction with the above graph, allows the 'average' molecular weight of peptides eluting in each fraction to be determined. The 'average' chain length of the peptides eluting in each fraction can also be calculated, assuming a molecular weight of 120 daltons for each amino acid.

Fraction	Molecular Weight	Peptide Chain Length
21	596	5.7
22	484	4.6
23	367	3.4
24	295	2.7
25	226	2.1
26	174	1.6
27	141	1.2

For convenience, instead of considering elution volumes, reference will be made to the G-15 fractions collected during elution.

The average chain length of peptides eluted in each fraction can be calculated (Fig. 5.2). Thus, nothing will elute in fractions 1-16, proteins and polypeptides of more than six residues in fractions 17-20, peptides of six or less residues in fractions 21-27, and amino acids in fractions 28-30. These will be referred to as the 'protein', 'peptide', and 'amino acid' fractions, respectively. It must, however, be remembered that the 'protein' fractions will also contain any peptides larger than hexapeptides present in the extracts.

5.3.3 Efficiency of Extraction

5M acetic acid has been shown to be a suitable extractant for amino acids and peptides from barley embryos (section 2.4.1). Ethanol or TCA extracts gave very similar 206nm absorption profiles to those obtained for acetic acid extracts, after separation of ion-exchange purified samples on the G-15 column. Only in the 'protein' fractions was a significant difference apparent, indicating different degrees of 'protein' extraction. As 206nm absorbance is proportional to peptide concentration, at least in the 'peptide' fractions (section 5.4.1.3), it seems likely that the similar levels of peptide extracted by each of the three solvents represent 100% recovery.

5.3.4 Efficiency of the Purification and Separation Procedures

At pH 2.0, amino acids and peptides will be positively charged and therefore bind to the ion-exchange resin, remaining bound during subsequent washing. However, many other cell components, in particular most carbohydrates, will be neutral and therefore pass straight through the column. Dansylation of samples of the washings showed that amino acids and peptides do indeed remain bound. Similarly, the bound amino acids and peptides were shown to elute from the column in 3M ammonia.

As an overall check on the efficiency of extraction and purification, three known peptides, dialanine, pentaglycine and Phe-Glu, were added to the acetic acid extractant. An extract of 3-day endosperm tissue was made and subjected to the entire purification and separation procedure. Dansylation of the final G-15 fractions showed better than 85% recovery of each peptide. In addition, each peptide eluted from the G-15 column in the same fraction as it would in the absence of extract. Thus, components of the extract do not seem to affect the separation of peptides by gel-exclusion chromatography.

The rapid grinding and boiling of the tissue during extraction was intended to minimize any enzymic peptide cleavage. In addition, little or no non-enzymic cleavage occurs during the extraction procedures, as evinced by the efficient recoveries of the added peptides obtained above.

Figures 5.3a-g: Peptides and Amino Acids in Endosperm Extracts from Barley Grains Germinated for Varying Periods of Time

Various methods were used to assay the peptides and amino acids in each G-15 fraction of endosperm extracts:

I (●) Absorbance at 206nm (section 5.2.3.1), expressed as nanomoles of peptide bond/fraction.

(▾) Dansyl chloride analysis after acid hydrolysis (section 5.2.3.3), expressed as nanomoles of 'bound' amino acids/fraction.

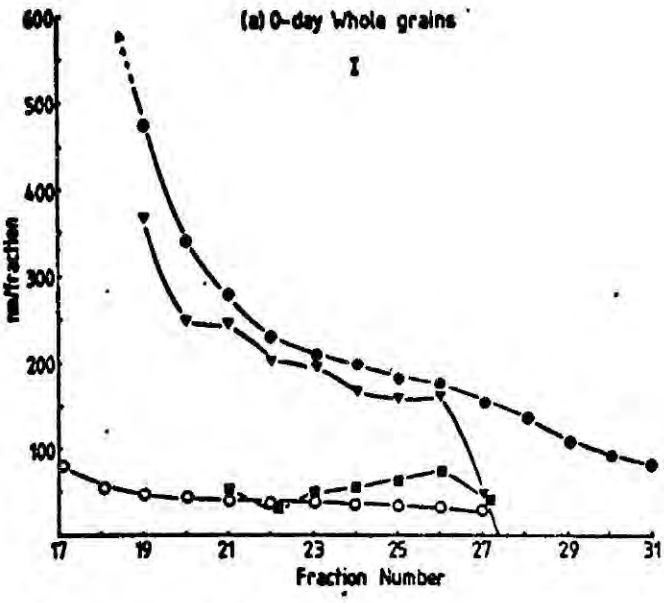
(○) Fluorescamine (section 5.2.3.2), expressed as nanomoles of free amino acid groups/fraction

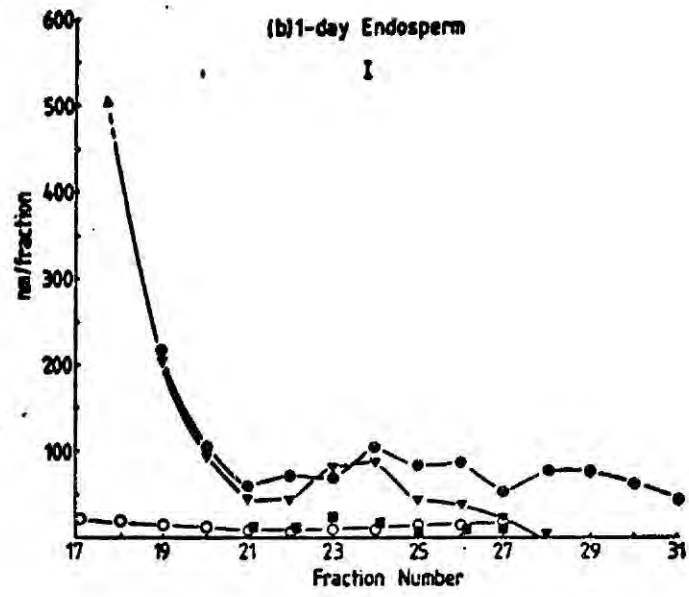
(■) N-terminal analysis (section 5.2.3.3), expressed as nanomoles of N-terminal amino acids/fraction.

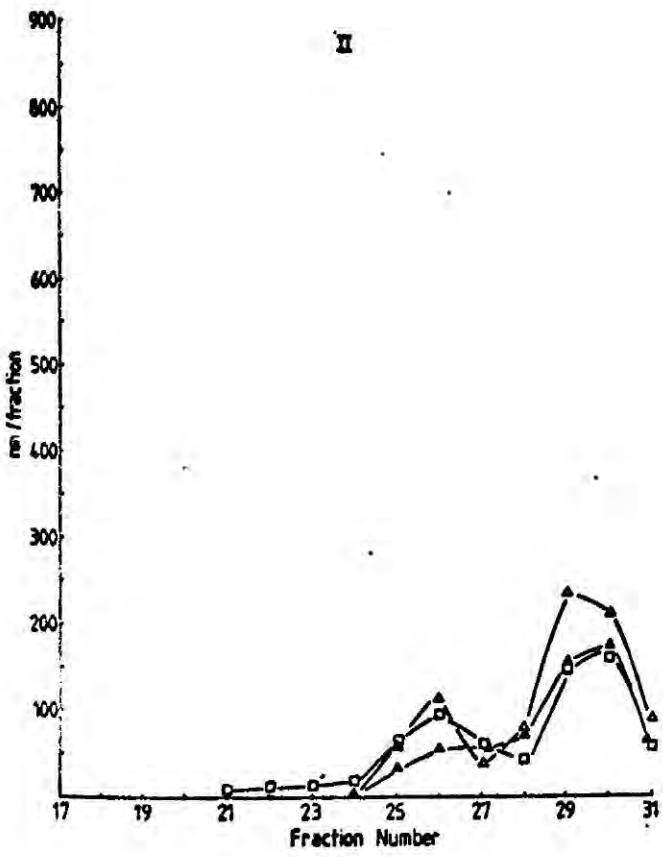
II (△) Dansyl chloride analysis (section 5.2.3.3), expressed as nanomoles of free amino acids/fraction.

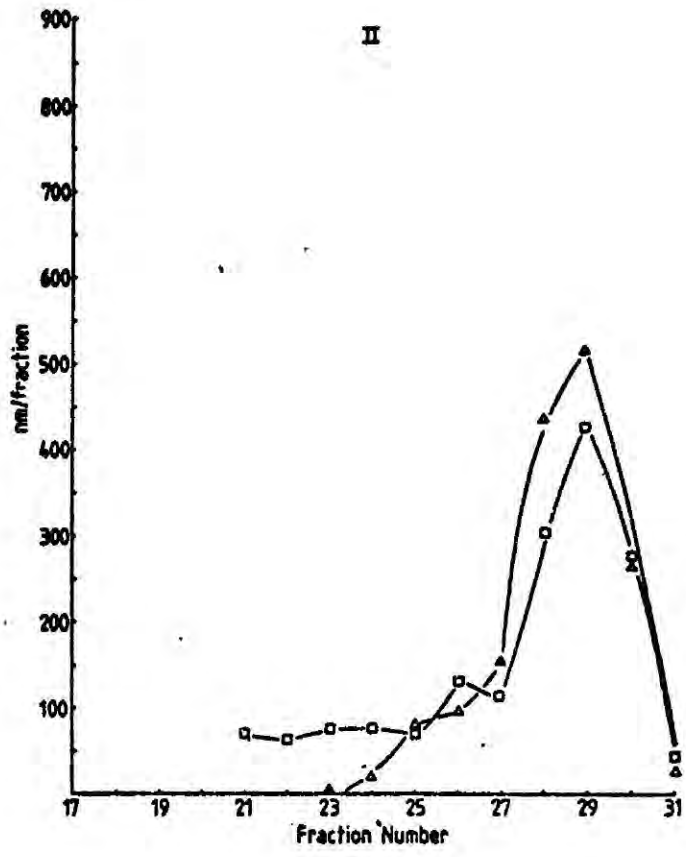
(▲) TNBS assay in the presence of Cu^{2+} ions (section 5.2.3.4), expressed as nanomoles of free amino groups/ fraction.

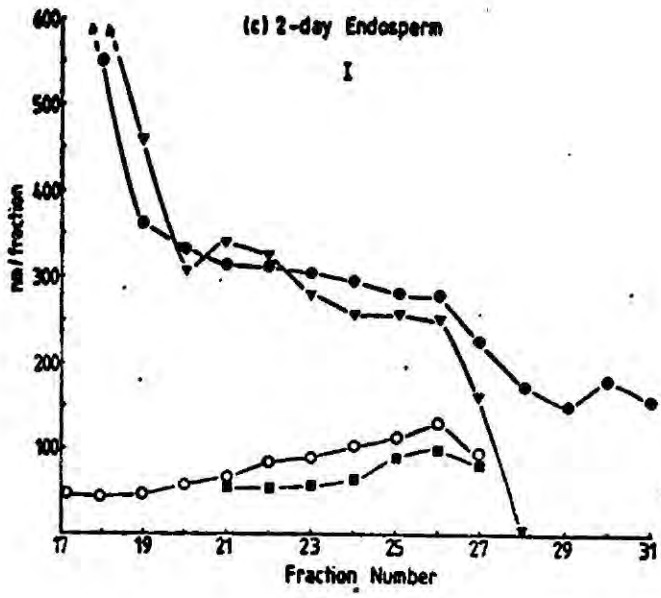
(□) TNBS assay in the absence of Cu^{2+} ions (section 5.2.3.4), expressed as nanomoles of free amino groups/fraction.


(a) 0-day Whole grains
I
ml/fraction
Fraction Number


(b) 1-day Endosperm
I
ml/fraction
Fraction Number


II
ml/fraction
Fraction Number

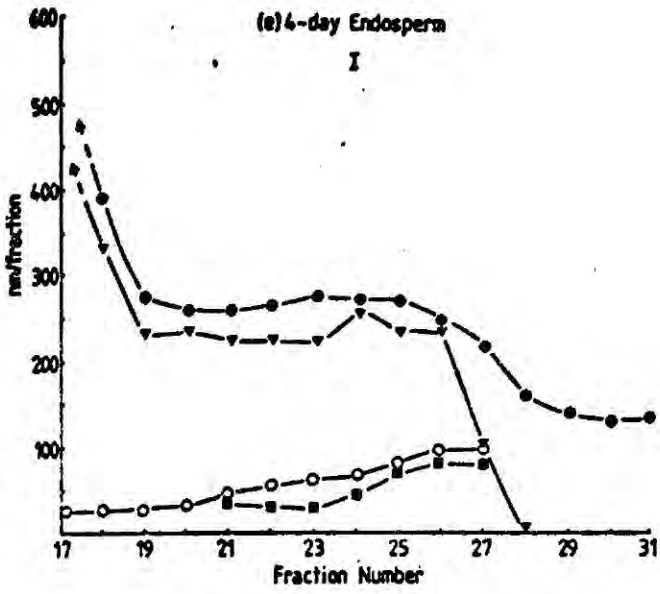

II
ml/fraction
Fraction Number

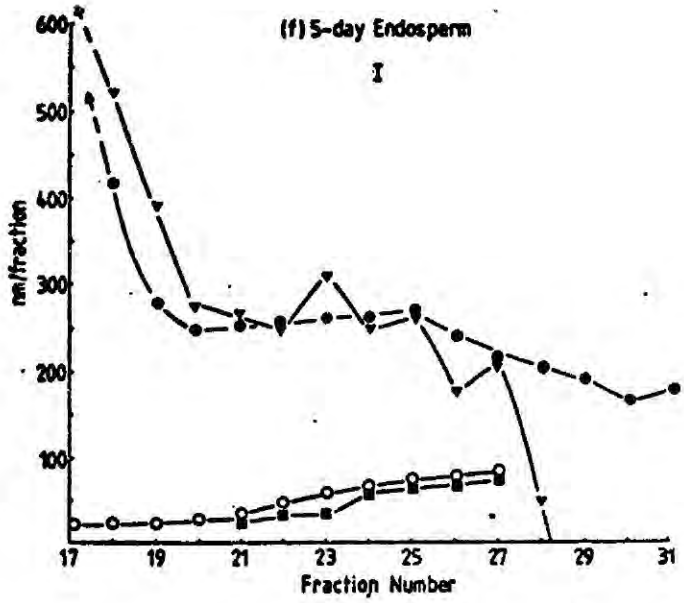

(c) 2-day Endosperm
I
nm/fraction
Fraction Number

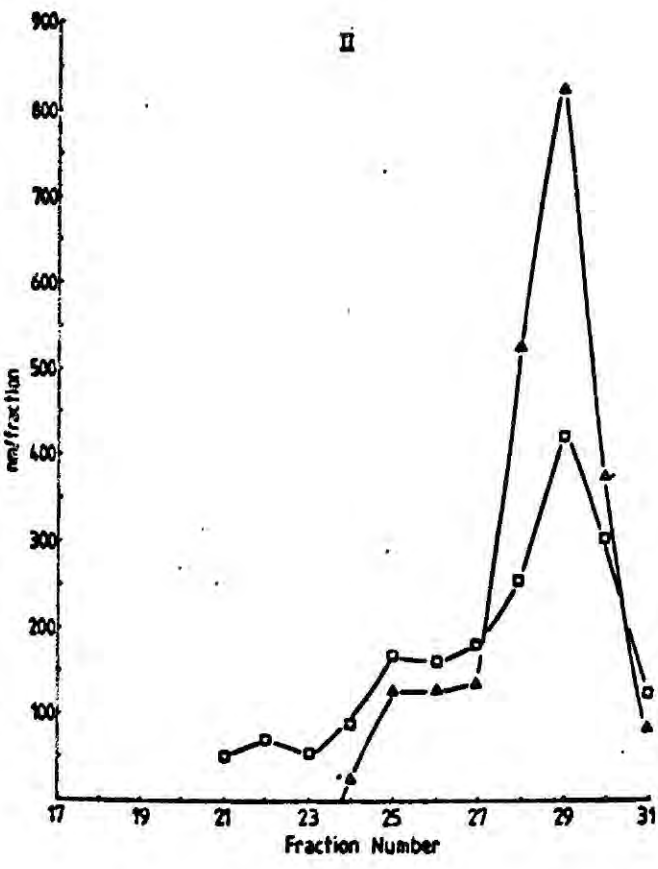

(d) 3-day Endosperm
I
nm/fraction
Fraction Number

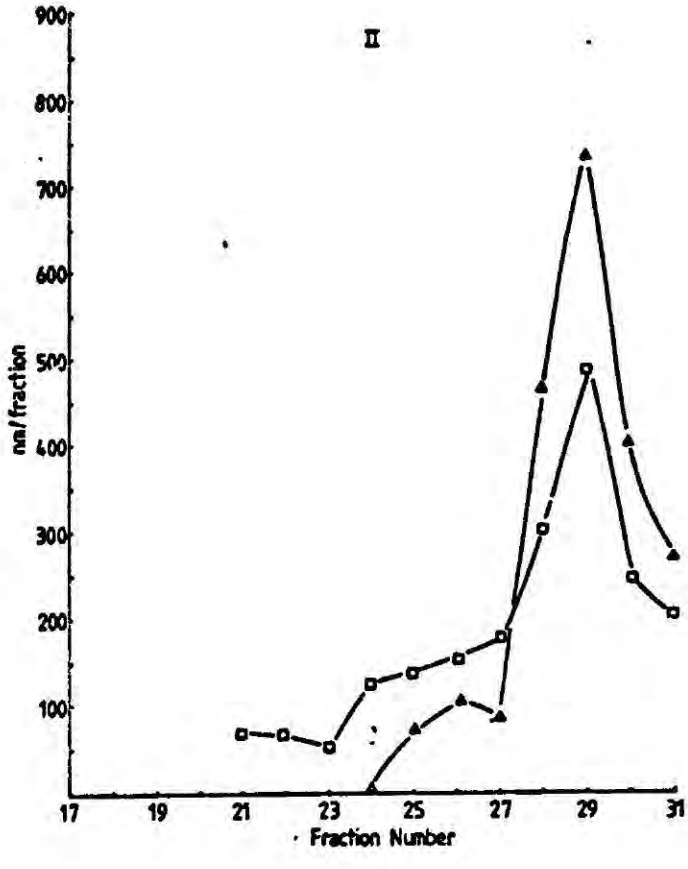
II
nm/fraction
Fraction Number

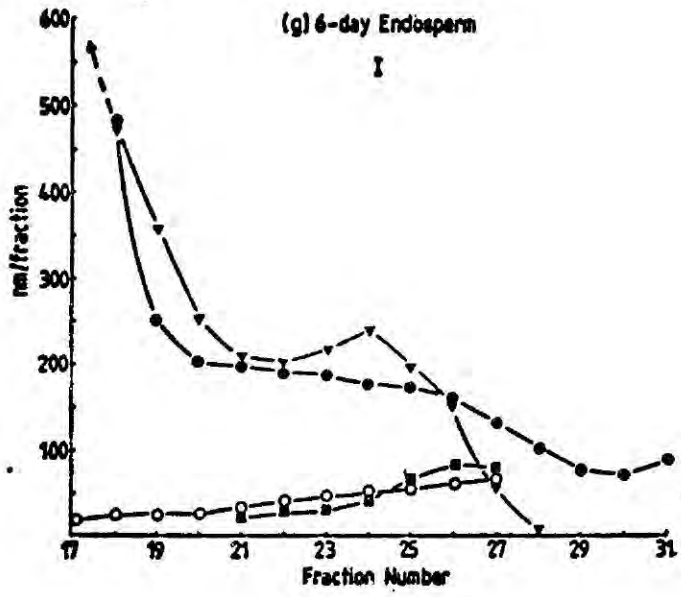
II
nm/fraction
Fraction Number

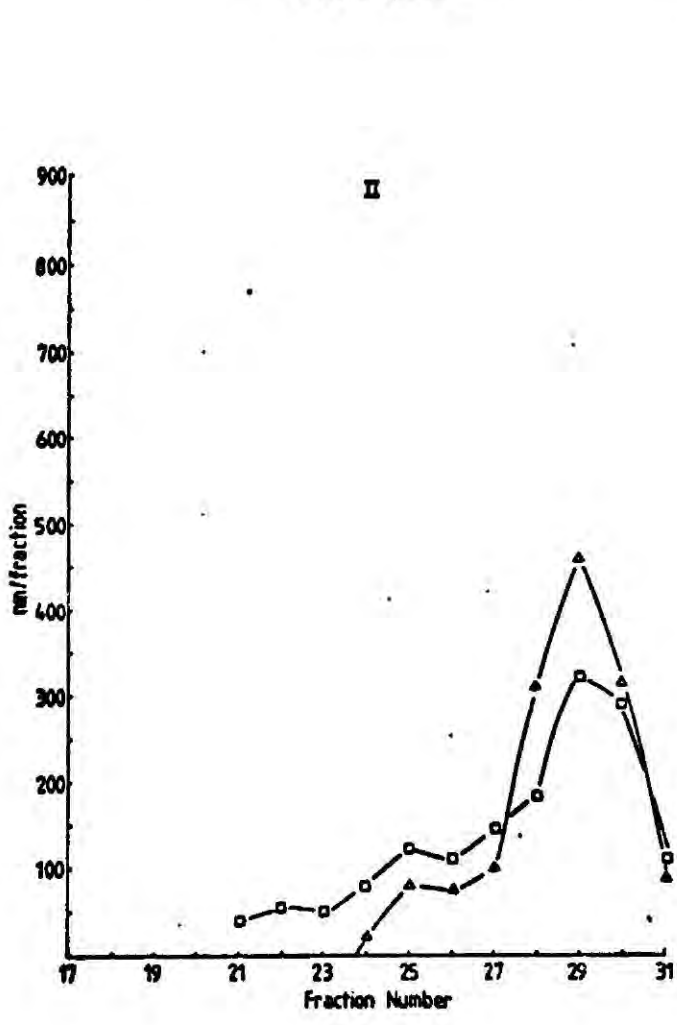

(e) 4-day Endosperm
I
nm/fraction
600
500
400
300
200
100
17
19
21
23
25
27
29
31
Fraction Number


(f) 5-day Endosperm
I
nm/fraction
600
500
400
300
200
100
17
19
21
23
25
27
29
31
Fraction Number


II
nm/fraction
900
800
700
600
500
400
300
200
100
17
19
21
23
25
27
29
31
Fraction Number


II
nm/fraction
900
800
700
600
500
400
300
200
100
17
19
21
23
25
27
29
31
Fraction Number


(g) 6-day Endosperm
I
nm/fraction
600
500
400
300
200
100
17
19
21
23
25
27
29
31
Fraction Number


II
nm/fraction
900
800
700
600
500
400
300
200
100
17
19
21
23
25
27
29
31
Fraction Number

Figures 5.4a-c: Peptides and Amino Acids in Embryo Extracts from Barley Grains Germinated for Varying Periods of Time

Various methods were used to assay the peptides and amino acids in each G-15 fraction of embryo extracts:

I (●) Absorbance at 206nm (section 5.2.3.1), expressed as nanomoles of peptide bond/fraction.

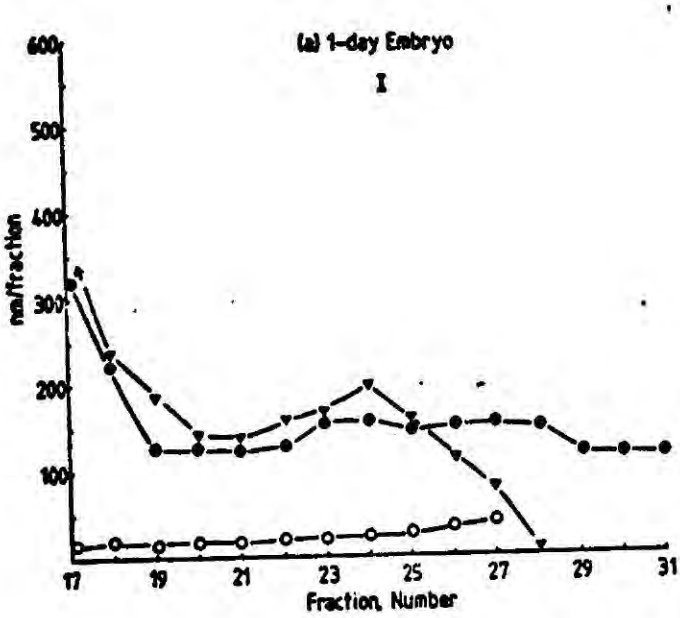
(▾) Dansyl chloride analysis after acid hydrolysis (section 5.2.3.3), expressed as nanomoles of 'bound' amino acids/fraction.

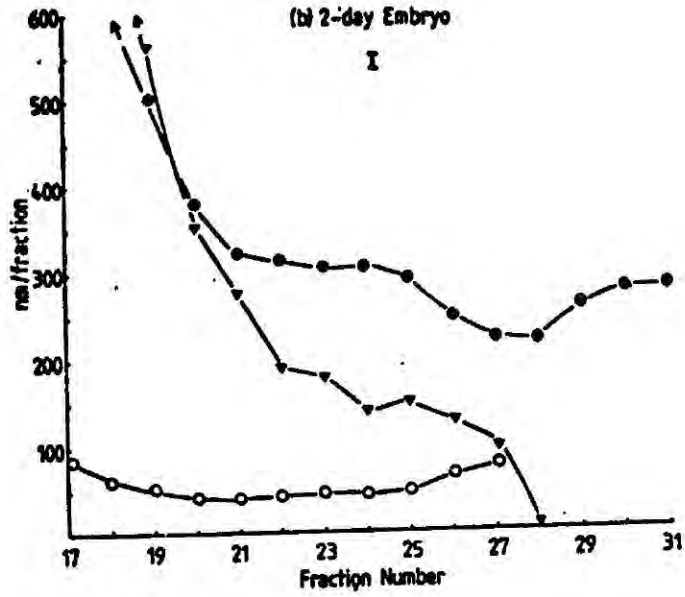
(○) Fluorescamine (section 5.2.3.2), expressed as nanomoles of free amino groups/fraction.

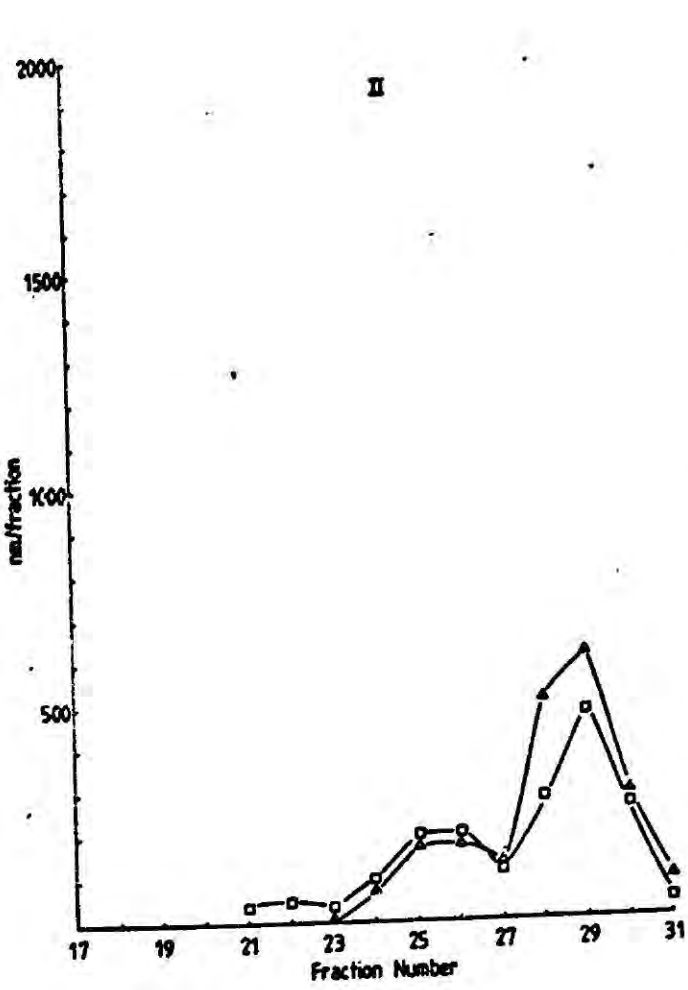
II (△) Dansyl chloride analysis (section 5.2.3.3), expressed as nanomoles of free amino acids/fraction.

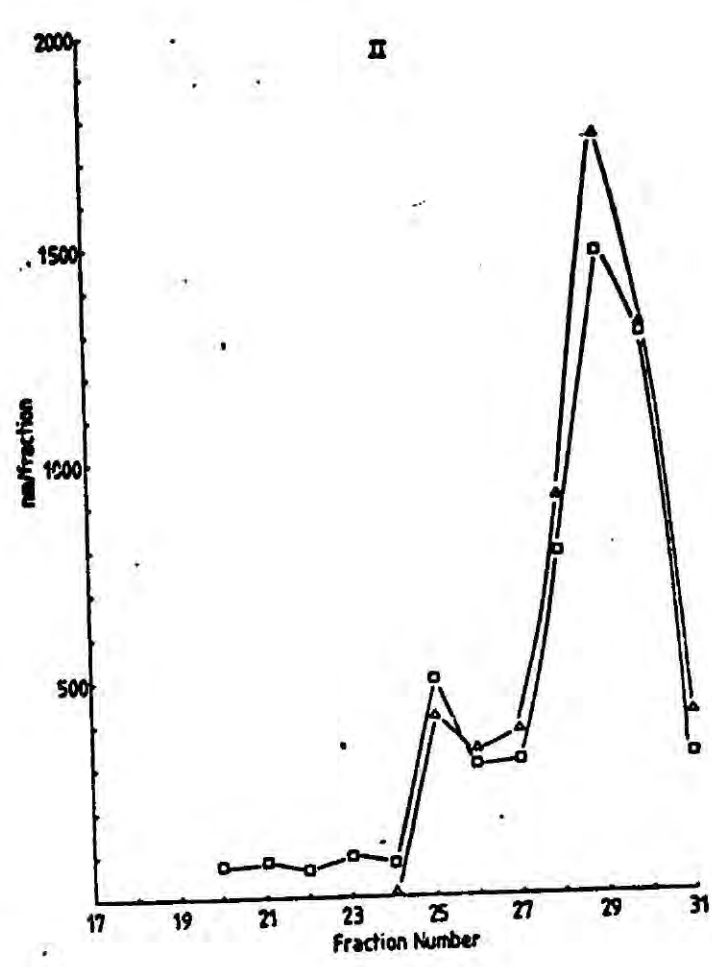
(□) TNBS assay (section 5.2.3.4), expressed as nanomoles of free amino groups/fraction.

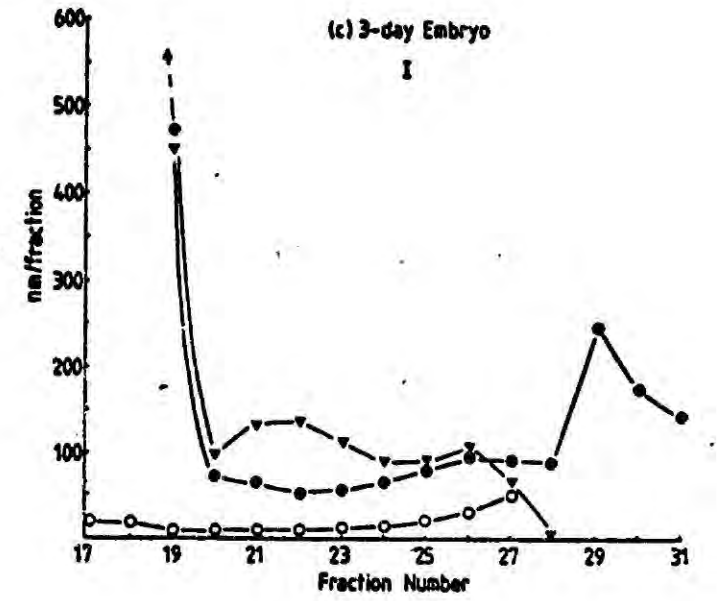
Note the difference in scales between I and II

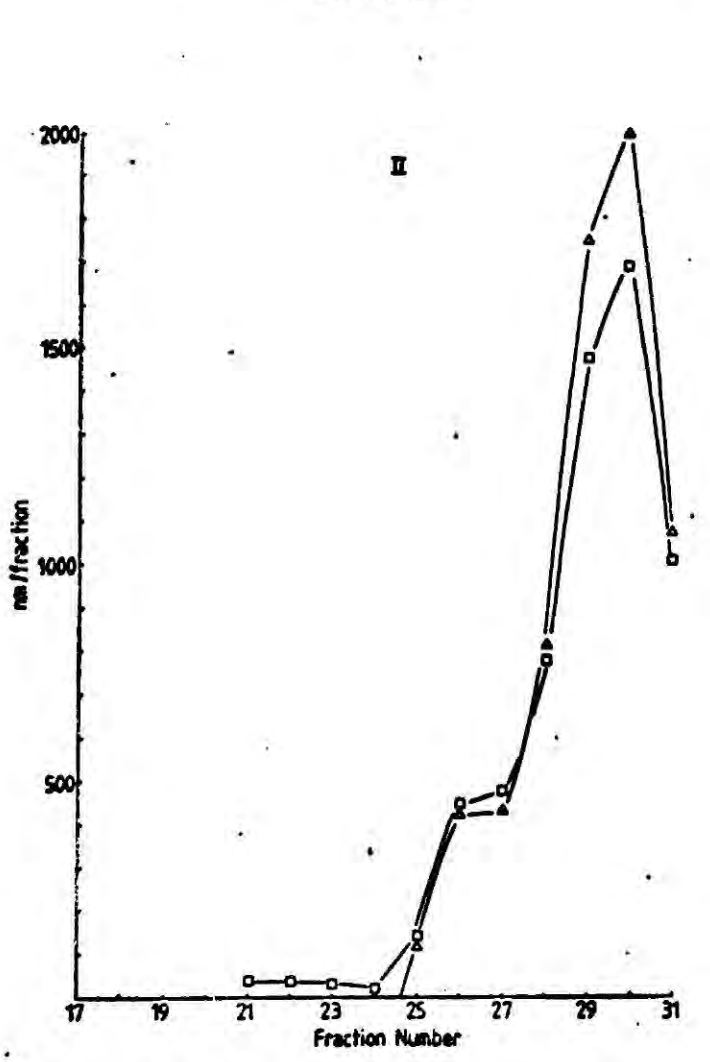

(a) 1-day Embryo
I
600
500
400
300
200
100
nm/fraction
17
19
21
23
25
27
29
31
Fraction Number


(b) 2-day Embryo
I
600
500
400
300
200
100
nm/fraction
17
19
21
23
25
27
29
31
Fraction Number


II
2000
1500
1000
500
nm/fraction
17
19
21
23
25
27
29
31
Fraction Number


II
2000
1500
1000
500
nm/fraction
17
19
21
23
25
27
29
31
Fraction Number


(c) 3-day Embryo
I
600
500
400
300
200
100
nm/fraction
17
19
21
23
25
27
29
31
Fraction Number


II
2000
1500
1000
500
nm/fraction
17
19
21
23
25
27
29
31
Fraction Number

5.3.5 Analysis of G-15 Fractions

Various analytical techniques were employed to identify and characterize the amino acids and peptides eluted from the G-15 column. In order to facilitate comparison of these methods, the results are presented together (figs. 5.3a-g and 5.4a-c).

5.3.5.1 Dansyl Chloride Analysis

The estimation of amino acids in the G-15 fractions, both before and after acid hydrolysis, has provided a considerable body of information on the concentration and composition of the amino acid and peptide pools of germinating barley grains.

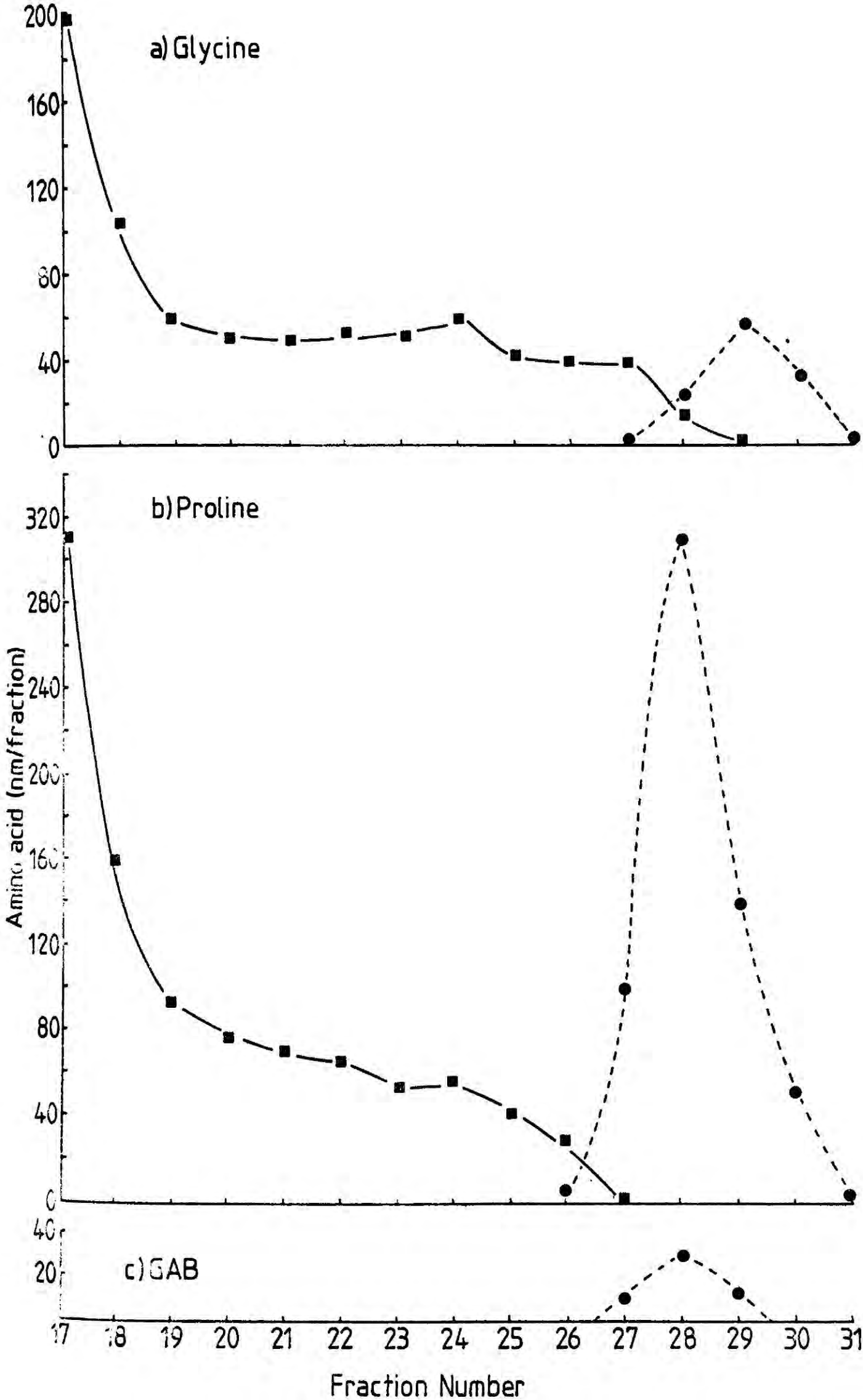
Preliminary investigations showed that ornithine was absent from the endosperm fractions; thus, it remained a suitable internal standard for the dansylation procedure.

Figures 5.5a-c show the estimated levels of three representative amino acids, glycine, proline and GAB, in each G-15 fraction from a 2-day endosperm extract, both before and after hydrolysis. Far more free proline is present in the extract than either glycine or GAB. However, when 'bound' amino acids (released by hydrolysis) are considered, the levels of proline and glycine are very similar. No 'bound' GAB could be detected. Similar results (not presented) were obtained for every amino acid in each of the endosperm and embryo extracts.

The total pool of free amino acids in each fraction was obtained by summing the amounts of each of the individual amino acids. A similar process gave the total

Figures 5.5a-c: Levels of Free and 'Bound' Amino Acids in each G-15 Column Fraction

The levels of free (●---●) and 'bound' (▫——▪) amino acids in each column fraction were estimated using dansyl chloride: a) glycine; b) proline; c) GAB. 'Bound' amino acids were determined after acid hydrolysis.


a) Glycine
b) Proline
c) GAB
Amino acid (nm/fraction)
Fraction Number
200
160
120
80
40
0
320
280
240
200
160
120
80
40
0
40
20
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31

pool of 'bound' amino acids in each fraction. These results are presented in figures 5.3a-g and 5.4a-c. Although no great accuracy is claimed for the levels of each individual amino acid, when summated to give the total pools, any errors will be minimized.

These results illustrate the efficient separation of free and 'bound' amino acids by gel-exclusion chromatography. Apart from certain anomalies, discussed below, no free amino acids elute in the 'protein' (17-20) or 'peptide' (21-27) fractions. As expected from their 'molecular size', free amino acids elute in fractions 28-31. Similarly, 'bound' amino acids (presumptive peptides) are only found in the 'protein' and 'peptide' fractions. However, certain amino acids do not behave as expected:

(i) Tyrosine, phenylalanine and histidine elute in fractions 32-36. The retardation of aromatic amino acids, as a result of their interaction with dextran gels, is well known (Porath, 1960; Canfield & Anfinsen, 1963). However, the extremely high levels of an unknown dansyl-reactive compound (an amino-sugar?) which also elutes in these fractions precludes any quantification of these three amino acids. Fortunately, the small proportion of aromatic residues in the 'bound' amino acid fractions (section 5.4.4.2) does not seem to affect the elution of these compounds from the G-15 column, under the conditions employed (section 5.4.1.1).

(ii) Three amino acids, glutamic acid, aspartic acid and lysine, elute rather early, peaking in fractions 25-26, 25-26 and 26-27, respectively. Presumably this is due to their exclusion from certain regions of the gel by virtue of their charge, although the high ionic strength of the eluent might have been expected to counteract such effects. Although the free and bound forms of these amino acids can be separated by the dansyl chloride technique (figure 5.6), the presence of these free amino acids in the 'peptide' fractions must be considered when interpreting the results of other assays.

(iii) Although other 'bound' amino acids cannot be detected in fractions 28-32, glutamic acid appears in these later fractions after hydrolysis. This is almost certainly due to the relative ease with which glutamate/glutamine can cyclize to form pyroglutamic acid (Moore and Stein, 1951; Meister, 1965). Pyroglutamic acid does not react with dansyl chloride and is therefore not detected as a free amino acid. However, on acid hydrolysis it is converted to glutamic acid (Cocking and Yemm, 1961) and therefore appears as 'bound' glutamic acid, eluting in the 'amino acid' fractions. These derivatives have been included with the free glutamate/glutamine pools.

(iv) Glutamine and asparagine were absent from any extracts, presumably the result of deamidation to the corresponding acids. Thus the acid/amide pools have been considered together. In addition, arginine was not estimated by the dansyl chloride method. It runs close

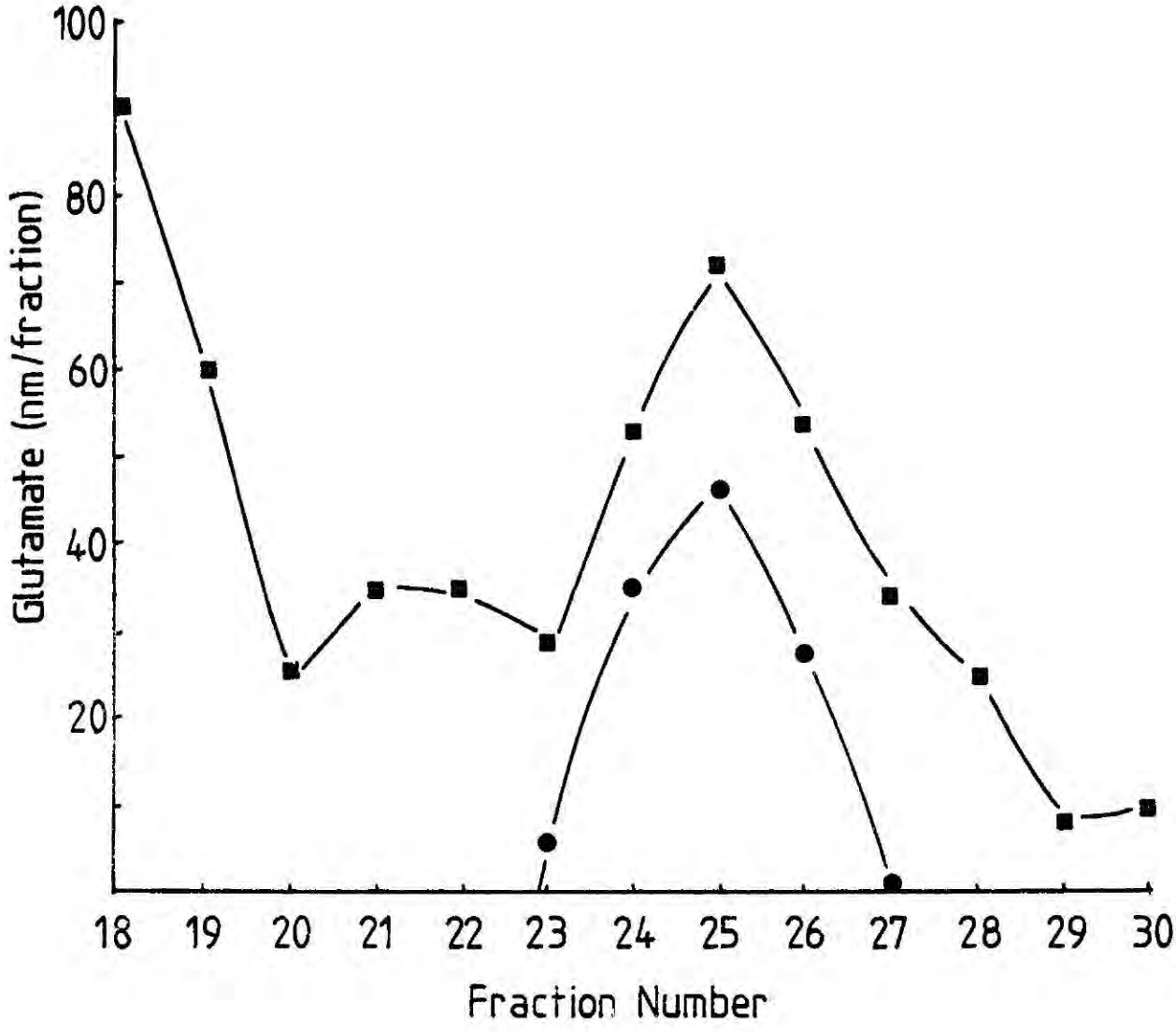


Figure 5.6: Free and 'Bound' Glutamic Acid in each G-15 Column Fraction

The levels of glutamic acid in each G-15 fraction, both before (●) and after (■) acid hydrolysis, were determined using dansyl chloride.

to the solvent front during chromatography and is consequently difficult to quantify.

(v) In addition to the protein amino acids, two unidentified dansyl-reactive compounds (presumably amino acids) were often present in embryo and endosperm extracts. Their locations on polyamide sheets, after chromatography, are shown in appendix 2.

Unknown 'E' is only found in the free form, never bound in peptides/proteins. It is not a peptide itself as it is unaffected by hydrolysis. It is also unlikely to be a derivative of one of the protein amino acids, formed during extraction, as its levels in the embryo do not parallel those of any other amino acid.

Unknown 'G' shows the same anomalous elution characteristics as glutamic and aspartic acids. In addition, it appears in the 'peptide'/'protein' fractions after hydrolysis. Thus, it cannot simply be a non-protein amino acid and is probably a derivative of glutamic or aspartic acid. Whether this derivative is formed during extraction or dansylation is not clear.

Dansyl chloride analysis also provides considerable information on the amino acid composition of the 'bound' and free amino acid pools. At any stage of germination the amino acid composition of the 'bound' amino acids eluting in each of the endosperm 'protein' fractions (17-20) is similar. In addition, the composition of these fractions does not vary as germination proceeds. Thus, the composition of the 'bound' amino acids eluting in the 'protein'

fractions, is presented as an average of all the 'protein' fractions from each stage of germination. A similar average is applicable to the 'bound' amino acids eluting in the endosperm 'peptide' fractions and both the 'protein' and 'peptide' fractions of the embryo.

The amino acid compositions of the 'protein' and 'peptide' fractions of the endosperm are presented in table 5.2, together with various amino acid analyses of barley reported in the literature. It must be remembered that arginine and tryptophan were not determined. Amino acid compositions from the literature have therefore been recalculated, excluding these two amino acids, to facilitate comparison. Similarly, table 5.3 shows the amino acid compositions of the 'bound' amino acids in the embryo 'peptide' and 'protein' fractions, together with two published analyses.

Unlike the 'bound' amino acid fractions, the compositions of the free amino acid pools of the embryo and endosperm vary during germination. Table 5.4 shows the composition of the free amino acid pool of barley endosperm at different stages of germination. In the embryo, the levels of one or two amino acids vary considerably during germination; expressing the composition of the pool in percentage terms then becomes meaningless. Thus, the composition of the free amino acid pool of barley embryos at different stages of germination is expressed both in percentage terms, and as nm of amino acid per embryo (table 5.5).

Table 5.2: Amino Acid Composition of the 'Proteins' and 'Peptides' of Barley Endosperm

	Determined in the present study				Recalculated from data reported in the literature[d]							
					Folkes and Yemm, 1956[c]					Whole Endosperm[a]	Whole Grains[a]	
	'Protein' Fractions[a]	'Protein' Fractions[b]	'Peptide' Fractions[b]	Whole Grains[a][d]	Hordein	Hordenin	Globulin	Albumin	Ave.[e]	Jones & Pierce 1967b	Pomeranz & Robbins, 1972	Rhoades & Mathers, 1974
Ala	8.6	8.9	8.0	4.6	3.1	9.2	1.1	9.2	5.0	6.8	5.0	4.5
Gly	15.5	16.1	19.9	4.9	2.4	7.2	17.8	8.6	6.4	8.3	4.8	4.2
Glu/Gln	15.9	15.8	12.9	28.4	33.1	16.2	11.4	10.5	22.9	21.8	25.2	25.2
Asp/Asn	8.7	8.7	8.4	7.1	1.7	6.5	9.3	10.3	4.9	6.9	8.0	7.0
Ser	7.2	6.7	7.4	5.0	4.5	5.8	6.5	5.3	5.1	6.3	4.4	4.8
Thr	5.1	4.6	5.4	3.2	2.7	4.3	4.0	4.4	3.4	4.4	4.0	3.9
Pro	13.9	15.5	13.6	11.7	21.7	9.2	4.5	5.4	14.0	12.4	11.5	13.3
Val	5.9	5.4	6.5	5.4	5.0	6.8	6.8	7.4	6.0	6.2	5.9	5.4
Cys	-	ND	ND	-	2.1	1.2	4.3	1.9	2.2	-	1.5	0.9
Met	-	ND	ND	1.7	-	-	-	-	-	1.8	2.6	2.1
Leu	5.5	5.3	5.9	8.0	6.5	8.1	7.5	7.3	7.0	8.1	7.6	7.6
Ile	3.1	2.5	3.4	3.8	5.1	4.9	3.7	5.3	4.9	3.9	4.1	3.8
Phe	1.9	1.9	1.3	6.3	5.1	3.7	3.5	3.8	4.4	4.4	5.4	6.5
His	2.0	2.6	3.4	2.6	3.1	6.0	5.2	5.5	4.3	2.1	2.6	2.3
Lys	5.5	3.7	2.7	4.0	1.1	6.7	10.5	10.1	4.9	4.0	4.4	4.5
Tyr	1.2	1.3	0.5	2.8	2.3	2.6	2.5	3.2	2.5	2.6	3.0	3.9
GAB	-	ND	ND	ND	-	-	-	-	-	ND	ND	0.2
G	-	1.2	0.8	-	-	-	-	-	-	-	-	-

All values are expressed as a percentage of the total amino acids recovered.

(a) Obtained using an amino acid analyser
(b) Obtained by dansyl chloride analysis
(c) Obtained by microbiological assay
(d) Using ungerminated grains
(e) Calculated from the compositions of each fraction, assuming the following contributions to the total seed protein: albumin, 15%, globulin, 13%; hordenin, 23%; hordein, 49% (Pomeranz, 1975)

ND = not detected. - denotes not determined

Table 5.3: Amino Acid Composition of the 'Proteins' and 'Peptides' of Barley Embryos

	'Protein' Fractions	'Peptide' Fractions	Whole Embryos[a] Jones & Pierce (1967b)[b]	Whole Embryos[a] Yemm & Folkes (1953)[c]
Ala	8.7	8.2	10.2	10.7
Gly	15.5	24.3	10.4	8.9
Glu/Gln	15.1	13.3	13.1	10.3
Asp/Asn	10.7	6.8	9.8	8.5
Ser	6.0	9.3	6.0	4.5
Thr	3.1	4.1	5.6	5.2
Pro	11.5	11.5	6.0	5.0
Val	6.6	5.1	7.1	6.6
Cys	ND	ND	-	1.2
Met	ND	ND	2.4	1.5
Leu	5.1	3.5	8.6	7.7
Ile	3.0	2.6	4.2	5.8
Phe	2.2	0.8	4.2	4.0
His	3.2	3.9	2.7	5.4
Lys	4.0	3.9	7.6	10.2
Tyr	3.5	1.7	2.8	3.0
GAB	ND	ND	0.7	-
G	1.8	1.1	-	-

Values, determined by dansyl chloride analysis, are presented as a percentage of the total amino acids recovered. ND = not detected. - denotes not determined.

(a) Recalculated from values reported in the literature.
(b) Determined using an amino acid analyser for 6-day embryos.
(c) Determined by microbiological assay on mature leaf tissue.

Table 5.4: Composition of the Free Amino Acid Pool of Barley Endosperm

Tissue	Whole Grain	Distal Half-Grain	Proximal Half-Grain	Endosperm					
Days of Germination	0	0	0	1	2	3	4	5	6
Ala	13.4	14.2	11.5	14.3	13.0	13.3	11.3	11.9	16.3
Gly	3.9	6.0	5.6	7.7	5.1	4.4	4.0	7.4	7.7
Glu/Gln	7.3	9.0	8.4	18.5	6.9	4.8	6.1	5.0	8.9
Asp/Asn	21.5	20.3	19.6	14.1	7.4	4.8	7.4	4.9	6.2
Ser	3.7	5.8	2.2	4.6	4.8	3.9	3.3	3.1	3.9
Thr	1.8	1.7	1.6	2.2	2.8	2.3	2.3	1.8	3.5
Pro	17.9	19.8	19.3	17.2	27.5	38.0	31.4	30.4	16.7
Val	5.4	5.9	6.6	5.5	8.2	8.8	10.0	9.0	8.3
Cys	ND	ND	ND	ND	ND	ND	ND	ND	ND
Met	ND	ND	ND	ND	ND	ND	ND	ND	ND
Leu	3.1	3.4	2.1	4.6	10.2	7.6	8.3	8.2	7.3
Ile	2.3	3.5	1.9	2.0	5.1	4.1	5.7	4.5	3.8
Phe	-	-	-	-	-	-	-	-	-
His	-	-	-	-	-	-	-	-	-
Lys	2.1	2.8	2.5	2.2	5.0	3.8	4.8	4.3	5.8
Tyr	-	-	-	-	-	-	-	-	-
GAB	4.0	3.1	5.6	3.9	2.6	3.3	4.1	8.6	10.6
G	1.3	1.8	1.6	2.0	0.7	0.4	0.7	0.8	1.0
E	12.3	2.9	11.7	1.2	0.4	0.3	ND	ND	ND

Values, determined by dansyl chloride analysis, are expressed as a percentage of the total amino acids recovered. ND = not detectable. - denotes not determined.

Table 5.5: Composition of the Free Amino Acid Pool of Barley Embryos

	Amino Acid Composition					
	nm/embryo			%(a)		
Days of Germination	1	2	3	1	2	3
Ala	24	67	92	12.0	8.1	9.2
Gly	13	29	31	6.5	3.5	3.1
Glu/Gln	19	37	89	9.5	4.5	8.9
Asp/Asn	10	165	162	5.0	19.9	16.2
Ser	9	40	45	4.5	4.8	4.5
Thr	5	29	37	2.5	3.5	3.7
Pro	49	90	118	24.6	10.8	11.8
Val	19	105	123	9.5	12.7	12.3
Cys	0.2	6	6	0.1	0.7	0.6
Met	ND	ND	ND	ND	ND	ND
Leu	6	103	100	3.0	12.4	10.0
Ile	8	67	56	4.0	8.1	5.6
Lys	12	50	41	6.0	6.0	4.1
GAB	9	30	80	4.5	3.6	8.0
G	6	6	9	3.0	0.7	0.9
E	10	6	9	5.0	0.7	0.9

Values were determined by dansyl chloride analysis. ND = not detected. Phe, His, Tyr, Trp and Arg were not determined.

(a) Expressed as a percentage of the total amino acids recovered.

5.3.5.2 N-terminal Analysis

The procedure used to analyse the N-terminal residues of peptides will detect any amino acid with a free amino group. Free amino acids eluting in the 'peptide' fractions are accounted for in calculating the N-terminal amino acids in each fraction. All other residues detected are referred to as N-terminal residues, although it must be remembered that they may actually be bound in other types of linkages.

N-terminal analyses of the endosperm 'peptide' fractions were performed with two aims in mind: (i) to obtain an estimate of the average peptide chain length, by comparison of the total number of residues released on hydrolysis with the number of residues occupying an N-terminal position, and (ii) to ascertain whether specific cleavage of the storage protein occurs, producing a non-random distribution of amino acids at the peptide N-termini.

Several problems were associated with such analyses:

(i) The low levels of peptide, and hence even lower levels of N-terminal amino acids, present in each fraction necessitated the dansylation of large volumes ($>400\mu l$) of each column fraction. The presence of considerable amounts of eluent salts in these volumes interfered somewhat with the dansylation and chromatographic procedures, making accurate quantification difficult: the value obtained for each amino acid is probably only accurate to about $\pm$ 30%. However, summation of all the N-terminal amino acids in each fraction should minimize any errors.

(ii) Certain dansyl-amino acids are not stable to acid hydrolysis. Although most show >90% recovery after hydrolysis, DNS-glycine gives only 80% recovery, DNS-serine and DNS-threonine less than 70%, and DNS-proline only 23% (Hartley, 1970). For all except proline (see (v) below), these figures were taken into account when calculating the absolute levels of each N-terminal amino acid.

(iii) Glutamic acid, aspartic acid and lysine elute from the G-15 column rather earlier than one would predict on the basis of their molecular weights (section 5.3.5.1); the free amino acids appear in the 'peptide' fractions. This masks any N-terminal glutamic acid, aspartic acid or lysine residues appearing in the same fractions.

(iv) Several amino acids (e.g. phenylalanine, histidine) only constitute a small proportion of the peptide amino acids, and therefore appear at the N-terminus in quantities too low to be detected by present methods. Thus, the absence of amino acids from the N-terminus only indicates they are present in amounts too small to detect.

(v) DNS-proline is almost completely degraded during acid hydrolysis. Thus, under standard conditions, no N-terminal proline residues will be detected. As proline is one of the major amino acids of barley storage protein, it is likely to be one of the more abundant N-terminal amino acids. In addition, it has been suggested that the barley peptidases may not be able to cleave bonds involving proline (sections 5.4.4.2). Thus, it is possible that proline might occupy an even higher proportion of N-terminal

sites than its general abundance would suggest.

Figure 5.7 shows the breakdown of DNS-proline during acid hydrolysis. After 4.5h about 70% remains intact. Within this period, a high proportion (about 70%) of peptide bonds involving proline are cleaved (fig. 5.8). Thus, the levels of N-terminal proline were determined after only 4.5h hydrolysis. The DNS-proline lost by hydrolysis, and the peptide bonds not cleaved during this period, were taken into account in calculating the number of N-terminal proline residues.

The total number of N-terminal residues recovered in each column fraction are presented in figure 5.3. The composition of these terminal amino acids did not vary significantly with the fraction number or during germination. Thus, the average amino acid composition of the N-terminal residues eluted in the endosperm 'peptide' fractions is presented in table 5.6.

5.3.5.3 Absorbance at 206nm

The absorbance of each column fraction at 206nm is shown in figures 5.3 and 5.4. Results are given as nm of peptide bond per fraction, using dialanine as a standard.

5.3.5.4 Fluorescamine Assay

The fluorescamine assay for peptides was performed at pH 7.0, rather than the standard pH 6.2 (section 2.4.3.1), in order to achieve increased sensitivity. Interference from amino acids is unimportant here as most free amino acids have already been separated from the 'peptide' fractions. Only glutamic and aspartic

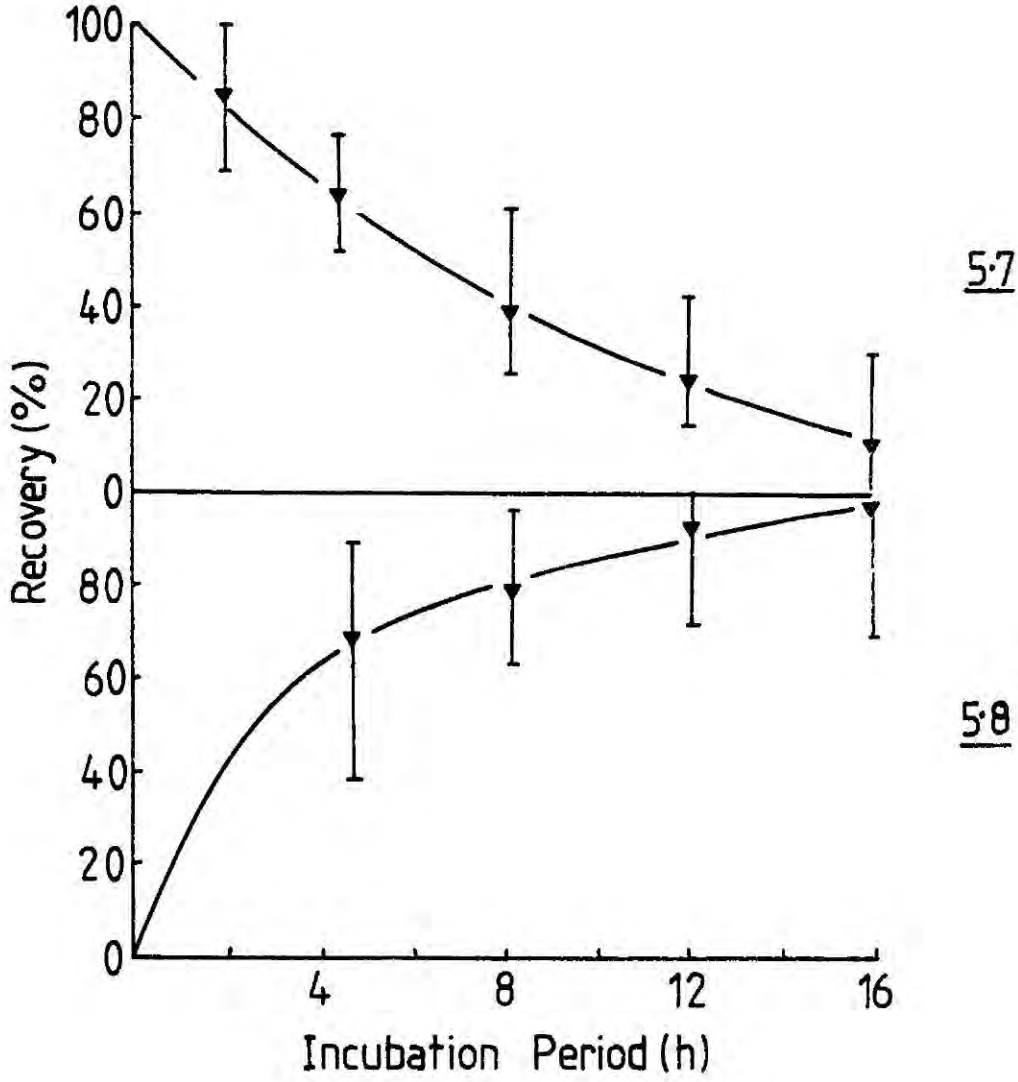


Figure 5.7: Acid Hydrolysis of DNS-Proline

The recovery of DNS-proline after varying periods of acid hydrolysis was monitored using dansyl chloride. Each value is the average of four separate determinations. Bars show the range of values obtained.

Figure 5.8: Acid Hydrolysis of Prolyl Peptides

The recovery of free proline from Pro-Gly or Pro-Gly-Gly after varying periods of acid hydrolysis was monitored using dansyl chloride. Each value is the average of four separate determinations for each peptide. No difference was observed in the release of proline from the two peptides. Bars show the range of values obtained.

Table 5.6: The N-terminal Amino Acids of Barley Endosperm Peptides

Amino Acid	N-Terminal Amino Acid Composition (%)(a)	Total Peptide Composition (%)(b)
Alanine	12.0	7.8
Glycine	11.6	19.4
Glutamate/Glutamine(c)	13.0	14.6
Aspartate/Asparagine(c)	9.7	8.2
Serine	8.1	7.2
Threonine	7.0	5.2
Valine	9.4	6.3
Proline	13.5	13.3
Leucine	6.3	5.7
Isoleucine	4.6	3.3
bis-Lysine(c)	2.6	3.1
ε-Lysine	2.0	-

(a) Each value is presented as a percentage of the total N-terminal amino acids recovered.

(b) Recalculated from Table 5.2, excluding those amino acids not detected at the peptide N-termini.

(c) Determined for those 'peptide' fractions where the free amino acids do not elute, and assumed to be the same for all other fractions (see section 5.3.5.1).

acids elute in the 'peptide' fractions (section 5.3.5.1) and these amino acids show negligible reaction at pH 7.0, due to the relatively high pK's of their α-amino groups.

Fluorescamine is potentially the most sensitive and specific of the assays employed, yet three possible limitations must be considered:

(i) Reaction with the ε-amino group of lysine. The problem of ε-amino groups is unlikely to be significant here. The high pK of the lysine ε-NH_2 group in peptides (Perrin, 1965) relative to the assay pH ensures that little, if any, reaction will occur (section 2.4.3.1). In addition, lysine only constitutes about 4% of the peptide-bound amino acids; this proportion remains constant in all fractions (sections 5.4.4.2).

(ii) Interference from secondary amines. Secondary amines, including proline and prolyl peptides, interfere with the reaction of fluorescamine with primary amines (section 2.4.3.6). As proline is one of the most abundant amino acids in barley storage proteins (Folkes & Yemm, 1956), as well as being the most important free amino acid (Jones & Pierce, 1967b; section 5.4.4.5), this will clearly be a problem. Figure 5.9 shows the fluorescence yields of 50µl and 200µl samples of the G-15 fractions of 1-day endosperm extracts. Fractions 21-27 show the expected four-fold difference in fluorescence yield between the 50µl and 200µl samples. However, in later fractions this relationship does not hold. Indeed, in some fractions a greater fluorescence yield is obtained with a 50µl sample

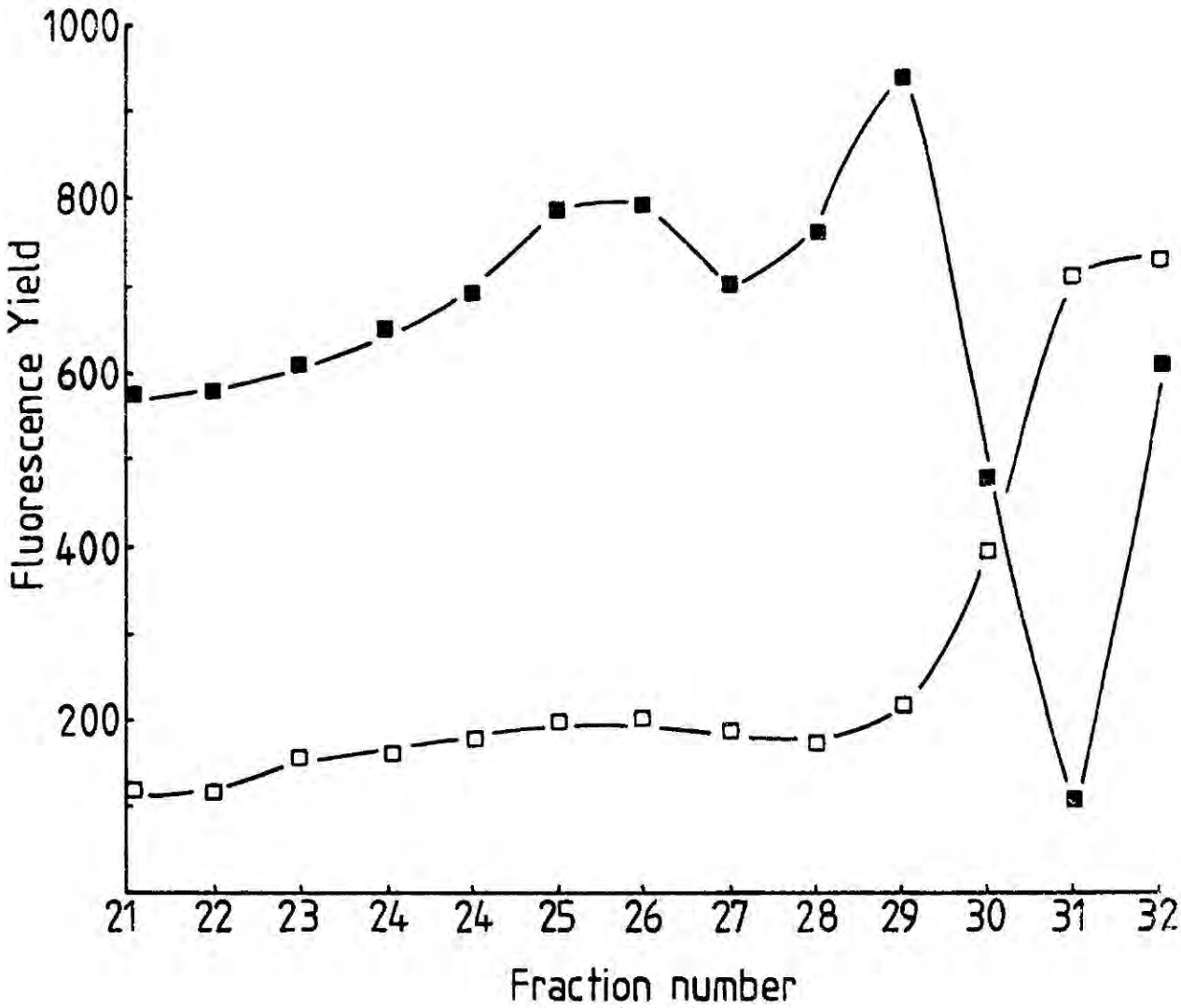


Figure 5.9: Fluorescamine Analysis of G-15 Column Fractions

The fluorescence yield obtained with 50µl (□) and 200µl (■) samples of G-15 column fractions of a 1-day endosperm extract were measured using standard procedures (section 2.3.2.2). Each value is the average of three separate determinations.

than a 200µl sample. This is almost certainly due to the high levels of free proline eluting in fractions 28-32 (section 5.3.5.1). Thus, the fluorescamine assay is only suitable for the 'peptide' fractions, in which little interference is apparent. Although peptides with N-terminal proline residues are present (section 5.3.5.2) they do not appear to interfere with the assay to any great extent. This is borne out by a control experiment in which it was shown that a similar fluorescence yield is obtained for a given amount of dialanine, whether the reaction with fluorescamine is performed in the presence or absence of a 200µl sample of any 'peptide' fraction.

(iii) Choice of a standard with which to calibrate the assay. Although an apparently meaningful fluorescence yield is obtained on analysis of any 'peptide' fraction, absolute quantification of α-amino groups is not possible. At any given assay pH, different peptides will give different fluorescence yields, depending upon the pK of their α-amino group (section 2.4.3.1). Diglycine was selected as a standard with which to calibrate the assay, as the pK of its α-amino group is in the middle of the range of values extended by physiological peptides. Thus, although fluorescamine results are expressed in terms of nm of primary amino groups, it must be remembered that this relates to a diglycine standard. Values can be used comparatively, although in absolute terms they may have little meaning.

The peptide α-amino groups present in each G-15 fraction, as estimated by the fluorescamine method, are presented in figures 5.3a-g (endosperm) and 5.4a-c (embryo).

5.3.5.5 TNBS Assay

TNBS (2,4,6-trinitrobenzene 1-sulphonic acid) reacts with primary amino groups of amino acids and peptides to form coloured TNP-derivatives (Okuyama & Satake, 1960; Satake et al., 1960). However, under the conditions employed, there is no reaction with proline or prolyl peptides (Payne, 1972b).

The assay conditions employed were based upon Binkley et al.(1968), although certain modifications were introduced to improve sensitivity.

TNP-amino acids in acidic solution show an absorbance maximum at 340nm with a secondary peak at 420nm (Satake et al., 1960). Acidification not only increases sensitivity at 340nm, but also stabilizes the colour produced. Thus, an acidification step has generally been included in the assay. However, it was found that although acidification increases the sensitivity at 340nm, it actually reduces sensitivity at 420nm (table 5.7). Maximum sensitivity is achieved by measuring the absorbance at 420nm in alkaline solution. Under these conditions, although the formation of TNP-amino acids is complete within 15 min, the colours of both the TNP-amino acids and the reagent blank are unstable (fig. 5.10). Thus, when employed routinely, precise incubation periods and the simultaneous running of reagent blanks are both essential.

Table 5.7: Sensitivity of the TNBS Assay Under Various Conditions

Wavelength (nm)	HCl[a]	Absorbance		
		Glycine	Reagent Blank[b]	Glycine (reagent blank subtracted)
420	-	0.733	0.097	0.636
420	+	0.355	0.090	0.265
340	-	1.067	0.814	0.253
340	+	1.187	0.790	0.397

Assays were performed as described in methods (section 5.2.3.4) using 0.1mM glycine as a standard. All absorbances were read against a distilled water blank. Each value is the average of three separate determinations.

(a) + and - denote presence and absence, respectively.

(b) Obtained by replacing the glycine solution with distilled water.

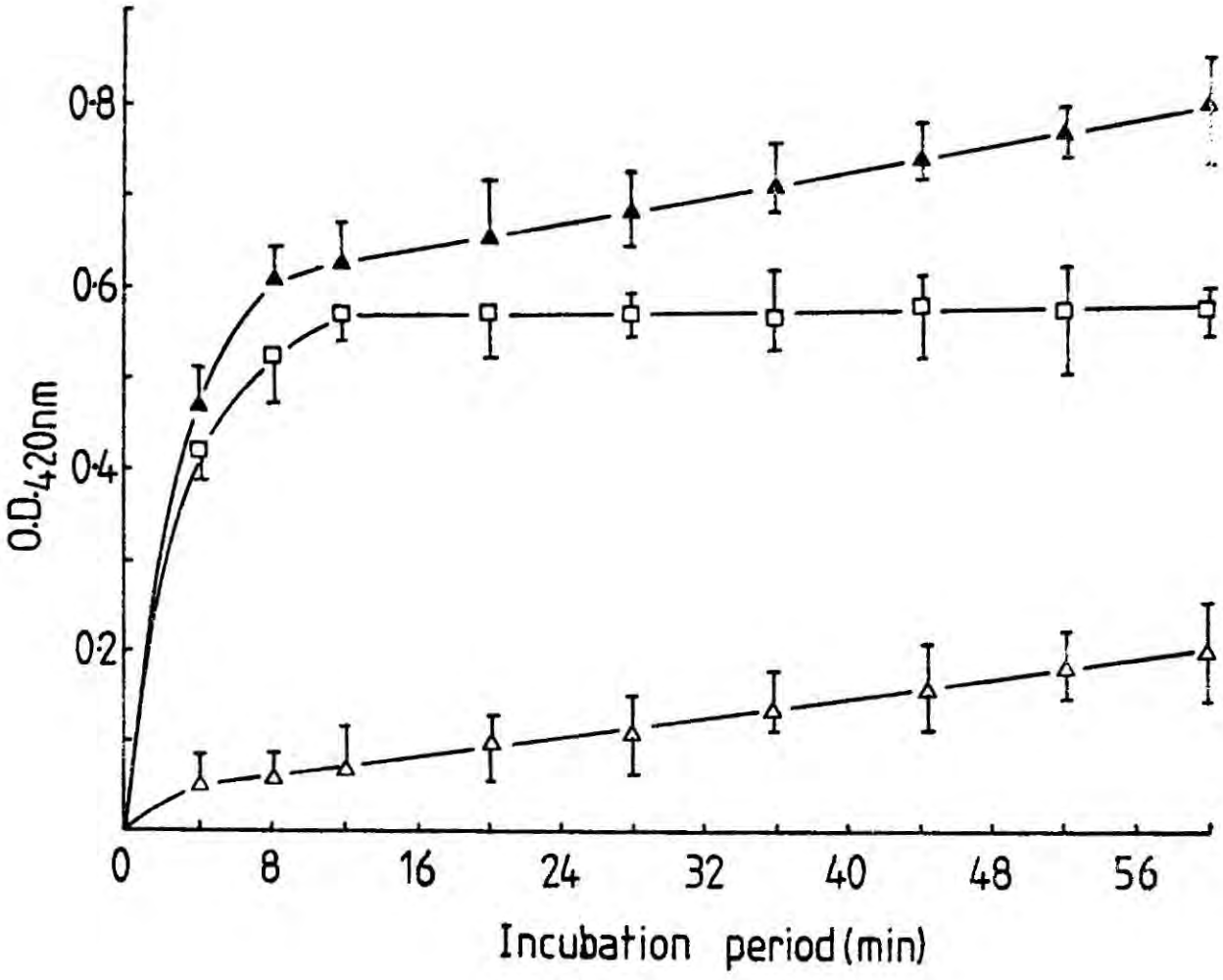


Figure 5.10: Time Course for the Formation of TNP-Glycine

The reaction of glycine (0.1mM) with TNBS was carried out under standard conditions (section 5.2.3.4). The O.D. at 420nm was measured against a distilled water blank: glycine (▲), reagent blank (△), glycine with reagent blank subtracted (□). Each value is the average of three separate determinations. Bars show the range of values obtained.

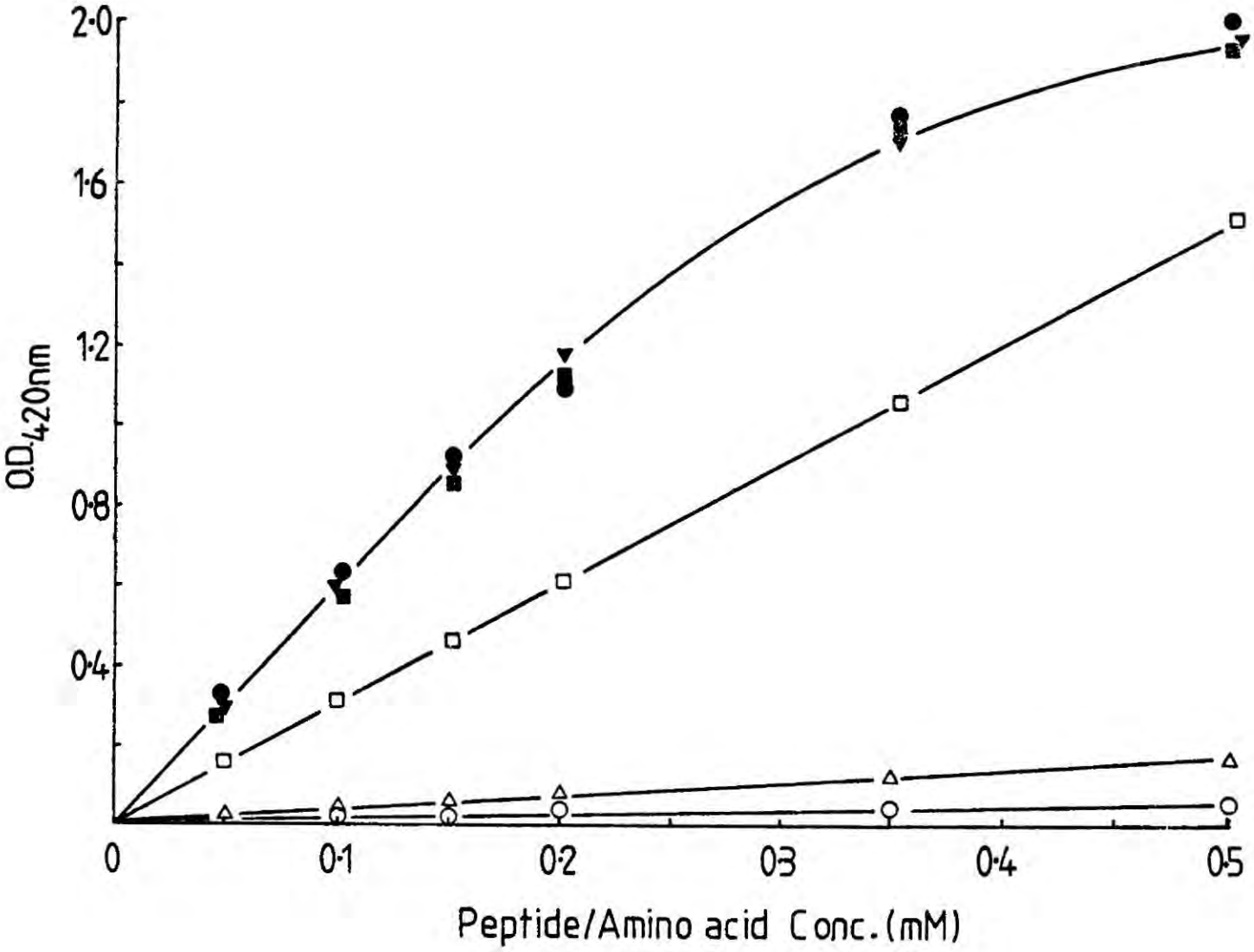


Figure 5.11: Effect of Cu^{2+} ions on the TNBS Assay

The TNBS assay was carried out under standard conditions (section 5.2.3.4), using varying concentrations of the following amino acids or peptides: glycine (■), diglycine (▼) or triglycine (●) in the absence of Cu^{2+} ions; glycine (□), diglycine (△) or triglycine (○) in the presence of Cu^{2+} ions. Each value is the average of three separate determinations.

Cu^{2+} ions have been reported to interfere with the reaction between TNBS and peptides (Binkley et al., 1968). This is illustrated by figure 5.11. In the absence of Cu^{2+} ions, amino acids, dipeptides and tripeptides give very similar colour yields. However, in the presence of Cu^{2+} ions, although the reaction with amino acids is slightly reduced, the reaction with peptides is almost completely inhibited.

Phosphate buffer in the G-15 fractions was found to interfere with the TNBS assay in the presence of Cu^{2+} ions. Thus, 1- and 3-day endosperm and embryo extracts were passed through the G-15 column using unbuffered 0.1M saline (adjusted to pH 7.0) as eluent. Judging from the 206nm absorbance profile, the lack of buffering capacity had little effect on the separation achieved. These fractions were used for the TNBS assay in the presence of Cu^{2+} ions.

The levels of amino acid and peptide in each column fraction, estimated by the TNBS assay, are presented in figures 5.3 and 5.4.

5.3.6 Separation of 'Protein' fractions on a G-50 column

The compounds eluting in the endosperm 'protein' fractions (17-20) were pooled, evaporated to dryness on a rotary evaporator, redissolved in 1.0ml PBS, and separated on a G-50 Sephadex column (section 5.2.6).

Figure 5.12 shows the 206nm absorbance profile of column effluent during elution of the 'protein' fractions from 3-day endosperm. The volumes at which a number of standards elute are also indicated (the insulin α- and β-chains

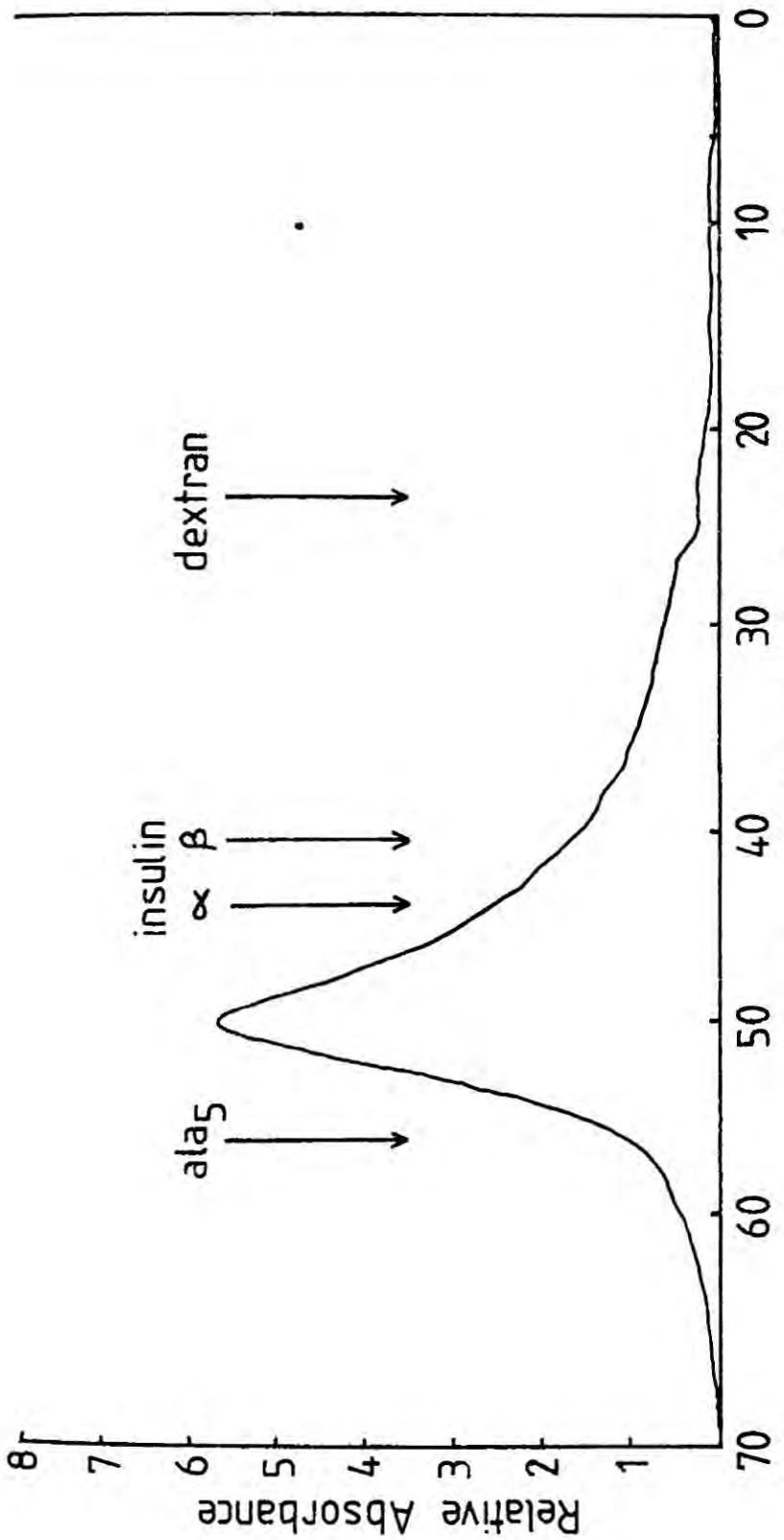


Figure 5.12: Separation of [illegible] of Endosperm 'Protein' Fractions by Gel-Filtration

The absorbance of column effluent was monitored continuously at 206nm during the separation of the pooled 'protein' fractions from a 3-day endosperm extract (see section 5.2.6 for methods). The positions at which a number of standards elute are also indicated.

contain 21 and 30 residues respectively). Although the secondary structure of polypeptides may affect their elution through the column, an approximate relationship between elution volume and chain length would still be expected. Assuming that the 'bound' amino acids eluting in the 'protein' fractions are peptide-bound, comparison with the insulin standards shows that the 'protein' fractions actually contain polypeptides of 5-20 residues; the average chain length is about 10 residues. Very few polypeptides/proteins with more than 20 residues are present in the extract. Compounds present in the 'protein' fractions of 1- and 6-day endosperm extracts showed a very similar size distribution, as judged by the 206nm absorption profile, although the absolute amounts present varied considerably.

5.3.7 Amino Acid Analyses

The amino acid composition of ungerminated barley, and of the 3-day endosperm 'protein' fractions, as determined on an amino acid analyser, are shown in table 5.2. In calculating the percentage of each amino acid, arginine was ignored, to aid comparison with data obtained by other methods.

5.4. Discussion

5.4.1 Methodology

The procedures employed for the isolation of barley peptides seem entirely adequate for the purpose; minimal losses occur during the extraction and purification steps (sections 5.3.3, 5.3.4). The extracted peptides would therefore seem to be representative of the pools present *in vivo*.

5.4.1.1 Peptide Separation

Separation of peptides by Sephadex G-15 gel-exclusion chromatography is also effective. Apart from those anomalies discussed previously (section 5.3.5.1), several lines of evidence indicate that separation is based upon molecular weight.

(i) No 'bound' amino acids (presumptive peptides) elute in the amino acid fractions (28-30). Thus, there is no retardation of 'peptide' movement in the column.

(ii) Known peptides, applied to the G-15 column in the presence of endosperm extract, elute in the expected fractions (section 5.3.4).

(iii) The average chain length of the 'peptides' in each fraction, as estimated from the ratio of the N-terminal amino acids to the total 'bound' amino acids (section 5.4.3), corresponds well with the values predicted from the column calibration (section 5.3.2).

(iv) In an 'average' protein, the majority of lysine and tyrosine residues will be located within the polypeptide chain, rather than at the N-terminus. Thus, N-terminal analysis will give extremely high ratios of DNS-o-tyr and DNS-ε-lys to the bis-derivatives. However, if the protein has been randomly cleaved to small peptides, this ratio

will be very much smaller. The absence of any detectable DNS-o-tyr, and no more DNS-ε-lysine than bis-DNS-lysine, in the 'peptide' fractions, is a good indication that no large polypeptides/proteins are present.

Thus, a number of independent lines of evidence indicate that adequate separation of peptides, proteins and amino acids is achieved: only peptides with less than about six residues will elute in fraction 21-27, the 'peptide' fractions.

5.4.1.2 Amino Acid Quantification

Most amino acids have no measurable absorbance at 206nm, and the high levels of free proline interfere with analysis using fluorescamine (section 5.3.5.4). Thus, only the TNBS and dansyl chloride methods were used for the assay of free amino acids. Both methods gave similar levels of free amino acids in each fraction. The somewhat lower values obtained with TNBS in certain fractions may reflect the lack of reaction of this reagent with proline.

5.4.1.3 Peptide Quantification

The most reliable estimates of the amount of peptide in each fraction might be expected to come from dansyl chloride analysis after acid hydrolysis. However, it is inadvisable to assume that all amino acids released by hydrolysis are, in fact, peptide-bound (section 5.1). In addition, in order to determine the peptide concentration, it is essential to know the 'average' chain length in each fraction. Although the theoretical values are known, from calibrating the G-15 column (section 5.3.2), it is

important to demonstrate that the peptides are indeed adequately separated. Thus, several additional methods were employed to estimate the peptide concentration in each 'peptide' fraction; together these show that the 'bound' amino acids detected using dansyl chloride are indeed small peptides.

Absorbance at 206nm: The close agreement between the levels of 'bound' amino acids (estimated by dansyl chloride) and the number of peptide bonds (estimated by 206nm absorbance) indicates that the 'bound' amino acids are indeed peptides. This being the case, little non-peptide material which absorbs at 206nm seems to elute in the 'peptide' fractions. Thus, 206nm absorbance can be used as a rapid scanning method to estimate the amount of peptide eluted in each fraction. However, 206nm absorbance estimates the peptide bonds present, while dansyl chloride estimates the peptide-bound amino acids. For large peptides, the presence of one more amino acid residue in each peptide than the total number of peptide bonds will not lead to any significant difference in the level of peptide estimated by each method. However, for smaller peptides, estimation by 206nm absorbance would be expected to give a considerable underestimate of the amount of peptide present, and for dipeptides, would only be expected to give 50% of the peptide level estimated by dansyl chloride. However, such differences between the two estimates are not observed. In fractions containing only very small peptides (26 and 27) the absorbance at 206nm may even exceed the

number of 'bound' amino acid residues estimated by dansyl chloride. Thus, in these later 'peptide' fractions (26 & 27), non-amino compounds interfere with peptide estimation by 206nm absorbance. Similarly, the high 206nm absorbance shown by the 'amino acid' fractions indicates that, here too, similar non-peptide compounds are eluted.

Fluorescamine, TNBS and N-terminal analyses: Both fluorescamine and TNBS give an estimate of the free amino groups present in each fraction. The close agreement between the two assays indicates that, although arbitrary, diglycine is in fact a good standard for the fluorescamine reaction (figs. 5.3 & 5.4).

In the 'protein' fractions, the ratio of free amino groups to the total number of 'bound' amino acids, is small. However, in the 'peptide' fractions this ratio is considerably larger; as the total number of 'bound' amino acids decreases (from fractions 17 to 27), the number of free amino groups actually increases. Assuming the 'bound' amino acids are all peptide-bound, the average chain length of the peptides in each fraction can be calculated by dividing the number of amino acids by the number of free amino groups. The values obtained correspond well with those predicted from calibration of the G-15 column (data not shown).

The number of N-terminal amino acids in each fraction is very similar to the number of free amino groups obtained using fluorescamine and TNBS. Consequently, the chain lengths of the 'peptides' in each fraction, calculated from the number of 'bound' amino acids and the number of

N-terminal residues, are also very similar to the predicted values.

Thus, it seems reasonable to assume that the 'bound' amino acids, determined using dansyl chloride, are indeed peptide-bound. The excellent agreement between the experimentally derived and the predicted peptide chain lengths in each fraction, seems unlikely to be an artefact in ten separate extracts. Besides, there is considerable independent evidence that peptides do indeed elute from the G-15 column in the fractions predicted on the basis of their molecular size (section 5.4.1.1).

5.4.2 Size of the Peptide and Amino Acid Pools

Assuming that the 'bound' amino acids are conjugated in peptide linkages, the total 'peptide' (fractions 21-27) and free amino acid pools (fractions 28-32), in both the endosperm and the embryo, can be calculated for each stage of germination. The size of the 'peptide' pool is calculated from the levels of 'bound' amino acids, assuming that the average peptide chain length in fractions 21-27 is three residues (see fig. 5.2). In a system like the germinating seed it is difficult to know the best form in which to express the amount of any individual component, due to the rapid changes in both water content and dry weight as germination proceeds. Thus, the sizes of the peptide and amino acid pools have been expressed in several different ways (tables 5.8 & 5.9). Figure 5.13 shows the variations in the sizes of these pools, in both the embryo and endosperm, as germination proceeds. A number of interesting features are apparent:

Table 5.8: Sizes of the Total Free Amino Acid Pools of Barley Grains During Germination

Tissue	Whole Grain	Distal half-grain	Proximal half-grain	Endosperm						Embryo		
Days of Germination	0	0	0	1	2	3	4	5	6	1	2	3
nx/grain	271	196	185	155	399	547	402	313	205	197	829	996
µx/g dry wt.	7.9	6.5	7.8	4.1	14.2	21.0	23.6	23.5	26.0	154	187	40
µx/g fresh wt.	7.2	6.0	7.1	2.5	7.1	8.6	8.3	6.6	5.1	31	16	28
µx/ml cell water	76.2	63.0	75	6.2	14.2	14.5	12.9	9.2	6.3	39	18	30

Table 5.9: Sizes of the Peptide Pools of Barley Grains during Germination

Tissue	Whole Grain	Distal half-grain	Proximal half-grain	Endosperm						Embryo		
Days of Germination	0	0	0	1	2	3	4	5	6	1	2	3
nm/grain	69	64	45	20	117	116	96	83	62	34	62	36
µm/g dry wt.	2.0	2.2	1.9	0.6	4.2	5.0	5.6	6.2	7.8	26.0	13.9	14.4
µm/g fresh wt.	1.9	2.0	1.7	0.4	2.1	2.0	2.0	1.8	1.5	5.3	1.2	1.0
µm/ml cell water	19.5	20.8	18.4	0.9	4.2	3.4	3.1	2.4	1.9	6.6	1.3	1.1

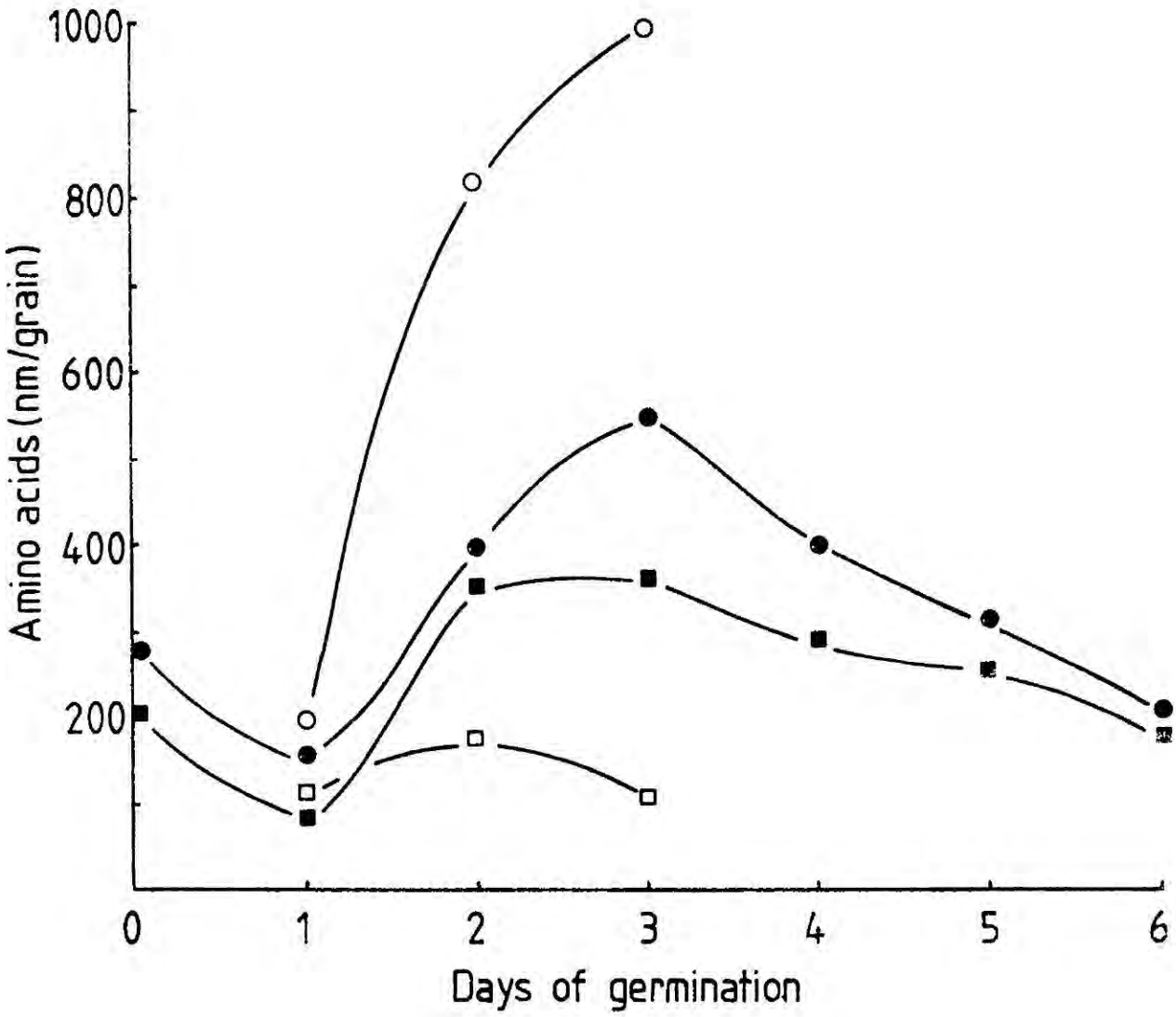


Figure 5.13: Changes in the Peptide and Free Amino Acid Pools of the Barley Endosperm and Embryo during Germination

Peptides (□,■) and amino acids (o,●) in the embryo (open symbols) and endosperm (closed symbols) were estimated using dansyl chloride. Peptide concentrations are expressed as nm of peptide-bound amino acids per grain.

(i) There are considerable amounts of peptide and free amino acids in the ungerminated barley grain. Comparison of the amount of peptide in the proximal and distal half-grains (table 5.9) indicates that most of the peptide is endospermal (especially considering the embryo only occupies a small proportion of the proximal half-grain). However, it seems that the free amino acids are at a higher concentration in the embryo than the endosperm (table 5.8). Jones and Pierce (1967b) have reported that 38% of the total free amino acids of ungerminated barley grains are in the embryo.

The high levels of peptide in the ungerminated grain may reflect the length of time the grains were stored. They were harvested at least three years prior to this study. Low levels of proteolytic activity in the ungerminated grains may have produced a pool of peptides; enzymic activity has been reported in unimbibed barley grains (Stevens & Stevens, 1976). Alternatively, these peptides may have been present as the grain matured and dried.

(ii) The levels of peptide in the endosperm decrease during the first 24h of germination. This may be due to the movement of peptides to the embryo via the peptide transport system, known to be active after only a few hours of germination (section 6.2). In addition, the overall loss of peptide from the grain (embryo plus endosperm) between days 0 and 1, indicates that peptidases are active.

(iii) The size of the peptide and amino acid pools in the endosperm increases to a maximum after 2-3 days of

germination, and subsequently declines (fig. 5.13). Similar changes in the soluble, non-protein nitrogen fractions (probably peptides), and the free amino acid pools, have been reported in barley (Folkes & Yemm, 1958) and maize (Ingle et al., 1964; Oaks & Beevers, 1964) endosperm.

(iv) The size of the peptide pool reaches a peak before the free amino acid pool. This might be expected if free amino acids are produced from peptides by hydrolysis.

(v) There are similar amounts of free and peptide-bound amino acids in the endosperm. This indicates that peptides might be at least as important as amino acids in the transport of nitrogen from the endosperm to the embryo.

The concentration of peptides in the endosperm reaches 3-4mM during germination (table 5.9). This value is, of course, a minimum concentration. Assuming all the peptides have been extracted, it is unlikely that they are evenly distributed throughout the endosperm in vivo; higher concentrations may be reached adjacent to the scutellum. These peptide concentrations are remarkably close to the K_t values obtained for the scutellar peptide transport system (section 3.1.3.6). Thus, peptides are present in the endosperm at adequate concentrations for the efficient operation of the peptide transport system. It can be calculated that the rate of peptide transport at these physiological concentrations of peptide can adequately account for the complete transfer of nitrogen from the endosperm to the embryo during germination (appendix 4). Similarly, the rates of amino acid transport are unable to account for this transfer.

(vi) Peptides are also detected in the embryo. This is consistent with a number of reports which indicate that peptides may be present in growing seedlings (section 1.2.3). These may arise from the movement of peptides into the embryo from the endosperm. Alternatively, they may be produced within the embryo, either as precursors of protein released from the ribosomes during extraction, or more probably from the degradation of storage proteins known to be present in the scutellum (Dure, 1960).

(vii) Unlike the peptide pool, the free amino acid pool of the embryo increases very rapidly during germination. Similar increases have been reported elsewhere (Folkes & Yemm, 1958; Ingle et al., 1964; Jones & Pierce, 1967b). However, although the total amount of amino acids per embryo increases markedly, the concentration of amino acids in the cell fluid remains relatively constant (table 5.8).

(viii) Although the total amount of peptide in each endosperm decreases between days 2 and 6 of germination, the amount per g dry weight actually increases (fig. 5.14). Assuming a seed protein content of 10%, at day 2 about 3% of the endosperm nitrogen is present as peptide or amino acid. This proportion can reach 15% by day 6, as nitrogen is removed from the endosperm.

5.4.3 Peptide Chain Length

At any particular stage of germination, peptides of all sizes are present in similar amounts; there is no preponderance of, say, di- or tripeptides. Thus, although

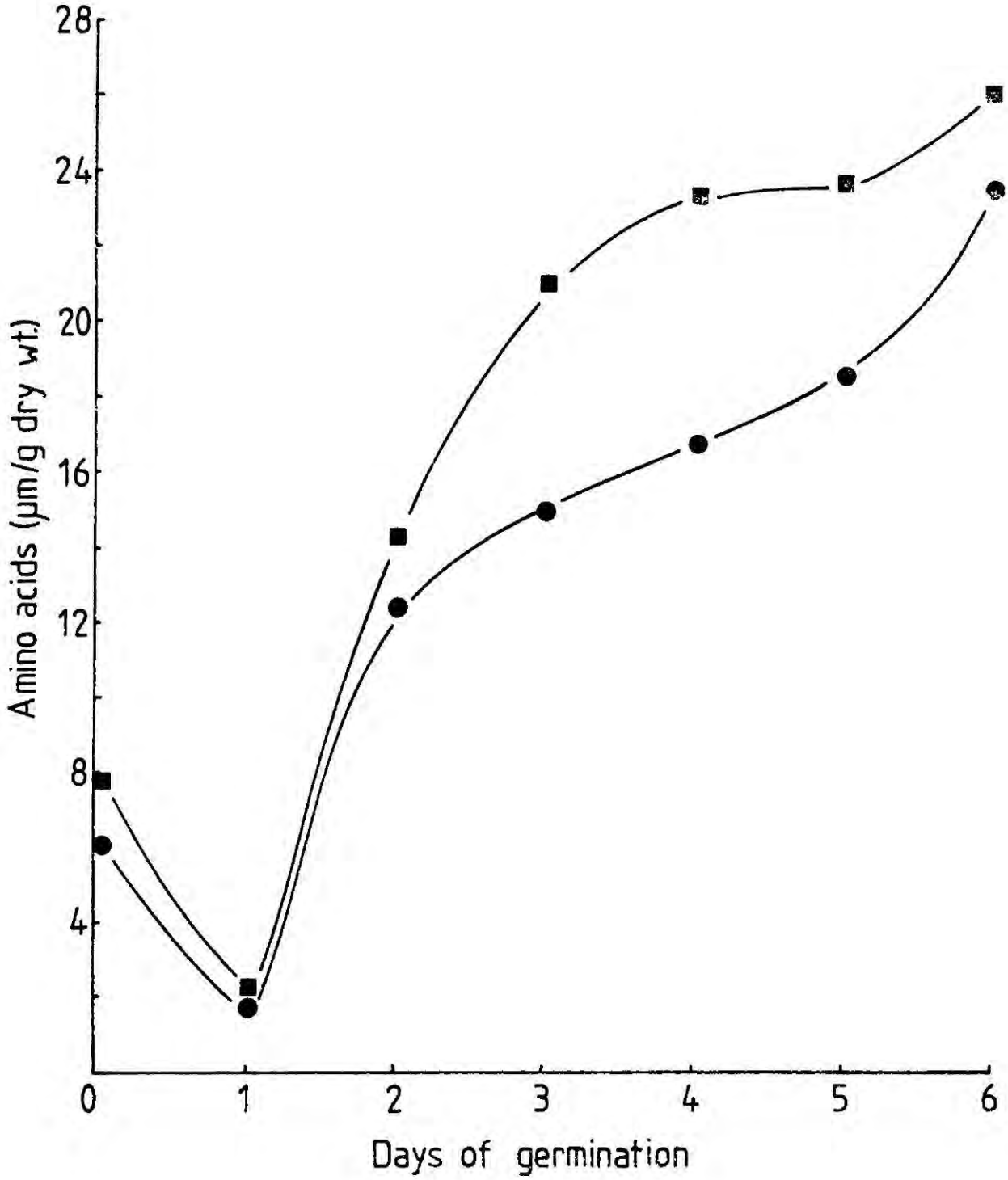


Figure 5.14: Changes in the Free Amino Acid and Peptide Pools of Barley Endosperm During Germination

Peptides (●) and amino acids (■) were estimated using dansyl chloride. Peptide concentrations are expressed as µm of peptide-bound amino acids per g dry weight.

small peptides are produced in the endosperm during germination they do not accumulate, and may even decline, despite the apparent absence of any peptidase activity (section 1.3.4). This is a good indication that peptides are being transported to the embryo.

5.4.4 Amino Acid Compositions

5.4.4.1 Endosperm 'Protein' Fractions

The amino acid composition of the endosperm 'protein' fractions remains relatively constant throughout germination (section 5.3.5.1). Although at day 0 this analysis includes the embryo, this is unlikely to affect the overall composition. The embryo contains less than 10% of the total barley protein (Jones & Pierce, 1967b) and anyway, the composition of the embryo 'protein' is similar to that of the endosperm (section 5.4.4.3).

However, the composition of the isolated 'protein' fractions differs from the amino acid composition of the ungerminated grain (table 5.2). This difference seems to be real:

(i) The composition of the 'protein' fractions is the same, whether estimated using dansyl chloride or an automatic amino acid analyser.

(ii) The total amino acid composition of the present strain of barley (Maris Otter, Winter) is very similar to all other strains (table 5.2).

(iii) Experimental error is unlikely to be responsible for the difference, as the same composition was obtained

independently for the endosperm at seven different stages of germination.

(iv) The difference is not simply due to the separation of peptides and amino acids from the 'protein' fractions. Peptides have the same composition as the 'protein' fractions (table 5.2), and only 2% of the total amino acids of the ungerminated grain are present in an unbound form (calculated from table 5.8, assuming a grain protein content of 10%).

(v) The non-digestible proteins (e.g. the husk) of the barley grain are unlikely to account for this difference, as the 'average' amino acid composition of the barley storage proteins is very similar to the total composition of the grain (table 5.2).

One would not necessarily expect the amino acid composition of the 'protein' fractions to resemble the overall amino acid composition of the grain, as no attempt was made to extract a representative sample of the endosperm proteins. However, the 'protein' fractions do not simply represent the specific extraction of one of the main storage proteins. Comparison of the amino acid composition of the 'protein' fractions with the composition of each of the major barley storage proteins (table 5.2) shows that no single protein has been specifically extracted. The high levels of glutamic acid and proline in the 'protein' fractions would suggest they are derived from hordein, yet the high levels of alanine and glycine show that hordenin, albumin and globulin must also be involved.

The extracted 'protein' fractions actually contain no protein, but a mixture of polypeptides varying from 6-20 residues long (fig. 5.12). Clearly, any proteins which might have been extracted have all been precipitated during the remainder of the isolation procedure. Although the amino acid composition of these polypeptides seems different from the total composition of the barley grain, the two compositions are in fact remarkably similar, except for two amino acids. The extracted polypeptides have very low levels of glutamate/glutamine and high levels of glycine. The low levels of glutamate/glutamine may be accounted for by the ease with which these two amino acids are converted to pyroglutamate, which may be lost during purification and separation (section 5.3.5.1). However, other differences, particularly the high level of glycine, presumably reflect the specificity of protease/peptidase activity (see section 5.4.4.2).

5.4.4.2 Endosperm 'Peptide' Fractions

The composition of the endosperm 'peptide' fractions is also constant throughout germination. The composition is very similar to the endosperm 'protein' fractions, the only important difference being the even higher proportion of glycine in the peptides (table 5.2). There is no evidence for the development of specific enzymic activities; protein/peptide cleavage is apparently uniform throughout germination. In addition, the composition of the peptide pool seems to be independent of peptide size, indicating that specific small peptides do not occur in large

quantities. For example, if γ-glutamyl peptides had been important, a higher proportion of glutamate in the dipeptide fractions might have been anticipated.

The amino acid composition of the 'peptides', like that of the 'protein' fractions, differs from the overall composition of the barley grain (table 5.2). Given that these differences are not artefactual (see section 5.4.4.1), three possible explanations may be invoked:

(i) Only certain of the storage proteins are being degraded to peptides. This is unlikely, as it seems that all the major storage proteins of barley contribute to the 'protein'/'peptide' fractions (section 5.4.4.1).

(ii) The unexpectedly high levels of peptide-bound glycine may reflect the rate of transfer from the endosperm to the embryo. In this context, it is interesting to note that glycine homopeptides are poorly transported by the scutellar peptide transport system (section 3.2.3.2). However, the high levels of glycine in the 'protein' fractions, which one would not expect to be available for transport, preclude this explanation, although the greater proportion of glycine in the 'peptide' than the 'protein' fractions may still reflect a poor affinity for transport.

(iii) Specific cleavage of storage proteins is occurring. Two main types of enzyme operate in the endosperm. Endopeptidases cleave the proteins to smaller polypeptides which can then be attacked by carboxypeptidases (section 1.3.4.5). It is possible that bonds including glycine residues are particularly resistant to attack by barley

carboxypeptidases and thus, the proportion of glycine in the peptide fractions is high. This view is also supported by the low levels of free glycine noted in the endosperm (section 5.4.4.5), and produced during endosperm breakdown in degermed malt, where metabolic removal of free glycine is unlikely (Jones & Pierce, 1966). Similarly, the rapid removal of glutamate/glutamine would account for their low levels in the peptides. In this respect it might prove fruitful to perform C-terminal analyses on the peptide fractions.

It is interesting to note that peptides isolated from wheat flour also have relatively high levels of glycine and low levels of glutamic acid. A similar high glycine/low glutamate profile was produced on proteolysis of soluble wheat flour protein (Grant & Wang, 1972). In maize, far more glycine is found in endosperm leachate (presumably containing peptides) than in the storage proteins (Oaks & Beevers, 1964).

Nothing is known about the specificities of barley carboxypeptidases for glycine and glutamic acid residues, although wheat carboxypeptidases seem to have a broad specificity (Preston & Kruger, 1977). However, these enzymes do have a restricted activity on peptide bonds involving a proline residue (Visuri et al., 1969; Moeller et al., 1970; Yabuuchi et al., 1973; Ray, 1976; Baxter, 1978). The fact that proline residues were not found to accumulate in peptides indicates that either carboxypeptidase activity is sufficient to release free proline, or that a

specific enzyme(s) for cleaving such bonds exists. Some evidence in favour of such an enzyme has been presented (Jones & Pierce, 1967a), and the carboxypeptidases of wheat will apparently cleave bonds involving proline (Preston & Kruger, 1976b, 1977).

5.4.4.3 Embryo 'Protein' Fractions

Although not determined, it seems probable that the embryo 'proteins' are also actually polypeptides of less than 20 residues. Their composition resembles the composition of the proteins from barley seedlings reported by other authors (table 5.3), although are rather high in glycine and proline and low in lysine. As for the endosperm 'protein' fractions, this may represent differential extraction, the specific degradation of scutellar storage proteins, or simply the fact that the young embryos analysed here do have a different amino acid composition (probably the result of scutellar storage proteins) from older seedlings.

5.4.4.4 Embryo 'Peptide' Fractions

The amino acid composition of the embryo 'peptide' fractions is very similar to the 'protein' fractions, except that they contain very much more glycine. This may be the result of the transport of glycine-rich peptides from the endosperm to the scutellum. On the other hand, it may be due to the lack of peptidase activity on glycine-containing peptides.

5.4.4.5 Free Amino Acid Pools

The absence of arginine, phenylalanine, histidine, tyrosine and tryptophan from the pools reflects the limitations of the methods employed (see section 5.3.5.1).

Changes in the composition of the free amino acid pools during germination, expressed in percentage terms, are often misleading due to the large variations apparent in the levels of one or two particular amino acids. In addition, the considerable differences in concentration between the amino acids eluting in any one fraction made accurate estimation by the dansyl chloride method difficult. However, comparisons can be made with the 'peptide' pools.

The composition of the endosperm amino acid pool remains relatively constant during germination, yet the composition is very different from that of the 'peptide' pool (table 5.4). The levels of leucine, valine, and particularly proline, are much higher than in the 'peptide' pool, while glycine and glutamic acid are relatively low. Presumably the amount of any particular amino acid in the endosperm reflects a balance between its rate of production by carboxypeptidase activity and its transfer to the embryo. In addition, two amino acids, 'E' and GAB, are found which are totally absent from the 'protein'/'peptide' fractions.

The free amino acid pool of the embryo is similar to that of the endosperm (table 5.5), although its overall size increases enormously during germination.

A number of interesting variations in composition, during germination, can be identified. The unknown amino

acid, E, is present in relatively large amounts in the ungerminated seed. Comparison of the composition of proximal and distal half-grains shows that E is predominantly located in the embryo. During germination, E rapidly disappears from the endosperm. In the embryo, however, although as a percentage of the total amino acid pool E seems to decrease, in terms of nm/grain it remains relatively constant. Thus, the apparent loss of E in the embryo is actually due to a constant level being maintained, while all other amino acids increase.

GAB increases more rapidly than any other amino acid, in both the endosperm and embryo. The accumulation of this amino acid in barley is well known, and has been discussed elsewhere (section 3.3.3.3). Changes in the levels of other individual amino acids will also not be considered further, as variations in the composition of the free amino acid pool, particularly proline, glutamic acid and alanine, are very dependent upon the conditions in which the barley grain is germinated.

5.5 Conclusions

During germination of the barley grain, a considerable pool of peptides is produced in the endosperm, similar in size to the free amino acid pool. It has been shown that this pool is adequate for the efficient operation of the peptide transport system. Indeed, peptides would seem to be at least as important as amino acids in the transfer of nitrogen from the endosperm to the embryo during germination.

In addition to the peptides in the endosperm, a pool of peptides has also been demonstrated in the growing embryo. Although there have been many indications that plant tissues may contain such a pool (section 1.2.3), the present results provide the first unambiguous evidence. It seems likely that the methods employed here could be applied to other plant tissues, with little or no modification, in an attempt to establish the general existence, or otherwise, of peptide pools.

If peptide pools in other tissues also prove to be of the same order of magnitude as the free amino acid pools, it would be interesting to consider their possible functions, if any. Does the pool have any specific metabolic or regulatory function? Is the pool important in regulating the supply of free amino acids for protein synthesis and other metabolic processes? In the absence of peptidase activity, peptides might serve to 'protect' amino acids from catabolism. Is the pool undergoing rapid turnover or are the peptides sequestered away from metabolic activity? Is the pool important in the movement of nitrogen within and between cells? Clearly, there is considerable scope here for further investigation.

CHAPTER 6

CONCLUDING REMARKS

The characterization of a peptide transport system in the germinating barley grain extends the range of organisms in which peptide transport is known to be important to all the major groups: bacteria, fungi, animals, and now plants. This apparently universal distribution emphasises the important role which peptide transport may serve in the nitrogen nutrition of cells. Furthermore, information obtained in higher plants may provide an alternative outlook on certain aspects of peptide transport, and thus, shed a new light on problems which have arisen as a result of studies on other groups of organisms.

6.1 The Role of Peptide Transport In Vivo

The ability of barley embryos to absorb peptides, together with evidence that peptides are produced in the endosperm, implies that the peptide transport system functions in the transfer of nitrogen from the endosperm to the embryo during germination. However, this gives no indication of the importance of the transport system in the germination process, or the proportion of nitrogen which it handles. Indeed, alternative roles, not involving the bulk movement of nitrogen, may also be envisaged. More light is shed on the role of the peptide transport system by a number of lines of evidence, both direct and indirect, presented above. All indicate that the peptide transport system is of considerable importance in the mobilization of nitrogen during the germination of barley grains.

(i) The peptide transport system is specifically located in the scutellum, the site of absorption from the endosperm.

(ii) The pH optimum for transport is low, but similar to the pH of the endosperm *in vivo*, the environment to which it is exposed.

(iii) The K_t of the transport system is very similar to the levels of peptide present in the endosperm during germination.

(iv) The distribution of peptidases in the barley grain indicates that peptides are absorbed by the scutellum prior to hydrolysis.

(v) Peptides and amino acids are produced at similar concentrations in the endosperm, yet at such concentrations, peptides are taken up by the scutellum rather faster than amino acids.

(vi) At peptide concentrations equivalent to those in the endosperm, the rate of peptide transport is sufficient to account for the known rate of nitrogen transfer from the endosperm to the embryo.

Further experiments designed to confirm this *in vivo* role should include the demonstration that the actual mixture of peptides isolated from barley endosperm can be handled at an adequate rate. Final proof will await the design of specific inhibitors of peptide transport or suitable systems for isolating mutant plant cells (section 6.5). Thus, growth (if any) of seeds lacking a functional peptide transport system can be compared with the parental strain.

6.2 Development and Regulation of Peptide Transport

Little attention has been paid during this study to the development and regulation of peptide transport. What evidence is available indicates that, once operative, there is little or no regulation of peptide uptake except through the supply of substrate. Thus, uptake and accumulation of intact peptide is linear over essentially indefinite periods: no evidence for feedback inhibition has been obtained. This is perhaps not surprising in view of the considerable metabolic sink provided by the growing embryo, and the additional possibility of removing any excess peptides/amino acids by sequestration within the vacuolar system.

There is some evidence to suggest that the peptide transport system increases in activity during germination (section 3.4.3). It is not known whether this is due to activation or to synthesis of additional transport proteins. The mechanism and regulation of this development warrants further investigation.

Preliminary experiments have indicated that the peptide transport system is present at very early stages of germination (section 2.4.1), and it is clearly active at 24h. Although this might seem surprisingly early, it is known that considerable metabolism occurs within the first few hours of imbibition (Collins & Wilson, 1975; Stevens & Stevens, 1976). De novo synthesis of protease begins after only 8h; 60% activity is achieved within 24h (Jacobsen & Varner, 1967). Thus, if transport is dependent upon activation or synthesis,

this must occur at very early stages of germination. The possibility of hormonal regulation (e.g. by gibberellic acid) might be considered.

6.3 Site of the Peptide Transport System and Peptidase Activity

It seems that the vacuolar system plays an important role in the utilization of peptides. Indeed, a peptide transport system is probably located in the surrounding membrane, possibly even the system characterised in this study (chapter 4). The existence of separate transport systems in an intracellular membrane and the plasmalemma may be confirmed, or otherwise, using isolated organelles. Similarly, the subcellular location of peptidases, clearly relevant to the utlization of extracellular peptides, may be identified. Recent techniques developed for the isolation of mature plant vacuoles (as opposed to protein bodies and other vacuole-like organelles) may allow such studies in the relatively near future (Wagner & Siegelman, 1975; Leigh & Branton, 1976).

6.4 Possible Applications of Peptide Transport Studies

The peptide transport system in barley, as in all other organisms studied to date, lacks specificity towards the amino acid side chains of a peptide. Thus, normally impermeant molecules can gain access to the cell if conjugated in a peptide which may be handled by the peptide transport system. Such derivatives may be useful in

scientific studies, allowing the metabolism (or other effects) of non-physiological substances to be studied *in vivo* by providing a means whereby such substances may enter the cell. An extension of this idea might be applied to the design of specific herbicides. Thus, peptides could be used as a carrier to allow normally impermeant toxic molecules to enter the cell. Alternatively, specific toxic peptides might be designed; a certain amount of success has already been achieved in the design of specific antimicrobial peptides (Lichliter *et al.*, 1976; Allen *et al.*, 1978; Ringrose, 1979). The possibility of differences between the peptide transport system(s) in plants and those of various microorganisms might also be exploited in the development of systemic fungicides etc. Thus, toxic peptides might be designed which will enter and kill a pathogenic microorganism, yet, due to the differences in transport specificity, be unable to enter the host plant cells, and consequently remain harmless.

6.5 Future Avenues for Study

Transport studies in plants have fallen well behind comparable work in animals and microorganisms. Part of the reason for this is the complex nature of plant cells and tissues which often complicates interpretation and experimental design. Recently, a number of studies of amino acid transport by cell suspension cultures has appeared (Maretzki & Thom, 1970; Francki *et al.*, 1971; King &

Hirji, 1975; King, 1976; Harrington & Smith, 1977; Berlin & Mutert, 1978; Blackman & McDaniel, 1978). Such studies eliminate problems caused by the non-uniformity of cells within a tissue and the need for solute to penetrate into the bulk of a multicellular organ. In addition, two recent studies have illustrated the advantages of using isolated protoplasts for transport studies (Guy et al., 1978; Ruesink, 1978): the absence of a cell wall reduces interference from the 'unstirred layer'.

While it has been extremely valuable to study peptide transport in intact tissue, in terms of identifying an in vivo role, it is clear that extension of these studies to cell suspension cultures or protoplasts will be essential for detailed kinetic studies and the elucidation of the molecular mechanisms of transport and energy coupling. In addition, recent work has indicated that it may be possible to obtain and maintain biochemical mutants of plant cells in culture (Bigot, 1978; Bourgin, 1978; Zryd, 1978). Indeed, amino acid analogues have been used to select cell lines with reduced rates of uptake (Widholm, 1974, 1976; Berlin & Widholm, 1978a,b). The advent of such techniques should prove extremely fruitful in studying the mechanism(s) of peptide, and all other plant transport processes.

6.6 Possible Roles for Peptide Transport in Plants

In view of the widespread importance of peptides in the nutrition of animals and microorganisms, and the present demonstration that active peptide transport can operate in plant cells, it is perhaps pertinent to speculate on other possible roles for peptide transport within the plant kingdom. In situations in which organic nitrogen is known to be transported by plant cells it has almost always been assumed, often with little justification, that transport is solely in the form of free amino acids. However, in many situations peptide transport could equally well operate and, in addition, may confer a number of advantages over the transport of free amino acids.

6.6.1 Utilization of Exogenous Peptides

6.6.1.1 Algae

While certain algae are able to transport and utilize exogenous amino acids as a nitrogen source, the possession of this facility is by no means universal. These differences presumably reflect adaptation to specific ecological niches in which organic nitrogen may or may not be prevalent. Peptide transport, if important, might therefore be expected to parallel the existence of amino acid transport systems.

Amino acids seem to be unimportant in the nutrition of blue-green algae (Smith, 1973). This is perhaps not surprising, in view of the ability of blue-green algae to fix atmospheric nitrogen. Thus, it also seems unlikely that peptide uptake will be important, although a role may exist

in species favouring highly eutrophic water. However, it is well known that blue-green algae, both free-living and in symbiotic associations, may release large quantities of extracellular peptides (section 1.2.2.2). Although there is little evidence to show that these peptides are released intact across the plasmalemma, this presumably is the case. A peptide transport system may therefore be present, although possibly with a restricted specificity rather than serving a general nutritional role.

Amongst the eukaryotic algae, the occurrence of amino acid transport systems is very variable. Many, though by no means all, marine phytoplankton are able to utilize amino acids present in sea water (North & Stephens, 1972; Stephens, 1972; Wheeler et al., 1974). Similarly, certain freshwater algae possess a general amino acid transport capacity (Bollard, 1966; Kirk & Kirk, 1978c) although many other species apparently lack such a facility (Kirk & Kirk, 1978a,b,c). Again, it seems likely that, where amino acid uptake is important, the simultaneous ability to absorb peptides will confer an additional advantage. It is also possible that, in certain species, peptide transport may occur to the exclusion of amino acids (as in certain bacteria: Pittman et al., 1967). It has been reported that certain strains of Chlorella will utilize peptides (Bollard, 1966), although the possibility of extracellular hydrolysis was not discounted. However, North (1975) has suggested (although by no means conclusively) that many marine phytoplankton are unable to utilize peptides present in sea water. Given

the rapidity of the fluorescence methods described here (chapter 2) it would be simple, and perhaps revealing, to screen a range of suitable algae for the capacity to absorb small peptides.

6.6.1.2 Plant Associations

Many examples of parasitic and symbiotic associations are known within the plant kingdom in which nitrogen is transferred from one species to another. Thus, parasitic plants, which absorb and utilize organic nitrogen from their hosts, may have a peptide transport system(s) in their haustoria. Such a system would be most appropriate in species secreting proteases (or increasing host proteolytic activity) and absorbing the hydrolytic products.

Although specific peptides may be transferred between two symbionts, it seems rather less likely that a general peptide transport system will be important. A mixture of amino acids and small peptides, such as might result from proteolysis, is unlikely to be the form in which nitrogen is transferred. More efficient would be the incorporation of fixed nitrogen into a limited number of chemical species, specifically for transport between symbionts. Most available evidence, although admittedly rather limited, suggests this to be the case. Often nitrogen is transported in an inorganic form, although there is evidence that amino acids (particularly alanine) may be involved in certain species (see Smith, 1974, 1975; Pate, 1976b). However, specific peptides may be important in some of the

many, diverse symbiotic associations. Indeed, peptides have been implicated in certain lichens and nitrogen-fixing root nodules (section 1.2.2.4). One particular advantage would be the protection of transported nitrogen from metabolism (especially in peptides with unusual linkages) until it reaches its metabolic sink.

6.6.1.3 Higher Plants

Unlike other organisms in which peptide transport has been characterized, higher plants are generally autotrophic. Thus, the utilization of external organic nitrogen sources will normally be unimportant. However, a number of interesting examples may be envisaged, although probably of minor importance considering the number of species involved.

(i) Uptake by plant roots: It seems unlikely that many terrestrial plant species will absorb organic nitrogen through their roots, due to the lack of suitable substrates in the soil. However, a number of reports of amino acid transport by plant roots have appeared (Wright, 1962; Watson and Fowden, 1975; Soldal and Nissen, 1978). Thus, the possibility that peptides are also involved cannot yet be discounted.

The roots of aquatic plants seem more likely candidates for a peptide transport system. Observations that the duckweed may absorb amino acids from aqueous media presumably reflect uptake by the roots (Borstlap, 1974, 1977, 1978; Novacky et al., 1978a). A single report that this species may also utilize peptides as a nitrogen source (Bollard,

1966) may be due to a peptide transport system. The possibility of peptide uptake by the leaves of totally submerged plants might also be considered, especially in species adapted to highly eutrophic conditions. Amino acids are absorbed by the leaves of Egeria densa (Petzold & Jacob, 1975).

(ii) Carnivorous Plants: It is well known that insects, protein, peptones, and other forms of organic nitrogen may be absorbed by the traps of carnivorous plants, resulting in increased growth (see review by Higgins & Payne, 1979). The secretion of proteases into the traps implies that intact proteins are not absorbed. However, the degree to which protein is digested prior to absorption is unknown. Complete degradation to amino acids has been claimed in pitcher plants (Plummer & Kethley, 1964; Amagase et al., 1972), although in the Venus-Flytrap extracellular peptidase activity is apparently absent (Scala et al., 1969), indicating that peptide uptake may be important. Similarly, while there is a certain amount of evidence to suggest that amino acids may be specifically absorbed by carnivorous plants (Hepburn et al., 1927; Plummer & Kethley, 1964; Lüttge, 1965; Chandler & Anderson, 1976; Simola, 1978a), peptides have also been implicated (Hepburn et al., 1927; Plummer & Kethley, 1964; Simola, 1978b), and in one case intact uptake actually demonstrated (section 1.2.4.2). It therefore seems likely that peptides, as well as amino acids, will be important in the absorption of nitrogen by many, if not all carnivorous plants.

6.6.2 Transport of Peptides Within the Plant

Unlike bacteria, on which the majority of peptide transport studies have been performed, higher plants are multicellular organisms. In addition to absorption from the external environment, transport of peptides within the plant body may also be of importance. Two convenient, although possibly rather arbitrary, categories may be considered; long-distance transport involving the phloem and xylem, and short distance, cell-to-cell transfer.

6.6.2.1 Transport in the Phloem and Xylem

Although there have been reports of peptides in both the phloem and xylem (section 1.2.2.3), it is generally considered that the bulk of nitrogen transported in these tissues is in the form of amino acids (see review by Higgins & Payne, 1979). The transport of amino acids conjugated as peptides might offer protection from metabolic degradation by enzymes known to be present in the phloem, although such a function has yet to be demonstrated.

Most studies of phloem and xylem exudates have been concerned with specific compounds. In many cases, a considerable proportion of the nitrogen may have been present as part of a general peptide pool, no single peptide being present in detectable quantities. Situations in which the long-distance transport of such a pool might be important can be envisaged, particularly during the bulk movement of protein degradation products (e.g. during leaf senescence; section 6.6.2.2.2).

6.6.2.2 Cell-to-Cell Transport

Two possible routes for short-distance, cell-to-cell transport must be considered; the extracellular compartment (apoplast; Läuchli, 1976) and the cytoplasmic continuum, involving plasmodesmata (symplasm; Spanswick, 1976). The relative importance of these two routes is still to be assessed, although both seem likely to play some role in the transport of metabolites within the plant.

The role of the symplasm in peptide transport clearly depends upon the subcellular location of peptidases within the cell; peptides may not remain intact within the cytoplasm. Apoplastic transport seems a less efficient mechanism for cell-to-cell transfer, as it entails the secretion and absorption of metabolites across the plasmalemma as they pass from one cell to the next, presumably an energy-dependent process. However, cells from a wide variety of tissues are able to absorb amino acids, often, if not always, via specific transport systems (see Higgins & Payne, 1979). In vivo, these transport systems must function to absorb metabolites from the extracellular space, indicating a role for the apoplast in the movement of compounds throughout the plant body. It would be interesting to know whether such tissues are also able to absorb peptides.

Although the transport of nitrogenous compounds between cells will clearly be of importance throughout the life of a multicellular plant, a number of situations may be envisaged in which peptide transport might be of particular significance.

6.6.2.2.1 Germinating Seeds

The present study has demonstrated that peptide transport is important in the movement of nitrogen during the germination of one particular seed, barley. However, most seeds store large amounts of nitrogenous material which must be transferred to the growing axis during germination. A possible role for peptides in these species should also be considered.

In addition to barley, the embryos of several other cereals, maize, wheat, oats and sorghum, have been shown to absorb Gly-Sar intact, against a concentration gradient (table 6.1). Preliminary experiments have indicated that these species are also able to absorb physiological peptides and that uptake shows an acidic pH optimum, similar to that of barley (results not presented). The situation in sorghum is particularly interesting. The major protease in the endosperm of this cereal is apparently specific for peptide bonds in which the carbonyl group is provided by an aspartic or glutamic acid residue (Garg & Virupaksha, 1970a,b). Thus, a large number of peptides with C-terminal aspartic or glutamic acid residues may be produced during germination, raising the possibility that a peptide transport system specific for such peptides may exist.

The structure of non-endospermic dicot seeds is rather different from the monocotyledenous cereals; the bulk of the protein reserves are located in the cotyledons. During germination, protein hydrolysis products enter the vascular tissue within the cotyledon and are transported directly to

Table 6.1: Uptake of Gly-Sar by Embryos or Cotyledons from Various Seeds

Species	Gly-Sar Uptake (µm/g fresh wt.)
Barley	8.3
Wheat	3.8
Oats	8.0
Maize	2.5
Sorghum	4.6
Castor Bean	0.2

Incubations were for 8h in 2mM Gly-Sar, under standard conditions (section 2.3.1). Gly-Sar uptake was determined by embryo extraction. 24h embryos were used, except for maize (48h) and castor beans (48h cotyledons). Each value is the average of two separate determinations.

the growing axis. There is no absorptive membrane equivalent to the scutellum. While little is known concerning the enzymes involved in proteolysis in these seeds, a number of factors are relevant when considering a possible role for peptide transport. Thus, the bulk of the nitrogen entering the vascular tissue is in the form of amino acids, particularly asparagine or glutamine (Capdevila & Dure, 1977; Miflin & Lea, 1977: Kern & Chrispeels, 1978); complete degradation to amino acids seems to occur within the cotyledon. In some species, considerable peptidase activity has been detected within the cotyledons, providing further evidence that proteolysis reaches completion _in situ_ (Ashton & Dahmen, 1967a,b, 1968). Thus, the only possible role for peptide transport would seem to be in the cell-to-cell transfer of hydrolytic products across the cotyledon, prior to entering the vascular tissue. Such a role would require an uneven distribution of peptidases throughout the cotyledon, such that peptide hydrolysis occurs immediately prior to, or during, entry into the vascular tissue. There is as yet no evidence for such differentiation.

In the endospermic dicots, epitomized by the castor bean, the bulk of the protein storage reserves are located in the endosperm. The cotyledons are adapted as absorptive organs, analogous (possibly homologous) to the scutellum (Kriedeman & Beevers, 1967a,b). Specific sucrose transport systems are located on the cotyledon surface (Komor, 1977; Hutchings, 1978a,b); clearly, nitrogenous materials must also be absorbed. Again, it is unclear

whether the endospermic enzymes are able to cleave the storage proteins to amino acids, or whether a mixture of amino acids and peptides remains. The only detailed study of the proteases/peptidases was based upon artificial substrates (Tully & Beevers, 1978), which may be misleading.

It has been suggested that glutamine is the main nitrogenous compound absorbed by the cotyledons of castor beans (Stewart & Beevers, 1967). Thus, a number of amino acids (alanine, aspartic acid, glutamic acid, glycine and serine) are apparently metabolized within the endosperm, the nitrogen being transferred to glutamine and the carbon skeletons converted to sucrose. Other amino acids may be absorbed directly by the cotyledon. However, as in barley, it would seem to be more efficient if peptides were transported. In an attempt to test this hypothesis preliminary experiments have so far failed to indicate the existence of an active peptide transport system in the castor bean cotyledon, although many possible explanations for this failure may be advanced (results not presented). Under optimal conditions for the barley peptide transport system, there is little or no peptide uptake by castor bean cotyledons. Thus, if a peptide transport system is present in the cotyledon it must be very different from the barley system.

One further group of seeds with an absorptive structure similar to that of barley are the conifers, in particular the pines. Here, protein is stored in the endosperm and must be absorbed by the cotyledons during germination.

Again, it might be more efficient for peptides to be absorbed, rather than free amino acids. However, unlike barley there seems to be considerable peptidase activity in the endosperm of these seeds, suggesting that hydrolysis might reach completion prior to absorption by the cotyledon (Salmia & Mikola, 1975, 1976; Salmia et al., 1978). There are, of course, many other seeds in which peptide transport may be important. In addition, a role for peptides in other protein storage organs (e.g. tubers) might also be considered.

6.6.2.2.2 Peptide Transport During Senescence

During the senescence of organs such as leaves, fruits and flowers, protein is degraded and transferred from the senescing tissue to other regions of the plant (Thimann, 1978). Little is known concerning the proteases/peptidases of senescing tissue. In senescing petals, proteases (Matile & Winkenbach, 1971) and several peptidases (Sopanen & Carfantan, 1976) are active, although in the absence of any understanding of their distribution it is not yet clear whether a pool of peptides will be produced.

In senescing oat leaves, the two most important enzymes are a carboxypeptidase and an endopeptidase (Drivdahl & Thimann, 1977). It has been claimed that these two enzymes could result in the complete degradation of leaf proteins to amino acids (Thimann, 1978), although the inability of most plant carboxypeptidases to handle small peptides suggests that a peptide pool might accumulate. The existence of such a pool has been claimed in senescing

barley leaves (Hendry & Stobart, 1977). However, reports of highly active peptidases in senescing leaves (Sopanen & Laurière, 1976), and the apparent absence of peptides from the vascular tissues (section 6.6.2.1), indicate that hydrolysis will reach completion within the senescing tissue. Thus, a possible role for peptides during senescence remains to be assessed.

6.6.2.3 Intracellular Transport

While the subscellular location of most peptides and peptidases remains unknown, the entry of intact peptides into various organelles must be considered. The possible absorption of peptides by plastids, vacuoles, etc. has been considered elsewhere (section 4.4.8). However, one further situation in which peptide transport across an intracellular membrane may be important, should be mentioned; the release of protein hydrolysis products from protein bodies, particularly during seed germination. In the barley endosperm, peptides must be transported across the protein body membrane unless the permeability barrier presented by this membrane is rapidly destroyed. In dicots, protein hydrolysis is initiated within the protein body (Harris & Chrispeels, 1975; Chrispeels *et al.*, 1976; Nishimura & Beevers, 1978, 1979), although it is not known whether hydrolysis reaches completion or whether peptides are released across the membrane to be hydrolysed in the cytoplasm. In the mung bean, it has been reported that 61% hydrolysis occurs in isolated protein bodies (Baumgartner & Chrispeels, 1977) although this might not

reflect the situation *in vivo*. Aminopeptidase and BAPAase, both of which hydrolyse small peptides in barley (section 1.3.4), are located in the cytoplasm of the mung bean cotyledon, indicating that hydrolysis may be completed outside the protein bodies. Considering the relative ease with which protein bodies can now be isolated, this possibility deserves attention.

Thus, many situations can be envisaged in which peptide transport might serve an important role in plant tissues. In the light of present evidence, such possibilities should not be ignored. In addition, certain assumptions concerning the role(s) of amino acid transport might now be reexamined. The demonstration that peptide pools may exist in plant tissues also provides positive support for a concept which has received little attention in the past, and the possibility that peptide pools may serve specific functions (e.g. regulatory) within the plant cell should now be considered. It is to be hoped that this study will emphasise the need for further investigations into the role(s) of peptides in the nitrogen metabolism and nutrition of plant cells.

APPENDICES

APPENDIX 1

ABBREVIATIONS

Amino Acids and Their Derivatives:

Ac-	acetyl-
ANA	∝-naphthyl acetate
DNS-	dansyl- (1-dimethylamino-naphthalene-5-sulphonyl-)
GAB	γ-aminobutyric acid
<Glu	pyroglutamic acid
$-NH_2$	-amide
-OEt	-ethyl ester
-OMe	-methyl ester
Sar	sarcosine
TNP-	2,4,6-trinitrophenyl-

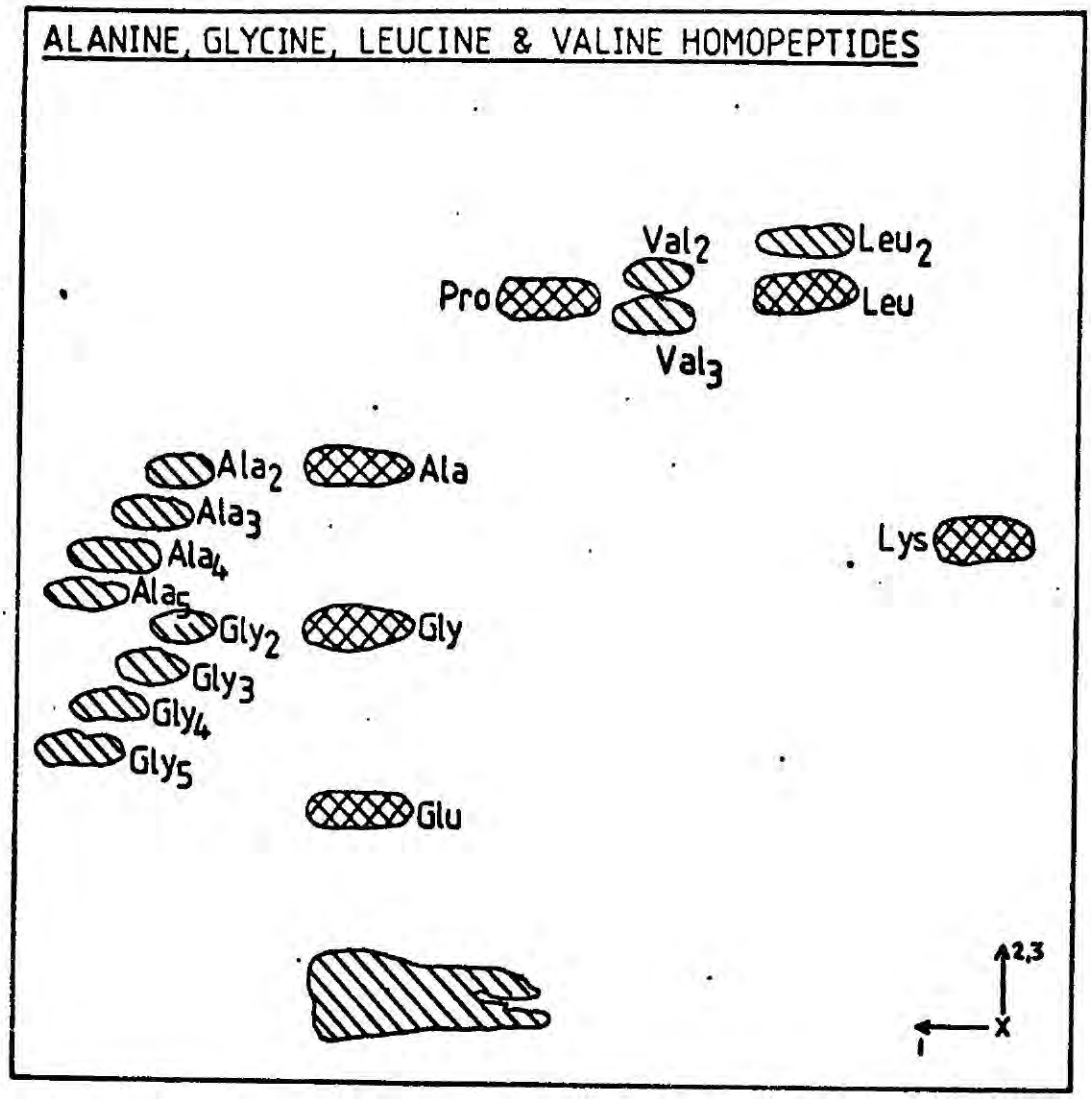
Others:

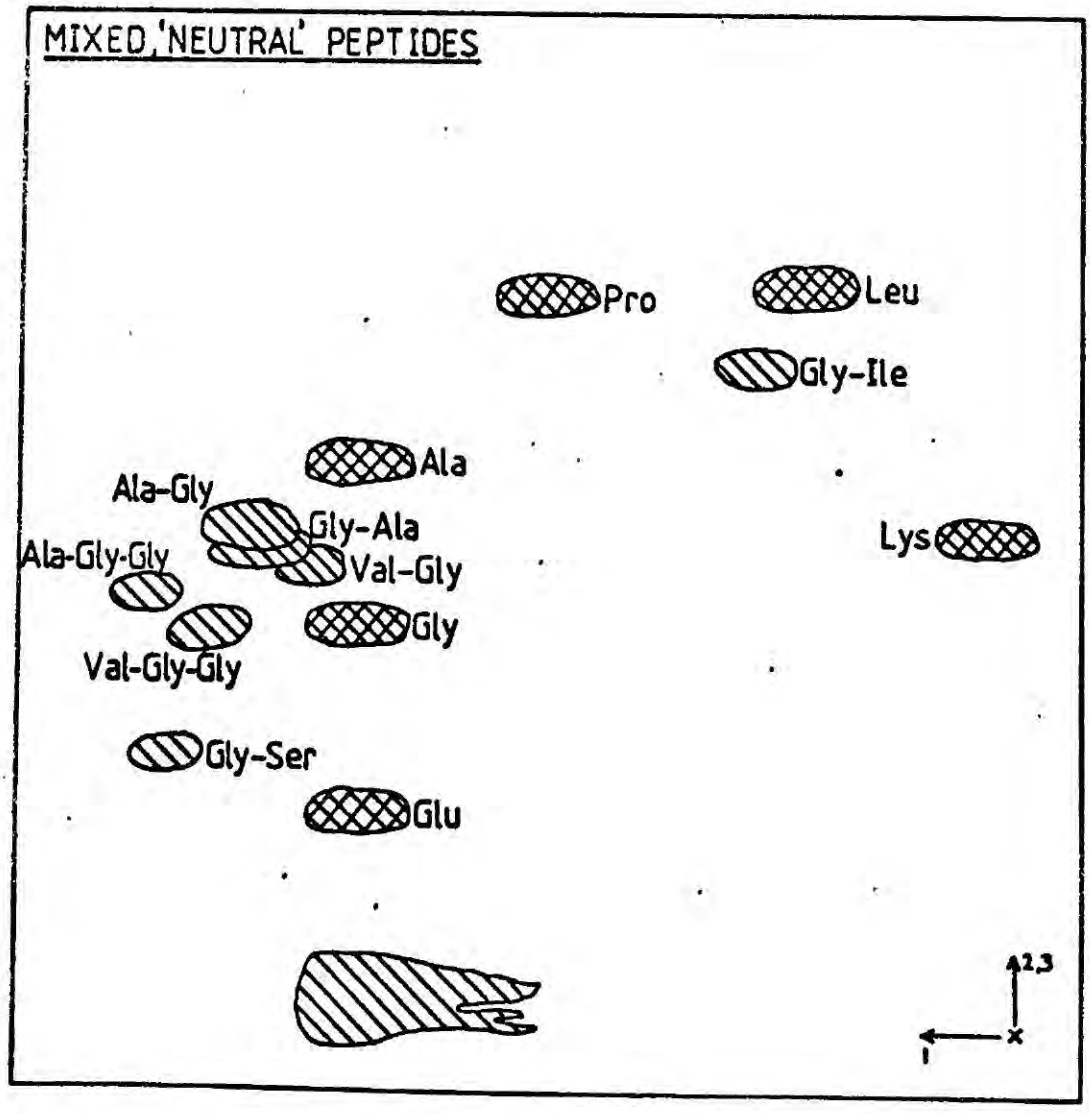
ABA	abscisic acid
ATP	adenosine 5'-triphosphate
BAEE	∝-N-benzoyl-L-arginine ethyl ester
BAPA	∝-N-benzoyl-L-arginine p-nitroanilide
CCCP	carbonylcyanide m-chlorophenylhydrazone
DNP	2,4-dinitrophenol
GA	gibberellic acid
IAA	indol-3-acetic acid
PBS	phosphate buffered saline
TCA	trichloroacetic acid
TNBS	2,4,6-trinitrobenzene 1-sulphonic acid
tris	2-amino-2-hydroxymethylpropane-1,3-diol

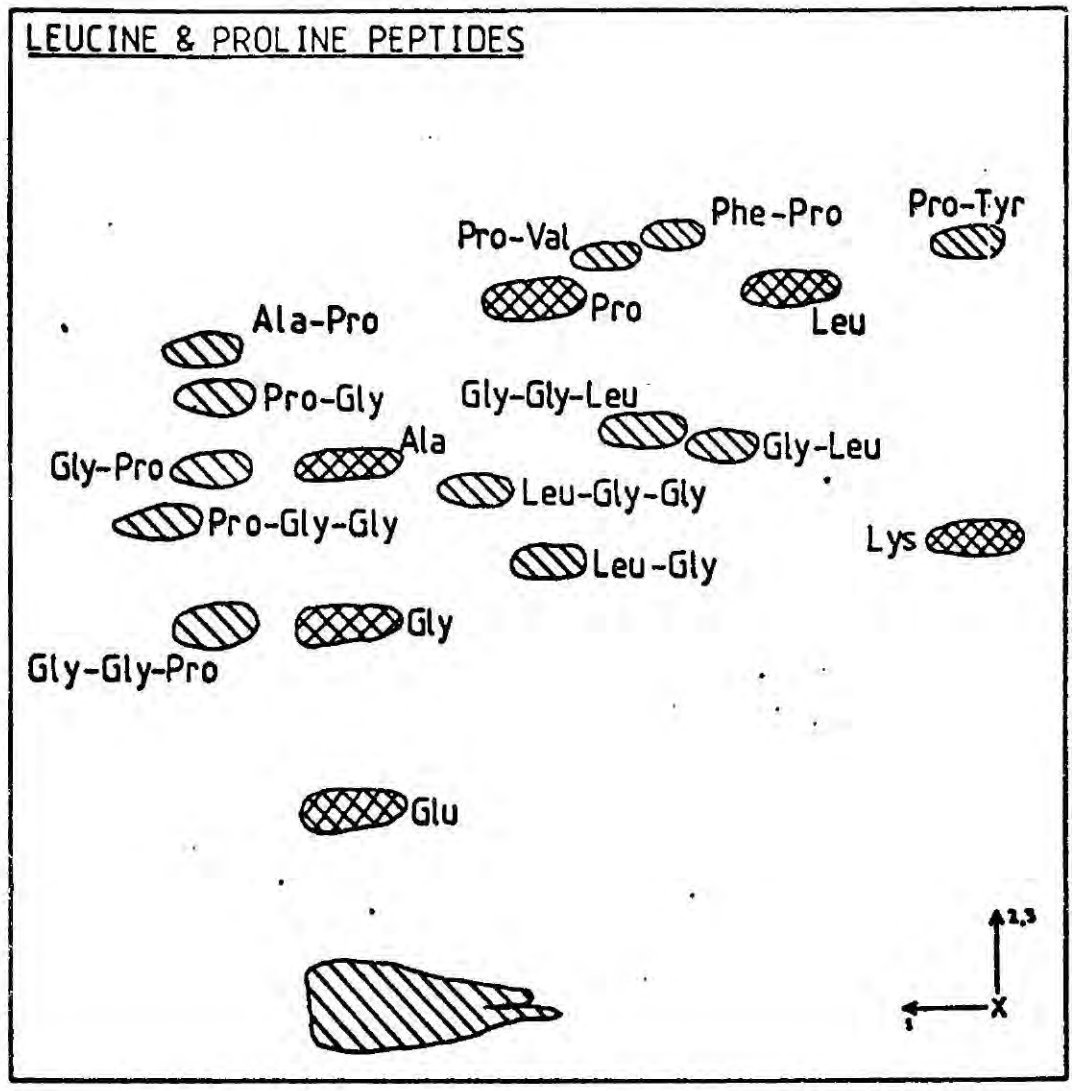
APPENDIX 2

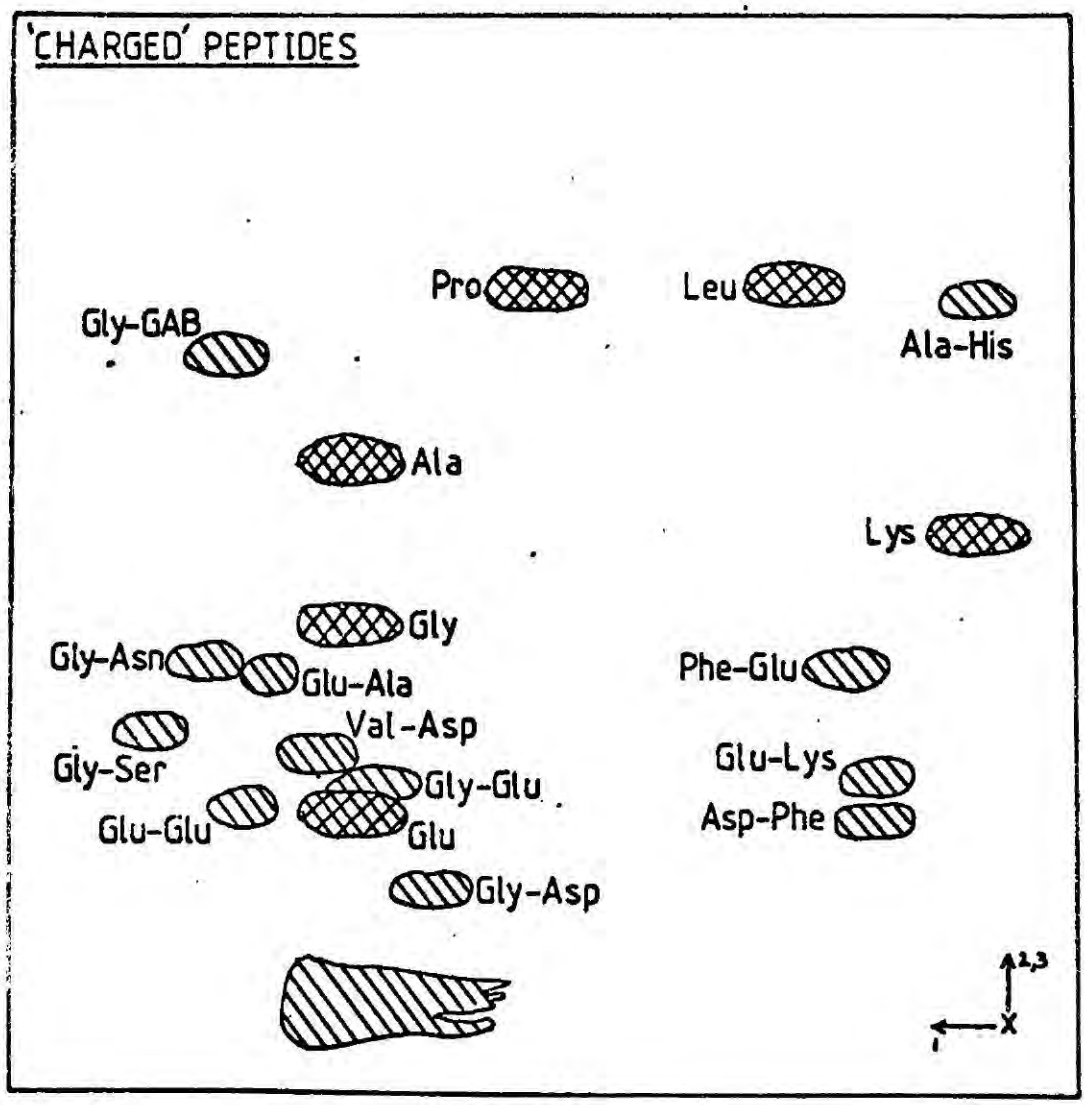
Diagrams Illustrating the Chromatographic Locations of the Dansyl-Peptides used in this Study

In each diagram the locations of six dansyl-amino acids, glutamic acid, glycine, alanine, proline, leucine and lysine, are indicated as reference points.

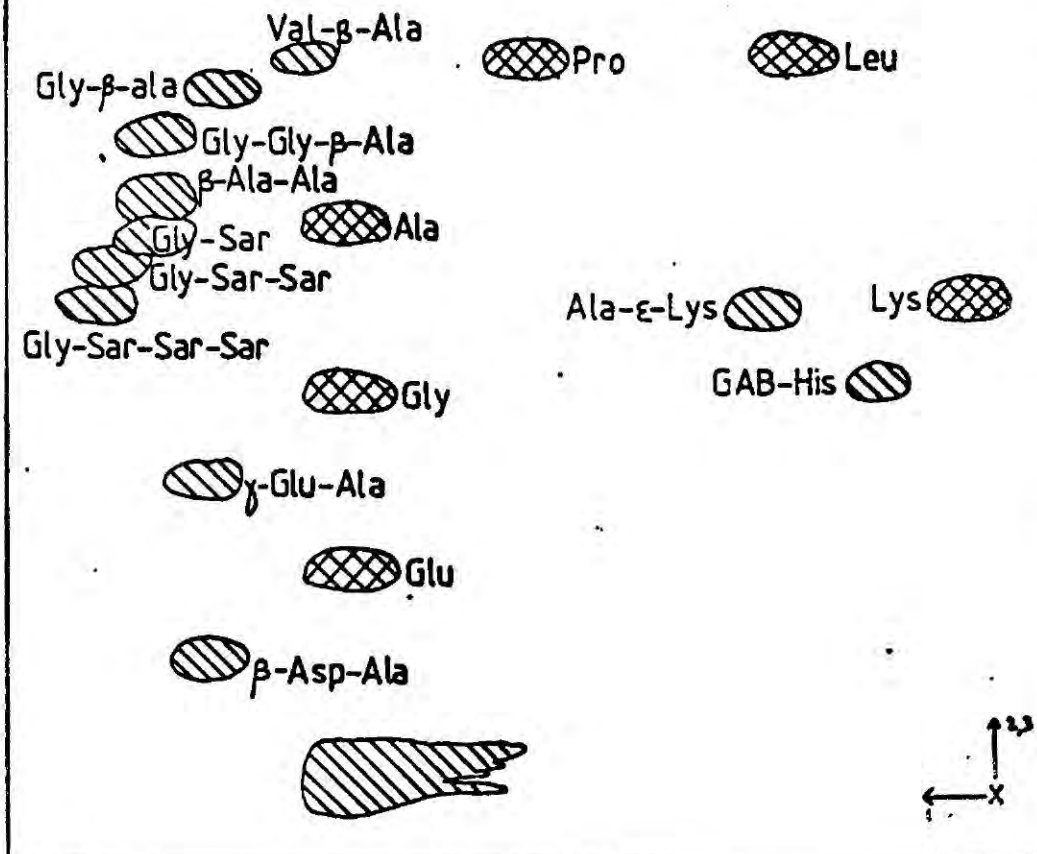

ALANINE, GLYCINE, LEUCINE & VALINE HOMOPEPTIDES
Val2
Leu2
Pro
Leu
Val3
Ala2
Ala
Ala3
Ala4
Ala5
Lys
Gly2
Gly
Gly3
Gly4
Gly5
Glu
2,3
X
1


MIXED 'NEUTRAL' PEPTIDES
Pro
Leu
Gly-Ile
Ala
Ala-Gly
Gly-Ala
Lys
Ala-Gly-Gly
Val-Gly
Gly
Val-Gly-Gly
Gly-Ser
Glu
2,3
X
1

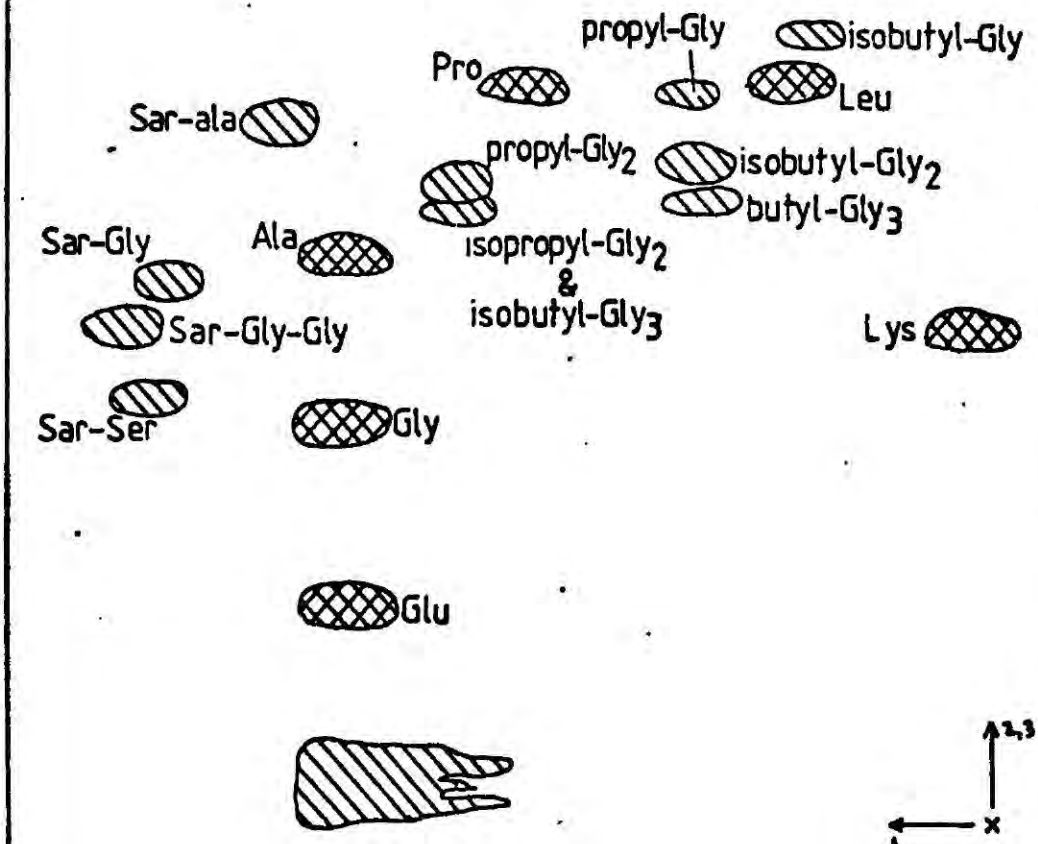

LEUCINE & PROLINE PEPTIDES
Pro-Val
Phe-Pro
Pro-Tyr
Pro
Leu
Ala-Pro
Pro-Gly
Gly-Gly-Leu
Gly-Leu
Gly-Pro
Ala
Leu-Gly-Gly
Pro-Gly-Gly
Lys
Leu-Gly
Gly
Gly-Gly-Pro
Glu
2,3
X
1

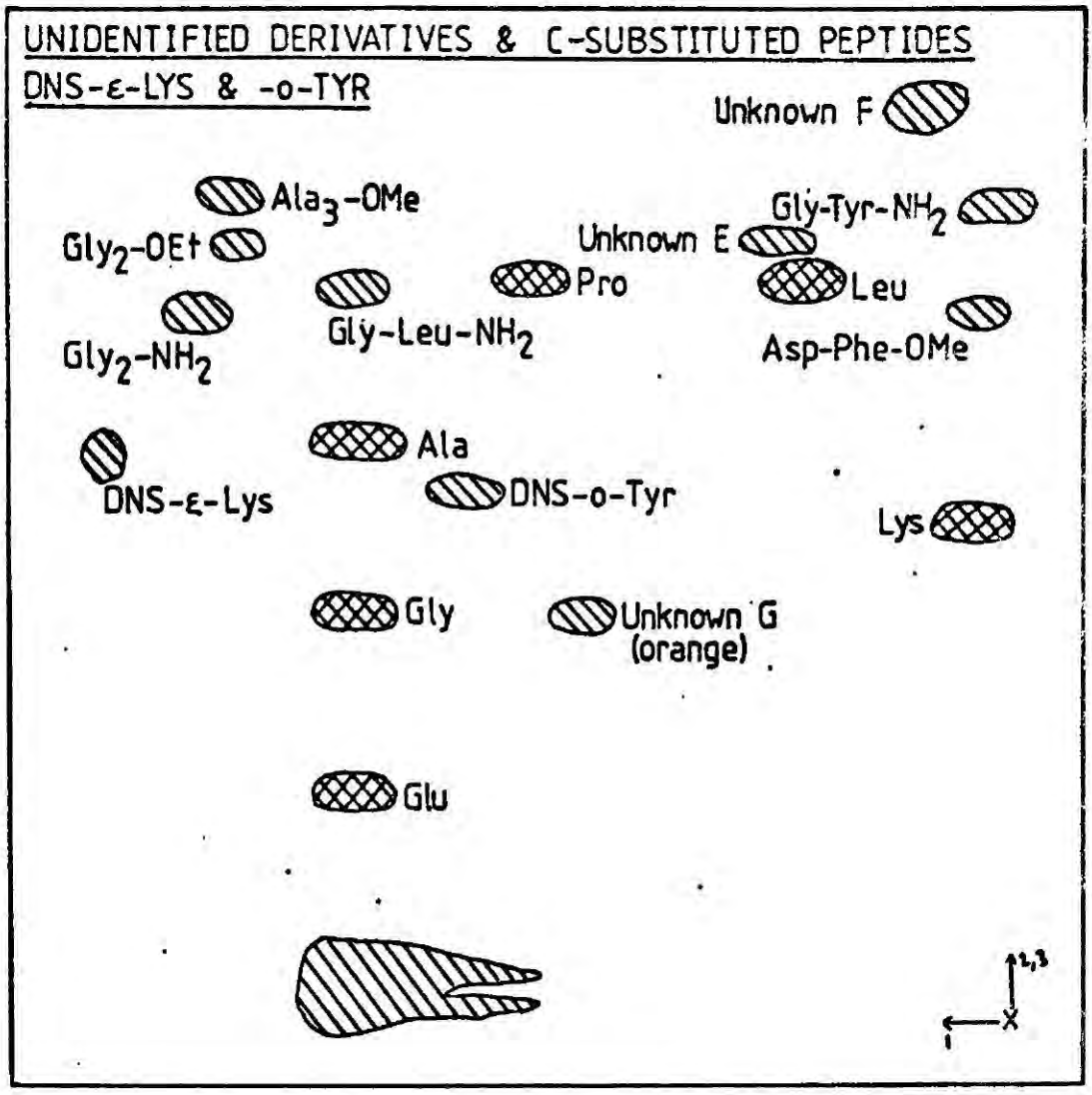

'CHARGED' PEPTIDES
Pro
Leu
Gly-GAB
Ala-His
Ala
Lys
Gly
Gly-Asn
Glu-Ala
Phe-Glu
Val-Asp
Gly-Ser
Glu-Lys
Gly-Glu
Asp-Phe
Glu-Glu
Glu
Gly-Asp
2,3
X
1

PEPTIDES WITH UNUSUAL LINKAGES



N-SUBSTITUTED PEPTIDES




UNIDENTIFIED DERIVATIVES & C-SUBSTITUTED PEPTIDES
DNS-ε-LYS & -o-TYR
Unknown F
Ala3-OMe
Gly-Tyr-NH2
Gly2-OEt
Unknown E
Pro
Leu
Gly-Leu-NH2
Gly2-NH2
Asp-Phe-OMe
Ala
DNS-ε-Lys
DNS-o-Tyr
Lys
Gly
Unknown G
(orange)
Glu
1,3
2
X

APPENDIX 3

Effect of Varying Concentrations of a Non-Competitive Inhibitor on Transport

Assume the uptake of substance A is mediated by a single transport system, the kinetics of which follow the Michaelis-Menten equation.

Let v_o be the rate of uptake in the absence of an inhibitor and v_i be the rate of uptake in the presence of a non-competitive inhibitor, I.

From the Michaelis-Menten equation we get

$$v_o = \frac{V_M [A]}{K_t + [A]}$$

$$v_i = \frac{V_M [A]}{(K_t + [A])(1 + \frac{[I]}{K_i})}$$

where [I] is the concentration of inhibitor and K_t and K_i are the Michaelis constants of the substrate (A) and the inhibitor (I), respectively.

$$v_o - v_i = \frac{V_M\ [A]}{K_t + [A]} \left(1 - \frac{1}{1 + \frac{[I]}{K_i}}\right)$$

But $\frac{V_M\ [A]}{K_t + [A]} = v_o$

$$\therefore \frac{v_o - v_i}{v_o} = 1 - \frac{1}{1 + \frac{[I]}{K_i}}$$

$$\therefore \frac{v_o}{v_o - v_i} = \frac{1}{1 - \frac{1}{1 + \frac{[I]}{K_i}}}$$

$$= \frac{K_i}{[I]} + 1$$

$$\therefore \frac{1}{1 - \frac{v_i}{v_o}} = \frac{1}{[I]} K_i + 1$$

As K_i is a constant, the Inui-Christensen plot, $\frac{1}{1 - \frac{v_i}{v_o}}$ vs $\frac{1}{[I]}$, will be linear, crossing the ordinate at 1.0. This is similar to the plot obtained in the presence of a competitive inhibitor (section 3.1.3.8; Inui & Christensen, 1966). However, the slope is dependent only upon K_i, and is independent of K_t and [A]. Thus, in the case of non-competitive inhibition, the Inui-Christensen plot will be independent of the transport substrate. As

different slopes were obtained experimentally, for the inhibition of Gly-Sar and Gly-Sar-Sar by dialanine, and for the inhibition of Gly-Sar and Gly-Sar-Sar uptake by trialanine (figs. 3.12 and 3.13, section 3.1.3.8), it seems that the inhibitory effects of di- and trialanine on transport are not compatible with simple non-competitive inhibition.

APPENDIX 4

Rate of Nitrogen Transfer from the Endosperm to the Embryo During Germination of Barley Grains

The maximum rate of nitrogen transfer from the endosperm to the embryo of barley grains during germination is about 150µg nitrogen/grain/day (Metevier & Dale, 1977). Using the usual conversion factor (6.25), this is equivalent to 940µg protein/grain/day. Assuming transfer occurs entirely as tripeptides (with an average molecular weight of 320), this gives a maximum rate of transfer of about 2.9µm peptide/grain/day.

The concentration of peptides in the endosperm is at least 3mM (table 5.9). The rate of trialanine uptake at this concentration is about 5.0µm/g fresh wt./h (fig. 3.7) and most other peptides seem to be transported at similar rates. One embryo weighs about 100mg at day 3 of germination (table 5.1). Thus, the rate of peptide transport is about 0.5µm/grain/h

$$= 12\mu m/grain/day$$

Peptide transport therefore occurs at a more than adequate rate to account for the complete transfer of nitrogen from the endosperm to the embryo during germination.

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