PHENOLIC METABOLISM IN RELATION TO CELL WALL BIOSYNTHESIS IN FESCUE AND MAIZE

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Declaration

I hereby declare that this thesis was composed by myself and that the work contained herein to be my own, except where otherwise indicated.

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Edinburgh 1993

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This thesis is dedicated to my Mother and to the memory of my Father

"Everybody's born to do a certain thing and
if you're dead jammy you find it, and if you're good
at it just keep doing it until you're fed up, then do something
else. We're just all here to make babies and
look after the place you know."

Billy Connolly

Abbreviations

AIR	alcohol insoluble residue
Ara	Arabinose
BAW	paper chromatography system butan-1-ol/acetic acid/water (12:3:5)
BEW	paper chromatography system butan-1-ol/ethanol/water (20:5:11 v/v/v), for 16 h.
BIS-TRIS	(bis[2-hydroxyethyl]imino-tris[hydroxymethyl]-methane
BPW	paper chromatography system butan-1-ol/pyridine/water (4:3:4)
Bq	Bequerels
BSA	bovine serum albumin
CAD	coniferyl alcohol dehydrogenase
cpm	counts per minute
DTT	dithiothreitol
EAW	paper chromatography system ethyl acetate/acetic acid/water (10:5:6)
EDTA	ethylenediamine tetra-acetic acid
g.p.c	gel permeation chromatography
MOPS	(3-[N-morpholino]propane sulphonic acid)
OMT	caffeate O-methyltransferase
PAL	Phenylalanine ammonia-lyase
POPOP	1,4-bis-(5-phenyloxazol-2-yl)benzene
PPO	2,5-diphenyloxazole
Rf	The distance migrated by a substance during paper or thin layer chromatography relative to the solvent front
rpm	revolutions per minute
SAM	S-adenosyl methionine

TAPS	(N-tris[hydroxymethyl]methyl-3-amino propane sulfonic acid),
TFA	Trifluoroacetic acid
t.l.c.	Thin layer chromatography
TRIS	tris[hydroxymethyl]methane
xyl	xylose
p.c.	paper chromatography
f.w.	fresh weight
C18 SPEC	C18 solid phase extraction cartridge

CONTENTS.

ABSTRACT

Phenolic acid residues e.g. feruloyl groups are found esterified to specific sugars of primary cell wall polysaccharides of plants and phenylpropanoid derivatives are incorporated into lignin. These phenolic moieties probably have profound effects on cell wall properties such as extensibility (and thus growth) and digestibility. The work described in this thesis is an investigation into some aspects of the biosynthesis of cell wall phenolic residues.

The site and mechanism of the feruloylation of primary wall polysaccharides has been a matter of some debate, although it is likely to be enzyme mediated. Fescue and maize cell cultures were supplied with radiolabelled precursors in order to ascertain the kinetics of this process and thus its likely sub-cellular site. [^{3}H]Arabinose uptake was negligible in maize cell cultures and labelling with [^{14}C]glucose resulted in very high background levels of radioactivity making analysis of feruloylated fragments difficult. However, radiolabelled [^{3}H]arabinose was rapidly taken up and incorporated into nascent polysaccharides of fescue cells and polymer bound [^{3}H]arabinose residues were beginning to be feruloylated within 5 minutes of the radiolabelled precursor being supplied. However, radiolabelled polymers were not secreted into the culture medium until 15-30 minutes after this point. Assuming that prior to the onset of secretion of radiolabelled polymers essentially all of the radiolabel was internal to the plasma membrane, the data presented show that feruloylation of polymer bound [^{3}H]arabinose residues must occur intraprotoplasmically.

As feruloylation was shown to occur intraprotoplasmically and polysaccharide synthesis occurs mainly in the Golgi bodies, endomembrane preparations from maize cell cultures were assayed to ascertain the presence

of feruloyl-CoA : polysaccharide feruloytransferase activity. Endomembranes were capable of incorporating radiolabel from [^{14}C]feruloyl-CoA into polymeric material and this activity appeared to be Co^{2+} dependent and was stimulated by addition of arabinoxylan during cell homogenisation. However, the incorporation of radiolabel into polymeric material was completely suppressed by the addition of catalase to assays, suggesting that the reaction was due to peroxidase activity and not a feruloyltransferase. The peroxidase activity towards feruloyl-CoA was inhibited by dithiothreitol, but this inhibition was reversed by the addition of Co^{2+}. These data demonstrate a serious artifact in the feruloyltransferase assay and the properties and the possible roles of membrane associated peroxidases are discussed.

Since lignin has a profound effect on the digestibility and pulpability of plant material, model systems have been developed in which to study secondary wall development and lignification. Within the graminaceous monocot internode there is a gradient of maturity from base to apex and this has been investigated as a model system with respect to the chemical and physical characteristics of the cell wall. Data are presented on the enzyme activities in maturing maize internodes and it appears that high phenylalanine ammonia lyase activity is correlated with rapid lignification and precursor demand. Caffeate O-methyltransferase and coniferyl alcohol dehydrogenase activities did not consistently correlate with any stage of development.

The significance of the finding is discussed.

CHAPTER 1
INTRODUCTION

1.1 The Plant Cell Wall.

The plant cell wall is a highly complex organelle that has several different roles in the control of plant metabolism and growth. The wall can be considered as the external limit of the cell and as such is responsible for cell to cell adhesion as well as governing the shape, size and growth rate of the cell and thus ultimately the morphology of the whole plant. The cell wall provides an external barrier to the entry of potential pathogens and is implicated in several disease resistance mechanisms (Ride 1980, Vance *et al.* 1980). Certain wall fragments (oligosaccharins) have been shown to exhibit biological activity - acting as elicitors for defence responses (Davis and Hahlbrock 1987, Aldington and Fry 1992),as growth regulators (York *et al.* 1984, McDougall and Fry 1988) and they may also act as molecular messengers in cell to cell signalling. Properties of the cell wall greatly influence the digestibility of forage (Chesson 1988, Akin and Chesson 1989) and affect our ability to exploit a number of non-food crops for fibre extraction and paper pulping.

In order to understand the mechanism of these functions and how they are controlled and thus how they can be manipulated, it is important to have an understanding of the structure and biosynthesis of the wall and the interactions that occur between individual wall components.

1.2. General Structure of the Cell Wall

Cell walls are generally divided into 3 different types. The middle lamella can be thought of as the initial cell wall as it is formed during cell division to separate daughter cells. It appears to be important as a cellular glue and is formed mainly from pectic polysaccharides. The primary cell wall is that which is synthesised by actively growing cells and in some cell types

e.g. colenchyma and parenchyma of the grass midrib area and the majority of epidermal cells, it is the only wall formed (Wilson 1993). The 1° wall is a biphasic structure consisting of a network of semi-crystalline insoluble cellulose microfibrils embedded in a gel-like matrix of polysaccharides and glycoproteins (Northcote 1972, Preston 1979, McNeil *et al.* 1984). These matrix polysaccharides vary greatly in their physical and chemical characteristics, and in their distribution between different taxa and different tissue types. For instance, there are considerable differences between the wall composition of dicots and that of graminaceous monocots.

The primary cell wall must be able to undergo plastic deformation to allow turgor driven growth (Cosgrove 1986). This is believed to occur through the cleavage of bonds restricting the movement of cellulose microfibrils and may be controlled by plant growth regulators (Cleland 1981, Fry 1989a) There presumably must be a corresponding decrease in cell wall extensibility to cause cessation of growth, which may occur through the formation of cross-links between polymers (Fry 1989b), decrease in cell wall lytic enzyme activity or an increase in deposition in the wall of inextensible structures e.g. lignin. The deposition of lignin has been correlated with a cessation of growth (Sauter and Kende 1992). Primary cell walls also contain a number of phenolic residues which will be discussed in greater detail below.

The secondary wall is synthesised after the cessation of cell growth and proceeds in 3 stages resulting in three discernable layers of secondary thickening - S1, S2, S3. The secondary cell wall comprises the vast majority of the dry weight of the mature plant and is most evident in differentiated cells such as sclerenchyma, xylem and tracheids. It provides thickening and support for such structures and has a different polysaccharide profile from that of the primary cell wall. Lignin occurs in greatest quantities in the secondary wall and certain tissue types can become very heavily lignified

during secondary growth. However, lignin is not confined to the secondary wall but extends through the primary wall and middle lamella.

1.3 Biosynthesis of wall polysaccharides.

Wall matrix polysaccharides appear to be synthesised by membrane bound polysaccharide synthases within the endomembrane system then transported via vesicles to the plasma membrane where they are excreted into the wall through exocytosis of the plasma membrane. Early microscopic work suggested a specific role for the Golgi bodies in the biosynthesis of slime polysaccharides and glycoproteins (Northcote and Pickett -Heaps 1966, Pickett-Heaps1967a,b) and cell free extracts were shown to synthesise cell wall polysaccharides from supplied precursors (Villemez *et al.* 1965,1966, Elbein 1969, Villemez 1971). Subsequent work demonstrated polysaccharide synthase activity in the endomembrane system, specifically the Golgi bodies (Bowles and Northcote 1974, 1976, Ray *et al.* 1976) although polysaccharide synthase activity has also been found in the endoplasmic reticulum (Dürr *et al.* 1979, Bolwell and Northcote 1983, Brett *et al.*1992).

Glycosyltransferases have been further localized to sub-fractions of the Golgi (Camirand *et al.* 1987, Brummell *et al.* 1990, Hobbs *et al.* 1991) and there is evidence that biosynthetic pathways for different types of polysaccharides may be spatially segregated (Moore *et al.* 1991, Zhang and Staehelin 1992).

In vitro, polysaccharide synthases are often stimulated by divalent cations and can utilise endogenous polysaccharide substrates in addition to utilising supplied nucleotide sugar precursors. Newly synthesised polysaccharides are then transported to the wall in secretory vesicles that are derived from the trans Golgi bodies. It is possible that further modifications are made to polysaccharides during this transport (Brummell *et al.* 1990).

Notable exceptions to this pattern are callose (ß(1→3)glucan) and cellulose (ß(1→4)glucan) synthesis. These polysaccharides appear to be synthesised at the plasma membrane directly into the wall (Delmer 1987, Giddings *et al.*1980) and there has been some debate as to whether both are synthesised by a single enzyme or enzyme complex, wounding or membrane peturbation causing a cessation of cellulose synthesis and inducing wound response callose instead (Carpita and Delmer 1980, Kauss 1987).

Polysaccharides are built from sugar nucleotide precursors that are synthesised by soluble enzymes in the cytoplasm outside the endomembrane system (Northcote 1985). Epimerases are present that interconvert sugar nucleotides (Grisebach *et al.* 1974, Dey 1984) and these have been shown to be loosely bound to membranes (Northcote 1985). Since the polysaccharide synthases are membrane bound the transport of nucleotide precursors into the lumen of the Golgi may provide a control point in polysaccharide biosynthesis. There is some evidence that there could be a control of synthesis at the level of nucleotide sugar supply since formation of certain polysaccharides are favoured at lower or higher concentrations of nucleotide precursors (Smith and Stone 1973, Henry and Stone 1982) and there are changes in enzymes producing polysaccharide synthase substrates that correlate with tissue differentiation (Dalessandro and Northcote 1977a,b, Longland *et al.* 1989).

Generally there is little known about what priming or termination reactions occur during wall polysaccharide biosynthesis. There is little evidence that lipid intermediates are used, although their use as intermediates in glycoprotein synthesis is well established (Maclachlan 1985) and the formation of lipid intermediates may represent a control point in the partitioning of sugars into wall or glycoprotein synthesis. The position with regards to protein intermediates in polysaccharide synthesis is less clear,

although there is some evidence that nascent polysaccharides are closely associated with protein, possibly acting as a primer molecule (Campbell *et al.* 1988, Baydoun *et al.* 1991).

There are considerable practical problems associated with the study of polysaccharide synthases. Impure endomembrane preparations are frequently used in such experiments, and one cannot be sure that a single enzyme is utilising exogenously supplied precursors - several glycosyltransferases manufacturing polysaccharides or glycoproteins may be competing for a single substrate. The presence of several epimerases in such preparations will result in supplied nucleotide sugar precursors being rapidly interconverted and incorporated into a wide range of products. Since the enzymes are membrane bound they may not obey Michealis -Menten kinetics, making a study of even simple kinetics difficult. There have been particular problems with attempted purification of glycosyltransferases and as yet no polysaccharide synthase has been purified sufficiently for its primary structure to be elucidated.

Recently the development of improved biological detergents has increased success in the initial stages of purification (Sloan *et al.*1987, Waldron *et al.* 1989, Fink *et al.* 1990, Hanna *et al.* 1991), although this process often leads to loss or reduction of activity. This reduction of activity has recently been shown, in bean, to be largely a result of the release of pyrophosphorylases and phosphatases that rapidly hydrolyse the nucleotide substrates (Rodgers and Bolwell 1992). Certain other methods of purification have been attempted, such as product entrapment (Wu *et al.*1991), photoaffinity labelling (Frost *et al.* 1990, Delmer *et al.* 1991) and the use of antibodies (Bolwell and Northcote 1984, Moore and Staehelin 1988, Dhugga and Ray 1991). It is likely that the development of new techniques and reagents will further enable the study and purification of membrane proteins.

The cell wall contains polysaccharides with extremely complex structures and it is likely that there will be sophisticated control over their biosynthesis. Most research has been directed towards the synthesis of the relatively simple heteropolymers consiting of a backbone of glycosyl residues to which one or more types of glycosyl units may be attached. In the simpler heteropolysacharides these side chains may consit of a single sugar residue (i.e. galactomannan, etc), whilst the most complex substituent groups may consist of several sugars, present in a regular and repeating pattern (e.g. the sidechains of rhamnogalacturonan II).

The study of the action of these synthases has often involved supplying radiolabelled precursors of polysaccharides to particulate membrane preparations and subsequent analysis of the product. There has been debate over the precision with which the polysaccharides are formed. At one extreme a highly imprecise mechanism, where only the glycosyl residues and their linkage types are pre-determined, would result in highly irregular polysaccharides e.g. substitution by side chains or non-glycosyl moieties will be irregular along the backbone and can occur to pre-formed polymers. Their structure will be variable. In a highly precise mode of synthesis, the primary structure of a polysaccharide will be controlled and all polysaccharides produced will be regular in structure and consisting of identical subunits and a homogenous polysaccharide product. For this to be achieved there would be expected to be considerable co-operation between individual glycosyltransferases and, since glycosyl residues would need to be added in strict order there would be an absolute requirement for all sugars present in a heteropolymer to be available as nucleotide-sugars during synthesis (Waldron and Brett 1985).

It is unlikely that either system is exclusively adopted by plants and the available evidence appears to suggest that whereas some parts of a

polysaccharide appear to be synthesised precisely, other moieties can be added in an imprecise manner. For example, studies monitoring the incorporation of sugars into xyloglucan suggest that UDP-xylose is essential for the continued incorporation of glucose from UDP-glucose into the xyloglucan backbone , but the fucose and galactose residues are not essential for backbone elongation and do not appear to have to be added during the synthesis of the main xyloglucan backbone (Maclachlan *et al.* 1992, Hanna *et al.* 1991)

1.4 Addition of Non-glycosyl Moieties.

Sugar residues of wall polysaccharides are often substituted to non-glycosyl moieties. The galacturonic acid residues of pectic polysaccharides are often methyl esterified. Etherified methyl residues have been reported as 2-O- and 3-O-Me-D-Xyl, 2-O-Me-Fuc, and 4-O-Me-GlcA.(Fincher and Stone 1981).

O-Acetylation of sugar residues has also been reported, occurring on xylose and arabinose residues of arabinoxylan (Bacic *et al.* 1988, Ishii 1991a) and galactose residues of xyloglucan (York *et al.* 1988), mannose residues of gymnosperm glucomannan (Tinnell 1964), and GalA of pectins.

The degree of methylation or acetylation of a polysaccharide will affect its physical conformation and chemical properties. Such changes are likely to affect interaction with other polysaccharides, the ability to form gels (Rees 1981, Jarvis 1984) or hydrogen bonding (e.g. xyloglucan to cellulose). Methylation and acetylation appear to occur as a post-polymerisation modification to nascent polysaccharides. Particulate membrane preparations were able to utilise S-adenosyl-methionine as a donor in the methyl esterification of polygalacturonic acid (Kauss and Hassid 1967), methyl etherification of xylose and fucose (Fincher and Stone 1981) and glucuronate

residues of glucuronoxylan (Kauss 1969, Baydoun *et al.* 1989a). The donor of acetyl groups is presumed to be acetyl-CoA, in a similar way to that during the biosynthesis of bacterial lipopolysaccharides (Tung and Ballou 1973).

It appears that such non-glycosidic groups are added directly to nascent polymers rather than to the sugar nucleotide sugar precursor. UDP-methyl-galacturonate does not act as a substrate for polygalacturonate synthetase, whereas the demethylesterified sugar nucleotide does (Villemez *et al.* 1966), and the addition of neither acetyl-CoA nor S-adenosyl-methionine to particulate membrane preparations stimulated the incorporation of glucuronic acid into glucuronoxylan, indicating that the addition of acetyl and methyl groups is not a pre-requisite for polysaccharide elongation (Baydoun *et al.* 1989b). This suggests that methylation and acetylation occur to preformed polysaccharides, possibly during transport in Golgi derived vesicles.The addition of acetyl-CoA to particulate membrane preparations inhibited the incorporation of radiolabel from S-adenosyl-[methyl-^{14}C]adenosine into polymeric material (Baydoun *et al.* 1989a), suggesting that there may be competition between the two donor substrates for substitution sites.

Certain polysaccharides are also substituted with phenolic acid derivatives and these are discussed in greater detail below.

1.5 Phenolic residues of the primary cell wall.

Many growing plant cell walls contain polysaccharides that bear a small proportion of hydroxycinnamoyl side chains (Smith and Hartley 1983). These wall bound phenolic residues have been found in a large number of plant families (Harris and Hartley 1980, Hartley and Harris 1981) but have been perhaps more wholly characterised in the gramineae. In primary cell walls residues derived from 3-methoxy-4-hydroxycinnamic (ferulic) acid are

the most abundant although p-coumaroyl, p-hydroxybenzoyl, sinapoyl and 5-hydroxyferuloyl residues have all been detected, albeit in small quantities (He and Terashima 1989, Ohashi *et al.* 1987) (fig 1.1).

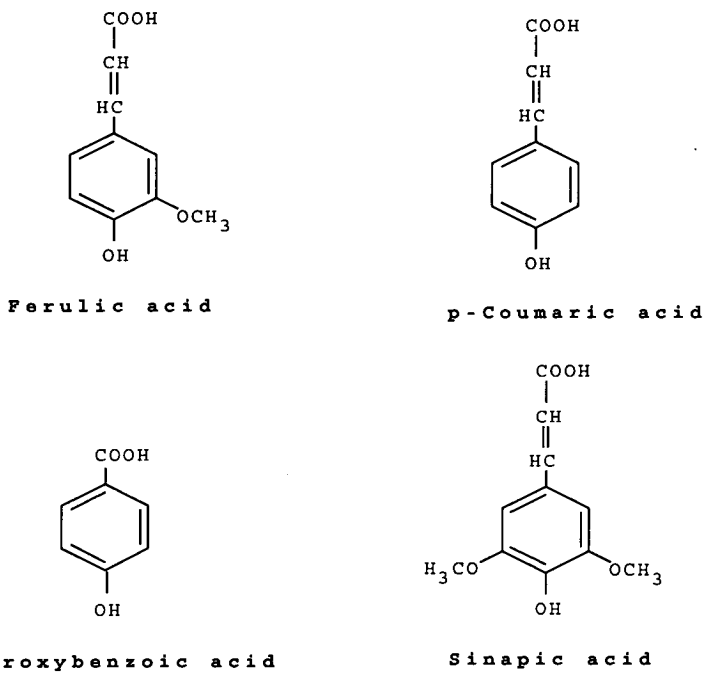


Figure 1.1 Phenolic acids commonly found esterified to wall polysaccharides

There is strong evidence that ferulic acid is esterified to specific residues of wall polysaccharides, namely arabinofuranose residues of arabinoxylan in graminaceous monocots and arabinose and galactose terminal residues of pectins in dicots (Fry 1982 & 1983, Kato and Nevins 1985, Mueller-Harvey *et al.* 1986, Rombouts and Thibault 1988). More recently it has been demonstrated that ferulic acid is also attached to xyloglucan in the wall of bamboo (Ishii *et al.* 1990). There are many reports of the isolation of feruloylated and p-coumaroylated fragments of wall polysaccharides (Fry 1982 & 83, Shibuya 1984, Kato and Nevins 1985, Ahluwalia and Fry 1986, Ford 1989, Hartley *et al.* 1990a, Ishii and Hiroi 1990) often through the use of fungal enzymes. These fragments have been

well characterised and have been used in physiological and biochemical investigations of the cell wall to determine the substitution patterns, amounts and biosynthesis of feruloylated polysaccharides (Fry 1987, Nishitani and Nevis 1990, Myton and Fry 1994).

1.6 Possible Roles for Primary Cell Wall Phenolics.

Wall bound hydroxycinnamic acids have been hypothesised to have a number of different functions in the wall.

Firstly they may help to control the extensibility of the cell wall by providing the opportunity for cross linking between wall polymers. This cross linking between phenolic residues could occur through oxidative coupling by peroxidase at the expense of H_2O_2 to form diferulic acid (fig. 1.2) (Markwalder and Neukom 1976, Fry 1982). Diferulic acid has been isolated from cell walls although in relatively small quantities in comparison to monomeric ferulic acid, and recently a diferuloyl hexasaccharide of arabinoxylan was isolated from bamboo (Ishii 1991b). This fragment showed clearly two arabinoxylan derived trisaccharides joined via a diferulic acid bridge, although it was not possible to show whether this was an inter- or intramolecular bridge. Such a diferulic acid bridge may also be able to link arabinoxylan and xyloglucan.

Since the formation of diferulic acid is assumed to be mediated by an essentially random one electron oxidation step a number of other products are possible. Some of these products, quinone methides, are susceptible to nucleophilic attack and may therefore cross link with hydroxyl groups of sugars to form an ether linkage, or the carboxyl groups of uronides to form an ester. In this way it is thereotically possible for a phenolic acid complex to tether three or four polysaccharides (fig. 1.3). In fact recent work on the oxidation of model feruloyl esters suggests that such quinone methides are

more abundant than diferulic acid (G. Wallace and S.C. Fry, University of Edinburgh, unpublished work).

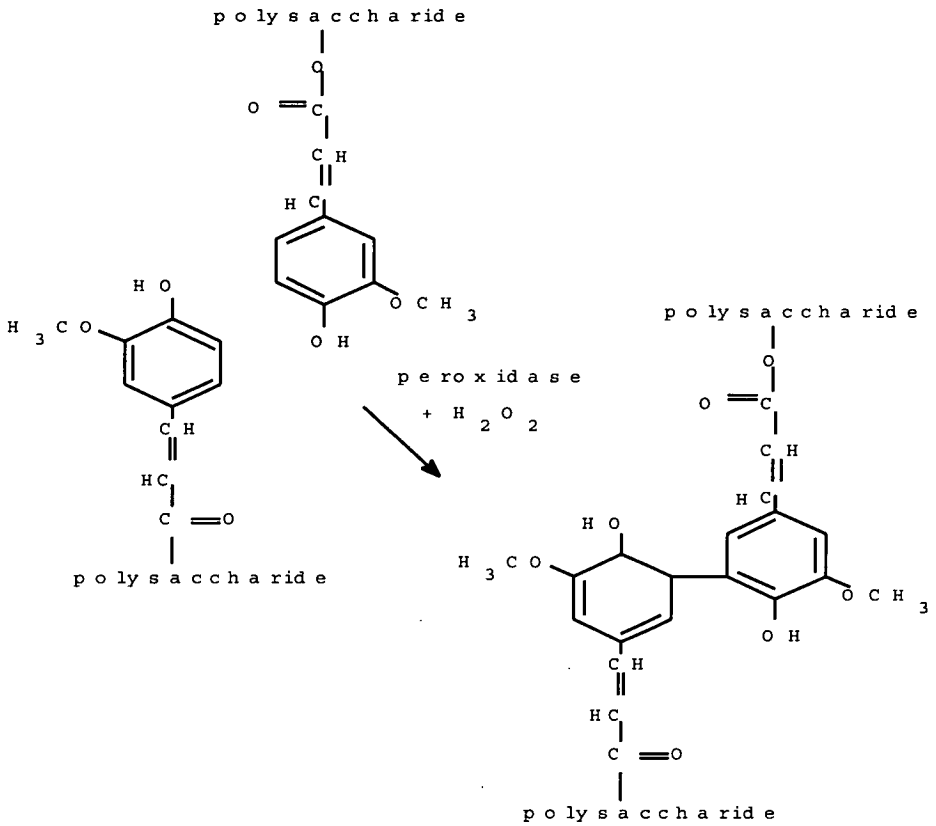
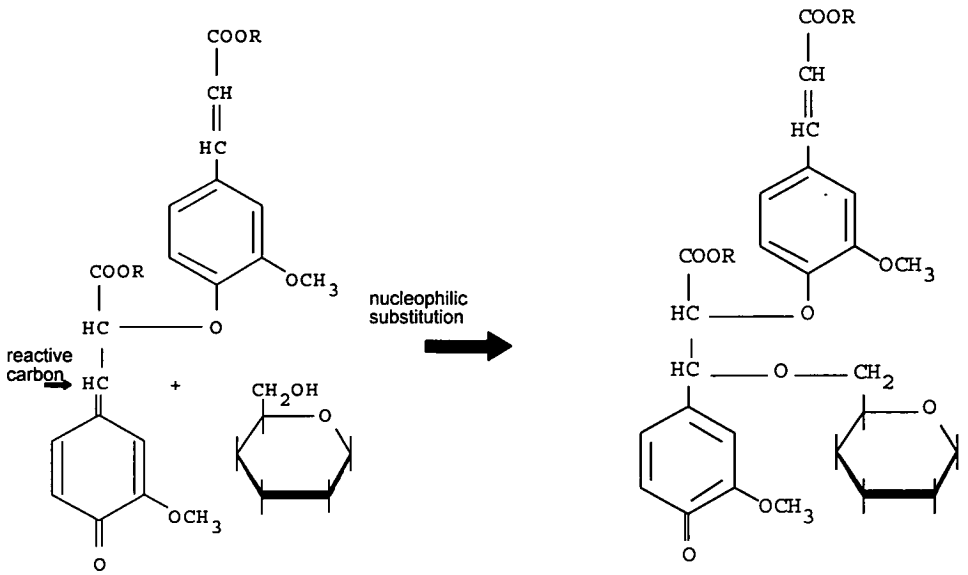


Figure 1.2 Possible mechanism for the formation of a diferuloyl cross-link between two adjacent cell wall polysaccharides

Coumaric and ferulic acids are known to photodimerise to form cyclobutane dimers (truxillic and truxinic acid derivatives) *in vitro* (Fig 1.4), and these dimers have been isolated from plant tissue (Hartley *et al.* 1990a,b, Tachibana *et al.* 1992). Further more, feruloylated and coumaroylated fragments of cell wall polysaccharides have been shown to photodimerize to produce fragments of polysaccharides joined via truxillic and truxinic acid cross links, with coumaroyl groups dimerising much more readily than feruloyl groups (Morrison *et al.*1992).



Where R= sugar residue of a polysaccharide.

Fig 1.3 Scheme by which a quinone methide derivative, formed through the action of peroxidase can tether two or more polysaccharides within the cell wall.

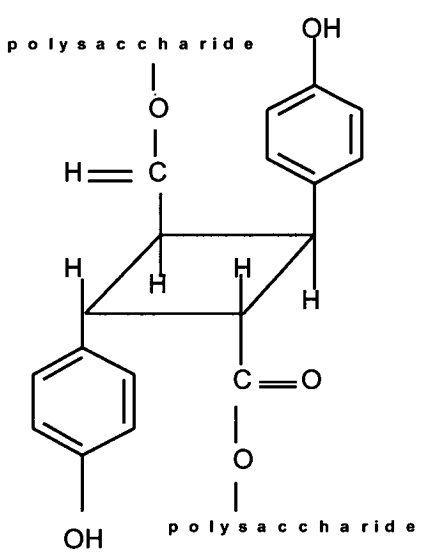


Fig 1.4 A truxillic acid bridge linking two wall polysaccharides.

Such cross links could restrict wall extensibility by preventing the movement of polysaccharides past one another. There is circumstantial

evidence for this role in the fact that in several systems both the absolute amount of ferulic acid and the degree of feruloylation of wall polysaccharides increases as tissue ages, (Yamamoto and Towers 1985, Nishitani and Nevins 1990). This negative correlation with growth implies a potential role for ferulic acid esters in elongation. There is also a positive correlation between the diferulic acid content of the wall and a decrease in wall extensibility, again suggesting a role for these cross links in the control of growth (Kamisaka *et al.* 1990, Tan *et al.* 1991). The fact that photodimerisation of phenolic residues could result in a decrease in wall extensibility may suggest a role in phototropism by creating differential growth rates in tissues exposed to unidirectional light (Towers and Abeysekera 1984).

Another proposed role for primary cell wall phenolics is as a link between the matrix polysaccharides and lignin. Recent Work (Lam, Iiyama and Stone 1992, Iiyama, Lam and Stone 1990) has shown that in mature tissue of wheat and *Phalaris* internodes the vast majority of ester linked feruloyl groups are also ether linked, and it is proposed that these ester-ether linked feruloyl residues act as a bridge between sugar residues (ester linkage) and lignin moieties (ether linkage) (fig 1.5).This type of bridging appeared to involve only ferulic acid, although it is coumaric acid which is more usually thought to be closely associated with lignin (Higuchi 1985). This fact raises the question of whether ferulic and coumaric acid actually have different physiological roles within the wall.

The firm anchoring of lignin into the polysaccharide fraction of the wall may well have a profound effect on the digestibility of forage and also on the extractibility of lignin in the pulping process.

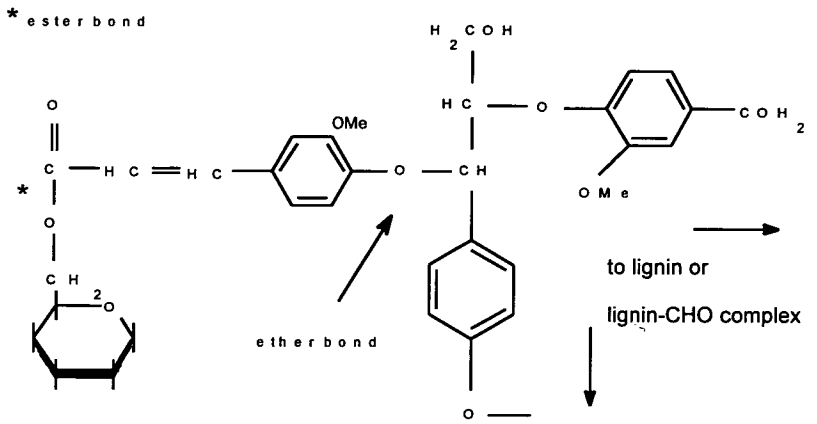


Fig. 1.5 Representation of a sugar-phenolic acid-lignin bridge linking wall polysaccharides to lignin via an ester and ether bond (after Iiyama *et al.* 1990)

It is also possible that phenolic acids laid down in primary growth acts as a template for lignin deposition. Lignification is known to begin in the middle lamella and primary wall, and the increase in feruloylated polysaccharides seen in ageing cells (Nishitani and Nevins 1990) could be hypothesised to be in preparation for subsequent lignification and formation of lignin-phenolic acid-polysaccharide bridges.

There is recent evidence that small feruloylated fragments of cell wall can act as anti auxins in rice lamina bioassays (Ishii and Saka 1992). This is similar to the growth regulatory effects shown by xyloglucan-derived oligosaccharins (York *et al.* 1984, McDougall and Fry 1988). However the concentration at which feruloylated fragments appeared to be biologically active is greater than other fragments (10-300 µM as compared to nM in XGO's). These biologically active feruloylated fragments consisted of only tri- and tetrasaccharides and the feruloyl moiety was essential for biological activity.

Primary wall phenolics may also play a defensive role against attack by pathogens. Feruloyl groups appear, *in vitro*, to protect the residues to which they are attached from digestion by fungal enzyme preparations (Fry

1984, Nishitani and Nevins 1988). This phenomenon may occur *in vivo* and thus provide a degree of protection from fungal invaders. The increase in peroxidase levels seen in the wall following fungal attack (Moerschbacher 1989, Flott *et al.* 1989) is thought to result mainly in the formation of defensive lignin type polymers and primary phenolics may be important in this process by either providing a base for subsequent deposition of phenolic material or by becoming oxidatively linked themselves.

1.7 The biosynthesis of primary cell wall ester linked phenolic acids.

Within the cell wall very specific sugar residues carry feruloyl groups, and their positioning would appear to be under strict control. Such precision suggests that an enzyme system is responsible for esterification of ferulate to polysaccharides.

There has been some debate over the site of feruloylation of primary wall polysaccharides. It is possible that feruloylation occurs as a co-synthetic modification to polysaccharides being manufactured in the Golgi bodies, in a similar way to the addition of methyl groups to pectic polymers (Kauss and Hassid 1967, Fincher and Stone 1981) or the addition of acetyl groups to lipopolysaccharides (Tung and Ballou 1973). Alternatively, feruloylation could occur in the wall to pre-formed polysaccharides, possibly mediated by a specific feruloyltransferase. The acyl donor is likely to be an activated form of ferulic acid such as feruloyl-CoA (Stöckigt and Zenk 1979) or a glycosyl ester of ferulic acid.

Evidence has been presented in support of both *in muro* and intraprotoplasmic feruloylation. Yamamoto and Towers (1985) presented work showing that in barley coleoptiles the number of esterified feruloyl groups in cell wall residues increased as tissue aged. Furthermore the

number of esterified feruloyl groups continued to increase even after the *net* deposition of wall polysaccharides had ceased. This was interpreted as being evidence for feruloylation occurring in the wall to preformed polysaccharides that had been secreted and integrated into the wall matrix. Nishitani and Nevins (1990) also reported increases in wall bound saponifiable feruloyl residues continuing after increases in wall polysaccharide material had ceased in maize coleoptiles.

However, Fry (1987) demonstrated that in spinach cell cultures arabinosylation and feruloylation of pectins occurred with similar kinetics and that both events were intraprotoplasmic phenomena. More recent work (Meyer *et al.* 1991) apparently demonstrated the ability of endomembranes of parsley cell cultures to transfer ferulate (and to a lesser extent coumarate) from its CoA derivative into polymeric materials. This polymeric material had several properties consistent with it being a pectic polysaccharide, and its formation would suggest that a membrane associated feruloyltransferase is feruloylating endogenous nascent polysaccharides. In the work of Meyer *et al.* (1991) the feruloyl groups appeared to be attached to galactose residues since saponification of fragments of the feruloylated product yielded galactose after acid hydrolysis. It was not been established whether this feruloylation is occuring directly to the polymer bound galactose residue or via feruloyl-UDP-galactose (fig 1.6). However, by analogy with the methylesterification of uronic acid residues, direct feruloylation of the sugar nucleotide would appear less likely.

Thus it appeared possible that dicots and graminaceous monocots employ different biosynthetic pathways to attach feruloyl groups to their wall polysaccharides, or that the contradictory evidence was due to a difference in the metabolic pathways between cell cultures and whole plants.

It was therefore my aim to investigate the kinetics of feruloylation of nascent polysaccharides in order to ascertain the sub-cellular site of this process in cell cultures of a graminaceous monocot.

a. feruloyl-CoA + Polysaccharide $\xrightarrow{(i)}$ feruloylated polysaccharide

b. feruloyl-CoA + UDP-Gal $\xrightarrow{(ii)}$ feruloyl-UDP-Gal $\xrightarrow{(iii)}$ feruloylated polysaccharide

Fig 1.6 The two possible pathways of feruloylation of polysaccharides. Scheme (a) the direct feruloylation of sugar residues of nascent polysaccharides. Scheme (b) feruloylation of the sugar-nucleotide before addition to the growing polymer. Enzymes involved are (i) feruloyl-CoA:polysaccharide feruloyltransferase, (ii) feruloyl-CoA:Sugar-nucleotide feruloyltransferase; (iii) UDP-(feruloylsugar):polysaccharide synthase. In (iii) the polysaccharide acceptor could also be arabinose or xylose residues (of pectin or hemicelluloses)

Data are presented demonstrating that feruloylation occurs within the protoplast in grass cell cultures, and it is likely that this process is occurring co- or postsynthetically to polysaccharides being synthesised within the Golgi or in transport vesicles.

A feruloyltransferase responsible for the feruloylation (and coumaroylation) of nascent polysaccharides is likely to play a key role in the biosynthesis of plant cell walls. It is likely to be a branch point in the phenylpropanoid pathway, perhaps controlling the distribution of phenolic acids between esterified moieties and lignin during differentiation from primary to secondary wall production. Several enzymes involved in phenylpropanoid metabolism also use feruloyl-CoA as a substrate and there is likely to be competition between the different branches of the phenylpropanoid pathway for substrate availablity. Primary cell wall

phenolics appear to be linked also to lignin, thus firmly anchoring lignin into the matrix polysaccharides. This will have an effect on the wall digestibility and lignin extractability and the enzyme responsible for the provision of these anchor points, i.e. feruloylation of polysaccharides, will be of considerable interest to those involved in the manipulation of lignin characteristics of forage and fibre crops. Thus, not only is this enzyme of intrinsic interest, but also a knowledge of its characteristics and properties is desirable for future manipulation of biochemical and agronomical features.

In order to investigate further the mechanism for the feruloylation of cell wall polysaccharides, I have therefore attempted to demonstrate the activity of a feruloyltransferase in maize, analogous to that recently described in parsley cell cultures.

The kinetics of feruloylation (chapter 3) suggest that feruloylation is likely to occur as a post synthetic modification to nascent polysaccharides within the Golgi system and so assays were conducted with membrane preparations of suspension cultured cells.

1.8 Other feruloyltransferases.

Higher plants contain a large number of hydroxycinnamoyl esters the formation of which is catalysed by transferases. Such transferases will utilise activated conjugates of hydroxycinnamic acids such as hydroxycinnamoyl-CoA thiolesters and hydroxycinnamoyl-β-glucosides. Hydroxycinnamoyl glucosides are synthesised from the free acid and the sugar nucleotide (Nagels *et al.* 1981) and will accumulate within the vacuole (Strack and Sharma 1985). Glucosyl derivatives of hydroxycinnamic acids are acyl donors in a number of transferase mediated reactions and their role in the transfer of hydroxycinnamoyl groups onto malate (Strack 1982, Strack *et al.* 1990), betacyanins (Bokern and Strack 1988) and quinate (Villegas and

Kojima 1986, Moriguchi *et al.* 1988) has been established, and a number of the enzymes involved have been purified (Villegas *et al.* 1987, Gräwe et al. 1992). The ubiquity of these hydroxycinnamoyl glucosides might suggest that these compounds are likely to participate as intermediates in many metabolic pathways.

A number of feruloyltransferases will also utilise CoA thiol esters of phenolic acids as acyl donors. In the synthesis of amides of hydroxycinnamic acids the CoA derivative is the preferred substrate and often such transferases will utilise several hydroxycinnamoyl-CoA's. The transferase responsible for the synthesis of hydroxycinnamyl-agmatine (a polyamine with the strucutre $NH_2(CH_2)_4NHC(NH)NH_2$) in barley will utilise coumaroyl-, feruloyl-, caffeoyl-, and sinapoyl-CoA, although with declining affinity (Bird and Smith 1981, 1983). This low substrate secificity can also extend to the acyl acceptor. The feruloyl-CoA:tyramine transferase in tobacco will use several aromatic amines along with tyramine as acyl acceptors, and it will also utilise a wide range of hydroxycinnamoyl-CoA thiolesters as acyl donors (Negrel and Martin 1984) However, such a lack of substrate specificity may indicate the existence of several isoenzymes (Fleurence and Negrel 1989). Feruloyl-CoA: tyramine transferase in tobacco is induced by inoculation by tobacco mosaic virus (Villeges and Brodelius 1990) and and its product (predominantly N-feruloyl-tyramine) is integrated into the cell wall by peroxidase (Negrel and Jeandet 1987, Negrel and Lherminier 1987) suggesting a role in the defence response. Phenolic acids are also esterified to polyamines (Meurer-Grimes *et al.* 1989), shikimic and quinic acids (Lofty *et al.* 1992), alkaloids (Strack *et al.* 1991) and sugar acids e.g. gluconate, galactarate, glucarate, (Strack *et al.* 1986, 1987) by the action of transferases utilising hydrocycinamoyl-CoAs as acyl donors. Hydroxycinnamoyl-sugar acids are further metabolised after a transient

accumulation, and it suggested that they may be involved in the formation of cell wall phenolic residues. This is hypothesised since the decline in soluble hydroxycinnamoyl-sugar esters is matched by a concomitant increase in insoluble material thought to be wall associated. However no further evidence for this hypothesis has been presented and the correlation described is not evidence that hydroxycinnamoyl-sugar acid esters are the direct precursors for wall phenolic material.

All the above enzymes are present in the soluble cytosolic fraction of the cell and it is likely that there must be some form of compartmentalisation of enzymes and substrates at the sub-cellular level, either within the vacuole or other organelles. The feruloyltransferase that acylates malate and its hydroxycinnamoyl product have been localised to the vacuole, where it is suggested the product is stored (Strack and Sharma 1985) , possibly as a deterrent to feeding insects (Neilson *et al.* 1984).

There is, however, little information about the exact location of these enzymes or where they are physiologically active.

1.9 Lignin and secondary cell wall phenolics.

Lignin is a complex heteropolymer formed through the oxidative coupling of three main phenylpropanoid units to form a coherent, integrated matrix. These units, namely coumaryl, coniferyl and syringyl alcohols (collectively known as monolignols) are produced within the cell via a complex multienzyme pathway then secreted into the wall. In the wall they are acted upon by peroxidases and laccases and the free radical thus formed polymerise to form lignin (Grisebach 1981, Higuchi 1985).

The composition of lignin varies depending on the type of tissues (Grand *et al.* 1979), the maturity of the tissue (Lam *et al.* 1990), between primary and secondary cell walls and between plant taxa (for a review see

Grand *et al.* 1982). Generally it is thought that gymnosperms have a high coniferyl type lignin, whereas angiosperms develop lignin derived from both syringyl and coniferyl alcohol. The graminaceous monocots have the most complex lignin consisting of residues derived from syringyl, coniferyl and coumaryl alcohols. However there is some debate as to how accurate these generalisations are as new techniques enable a more thorough analysis of lignin structure (Lewis and Yamamoto 1990).

Lignification occurs in cells with secondary walls and the onset of lignification is tightly co-ordinated with other events in wall formation in particular polysaccharide synthesis and deposition. The process of lignification appears to be initiated in the primary wall and middle lamella during the early phase of secondary wall deposition, but rapidly extends to the secondary wall (Takabe 1989). The concentration of lignin is higher in the primary wall/middle lamella than in the secondary wall, but because of its thickness the secondary wall contains most of the lignin present in the plant. In a few cell types e.g. collenchyma and phloem fibres in legumes, secondary thickening is present but lignification even in mature tissue does not occur (for a review see Wilson 1993).

1.10 Roles of lignin.

Lignin provides the plant with support, enabling an upright growth habit in the terrestrial environment, and strengthens tissues to allow a greater load bearing capacity. In xylem the characteristic rings of highly lignified wall material that form during differentiation allow the transport of water that is under considerable tension by preventing the collapse of the vessels. Lignin renders plant tissue highly resistant to enzymatic degradation and there is much evidence to show that high lignin content decreases the digestibility of forage plants, particularly with respect to ruminal fermentation (for reviews

see Akin and Chesson 1989, Jung and Deetz 1993). This fact has obvious implications for agriculture and food production and there have been considerable research efforts directed towards the improvement of the nutritive value of forage through the manipulation of lignin characteristics. The same research efforts will have implications in the pulping industry where the removal of lignin in paper production uses large amounts of energy and produces environmental pollutants.

Lignification, or the production of lignin type polymers, is induced as a response to fungal attack (Moerschbacher 1989). This response has been characterised and there is an increase in the activity of those enzymes responsible for the production of lignin precursors e.g. phenylalanine ammonia-lyase, caffeate O-methyl transferase, 4-coumarate-CoA ligase (Maule and Ride 1983) and often a concomitant increase in the levels of peroxidase activity, which has been implicated in the formation of lignin *in muro* (Flott *et al.* 1989).

1.11 The biosynthesis of monolignols.

Monolignols are formed via a complex multienzyme mediated process, generally referred to as the phenylpropanoid pathway since the majority of products and intermediates have a C_6-C_3 structure (fig 1.7)(Lewis and Yamamoto 1990). Although the steps within this pathway have generally been elucidated, and a number of the enzymes involved have been well characterised, there still remains a lack of knowledge regarding the control of this pathway. The situation is complicated by the fact that several other secondary metabolic pathways remove intermediates from phenylpropanoid metabolism, e.g. flavanoid production and phytoalexin biosythesis, and several enzymes may act as control points for three or four separate biosynthetic routes.

The first committed step in phenylpropanoid biosynthesis is the conversion of phenylalanine to cinnamic acid by the enzyme phenylalanine ammonia lyase (PAL, E.C. 4.3.1.5). The properties of PAL from several species have been investigated and in some species several isoforms have been identified (Bolwell *et al.* 1985). These isoforms have molecular weights of 250-330 kDa and appear to be composed of 4 sub-units. PAL activity is induced during the disease response with an increase in transcription and translation (de Sà *et al.* 1992, Cuypers *et al.* 1988). This is presumably linked not only to lignification but also to phytoalexin biosynthesis since transcription of genes encoding the first two steps in the phytoalexin branch pathway (chalcone synthase and chalcone isomerase) are induced concomitantly (Cramer *et al.* 1985). PAL is also induced in thigmomorphogenic responses (de Jargeur 1985). Several cDNAs encoding PAL have been isolated and cloned. A number of inhibitors of PAL have been identified (Amrhein *et al.* 1983), and appears to be inhibited by its product, cinnamic acid (Bolwell *et al.* 1986). Use of such inhibitors results in decrease in lignin and phytoalexin synthesis (Ingold *et al.* 1990) and PAL is considered to be a key enzyme in the metabolism of a number of phenylpropanoid pathways.

Hydroxylations of cinnamate to p-coumarate, coumarate to caffeate and ferulate to 5-hydroxyferulate are catalysed by the cytochrome P450-dependent, microsomal enzyme trans-cinnamate-4-monooxygenase (CMO, EC 1.14.13.11) which requires oxygen and NADPH (Grand 1984). Methoxylation of caffeic and 5-hydroxyferulic acid are catalysed by O-methyl transferase (OMT, E.C. 2.1.1.68) which utilises S-adenosyl methionine as a methyl donor and exists as isozymes that exhibit different substrate specificities. OMT purified from gymnosperms mainly catalyse the conversion of caffeic acid to ferulic acid with only a low rate of reaction for the conversion of 5-hydroxyferulic acid to sinapic acid (Kuroda *et al.* 1975). However OMT from

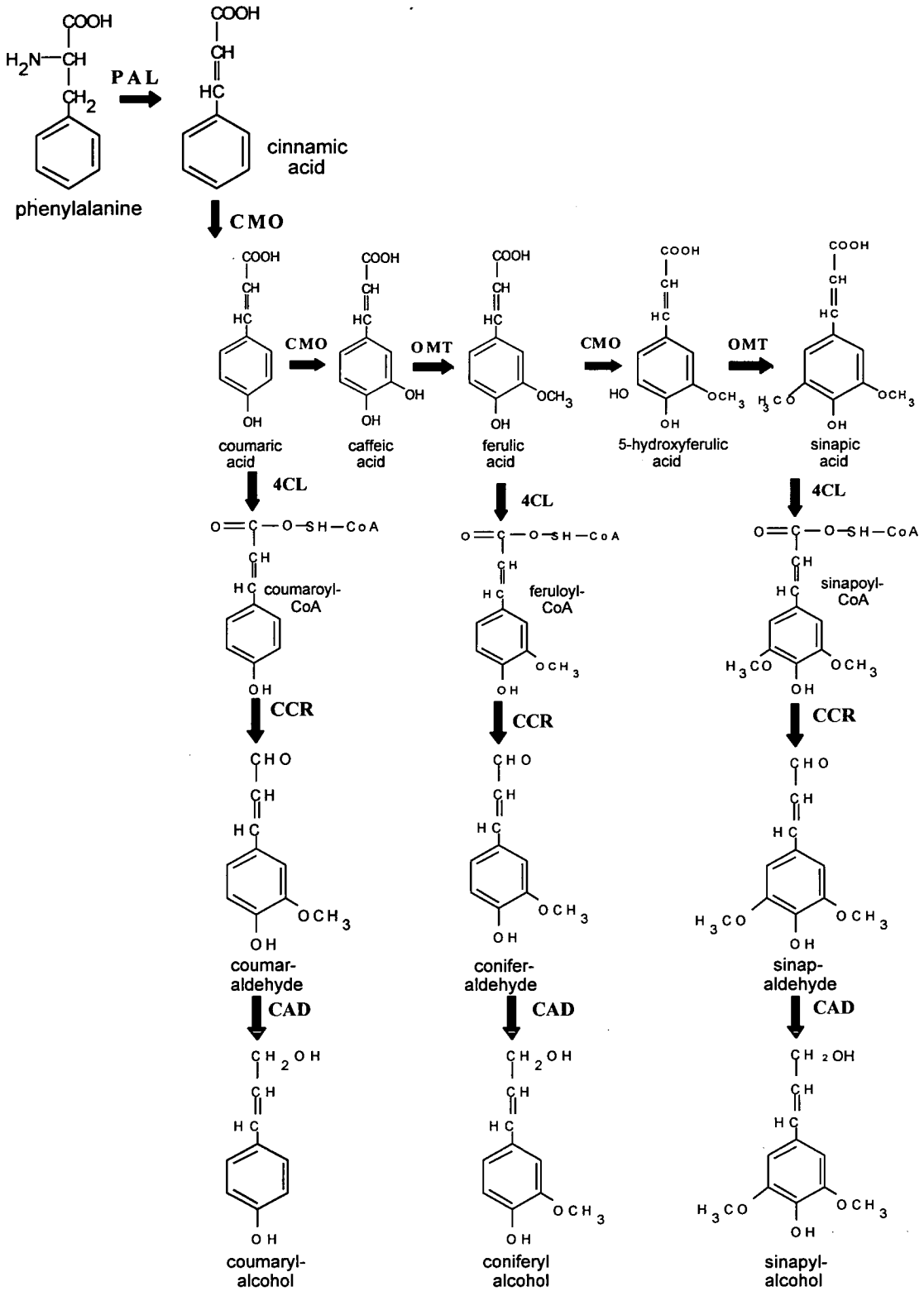


Fig 1.7 Pathway for synthesis of monolignol precursors from L-phenylalanine. PAL: L-phenylalanine ammonia lyase (EC 4.3.1.5), CMO: trans-cinnamate-4-monooxygenase (EC 1.14.13.11). OMT: caffeate-O-methyltransferase (EC 2.1.1.68), 4CL: 4-coumarate CoA ligase (EC6.2.1.12), CCR: cinnamoyl-CoA (EC 1.2.1.44), CAD: cinnamyl alcohol dehydrogenase (EC 1.1.1.195)

angiosperms will methylate both caffeic and 5-hydroxyferulic acid (Gross 1985). This difference in OMT substrate specificities may be responsible for the differences in the proportion of lignin monomers found in gymnosperms and angiosperms. Certain brown midrib mutants of maize have been shown to have a lowered OMT activity and a corresponding reduction in the proportion of methoxylated units in the core lignin (Grand *et al.* 1985a). However this decrease in OMT activity does not appear to result in a decrease in the amount of esterified methoxylated units, suggesting that this mutation may be regulating a modified distribution of phenylpropanoids between polymeric and none polymeric forms. OMT is induced during defence responses to fungal pathogenesis (Legrand *et al.* 1976).

Coumarate 4-CoA ligase (EC 6.2.1.12) is responsible for the formation of CoA esters of phenolic acids and can exist as several different isozymes (Grand *et al.* 1983), each with different substrate specificities. 4CLs isolated from different taxonomic groups vary in their substrate specifities, with sinapic acid being utilised only by leguminous and graminaceous 4CL (Kutsuki *et al.* 1982a). The distribution of isoenzyme appears to vary within a plant between different tissue types (Grisebach 1981).

The next step in the pathway is the reduction of CoA dervatives of phenolic acids to the corresponding aldehyde, catalysed by cinnamoyl-CoA reductase (CCR, EC 1.2.1.44). This step is specific for lignin biosynthesis and is in competition with a number of other enzymes that utilize CoA derivatives of phenolic acids. Examples are chlorogenic acid biosynthesis (Stöckigt and Zenk 1974), flavanoid production (Hahlbrock and Scheel 1989), formation of hydroxycinnamic amides of aromatic amides (Negrel and Martin 1984, Vilegas and Brodelius 1990). Another enzyme utilizing CoA derivatives of phenolic acids is the putative feruloyltransferase involved in the formation of polysaccharide-phenolic esters of the primary cell wall (Meyer *et al.* 1991).

Thus this step in the phenylpropanoid pathway could represent a control step in the partition of phenolic acids during transition from primary to secondary cell wall production. The mechanisms regulating the division of cinnamoyl-CoA esters between these competing pathways are, however, unknown.

The final step in the formation of monolignols is the reduction of cinnamyl aldehydes to alcohols catalysed by the enzyme cinnamyl alcohol dehydrogenase (CAD, EC 1.1.1.195). CADs isolated from different species have varying affinities for the three substrates available (Kutsuki *et al.* 1982b) and isoforms of this enzyme have been isolated from wheat that exhibit marked differences in substrate affinities (Pillonel *et al.* 1992). These different specificities may in part account for differences in lignin composition and CAD has been implicated as a controlling enzyme in lignification.

Inhibitors of CAD significantly reduce the incorporation of radiolabel from cinnamic acid into lignin in isolated xylem tissues (Grand *et al.* 1985). Plants transformed with CAD anti-sense mRNA have been obtained and severe down-regulation of CAD activity results in changes in lignin composition, with cinnamyl aldehydes being incorporated directly into the lignin core instead of the corresponding alcohol (Halpin *et al.* 1993). Lignins with higher than normal aldehyde content are also produced in the brown midrib (bm_6) mutant of sorghum in which CAD activity is suppressed (Pillonel *et al.* 1991).

1.12 The use of model systems in which to study phenylpropanoid metabolism and lignin biosynthesis.

Although many enzymes in the phenylpropanoid pathway have been isolated, purified and characterised, our understanding of the interactions of enzymes within the pathway and how the supply and availability of lignin precursors is controlled in whole plants is still far from clear.

In order to investigate the interactions between individual enzymes and the flux of substrates through different parts of the phenylpropanoid pathway during cell differentiation and lignification model systems have been developed. One of the most widely used systems is the *Zinnia elegans* tracheary element culture in which isolated mesophyll cells from *Zinnia* are induced to differentiate into tracheary elements (Fukuda and Komamine 1980). Such differentiation is accompanied by an increase in cell wall carbohydrate, particulaly hemicellulose (notably xylans) and cellulose (Ingold *et al.* 1988). Lignification of differentiating cells occurs after a short lag and this active lignification is marked by concomitant increases in PAL, 4CL and peroxidase activities (Fukuda and Komamine 1982, Church and Galston 1988). The increase in PAL activity appears to be due to an increase in *de novo* synthesis, controlled at the level of transcription (Lin and Northcote 1990).

Although the *Zinnia* cell culture systems have proved useful in measuring the flux of intermediates through individual steps of the pathway, equivalent systems have not been established for other dicots or monocots, although some embryogenic monocot cultures have been established which may be developed into model systems. It is debatable to what extent cell cultures accurately reflect processes occurring in whole plants during normal (as opposed to wound response) growth. It is unlikely that any cell culture system can model the latter stages of secondary cell wall formation that occur in mature plants due to lack of structural organisation in tissue cultures.

Mutant plants with altered phenylpropanoid metabolism have provided interesting systems in which to study lignification. These have consisted mainly of the natural brown midrib mutants of maize , sorghum and millet, although cell wall mutant *Arabidopsis* plants are now being produced that may prove extremely useful in the future (Reiter *et al.* 1993). In the brown

midribs considerable attention has been paid to mutations which result in alterations in forage digestibility or nutritive value and such work has provided some insight into the effects of reducing single enzyme activities on the physiology, biochemistry and cell wall composition of the plant.

Another model which has been promoted for the study of tissue maturation is the graminaceous monocot internode. The flowering stalk of graminaceous monocots consists of several internodes each of which is produced by an intercalary meristem near its base. During reproductive growth the internodes elongate rapidly in acropetal succession with two or more internodes elongating together or in a slightly staggered manner (Cherney *et al.* 1989). Each develops from the meristem near the node at its base and this meristem remains active until the final stage of elongation is complete. Thus within an individual internode, cells produced by cell division are displaced upwards, elongate and begin to mature whilst new cells continue to be formed from the meristem. Therefore, within an internode there is a gradient of maturity with the first formed (oldest) tissue at the apex, and the last formed (youngest) tissue at the base. The newly elongated internode provides spatial resolution of events occurring during secondary cell wall deposition and tissue maturation.

This model has proved useful in studies investigating changes in the structure and composition of cell wall polymers during maturation and secondary growth (Joseleau and Barnoud 1976). It has also been used to investigate changes in lignin chemistry during maturation of wheat and *Phalaris* stems (Iiyama *et al.* 1990, Lam *et al.* 1990,). Rice internodes have been used as experimental material to investigate the deposition of β-glucans and lignin during their rapid elongation and subsequent cessation of growth (Sauter and Kende 1992).

Recently a group of workers have used an internode model to investigate the biochemical and agronomical characteristics of forage maize.

Within this collaboration the physical, chemical and degradability qualities of the newly extended maize internode have been characterised. Phenolic material (saponifiable phenolics and lignin) is deposited along much of the internode, with the greatest increases in total phenolics (rate of lignification) occurring in the lower third of the internode, whilst net accretion of carbohydrate reaches a maximum in the lower third of the internode and thereafter remains essentially constant. Only the very lowest section of the internode has a polysaccharide profile similar to that of the primary cell wall, with all older tissues containing sparsley substituted xylans and 1-4 linked glucose typical of the secondary wall (Scobbie *et al.* 1993). The cell walls, particularly of the schlerenchyma and epidermis thicken in an approximately linear fashion as tissue matures (Travis *et al.* 1993). There is a substantial decrease in degradibility of tissue towards the top of the internode, consistent with the prevalent view that lignin content is a major limiting factor in tissue degradability (Joseleau 1992).

The maize internode has been shown to provide a good model in which to study secondary cell wall formation, composition and degradability It was my aim to investigate the value of this system in the monitoring of changes in activities of those enzymes involved in lignification during tissue maturation.

Previous work in the Forage Maize Workshop had been conducted on two inbred lines W401 and Co125, which differ in their agronomic characteristics and slightly in their internodal development. Accordingly plants of these cultivars were grown and used in enzyme assays.

PAL, OMT and CAD have been demonstrated as influential enzymes in the lignification process and so the activities of these

enzymes were monitored along newly extended internodes in order to assess the internode as a system in which to study the control of enzyme activity during tissue maturation.

CHAPTER 2.
MATERIALS AND METHODS.

2.1 Plant material.

2.1.1 Cell cultures.

Cell suspension cultures of tall fescue grass (*Festuca arundinacea* Schreber) and maize (*Zea mays* L.) were used as experimental material. Fescue cultures were incubated at 25°C with photon flux 20 µmol m^{-2} s^{-1} and rotation on a horizontal plane 100 rpm with an amplitude of 1.5cm. Cultures were sub-cultured into fresh medium (see appendix) every 2 weeks. Maize cultures were maintained at 24°C with a photon flux density of 1.5 µmol m^{-2} s^{-1}, rotation on a horizontal plane of 210 rpm with an amplitude of 2.0 cm. Maize cultures were sub-cultured into fresh medium (see appendix) every week.

All media and glassware used in the manipulation and maintenance of cell cultures were sterilised by autoclaving at 121°C for 18 min. Aseptic manipulation of cultures was carried out in a laminar flow cabinet.

2.1.2 Glasshouse grown material.

Plants of the two inbred lines of maize, W401 and Co125, were grown in the glasshouses at Jealott's Hill Research Station, Bracknell, Berks during the months October to January 1991-1993 and October to January 1992-1993. Plants were grown in a 16 hour daylength with supplementary lighting supplied by metal halide lamps (type PR64, Thorn lighting).

During the growth periods the average minimum temperature was 16.5 ±0.1°C and the average maximum temperature was 26.3 ±0.3°C (data monitored over 30 weeks, variation is standard error of 30 weekly measurements). The average light intensity during the same periods of time were minimum 2.4 klux and maximum of 21.4 ± 0.8 klux. Plants were watered daily.

2.1.3 Harvest of glasshouse grown material for enzyme assays.

Mature maize plants were harvested at 5 days post silking (5 days after the emergence of silks from the developing cobs). Internodes were numbered from the top of the plant, internode 1 being that which bears the tassel. Internodes 2 and 4 were removed from the outer leaf sheaths and divided into 5 approximately equal sections. Sections were weighed, labelled A (apex) to E (base) and frozen, first in dry ice and then at -80°C.

2.2 Techniques for analysis of cell walls.

2.2.1 Driselase Digestion.

Driselase (Sigma Chemical Co.) is a fungal enzyme preparation from *Irpex lacteus* able to digest most cell wall polysaccharides, releasing characteristic feruloylated digestion products.

Driselase was partially purified by precipitation in 52% (w/v) ammonium sulphate. The precipitate was collected by centrifugation, dissolved in water, desalted on Sephadex G-25 and lyophilized. The purified driselase was stored dry at -20°C. Routinely, dried samples were suspended in 0.5% (w/v) Driselase in pyridine/acetic acid/water (1:1:98 v/v/v) at pH 4.7, containing 0.5% chlorbutanol to suppress microbial growth. Enzyme mixtures were incubated at 24°C for 16 hours unless otherwise stated.

2.2.2 Hydrolysis by trifluoroacetic acid.

Virtually complete hydrolysis of non-cellulosic glycosidic bonds was achieved by treatment of the dried material in 2 M TFA for 1 h at 120°C. Alternatively samples were heated in 0.1 M TFA at 100°C for 1 h. This milder treatment will preferentially cleave furanose bonds leaving most pyranose

and feruloyl ester bonds intact, thus releasing from arabinoxylan, side chains attached *via* a arabinofuranose bond.

2.2.3 Paper chromatography.

Samples were applied to Whatman 3MM chromatography paper (Whatman International Ltd.) as spots or streaks. Sugars (0.05 mg) and alditols (0.5 mg) were used as external markers. Chromatograms were developed by descending chromatography in the following solvent systems.

BAW; butan-1-ol/acetic acid /water (12:3:5 v/v/v), for 16h.

BEW; butan-1-ol/ethanol/water (20:5:11 v/v/v), for 16 h.

EPW; ethyl acetate/pyridine/water (8:2:1 v/v/v) for 24 h.

EAW; ethyl acetate/acetic acid/water (10:5:6 v/v/v) for 16 h.

BPW; butan-1-ol/pyridine/water (4:3:4 v/v/v) for 16 h

The position of a compound on a paper chromatogram is given as an Rf value, that is, the position of the compound relative to the solvent front.

$$Rf = \frac{\text{distance of compound from origin}}{\text{distance of solvent front from the origin.}}$$

The mobility of a particular compound can also be described relative to another known compound within a sample.

e.g. $$R_{Ara} = \frac{\text{distance moved by compound}}{\text{distance moved by arabinose}}$$

2.2.4 Paper electrophoresis.

Samples were spotted onto Whatman No.1 chromatography paper and separated by electrophoresis in borate buffer (1.9% $Na_2B_4O_7.10H_2O$ adjusted to pH 9.4) at 2000 V for 4 hours. At this pH, in the absence of borate, neutral sugars and alditols are generally immobile. However borate ions will

reversibly bind to pairs of adjacent hydroxyl groups and since such pairs occur in most neutral sugars and polysaccharides they will aquire a charge and thus electrophoretic mobility.

2.2.5 Staining of sugar and alditol standards.

Reducing sugars were stained using aniline hydrogen-phthalate - 1.6% phthalic acid dissolved in acetone:diethyl ether:water 49:49:2 v/v/v added to aniline (0.5% v/v) immediately before use. Papers were dipped through the stain, allowed to dry for a few minutes and then heated at 105°C for 5 minutes. Samples are visible as: uronic acids - orange, pentoses - red, hexoses - brown.

Sugars and alditols that had been run in solvents containing borate were stained by dipping through a solution of 0.7% $AgNO_3$ in acetone, allowing to dry and then spraying with 2% NaOH and 4% pentaerythritol in 80% ethanol. Samples were visible as dark brown/orange spots on a light brown background.

2.2.6 Detection of radiolabel.

Aqueous radioactive samples were mixed with 10 volumes of 'Triton scintillant' consisting of toluene/Triton X-100 (2:1 v/v) containing 0.33% w/v PPO (2,5 - diphenyloxazole) and 0.033% w/v POPOP (1,4 bis-(5-phenyloxazole-2-yl)benzene)). Quench curves were constructed using [^{3}H]- and [^{14}C]toluene as radioactive standards, progressively quenched by the addition of acetone (Smith 1992)

Non-aqueous samples (eg. strips of paper chromatograms) were soaked in a toluene scintillation cocktail (0.5% w/v PPO, 0.05% w/v POPOP in toluene). In some cases the counting efficiency for ^{3}H was improved by

soaking the paper strips in 0.5 ml water before addition of 5 ml 'Triton scintillant'.

Samples in organic solvents (eg. toluene and ethyl acetate from PAL and OMT assays) were added to 5 ml 'Optiphase Safe' ready prepared scintillation cocktail (LKB-Pharmacia).

2.2.7 Assay for reducing sugars (Lever 1972)

Reducing sugars were detected using the p-hydroxybenzoic acid hydrazide (PAHBAH) test. 0.25 ml aqueous samples were added to 0.75 ml of a solution consisting of 10 ml 5% p-hydroxybenzoic acid hydrazide in 0.5 M HCl, freshly mixed with 40 ml 0.5 M NaOH. Samples were heated in a boiling water bath for 5 minutes, cooled and the absorbtion at 410 nm was measured.

2.2.8 Custom synthesis of L-[1-^{3}H]arabinose.

A pure authentic sample of L-arabinose was custom tritiated by Amersham International Plc. using the T-7 technique. This uses $^3H_2$ and a catalyst to replace the reducing terminus hydrogen with tritium. The specific activity of the [^{3}H]arabinose was 92 TBq/mmol.

2.3 Experiments to determine the sub-cellular site of feruloylation.

In vivo radiolabelling of maize and fescue cell cultures.

Early experiments (not reported) showed that maize cell cultures do not incorporate exogenously supplied arabinose into nascent polysaccharides in sufficient amounts to enable the desired experiments to be conducted.

Therefore maize was supplied with radiolabelled glucose and fescue cultures were used in arabinose feeding experiments.

2.3.1 Kinetics of feruloylation using [^{14}C]glucose-fed maize cell cultures.

Maize cultures were incubated in a 1% glucose:1% glycerol medium (see appendix) for two weeks before being subcultured into a 1% glycerol medium to starve the cells of glucose. Cultures were then incubated for 24 hours in this fresh medium under standard conditions. Aliquots of culture (10 ml) were adjusted to a settled cell volume of 20%, allowed to incubate for a further two hours and then supplied with D-[U-^{14}C]glucose (final concentration in culture, 50 kBq ml^{-1}, >8.5 GBq/mmol, Amersham International Ltd.). Samples (750 µl) were removed at 3 minute intervals for 30 min and thereafter every 15 mins. During this time cultures were rotated on a bench top shaker.

Samples were placed in 'Polyprep' columns (Biorad, Richmond, CA), rapidly filtered and killed by the addition of 5 ml ethanol. An aliquot (30µl) of spent medium from each time point was chromatographed on paper in BAW and the material remaining at the origin asssayed for radioactivity. This was to monitor the incorporation of radiolabel into soluble extracellular polymers.

Cell samples were rinsed in 80% ethanol until the radioactivity in the washings had stabilized at a constant low level (200-600 cpm per ml) and the alcohol insoluble residue (AIR) produced was air dried.

2.3.2 The kinetics of feruloylation in *Festuca* cell cultures

Fescue cell cultures were treated in essentially the same way as maize cultures, except that 2 and 5 day old cultures were supplied with L-[1-^{3}H]arabinose (to a final concentration in culture of 2 MBq/ml, 92 TBq/mmol). 30µl of spent medium was chromatographed in BAW and the material

remaining at the origin assayed for radioactivity. Alcohol insoluble residues of fescue samples were produced in the same way as for maize.

2.3.3 Analysis of alcohol insoluble residues.

2.3.3.1 Analysis of alcohol insoluble residue derived from maize cultures.

The maize AIR was incubated with Driselase (section 2.2.1) and solubilised products were passed through a C18 solid phase separation column ('BondElut', Varian, Analytichem, Harbour City, CA). The column was washed with 3 aliquots of 1 ml H_2O and the retained (aromatic) solutes eluted with 3 aliquots of 1 ml 50% MeOH. The eluates were combined to make two fractions (water fraction and methanol fraction) which were dried *in vacuo* and then chromatographed in BAW. Feruloylated compounds in the methanolic fraction were identified by their turquoise fluorescence under 366 nm UV light (Fry 1982). The aqueous fraction was further chromatographed in BPW, and all chromatograms were cut into 1 cm strips and assayed for radioactivity.

After scintillation counting, scintillant was removed by washing in toluene and low molecular weight feruloylated products were eluted from the paper strips and dried in two halves. One half was treated with 0.5 M NaOH for 1 h at 24°C then neutralised with HOAc to yield the de-feruloylated sugar moiety whilst the other half was retained as a control. Both halves were re-chromatographed in BAW and chromatograms assayed for radioactivity. The radiolabelled sugar moiety released after saponification of the feruloylated compound was re-eluted, dried *in vacuo* and treated with 2 M TFA for 1 hr at 120°C to cleave glycosidic bonds. The hydrolysed product was chromatographed in EPW for 24 hours and the chromatogram assayed for radioactivity.

2.3.3.2 Analysis of alcohol insoluble residue derived from fescue

The fescue AIR was treated in 2 ways. Initially driselase digestion was employed but in later experiments the AIR was hydrolysed by 0.1M TFA. The AIR was digested with driselase (section 2.2.1) and the solubilized products were passed through a C18 SPEC and chromatographed as above (section 2.3.3.1)

Solublised products of TFA hydrolysis were spotted directly onto paper chromatograms and developed in BAW; feruloylated products were identified by fluorescence and the chromatograms cut into 1 cm strips and assayed for radioactivity. Low molecular weight feruloylated products from later time points were eluted from the paper in 50% MeOH and subjected to saponification as described above (section 2.3.3.1).

2.3.4 Partial identification of the products of mild acid hydrolysis of fescue AIR. (with G. Wende, University of Edinburgh).

2.3.4.1 Bulk preparation of products.

Treatment of fescue AIR with 0.1 M TFA yielded two fluorescent low molecular weight products. In an attempt to characterise these compounds, larger preparations were made by supplying fescue cultures (250 ml 14% settled cell volume) with 47 MBq L-[1-^{3}H]arabinose under standard conditions for 5 hours. The cells were then percolated overnight with 2 litres of 80% ethanol to produce an alcohol insoluble residue (yield 220 mg).

One portion (40 mg) was treated with 2 ml 0.1 M TFA at 100°C for 1 h and the cooled hydrolysate subjected to preparative p.c. in BAW. Compounds A and B were identified and prepared as previously (section 2.3.3.2) and further purified by p.c. in BEW. They were eluted, dried and subjected to

alkaline hydrolysis to yield the de-feruloylated sugar moieties A_S and B_S respectively. A_S and B_S were rechromatographed in BAW followed by EPW (16 hours each).

2.3.4.2 Acid hydrolysis of B_S.

A sample of B_S was subjected to hydrolysis in 2 M TFA, 1 h at 120°C, and the resulting monosaccharides were separated by p.c. in EPW for 24 hours.

2.3.4.3 Gel permeation chromatography of B_S.

In addition a portion of B_S was passed through a column of Bio-Gel P-2 (in HOAc/Py/H_2O 1:1:98 by vol., pH $\approx$ 4.7) along with non-radioactive markers (2 mg each of xylose, xylobiose, glucose, maltose, maltotriose). The column eluate was collected and an aliquot of each fraction assayed for radioactivity. A further portion of each fraction was chromatographed on paper in BAW and marker sugars were located by staining with aniline hydrogen phthalate (section 2.2.5).

2.3.4.4. Reduction of compounds A_S and B_S with sodium borohydride.

Dried samples of B_S and A_S were dissolved in 200µl 0.5 M sodium borohydride (in 1 M ammonia soln) and incubated at 25°C for 4 h. This treatment converts reducing termini to the corresponding alditol. Acetic acid (30µl) was added and the sample applied to a column of Dowex 50 (bed volume 1.5 ml) previously washed in 1 M HCl and equilibrated with H_2O. The sample was eluted from the column in 3 ml water and then dried *in vacuo*. The reduced material was repeatedly dried from MeOH:HOAc (9:1 v/v) to remove boric acid as its volatile methyl ester, and subjected to 2 M TFA acid hydrolysis (section 2.2.2)

2.3.4.5 Chromatography of the products of borohydride reduction.

The cooled hydrolysates of reduced A_S and B_S were chromatographed in EPW to separate xylose from a lower Rf zone containing arabinose, xylitol and arabinitol. Xylose (identified by comparison to authentic markers) was assayed for radioactivity and components from the second zone were eluted, dried *in vacuo* and analysed by paper electrophoresis in borate buffer (section 2.2.4). Sugars and alditols were identified by comparison with authentic external markers and assayed for radioactivity.

2.3.4.6 Determination of specific activity of [^{3}H]arabinose and [^{3}H]xylose residues of fescue AIR.

In order to determine the specific activity of the [^{3}H]arabinose and [^{3}H]xylose residues in the AIR from which compounds A and B had been derived, a further portion (110 mg) of bulk AIR was subjected to hydrolysis in 2 M TFA (section 2.2.2) and the products were separated by p.c. in EPW for 24 h (section 2.2.3). The zones co-migrating with external marker arabinose and xylose were eluted in water and assayed (i) by scintillation counting in Triton scintillant (section 2.2.6), and (ii) for reducing sugars by the p-hydroxybenzoic acid hydrazide (PAHBAH) test (Lever 1972), using L-arabinose and D-xylose dried in a vacuum desiccator as standards.

2.4. Experiments to investigate putative feruloyltransferase activity in the endomembranes of maize.

2.4.1 Preparation and purification of [methoxy-^{14}C]feruloyl-CoA.

Ferulic acid specifically labelled in the methoxyl group was manufactured at ICI Radiochemicals Lab., Jealott's Hill Research Station, Bracknell, Berks. The radiolabelled ferulic acid was esterified to Co-enzyme A via a hydroxysuccinimide ester intermediate (for method see Stöckigt and Zenk 1967). This preparation was stored at -80°C at pH<7. Ferulic acid (≈15% of radiolabel) was present as a contaminant and the feruloyl-CoA was purified before use by p.c. in BAW. Feruloyl-CoA was identified by its fluorescence, UV absorption spectrum and radiolabel and eluted from the paper in H_2O. Specific activity was 2.11 MBq/mmol.

Non radiolabelled feruloyl-CoA was synthesised at Edinburgh using the same technique and identified by fluorescence and UV spectrum. NMR analysis strongly supported its identity (see appendix).

2.4.2 Preparation of endomembranes for enzyme assays.

Routinely, 3-4 day old cell cultures were filtered under reduced pressure in a Büchner funnel onto Whatman wet strengthened filter paper and rinsed in 400 ml ice cold 10 mM 3-[N-morpholino]propane sulphonic acid (MOPS), 1 mM dithioithreitol (DTT), pH 7.2. Cells (ca. 40 g) were homogenized in a teflon Potter type blender on ice for 5 minutes at 300 rpm in 50 mM MOPS, 1 mM DTT, 1 mM ethylenediamine tetra-acetic acid (EDTA), 0.4M sucrose, 0.5% bovine serum albumin (BSA), pH 7.2. The homogenate was filtered through 2 layers of muslin and then centrifuged at 20,000 g, 4°C for 15 min (12,000 rpm, Sorvall SS34 fixed angle rotor). The

supernatant was removed and re-centrifuged at 100,000 g, 4°C for 25 min (27,000 rpm in an AH629 swing out rotor (Sorvall Instruments)).

The supernatant was discarded and the pellet resuspended in a clean centrifuge tube in buffer as above except lacking EDTA and BSA. The washed microsomal fraction was recentrifuged at 100,000g, 25 min. The repelleted microsomes were routinely resuspended in 50 mM MOPS, 1 mM DTT, pH 7 and this suspension was used as a particulate membrane preparation in feruloyltransferase assays.

Endomembranes were used on the day of preparation. Protein contents, where shown, were determined by an adapted Lowry assay (after Peterson 1977).

2.4.3 Standard assay procedure.

Aliquots (75-100 μl) of endomembrane preparations were routinely supplied with [methoxy-^{14}C]feruloyl-CoA and incubated at room temperature (18-22°C). Assays were started by the addition of the membranes to other constituents of the assay and terminated by the addition of 10μl formic acid. The incorporation of radiolabel from [methoxy-^{14}C]feruloyl-CoA into polymeric material was monitored by the increase in radiolabel with zero mobility on p.c. in BAW, or that material that remained adsorbed to Whatman 3MM filter paper after 4 hours of washing in running water.

2.4.3.1 The effect of addition of arabinoxylan to the homogenization medium on the incorporation of radiolabel from [methoxy-^{14}C]feruloyl-CoA into polymeric material.

15 g washed maize cells were homogenized in buffer as above with or without the addition of 10 mg ml^{-1} arabinoxylan (prepared from wheat flour, Megazyme Inc., Australia). Membrane preparations produced were

resuspended in 50 mM MOPS, 1 mM DTT at pH 7. Assays were conducted using 0.5 kBq [methoxy-^{14}C]feruloyl-CoA, 5 mM $CoCl_2$, 100 µl membrane preparation in a total volume of 120 µl. After 15 minutes incubation assay mixtures were streaked onto Whatman 3MM chromatography paper and developed in EAW for 16 hr, and the origins assayed for radioactivity.

2.4.3.2 The effect of exogenously supplied divalent cations on the incorporation of radiolabel from [methoxy-^{14}C]feruloyl-CoA into polymeric material.

Endomembranes were prepared as above (section 2.4.2) and assayed using 0.5 kBq [methoxy-^{14}C]feruloyl-CoA, 70µl membrane preparation, with a range of concentrations (1-10 mM) of $CoCl_2$, $MnCl_2$, $CaCl_2$, $MgCl_2$, $ZnSO_4$, $ZnCl_2$. 1 mM EDTA was added to some assays as a negative control. After 15 minutes incubation assays were terminated by the addition of 10 µl formic acid, assay mixtures spotted onto 3 by 4 cm strips of Whatman 3MM paper and washed in running water for 4 hours. Papers were dried and assayed for radioactivity.

2.4.3.3 Changes in the incorporation of radiolabel from [methoxy-^{14}C]feruloyl-CoA into polymeric material by membranes from cultures of different ages.

Membranes extracted from cell cultures 24-192 h after sub-culturing were assayed for their ability to transfer radiolabel from [methoxy-^{14}C]feruloyl-CoA into polymeric material. Endomembranes were prepared as described (section 2.4.2)and assayed using 0.5 kBq [methoxy-^{14}C]feruloyl-CoA, 5 mM $CoCl_2$, 50 µl membrane preparation in a total volume of 70 µl per assay. Assays were terminated by the addition of 10 µl formic acid and the

incorporation of radiolabel into polymeric material was monitored as described.

2.4.3.4 Incorporation of radiolabel from [methoxy-^{14}C]feruloyl-CoA into polymeric material at different temperatures.

Endomembranes were prepared as described (section 2.4.2). Assays were conducted at different temperature using 0.5 kBq [methoxy-^{14}C]feruloyl-CoA, 5 mM $CoCl_2$, 50 µl membrane preparation per assay in a total volume of 70 µl. Another aliquot of the membrane preparation was boiled for 10 minutes before being assayed at 22°C. Assays were terminated by the addition of 10 µl formic acid, dried onto Whatman 3MM paper and treated as described.

2.4.3.5. Incorporation of radiolabel from [methoxy-^{14}C]feruloyl-CoA into polymeric material at different pH.

Endomembranes were prepared as before except that washed membranes were divided into 4 aliquots before final centrifugation at 100,000 g. The membrane pellets were resuspended in 0.2M BIS-TRIS (bis[2-hydroxyethyl]imino-tris[hydroxymethyl]methane), 0.2 M TAPS (N-tris[hydroxymethyl]methyl-3-amino propane sulfonic acid), 0.4 M Acetate (1:1:1, v/v/v) adjusted to various pHs by addition of 10 M KOH. This buffer was used to ensure that the ionic strength of the buffer remained constant regardless of pH to ensure that any possible effects of changing ionic strength were minimised. Membrane preparations were assayed as described above, spotted onto Whatman 3MM, washed for 4 hours, dried and assayed for radioactivity.

2.4.3.6 The effect of catalase on the transfer of radiolabel from [methoxy-^{14}C]feruloyl-CoA and [methoxy-^{14}C]ferulic acid into polymeric material.

There have been reports of peroxidases being associated with endomembranes (Penel 1991), and membrane preparations can polymerize free phenolic acids, presumably through oxidation by peroxidase (Rogers *et al.* 1993, Gröger *et al.* 1993). In order to ascertain whether there was any feruloyl-CoA-oxidising peroxidase activity associated with endomembrane preparations from maize cultures several experiments were conducted using inhibitors of peroxidase activity.

Catalase will degrade H_2O_2 which is required as an electron acceptor for peroxidase and therefore its addition to membrane preparations should minimise peroxidase activity. Membranes were prepared as described and re-suspended in 50 mM MOPS, 1 mM DTT, pH 7.2. Assays contained 75 µl membrane preparation, 2 mM $CoCl_2$, and were conducted with or without 55 units of catalase (where 1 unit will decompose 1.0 µmol of H_2O_2 per minute at 25°C). The substrate provided was either 0.5 kBq [methoxy-^{14}C]feruloyl-CoA per assay or 0.5 kBq [methoxy-^{14}C]ferulic acid per assay. Total volume was 105 µl. Incubations (0-20 min) were terminated by the addition of 10 µl formic acid and the assay mixtures spotted onto Whatman 3MM chromatography paper, washed in running water for four hours, dried and scintillation counted.

2.4.3.7 Effect of sodium azide on the incorporation of radiolabel from [methoxy-^{14}C]feruloyl-CoA into polymeric material.

Sodium azide is reputed to be a specific inhibitor of haem enzymes at millimolar concentrations (Dawson *et al.* 1986) and as such should inhibit peroxidase activity. Endomembranes were prepared as described and

assays conducted using 0.5 kBq [methoxy-^{14}C]feruloyl-CoA, 5 mM $CoCl_2$, 10 mM NaN_3, 75µl membrane preparation in a total volume of 105 µl. Assay mixtures were spotted onto Whatman 3MM, washed for 4 hours, dried and assayed for radioactivity.

2.4.4. Analysis of the product of assays containing catalase.

To enable analysis of the radiolabelled product of these assays, long term assays (45 min) were conducted. Endomembranes were prepared as before and assayed with 2 mM $CoCl_2$, 2 kBq [methoxy-^{14}C]feruloyl-CoA, 300 µl membrane preparation and 100 units of catalase in a total volume of 400 µl. After 45 min polymeric material was precipitated by the addition of 2 ml 1 M HOAc:EtOH (3:2 v/v), and pelleted by microcentrifugation at 10,000g for 20 minutes. To remove unreacted feruloyl-CoA the pellet was further washed thrice in 1 M HOAc:EtOH (3:2 v/v) with the pellet being resuspended by sonication in each wash.

The polymeric material was then treated with 0.1 M TFA, 1 h, 100°C and authentic feruloyl-[^{3}H]arabinose was added to the hydrolysed product as an internal marker. The mixture was chromatographed on paper in several different solvent systems and the chromatograms were cut into 1 cm strips and scintillation counted.

2.4.5. Preliminary investigations into the peroxidase activity of membrane preparations.

2.4.5.1 Experiment to investigate the peroxidase activity of membrane preparations against guaiacol.

Guaiacol is often used as a substrate in peroxidase assays, being oxidised to a coloured product by peroxidase in the presence of H_2O_2.

Membranes were prepared as described and 100 μl of microsomal preparation was added to 4 mM guaiacol, 2 mM H_2O_2 in phosphate buffer at pH 7.5 to a total volume of 1 ml. The absorbance at 470 nm was monitored.

2.4.5.2 The effect of dithiothreitol on the peroxidase activity of membrane preparations.

The effect of dithiothreitol on the oxidation of guaiacol was investigated by the inclusion of 1 mM DTT into assay mixtures consisting of 100 μl membrane preparation, 4 mM guaiacol, 2 mM H_2O_2 in phosphate buffer at pH 7.5 (total volume 1 ml).

2.4.5.3. The activity of membrane preparations against [methoxy-^{14}C]feruloyl-CoA in the absence of DTT and cobalt ions.

In order to investigate the effect of cobalt and DTT on the ability of membranes to incorporate radiolabel from [methoxy-^{14}C]feruloyl-CoA into polymeric material, membranes were prepared as previously but re-suspended in 50 mM MOPS without the addition of DTT. Assays were conducted consisting of 75 μl membrane preparation and either (i) 0.5 kBq [methoxy-^{14}C]feruloyl-CoA, or (ii) 0.5 kBq [methoxy-^{14}C]feruloyl-CoA with the addition of 1 mM DTT, (iii) 0.5 kBq [methoxy-^{14}C]feruloyl-CoA with the addition of 1 mM DTT and 2 mM $CoCl_2$. Assays were terminated by the addition of 10 μl formic acid, spotted onto Whatman 3MM filter paper, washed for 4 hours in running water and then scintillation counted.

2.5. Assays of enzymes involved in lignification using maize internode tissue.

2.5.1. Extraction and preparation of enzymes from maize internode segments.

Individual segments of maize internode (Section 2.1.3) were ground to a powder in liquid nitrogen using a pestle and mortar, and further homogenised in 100 mM TRIS, pH 7.4 containing 1% polyvinylpyrrolidone and 15 mM 2-mercaptoethanol. The homogenate was filtered through 50 μm nylon mesh and centrifuged at 40,000g, 15 min to pellet cell wall debris. The supernatant was made to 70% ammonium sulphate saturation and left on ice for 1 hour.

Precipitated proteins were pelleted by centrifugation at 40,000 g for 15 mins and then resuspended in 1 ml 100 mM TRIS, pH 7.4. Samples were de-salted through Sephadex-G25 (ready prepared column, PD-10, NAP-5, Pharmacia Ltd., equilibrated before use with 100 mM TRIS) and eluted in 1 or 3.5 ml 100 mM TRIS. This protein solution was used as the source of soluble enzymes in the following assays and used on the day of preparation.

2.5.2. Assay for phenylalanine ammonia lyase (PAL) activity.

PAL activity was assayed using a method adapted from de Cunha (1987), using L-[^{14}C]phenylalanine as a substrate. Routinely assays consisted of 75 μl enzyme preparation,1 mM phenylalanine (containing 3 kBq [^{14}C]phenylalanine per assay) in 50 mM borate buffer pH 8.8 in a total volume of 300 μl. Assays were incubated at 30°C for 2 h and terminated by the addition of 100 μl 1M HCl containing 0.5 mM cinnamic acid. Time zero control assays were conducted by addition of acid before addition of enzyme preparation.

The assay mixture was partitioned against 600 μl toluene by vigorous vortexing and then microfuged for 2 min at c 10,000g. An aliquot (200μl) of the organic phase was removed and assayed for radiolabel.

2.5.3. Assay for O-methyl transferase (OMT) activity.

OMT was assayed radiometrically using S-adenosyl-L-[methyl-^{14}C]methionine (SAM) as a methyl donor (Fukada and Komamine 1982). Routinely assays consisted of 65μl enzyme preparation, 1 mM $MgCl_2$, 1 mM caffeic acid (added in 5 μl ethylene glycol monomethyl ether) 10 mM sodium ascorbate, 1 mM SAM (containing 3 kBq ^{14}C labelled SAM per assay), 50 mM phosphate buffer, pH 6.5, in a total volume of 300 μl. Reactions were started by the addition of the protein preparation and terminated by the addition of 100 μl 1M HCl containing 0.1 mM ferulic acid.

Assay mixtures were partitioned against 600 μl ethyl acetate, vortexed and microfuged for 2 min at c.10,000g, and an aliquot (200 μl) of the organic phase was assayed for radioactivity.

2.5.4 Assay for cinnamyl alcohol:NADP+ oxidoreductase (CAD)

In vivo CAD catalyses the conversion of coniferyl aldehyde into coniferyl alcohol, which is then transported to the wall for incorporation into lignin. This reaction is fully reversible and *in vitro* it is convenient to measure the reverse reaction since coniferyl aldehyde is yellow at alkaline pH, and thus its production can be easily determined spectrophotometrically by monitoring A_{400}.

Assays were conducted with 5 mM NADP+ (Boehringer Mannheim) 200 μM coniferyl alcohol (Sigma Chemical Co.) in TRIS buffer at pH 8.8. The enzyme preparation was added to the $NADP^+$ and left for a minute before addition of the coniferyl alcohol. This was to allow any minor $NADP^+$

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dependent reactions that may occur in a crude protein preparation to run to completion before monitoring CAD activity. Such reactions were recorded as a slight decrease in A_{400} until a constant absorption was reached, and a negligible reduction in NADP+ was measured (absorption at 410 nm). After addition of the coniferyl alcohol A_{400} was measured for several minutes (Perkin Elmer, Llambda 3B UV/VIS spectrophotometer) whilst the temperature was maintained at 30°C. The initial linear rate of reaction was determined by taking tangents to the trace recorded by a chart plotter.

2.5.5 Determination of Km of PAL for phenylalanine.

In order to calculate the Km for PAL, assays were conducted using different final phenylalanine concentrations from 10 -100 μM. Appropriate quantities of [^{14}C]phenylalanine were added to maintain the total specific activity of phenylalanine in the assay at 1 kBq nmol^{-1}. Assay conditions were as in section 2.5.2, except that 100 μl of enzyme preparation was used per assay (from 7 ml preparation derived from 20.7 g fresh tissue) in a total volume of 0.5 ml. Initial reaction velocities were calculated.

2.5.6. Determination of Km of OMT for SAM.

The Km for OMT was determined by conducting assays (section 2.5.3) at concentrations of S-adenosyl-methionine from 10 -100 μM. Radiolabelled SAM was added in quantities to ensure that the specific activity of total SAM was maintained at 0.1 kBq nmol^{-1}. The enzyme preparation used was taken from 7 ml derived from 20.7 g fresh weight tissue.

2.5.7 Determination of Km of CAD for coniferyl alcohol.

Assays were conducted as described above (section 2.5.4) using a range of coniferyl alcohol concentrations. A_{400} was monitored for several

minutes and the initial reaction velocities were calculated by taking tangents to the trace on a chart plotter. The enzyme preparation used was taken from 7 ml which was derived from 20.7 g fw.

2.5.8. Experiment to ascertain linearity of PAL reaction with respect to time.

Assays were conducted as above (section 2.5.2) but allowed to incubate for up to 4 hours, with samples taken at 30 min, 1, 2, 3 and 4 hours.

2.5.9 Rate of conversion of phenylalanine by PAL with respect to increasing protein concentration

The activity of PAL was measured with respect to protein concentration. Assays were conducted as described (section 2.5.2) with increasing amounts of enzyme preparation. The protein concentration of the enzyme extract was measured by the Bio-Rad Bradford protein assay.

2.5.10. Rate of conversion of SAM by OMT with respect to increasing protein content.

Assays of OMT activity were conducted as described (section 2.5.3) with increasing amounts of enzyme preparation. The protein content of the enzyme preparation was measured using the Bio-Rad Bradford protein assay.

CHAPTER 3
RESULTS OF *IN VIVO* RADIOLABELLING EXPERIMENTS

THE KINETICS OF FERULOYLATION IN CELL CULTURES.

Experiments were conducted using radiolabel feeding techniques to ascertain the sub-cellular site of feruloylation, i.e. at what point in its career a cell wall polysaccharide gains its feruloyl groups. The supply of a radiolabelled precursor of polysaccharide sugar residues to growing cells enables us to follow the uptake, incorporation and feruloylation of a sugar moiety and thus consider the kinetics of this process.

3.1 *In vivo* radiolabelling of maize cultures with [U-^{14}C]glucose.

Alcohol insoluble residues (AIR) were prepared from cells supplied with [^{14}C]glucose over a period of time (0-90 minutes) and these AIRs were treated with Driselase to allow analysis of individual cell wall components. Driselase (Sigma Chemical Co.) is a fungal enzyme preparation from *Irpex lacteus*. It is a mixture of endo- and exo-polysaccharidases that effectively digest cell walls, releasing a mixture of mono- and di-saccharides. However, it lacks feruloyl esterase activity and will release characteristic feruloylated di- and trisaccharides from many cell wall preparations. These fragments are well characterised - monocots yield feruloyl-arabinosyl-xylose and feruloyl-arabinosyl-xylosyl-xylose (Kato and Nevins 1985), and there have been reports of the release of a feruloylated disaccharide of xyloglucan from bamboo (Ishii and Hiroi 1991).

The Driselase digestible material was separated by reverse phase chromatography into a polar (eluted with water) and non-polar (eluted with methanol) fraction, which were expected to contain simple sugars and feruloylated material respectively. The incorporation of radiolabel from the glucose into various cell wall components in each fraction was monitored.

[^{14}C]Glucose was incorporated into driselase digestible polymers (data not shown) and polymer bound arabinose residues began to accumulate radiolabel after a short lag period of ≈2.5 min (Fig 3.1.1.a). In the methanolic fraction an unknown compound (compound GS, Rf≈0.55), co-chromatographing with authentic feruloyl-arabinosyl-xylose (a product of driselase digestion of graminaceous cell walls), began to incorporate radiolabel after a lag of about 4 minutes (fig 3.1.1b).

Compound GS was eluted from the paper chromatograph and samples from individual time points were pooled and treated with 0.5 M NaOH to saponify esters. This treatment resulted in ≈20% of the radiolabel appearing in a compound with an Rf 0.3, consistent with it being a disaccharide (Fig 3.1.2). Acid hydrolysis (2 M TFA, section 2.2.2)resulted in the release of approximately equal quantities (on a ^{14}C basis) of radiolabelled arabinose and xylose and a trace of glucose (data not shown).

In cell cultures, non-cellulosic polysaccharides can fail to be retained by the wall and appear in the culture medium (Fry 1987, Bacic *et al.* 1987), The incorporation of radiolabel into these extracellular polysaccharides was determined by monitoring the incorporation of radiolabel into material remaining at the origin on p.c. in B.A.W. (section 2.2.3). There was a lag period of approximately 18-20 minutes before extracellular polysaccharides began to accumulate radiolabel, followed by rapid incorporation over the subsequent 60 minutes (fig 3.1.3). This would indicate that new polysaccharides are synthesised and secreted within an estimated period of 20 minutes.

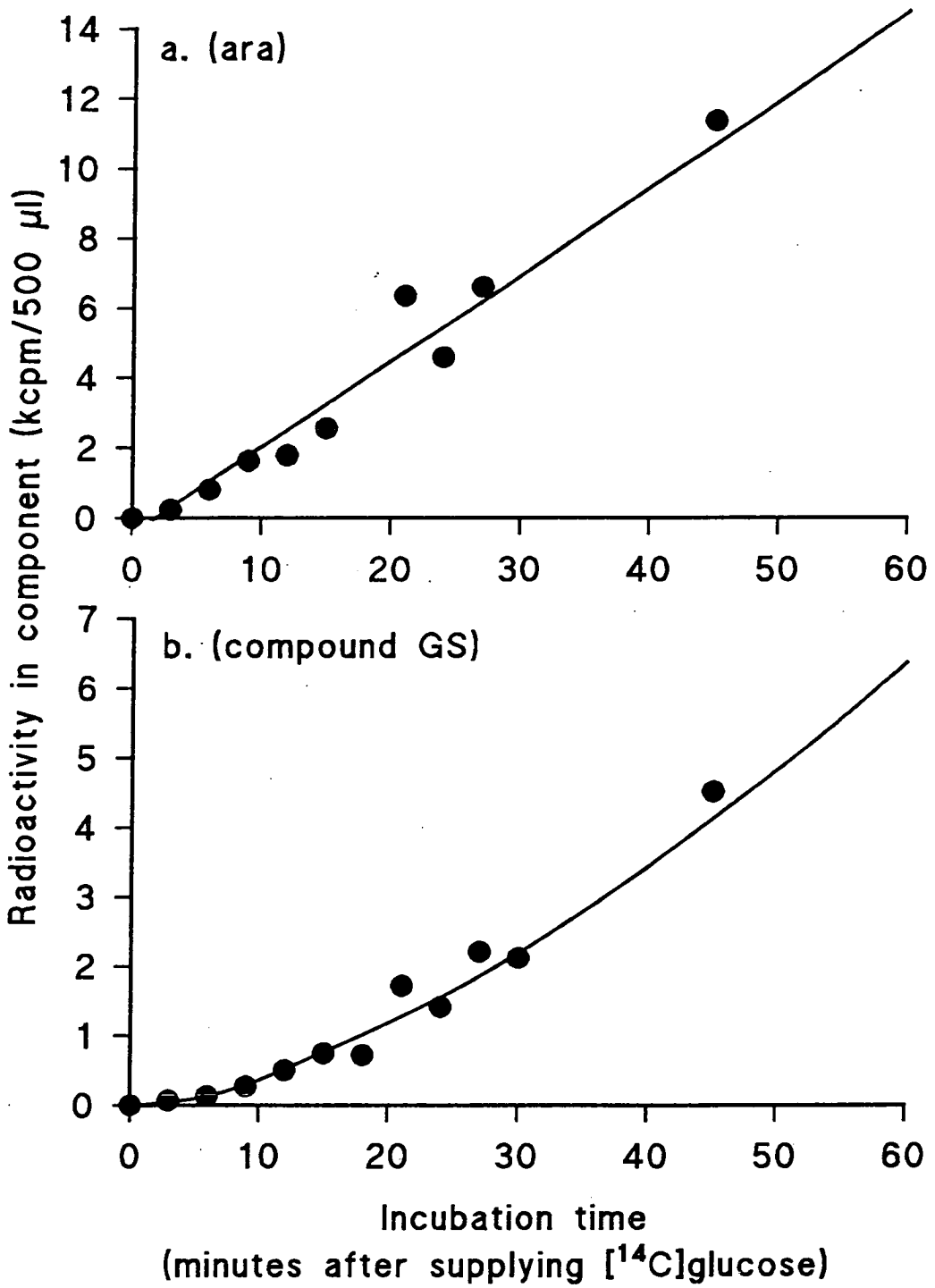


Fig 3.1.1 The incorporation of radiolabel from exogenously supplied [U-^{14}C]glucose into (a) polymer bound arabinose residues and (b) polymer bound compound GS by maize cell cultures. Data are for 750 µl of culture.

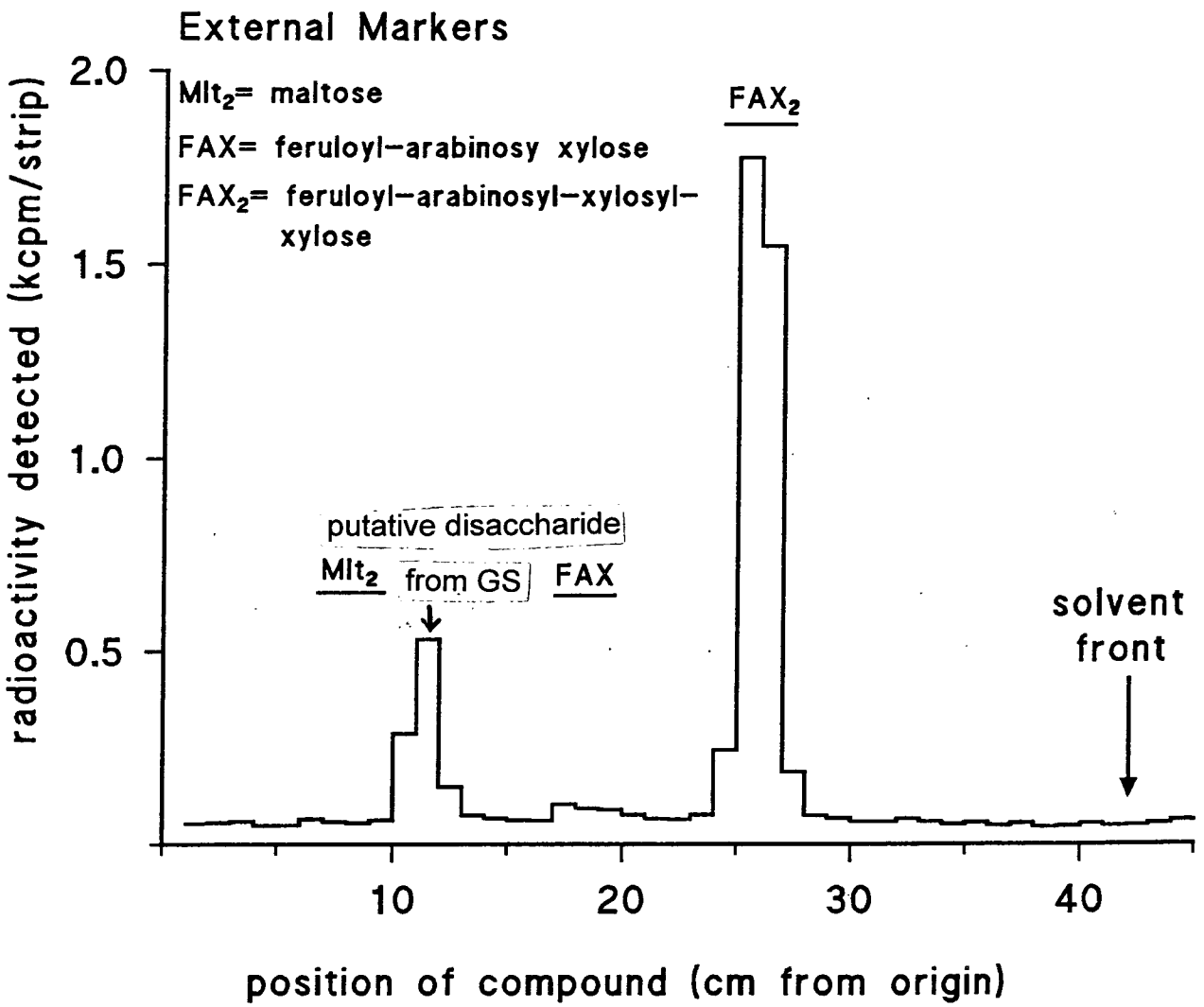


Fig 3.1.2 Saponification of compound GS. Compound GS, released by Driselase digestion of maize cell walls and retained by a C-18 solid phase extraction cartridge in water, was treated with 0.5 M NaOH. Chromatography of the products in BAW resulted in approximately 20% of the radiolabel appearing in a compound with an Rf consistent with that of a disaccharide.

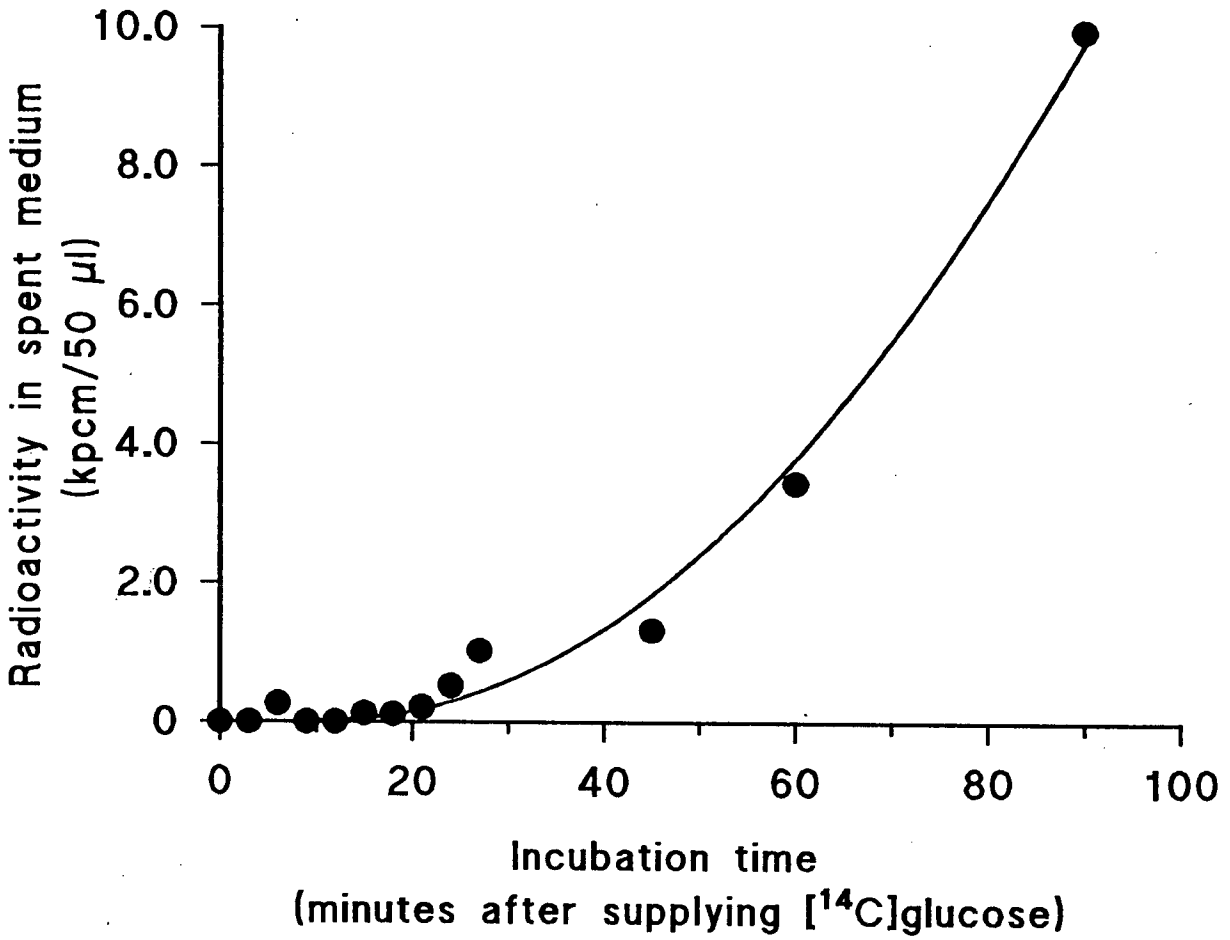


Fig 3.1.3 The appearance of radiolabelled extracellular polymers in the culture medium of maize cell cultures supplied with [U-^{14}C]glucose. Data are for material with Rf 0.0 on paper chromatography in BAW per 50 µl of spent medium.

3.2 *In vivo* radiolabelling of fescue cell cultures with [^{3}H]arabinose.

3.2.1. Driselase digestion of fescue alcohol insoluble residues.

Radiolabelled fescue cell wall alcohol insoluble residue was produced by supplying cell cultures with [^{3}H]arabinose. Initially this AIR was treated with Driselase (section 2.2.1) which is expected to release highly characteristic feruloylated fragments from monocot cell walls. Radiolabel was rapidly incorporated into arabinose residues identified following this treatment (fig. 3.2.1.1). However, on chromatography of the methanolic fraction (that material that is retained by a C18 SPEC in H_2O), it became apparent that very little low molecular weight (high Rf), feruloylated (fluorescent) material had been released by Driselase digestion and the majority of fluorescent material remained streaked within 3 cm of the origin.

Further digestion tests using non-radioactive fescue AIR and freshly purified Driselase showed that this phenomenon was not an artifact and that for some reason Driselase is ineffective at releasing low Mr feruloylated material from fescue cell walls. However, the fluorescent hydrophobic material that remained at the origin began to incorporate radiolabel very rapidly after a short lag (fig 3.2.1.2), indicating that radiolabelled arabinose and/or xylose residues were becoming linked to a non-polar moiety (possibly ferulate) shortly after their incorporation into nascent polysaccharides.

3.2.2 Treatment of Fescue AIR with 0.1 M TFA.

In subsequent experiments AIR produced in the time course feeding of fescue cultures were subjected to mild acid hydrolysis (Section 2.2.2). This treatment would be expected to break furanose bonds thus releasing, from a graminaceous monocot, arabinose and the short oligosaccharide chains previously attached to polysaccharides through arabinofuranose bonds and their feruloylated derivatives.

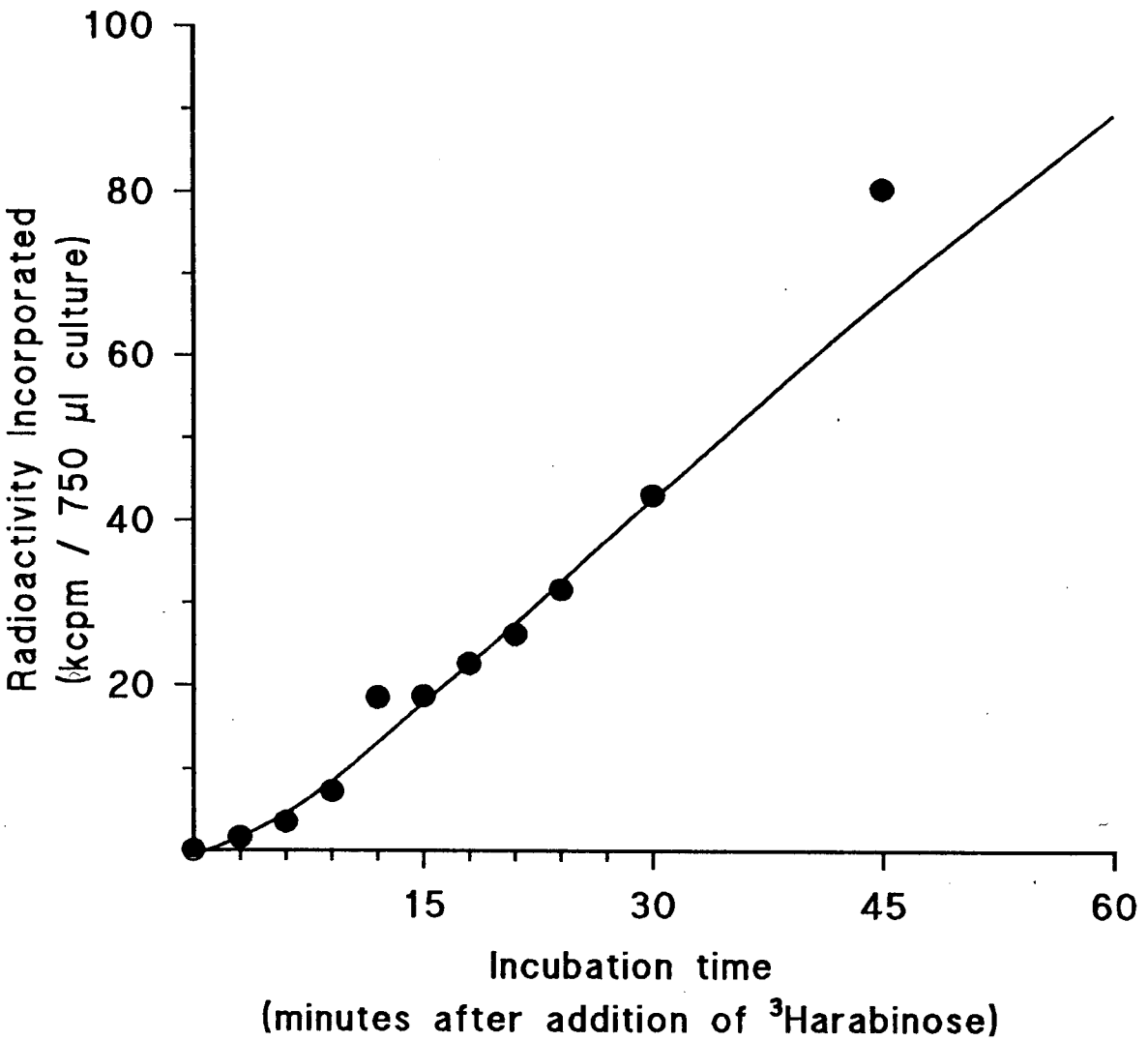


Fig.3.2.1.1 The incorporation of radiolabel from exogenously supplied [1-^{3}H]arabinose into polymer bound arabinose residues released from fescue cell walls by Driselase digestion. Data are for 750 µl of cell culture and each point represents the mean of duplicate experiments.

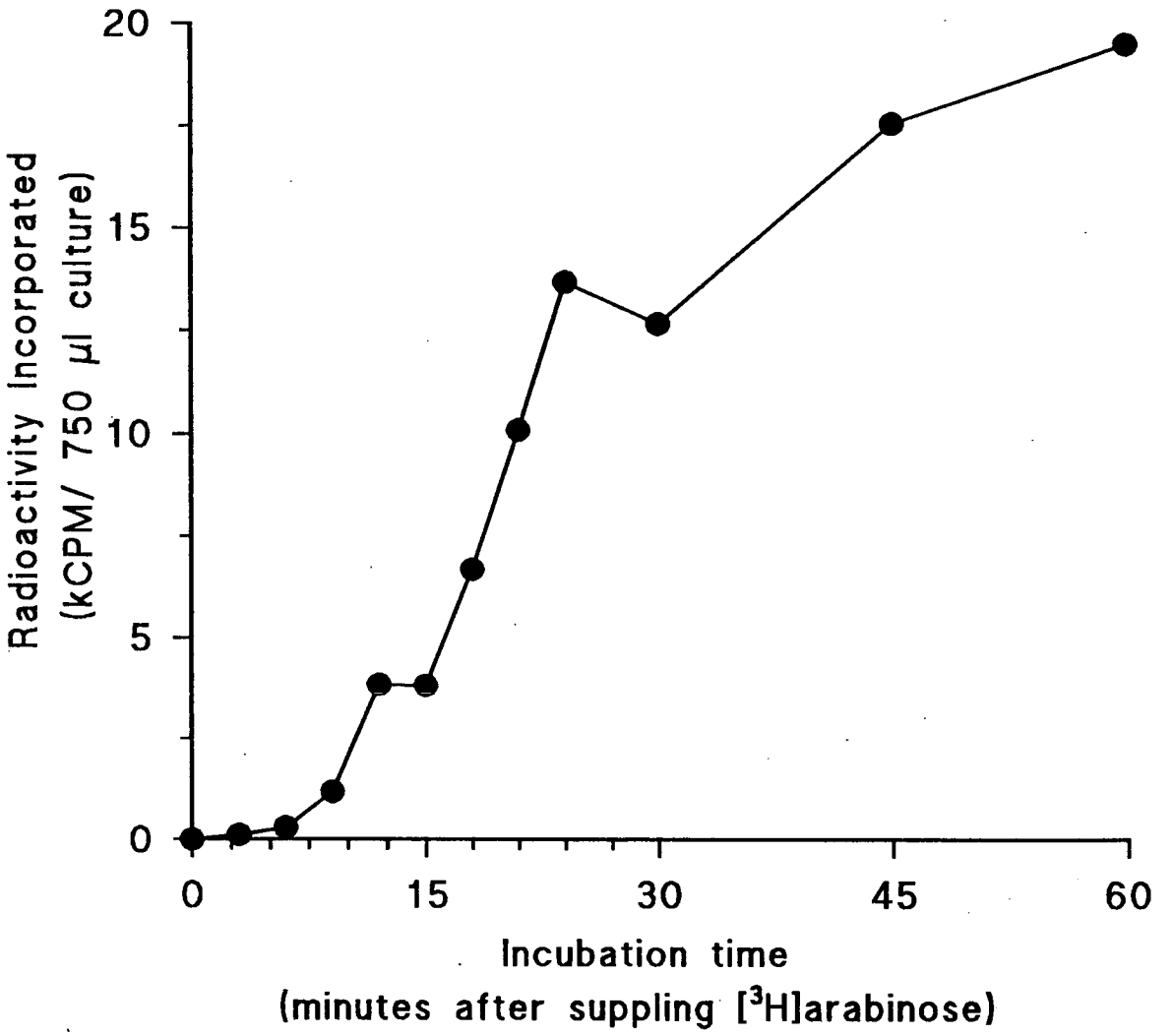


Fig. 3.2.1.2 Incorporation of radiolabel from exogenously supplied [1-^{3}H]arabinose into hydrophobic high molecular weight fluorescent material by fescue cell cultures. Data for the origin and first 2 cm of the paper chromatogram are shown and are for 750 µl cell culture. Each point represents the mean of duplicate experiments.

3.2.2.1 Incorporation of ^{3}H into soluble extracellular polysaccharides.

Radiolabel incorporated into soluble extracellular polysaccharides is that radiolabel from the spent culture medium that remained at the origin on p.c. in BAW. This fraction began to accumulate radioactivity after a lag of ≈20 minutes in 5 day old cultures and ≈30 minutes in 2 day old cultures (fig 3.2.2.1)

3.2.2.2 Incorporation of ^{3}H into cell wall components.

On treatment with 0.1 M TFA the ^{3}H labelled AIR yielded arabinose and two blue fluorescent spots (Rf 0.75 and Rf 0.64, feruloylated compound A and B respectively). Negligible xylose was detected.

Polysaccharide bound arabinofuranose residues began to incorporate radiolabel after a very short lag of ≈2 minutes in two day old cultures and a lag of ≈2.5 minutes in 5 day old cultures, indicating a rapid uptake and incorporation of [^{3}H]arabinose into pentose residues (Fig. 3.2.2.2 a and fig. 3.2.2.3 a). Incorporation proceeded in an approximately linear fashion over the time course of both experiments.

Compounds A and B displayed blue fluorescence under 366 nm UV light, becoming more intense and blue green when fumed with ammonia which is characteristic of feruloyl esters. Compound A had an Rf value in BAW of 0.75 and compound B was slightly slower with Rf 0.64.

Radiolabel was incorporated into compounds A and B after a short lag of ca. 5 minutes. (figs 3.2.2.2 b, fig 3.2.2.3 b, fig 3.2.2.4 a and b). These data show that within 5 minutes of being supplied to cell cultures [^{3}H]arabinose had been taken into the cell, had been incorporated into polymeric material and was beginning to be feruloylated.

Saponification of compounds A and B from samples taken during the time course resulted in most radiolabel appearing in compounds with Rf

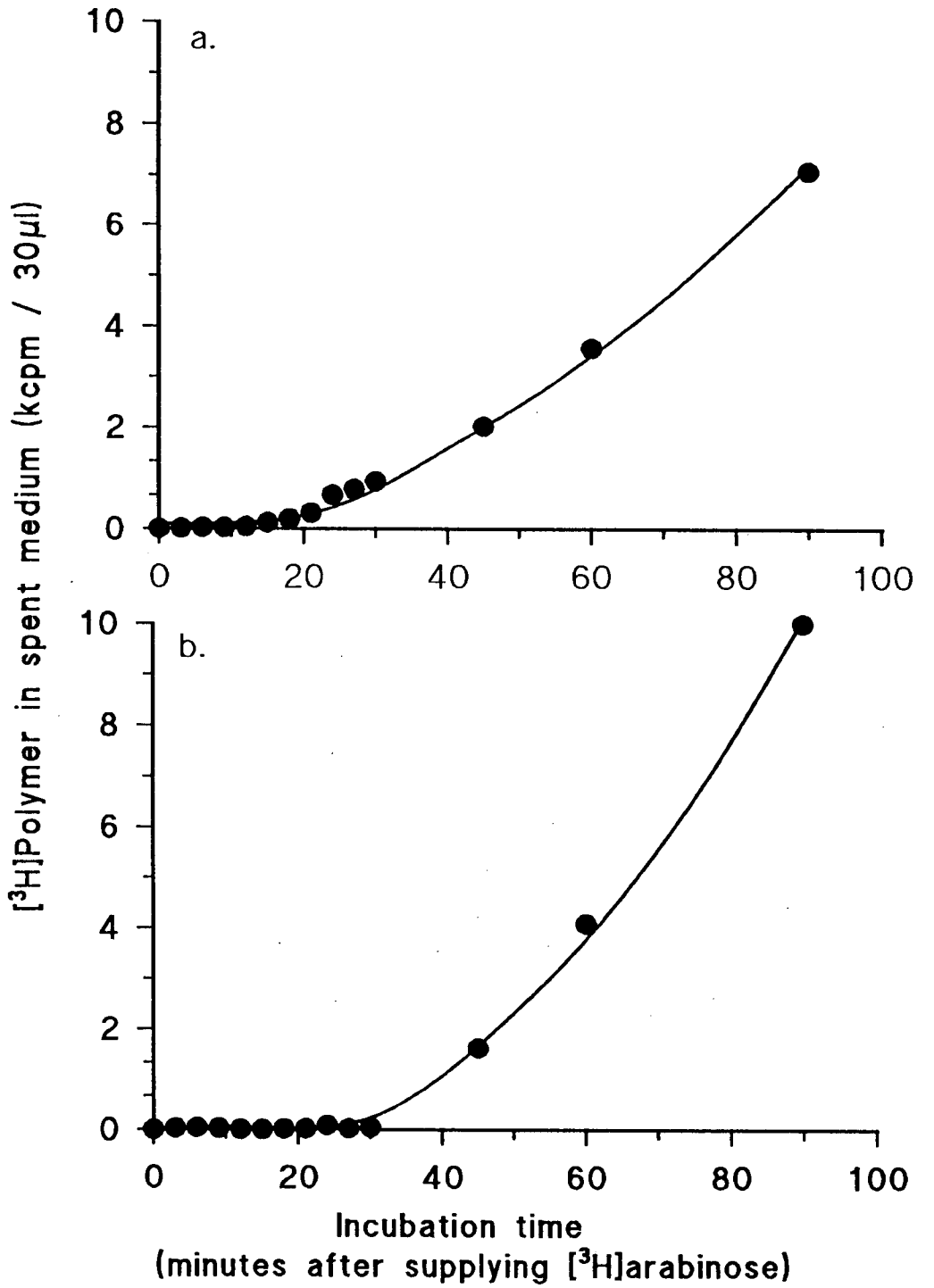


Fig. 3.2.2.1 The appearance of radiolabelled extracellular polymers in the culture medium of fescue cell cultures supplied with [1-^{3}H]arabinose. Data are for (a) 5 day old cultures, (b) 2 day old cultures and represent material with Rf 0.0 on paper chromatography in BAW for 30 µl of spent medium.

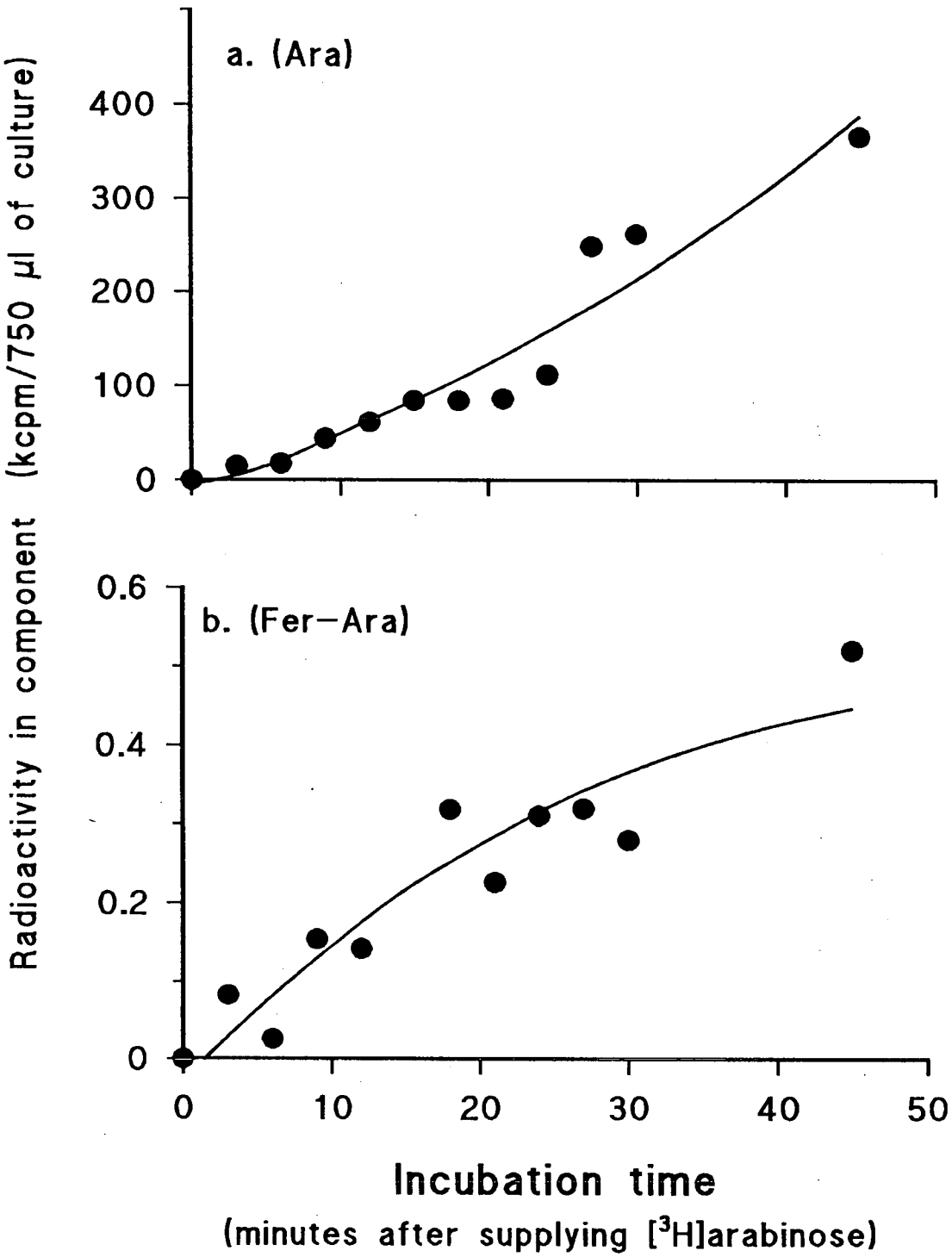


Fig 3.2.2.2. 2 day old fescue cultures. Incorporation of radiolabel from exogenously supplied [1-^{3}H]arabinose into a) polymer-bound arabinofuranosyl residues and b) polymer bound feruloyl-arabinose. Each point represents the mean of duplicate experiments

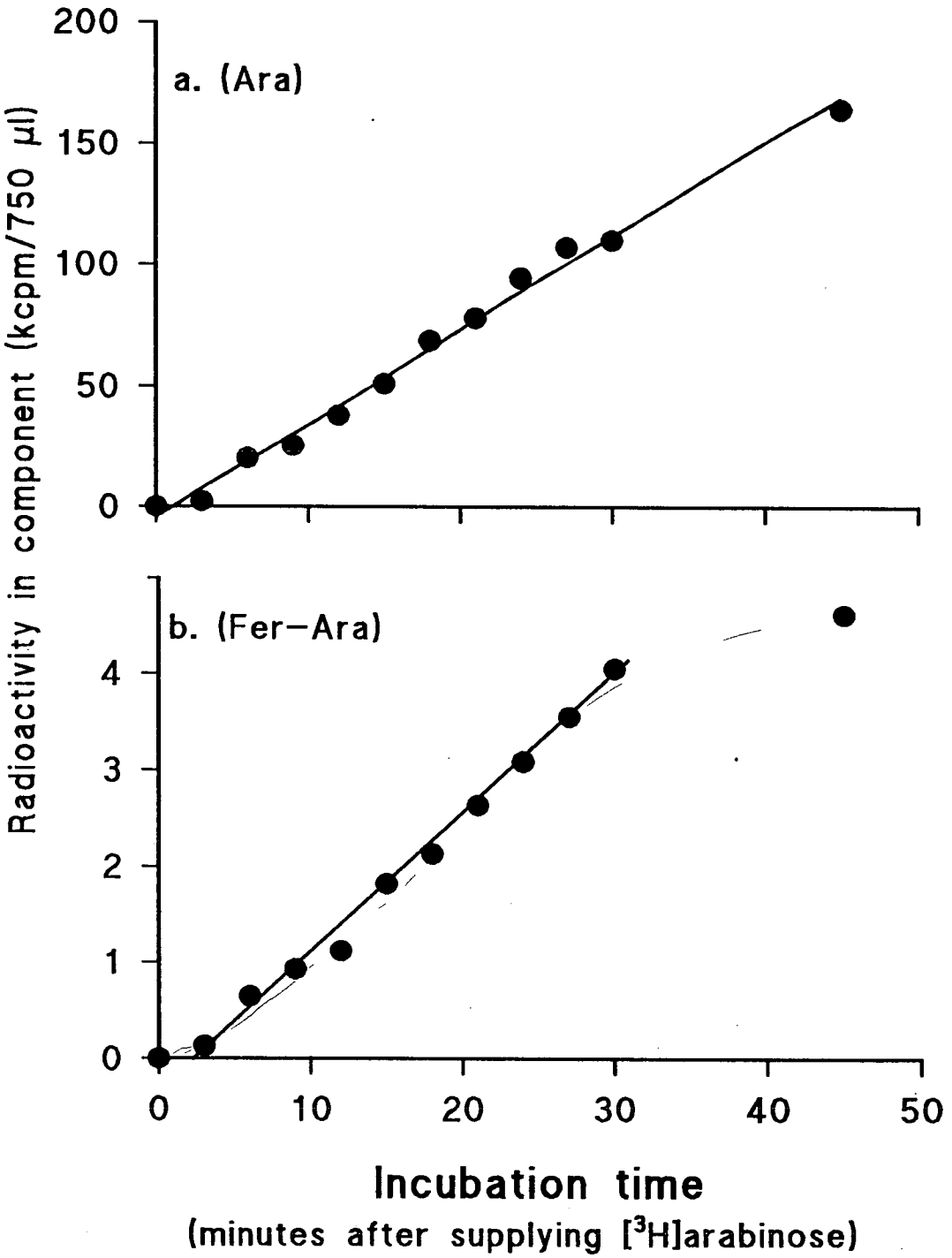


Fig. 3.2.2.3 Five day old fescue cultures. The Incorporation of radiolabel from exogenously supplied [1-^{3}H]arabinose into a) polymer bound arabinofuranosyl residues and b) polymer bound feruloyl-arabinose. Each point represents the mean of two duplicate experiments

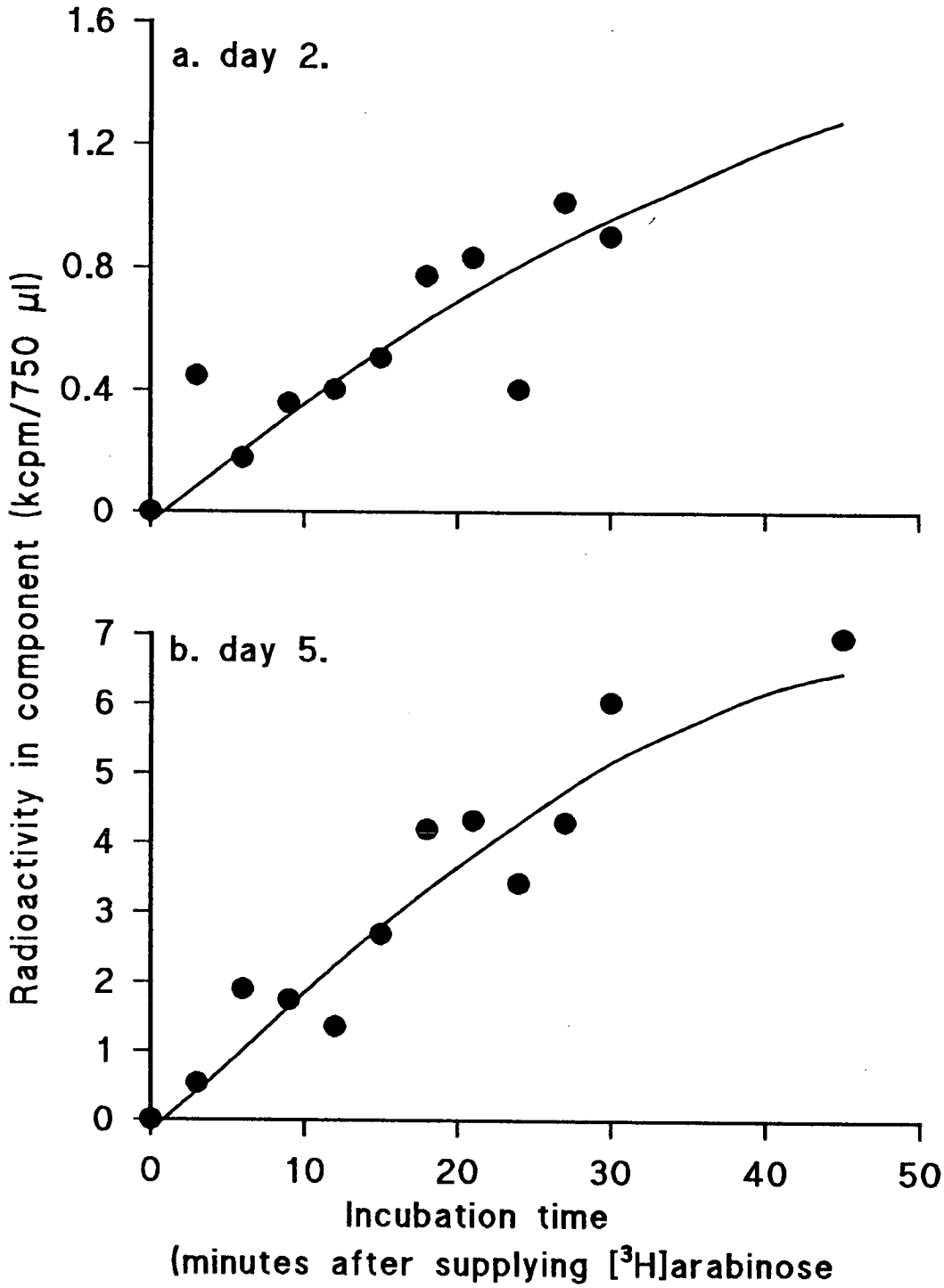


Fig. 3.2.2.4. Compound B. Incorporation of radiolabel into polymer bound compound B (feruloyl{xylosyl-arabinose}) in a) two day old cultures and. b) five day old cultures. Each point represents the mean of two duplicate experiments.

values consistent with arabinose and a disaccharide respectively.

3.2.3 Partial characterisation of compounds A and B.

Treatment of bulk ^{3}H labelled fescue AIR (section 2.4.3) released compound A and B in sufficient quantities to enable further analysis of their structure.

3.2.3.1 Saponification of Compounds A and B.

Treatment of A and B with 0.5 M NaOH for 1 hr at 25°C resulted in the release of the de-feruloylated sugar moiety, A_S and B_S.

A_S co-chromatographed with authentic arabinose in two paper chromatography solvent systems (BAW and BEW, 16 hr each). This decrease in mobility on treatment with NaOH suggests the presence of a relatively hydrophobic residue attached to the sugar moiety through an ester bond. The intense fluorescence of the parent compound in 366 nm UV light is highly indicative of feruloyl esters (Fry 1982, 1987) suggesting the identity of Compound A to be feruloyl-arabinose. Compound B_S had an R_{Ara} of 0.70 in BAW and 0.27 in EPW, indicating that it was an oligosaccharide.

3.2.3.2 Determination of specific activity of arabinose and xylose residues.

The specific activity of [^{3}H]arabinose residues released from bulk cell walls was calculated to be 5.14 TBq $mmol^{-1}$ and that of [^{3}H]xylose to be 2.38 TBq $mmol^{-1}$. This indicates that the 3H from exogenous [^{3}H]arabinose, after entering the pool of UDP-[^{3}H]arabinose via a scavenger pathway (Fry and Northcote 1983), did not fully equilibrate with the pool of UDP-xylose that is formed from the major carbon source, non-radioactive glucose.

3.2.3.3 Acid Hydrolysis of B_S

Total acid hydrolysis of B_S resulted in the release of [^{3}H]arabinose and [^{3}H]xylose in the ratio of 2.29:1 on a ^{3}H basis (data not shown), which corresponds to 1.06:1 on a molar basis if the specific radioactivities of the pentose residues in compound B_S are the same as in bulk cell walls. This suggests that B_S is a disaccharide of xylose and arabinose (presumably D- and L- respectively as these are the naturally occurring isomers), although the labelling data alone do not eliminate the possibility of there being a non-labelled sugar also present.

3.2.3.4 Gel permeation chromatography of B_S

G.P.C. of B_S (section 2.3.4.3) resulted in the elution of a single peak of radioactivity co-incident with the internal marker maltose (fig 3.2.3.1), supporting the hypothesis that B_S is a disaccharide.

3.2.3.5 Sodium Borohydride reduction of A_S and B_S.

After reduction (section 2.3.4.4) all reducing termini were assumed to be in the alditol form . Paper chromatography of the products (EPW, 24 h) separated xylose from a lower Rf zone containing markers of arabinose, arabinitol and xylitol. These 3 components were separated by p.e. in borate buffer.

Borohydride reduction followed by acid hydrolysis of A_S yielded only arabinitol, confirming that the arabinose possessed a reducing terminus. Reduced B_S released xylose and arabinitol but no arabinose or xylitol, suggesting that compound B had been linked to the xylan backbone *via* its arabinose residue. Since compound B was released by mild acid hydrolysis it is likely that its reducing terminal sugar moiety had been furanosidically linked within the parent polymer. Since arabinofuranose residues are abundant

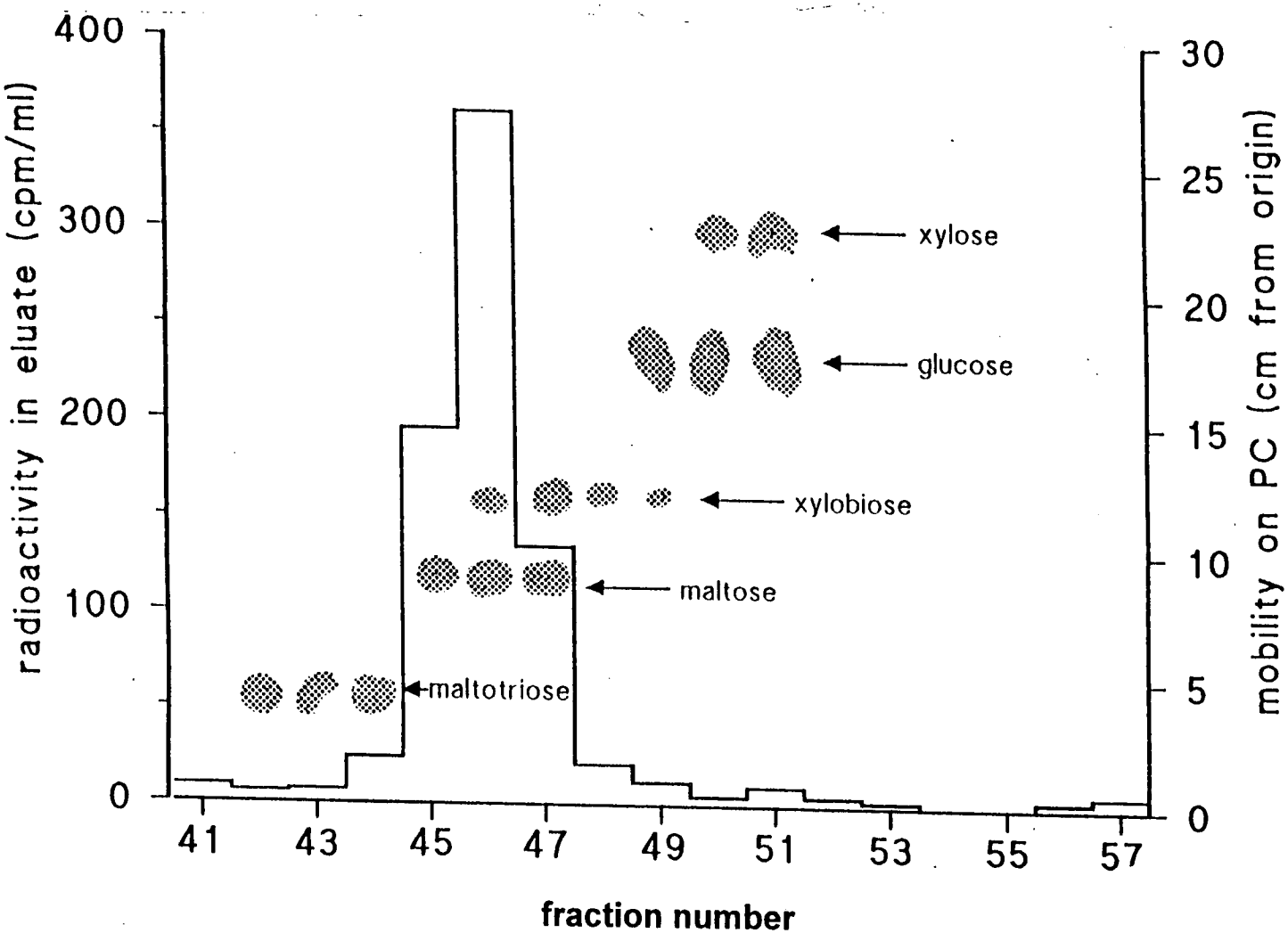


Fig. 3.2.3.1 Gel permeation chromatography of compound Bs on Bio-Gel P2. The histogram shows the distribution of radioactivity between Bio-Gel fractions, and the shaded spots show the location of non-radioactive internal markers after subsequent paper chromatography..

whereas xylofuranose residues are unknown in plant cell walls, the constitution of compound B_S is concluded to be D-xylopyranosyl-L-arabinose.

3.3 Discussion of results.

3.3.1 Glucose feeding of maize cell cultures.

There are considerable problems with using glucose as a radiolabelled precursor when analysing quantitatively minor specific components of cells. Glucose is a general precursor and was the sole carbon source in my cell cultures. Therefore, although exogenously supplied [^{14}C]glucose was rapidly taken up and incorporated into polysaccharides by the cells this would also result in almost every cell component becoming radiolabelled. Consequently background levels of radioactivity on chromatograms were very high and there would have been considerable contamination of alcohol insoluble residues by radioactive cytoplasmic polymers. Thus the analysis of the incorporation of radiolabel into a quantitatively minor cell wall component such as a feruloylated di- or trisaccharide becomes more difficult.

However, the data presented show that polymer bound arabinose residues rapidly incorporated radiolabel from [^{14}C]glucose (fig. 3.1.1.a). Radiolabel was also incorporated into an unknown compound (compound GS, fig. 3.1.1.b), which was released from cell wall preparations by Driselase digestion, adsorbed to a C18 SPEC, exhibited turquoise fluorescence under 366 nm UV light, becoming more intense and blue-green on exposure to ammonia vapour and had an Rf value on paper in BAW of ≈0.55. Compound GS therefore had several properties consistent with it being a feruloylated di- or tri-saccharide. However, saponification in 0.5 M NaOH only results in the release of ≈20% of the total radiolabel in compound GS into a product with a lower Rf value (consistent with being a disaccharide, fig. 3.1.2). This result

suggests a number of possibilities. Firstly compound GS may be an authentic feruloyl-ester but treatment with 0.5 M NaOH for 1 hour at 24°C is not sufficient to saponify it. Certainly, stronger alkaline treatments are used when removing phenolic acid residues from more mature cell walls, but evidence shows that this mild treatment is sufficient to saponify feruloylated di- or tri-saccharides (Fry 1982). Another possibility is that there is an unknown radiolabelled contaminant that is co-chromatographing with compound GS and which is not susceptible to saponification. Such a contaminant would need to be derived from a Driselase digestible polymer, and be relatively hydrophobic. Such a compound could conceivably be an aromatic amino acid or oligopeptide (Driselase has slight protease activity, Biggs 1988), feruloylated amines (Negrel and Lherminier 1987, Negrel and Jeandet 1987) or another phenylpropanoid derivative. Since glucose is a universal precursor, miscellaneous cell wall components or cytoplasmic contaminants will become labelled and seriously complicate the analysis of feruloylated cell wall components. Clearly glucose is not an ideal precursor for these experiments.

3.3.2 *In vivo* radiolabelling of *Festuca* cell cultures with [^{3}H]arabinose.

In these radiolabelling experiments where it is desirable to label the sugar residues of arabinoxylan, [^{3}H]arabinose is a more suitable precursor since it is taken up via a scavenger pathway and converted, by the action of an nucleotide epimerase into xylose (fig. 3.3.2).

UDP-xylose is produced from UDP-arabinose and is not further converted into other sugar nucleotides (Fry and Northcote 1983). Thus in the cell wall of cells supplied with [^{3}H]arabinose only the pentose residues will be radiolabelled.

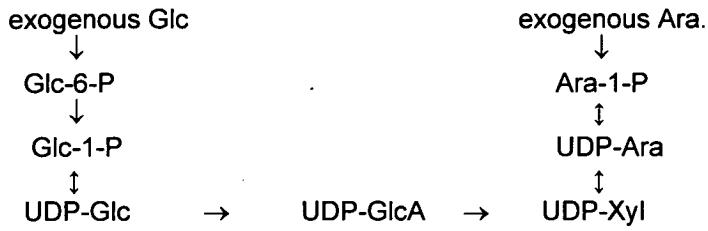


fig.3.3.2. Pathway of the conversion of exogenously supplied arabinose and glucose into UDP-pentoses

As stated earlier, maize does not incorporate exogenously supplied arabinose into polysaccharides in sufficient quantities to enable analysis of radiolabelled wall fragments. This may be due to a lack of an active scavenger pathway in maize, or to the existence of large pools of UDP-arabinose. The latter would result in a slow turnover rate and incorporation of [^{3}H]arabinose into the pools and thus into nascent polysaccharides. Certainly the lack of uptake of exogenous arabinose is not a universal characteristic of monocots since fescue cell cultures do rapidly take up and incorporate exogenous [^{3}H]arabinose, and cell walls of sufficient specific activity were produced to enable analysis of individual components.

As described previously, radiolabelled sugars are taken into the cell, converted into UDP-sugars and incorporated into nascent polysaccharides, which are then transported via the endomembrane system into the cell wall. In cell cultures, non-cellulosic polysaccharides can fail to be retained by the wall and pass into the medium. With radiolabelling experiments, the appearance of radiolabel into these soluble extracellular polysaccharides provides a maximum estimate of the time taken for this process of incorporation and secretion to occur.

In fescue cell cultures this time was ≈20-35 minutes, depending on the age of the culture (fig 3.2.2.1a and b). The lag time between the provision of [^{3}H]arabinose and its appearance in extracellular polysaccharides was

shorter in 5 day old cultures that in 2 day old cultures. This could be due to a decrease in transit time of secretory vesicles from Golgi body to plasmamembrane in older cell cultures.

These data can be compared to the data for the kinetics of feruloylation. Figures 3.2.2.2, 3.2.2.3, 3.2.2.4 show clearly that, within 5 minutes of supply, [^{3}H]arabinose had been incorporated into nascent polysaccharides and was beginning to be feruloylated. This is well before any radiolabel had appeared in extracellullar polymers. If we take the lag for secretion of radiolabelled polysaccharides into the medium as being the time before which essentially all the radiolabel is within the cytosol, then feruloylation must be beginning intraprotoplasmically. These data are consistent with those from dicots (Fry 1987, Meyer *et al.* 1991). There does not appear to be an increase in the rate of incorporation of radiolabel into feruloylated products after 25-30 minutes (the time after which radiolabelled polysaccharides would be present *in muro*) and this would therefore suggest that continued feruloylation does not occur at a comparable rate in the wall.

These results however are contradictory to previous work in monocots (Yamamoto and Towers 1985, Nishitani and Nevins 1990). The principal argument in these reports has been that feruloyl content of cell walls continues to rise after the increases in cell wall dry weight and net deposition of polysaccharides has ceased. This has been interpreted as indicating that there may be a modification to the wall bound polysaccharides, i.e. *in muro* feruloylation, to account for such a continued increase in feruloyl groups. There are, however, other explanations for such observations.

It is feasible that as cells age there is an increase in the degree of feruloylation of the polysaccharides being synthesised *de novo*, and this would be in agreement with the reports that the proportion of feruloylated polysaccharide fragments that can be released from the wall by fungal

enzyme digestion increases in older coleoptiles (Nishitani and Nevins 1990). There is indirect evidence for an increased rate of feruloylation with age from the data presented here. From the experimental data reported here we can approximate the degree of feruloylation by comparing the ratio of feruloyl-[^{3}H]arabinose and feruloyl-[^{3}H]{xylosyl-arabinose} (compound B) to [^{3}H]arabinose in the two ages of culture used. In 5 day old cultures the ratio is relatively constant at 0.075 suggesting that one in every 13.3 [^{3}H]arabinofuranosyl residues is feruloylated. In 2 day old cultures however this ratio is approximately 7 fold lower at 0.011, suggesting that only 1 in 90 [^{3}H]arabinofuranosyl residues are being feruloylated. These figures do not account for the hydrolysis of feruloyl-ester by hot acid and the true ratio is liable to be slightly greater than calculated by the data. However, assuming the proportion of feruloyl ester linkages cleaved is the same in both day 2 and day 5 samples, these data suggest that non-cellulosic polysaccharides in older cell cultures are more highly substituted with feruloyl groups than in younger tissue.

Thus there appears to be a temporal increase in the degree of feruloylation, resulting, in older tissue, in the deposition of highly feruloylated polysaccharides after the deposition of total, poorly feruloylated polysaccharides has ceased and perhaps been exceeded by degradation. In coleoptile tissue the increases in ferulate content after the net increases in cell wall weight have ceased could be accounted for by this process.

Wall polysaccharides can undergo enzyme mediated turnover (Labavitch 1981, Darvill *et al.*1978) involving both the cleavage of the polysaccharide backbone by endopolysaccharidases and the removal of single non-reducing terminal monosaccharides by exo-polysaccharidases. In maize coleoptiles terminal (1-2) or (1-3) linked arabinose residues of arabinoxylan are particularly susceptible to cleavage from the xylose

backbone, although arabinose residues which were previously *also* substituted in the 5-position are apparently resistant to degradation (Darvill *et al.* 1978). These substituted arabinose residues may have been esterified to a feruloyl group before extraction and analysis. It is feasible that feruloyl groups may protect the arabinose residues to which they are esterified from enzymatic degradation within the wall in a way similar to the protection they afford from fungal polysaccharidases (e.g. Driselase, feraxanase).

One possible result of this selective turnover would be that non-feruloylated regions of polysaccharides would be degraded to a greater degree than feruloylated sections, thus leaving a higher proportion of feruloylated sugar residues. This would have the effect of increasing the amount of ferulate per unit dry weight of cell wall, and thus the degree of feruloylation of degraded polysaccharides, explaining the gradual increase in ferulate content of tissues observed during tissue ageing (Nishitani and Nevins 1990).

Yamamoto *et al.* (1989) reported that cell wall preparations incubated with [^{14}C]feruloyl-CoA incorporated radiolabel (up to 1%) into the cell wall and that this radiolabel could subsequently be released as low molecular weight fragments through the action of Driselase. Feruloyl-CoA was reported as a more effective precursor for this reaction than either feruloyl-glucoside or ferulic acid. This was suggested to demonstrate the *in vitro* feruloylation of *isolated* cell walls, although the bonding patterns of the feruloyl residues to the wall have yet to be established and the release of radiolabel as ferulate by NaOH was not reported. Peroxidases can incorporate aromatic material from feruloyl-CoA into a polymeric product (see section 4.1.6) and it is probable that peroxidases present in such cell wall preparations are responsible for this apparent feruloylation.

From a bioenergetics point of view it is less probable that CoA derivatives of cell wall precursors would be secreted into the wall as it is unlikely that the CoA moiety could be reclaimed by the cell after use of the feruloyl-CoA. If such *in muro* feruloylation does occur, it is more feasible that feruloyl-glucoside would be used as an acyl donor and this could be secreted in a similar manner to that hypothesised for monolignol-glucosides . Thus, although data have been presented that are consistent with *in muro* feruloylation, the observations of Yamamoto and Towers (1985) and Nishitani and Nevins (1990) can be attributed to other processes and cannot be taken as evidence for feruloylation occurring in the wall.

The kinetics of feruloylation as presented here show that feruloylation begins intraprotoplasmically, and although it is possible that feruloylation continues via a different mechanism in the wall there was no evidence for such a process arising from the experiments above. There is, however, evidence that if *in muro* feruloylation did occur it was at a much lower rate than intraprotoplasmic feruloylation

3.3.3 Analysis of compounds A and B.

Compound A and B were released from fescue cell walls by mild acid hydrolysis, indicating that their attachment to the parent polymer was via a furanose bond. Both compounds exhibited blue fluorescence under 366 nm UV light, intensifying and turning blue green on exposure to ammonia vapour. This behaviour is highly diagnostic of feruloyl esters. Upon saponification, compound A yielded a single radiolabelled product that co-chromatographed with authentic arabinose in several chromatography systems. Sodium borohydride reduction yielded only [^{3}H]arabinitol, indicating that A was an O-feruloyl-L-arabinose derivative. Such a derivative, 5-O-feruloyl-L-arabinose

has been reported from many graminaceous cell walls (Gubler *et al.* 1983, Kato and Nevins 1985, Ahluwalia and Fry 1986, Mueller-Harvey *et al.* 1986).

After saponification compound B yielded a product with similar chromatographic properties to a disaccharide (sections 3.2.3.1 and 3.2.3.4), which yielded arabinose and xylose a molar ratio of 1.06:1 after hydrolysis (section 3.2.3.3), and arabinitol and xylose after sodium borohydride reduction and hydrolysis (section 3.2.3.5). From these data we can conclude that the carbohydrate moiety of compound B is D-xylopyranosyl-L-arabinofuranose. Compound B is a feruloyl ester but no conclusions can be drawn from these data as to the position of the feruloyl group within the molecule. The origin and structure of compounds A, A_S, B and B_S as elucidated from these data are summarised below (fig. 3.3.3.1).

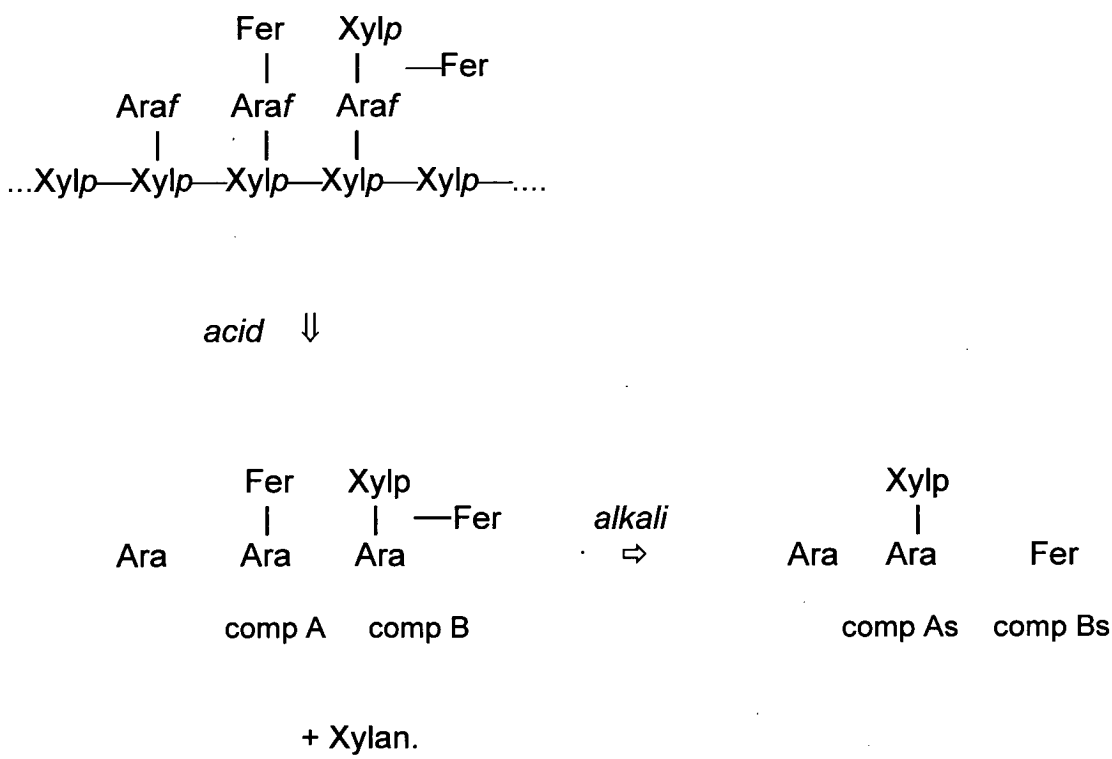


Fig. 3.3.3.1 Proposed effect of mild acid hydrolysis on fescue arabinoxylan, and the effect of mild alkaline hydrolysis on compounds A and B.

Xylans have been reported as possessing a wide variety of side-chains, the most common of which are terminal arabinofuranosyl and terminal glucuronosyl, which may be methylated at the 4-O position (for reviews see Darvill *et al.* 1980, McNeil *et al.* 1984). The disaccharide Xylp-(β1-2)-Araf- has been reported as attached to the xylans of bamboo (Wilkie and Woo 1977), guinea grass (Buchala 1974) and oat (Buchala *et al.* 1972), in each case to the C-3 of xylose residues. There have not, however been reports of this side chain being feruloylated. This lack of detection may be due to degradation during extraction techniques rather than an absence of such a structure in walls previously examined. Treatment of fescue cell walls with mild acid results in the release of a series of feruloylated oligosaccharides with increasing molecular weight (G. Wende and S.C. Fry, unpublished results). These feruloylated oligomers have presumably been previously linked to the polysaccharide through an arabinofuranosyl bond and may represent a sequence of complex side chains of fescue glucuronoarabinoxylan. The presence of such complex side chains may result in fescue cell walls being Driselase resistant.

CHAPTER 4
RESULTS OF ASSAYS FOR A PUTATIVE FERULOYLTRANSFERASE.

4.1 Results of assays for a putative feruloyltransferase.

Previous results had indicated that feruloylation of arabinoxylan in graminaceous monocots is a protoplasmic rather than cell wall located process. It was thus my intention to investigate the existence of a feruloyltransferase responsible for the feruloylation of polysaccharides. As outlined above (section 1.7) such a transferase may be expected to be associated with the membrane fraction of cells and to co- or post-synthetically modify nascent polysaccharides. To ascertain whether such membrane bound activity was present in graminaceous monocots, membranes were prepared from homogenised cell cultures of maize and supplied with a putative acyl donor, feruloyl-CoA, and the incorporation of radiolabel into polymeric material was monitored.

4.1.1 The effect of addition of arabinoxylan to the homogenisation medium on the incorporation of radiolabel from [methoxy-^{14}C]feruloyl-CoA into polymeric material.

A polysaccharide feruloyltransferase in monocots might be expected to utilise nascent arabinoxylan molecules as acyl acceptors. Meyer *et al.* (1991) observed a decrease in feruloyltransferase activity with time during enzyme assays and suggested that this may be due to the exhaustion of endogenous nascent polysaccharide acceptors in the membrane preparations. During homogenisation it is conceivable that added arabinoxylan would become associated with membranes either through entrapment within the lipid bilayer or encapsulation in vesicles and thus be available as acceptors for feruloyl groups.

The addition of arabinoxylan during homogenisation of cells appeared to increase the rate of reaction (fig 4.1.1) and prolonged the incorporation of radiolabel from [methoxy-^{14}C]feruloyl-CoA into polymeric material. In

subsequent preparations arabinoxylan was routinely added to the homogenisation medium at 0.5 mg ml^{-1}

4.1.2 The effect of divalent cations on the incorporation of radiolabel from [methoxy-^{14}C]feruloyl-CoA into polymeric material.

Membrane preparations were supplied with a number of divalent ions as potential co-factors at a concentration of 2 mM. No incorporation of radiolabel into polymeric material occurred unless cobalt was present in the assay mixture and this had an optimum concentration at 5 mM (fig. 4.1.2). When EDTA was present in the assay mixture at 10 mM activity was almost completely inhibited. Cobalt has been shown to replace zinc as a co-factor in some metalloenzymes, often with a stimulatory effect (Auld and Vallee 1970, Bond and Benyon 1985). However, in this system there was no activity when zinc was supplied, at a range of concentrations, as either zinc chloride or zinc sulphate (data not shown)

On addition of the membrane preparation to $CoCl_2$ (final concentration, 1-5 mM), a deep maroon colour developed as a result of the interaction between DTT and the cobalt II ion (Cullis *et al.* 1968). This colour change was not seen in those preparations containing EDTA or 'time zero' assays which had formic acid added before the addition of the membrane preparation.

4.1.3 Incorporation of radiolabel from [methoxy-^{14}C]feruloyl-CoA into polymeric material by membranes from cultures of different ages.

It has been shown that as maize coleoptile tissue ages there is an increase in the degree of feruloylation of certain polysaccharides. As discussed previously (section 3.3.2) this may be as a result of increased feruloylation of the

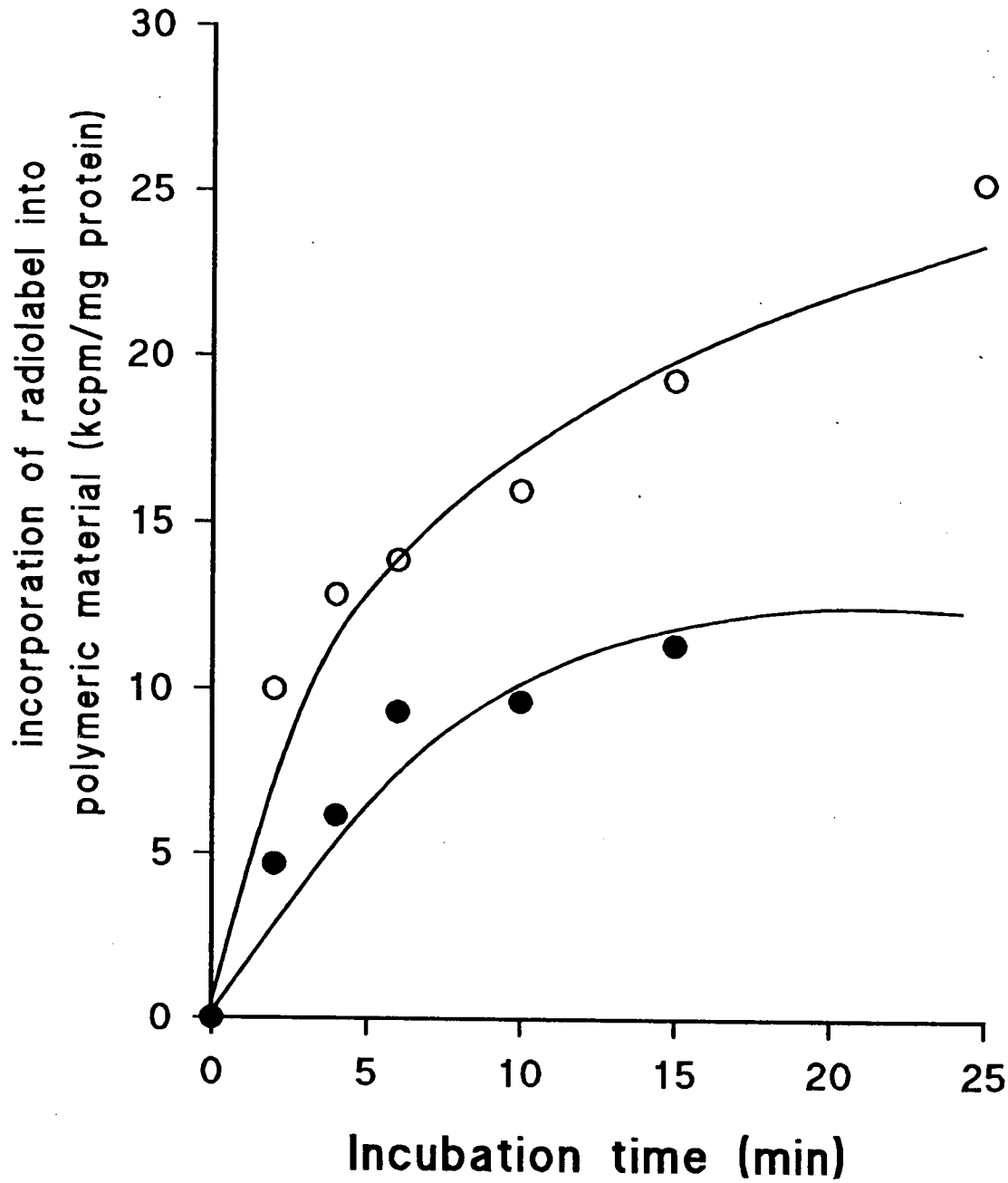


Fig. 4.1.1 The effect of arabinoxylan on the incorporation of radiolabel into polymeric material. Cell cultures were homogenised in the presence (O) or absence (●) of arabinoxylan (10 mg/ml homogenisation medium). Assays contained 5 mM $CoCl_2$, 0.5 kBq [methoxy-^{14}C]feruloyl-CoA, 75 µl membrane preparation, pH 7. Each point represents the mean of three replicate assays from two membrane preparations.

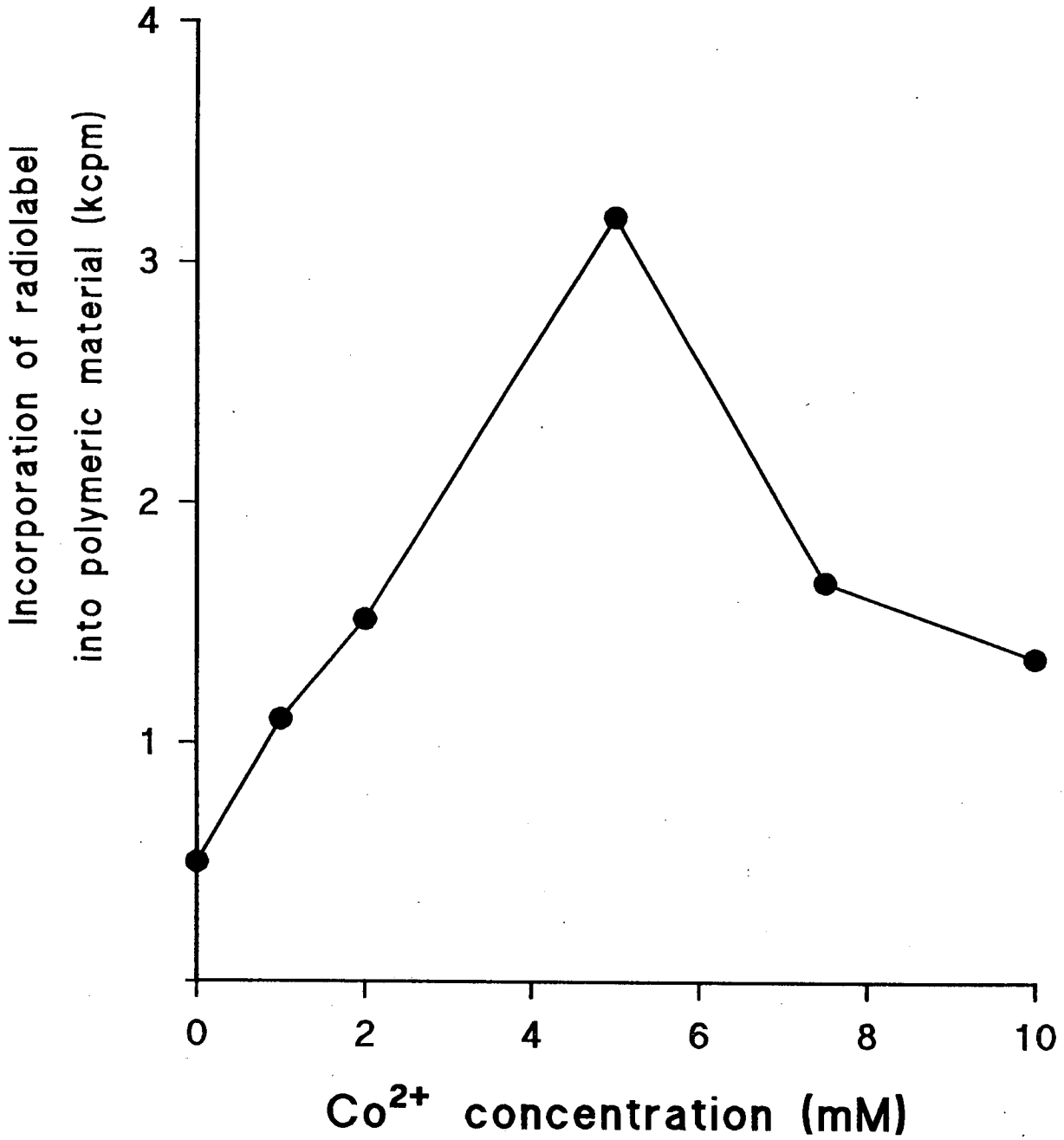


Fig. 4.1.2 The effect of increasing cobalt concentration on the incorporation of radiolabel into polymeric material. Points shown are means of triplicate assays conducted for 15 minutes. Assays contained 0.5 kBq [methoxy-^{14}C]feruloyl-CoA, pH 7, 75 µl membrane preparation. Points represent the mean of three replicate assays from two membrane preparations.

polysaccharides synthesised in older tissues. Such an increase could be effected in several ways e.g. enhanced precursor supply, compartmentalisation of substrates within different parts of the endomembrane system or control of movement of substrates into the lumen of the Golgi bodies. It is feasible that a feruloyltransferase could act as a control enzyme for this step in polysaccharide synthesis, and thus its activity may change during ageing of the tissue.

Membranes extracted from cell cultures 24-192 h after sub culturing were assayed for their ability to transfer radiolabel from [methoxy-^{14}C]feruloyl-CoA into polymeric material. There was little change in the specific activity over the eight day period. However, there may have been a slight decline in specific activity during the first 120 hours after sub culturing, rising again up to 192 hours (Fig. 4.1.3). There was no dramatic difference in the enzyme activity during exponential growth and during ageing of the cell cultures.

4.1.4 The effect of temperature on the incorporation of radiolabel from [methoxy-^{14}C]feruloyl-CoA into polymeric material.

Routine assays were conducted at different temperatures and membranes that had been boiled for 5 min were used as a negative control. Membrane preparations were added to feruloyl-CoA and allowed to incubate for 10 minutes before the reaction was stopped with formic acid. The reaction rate increased with temperature with the maximum seen at 40°C (fig. 4.1.4). Some slight activity was detected at 80°C, and there was no detectable activity in boiled preparations (data not shown).

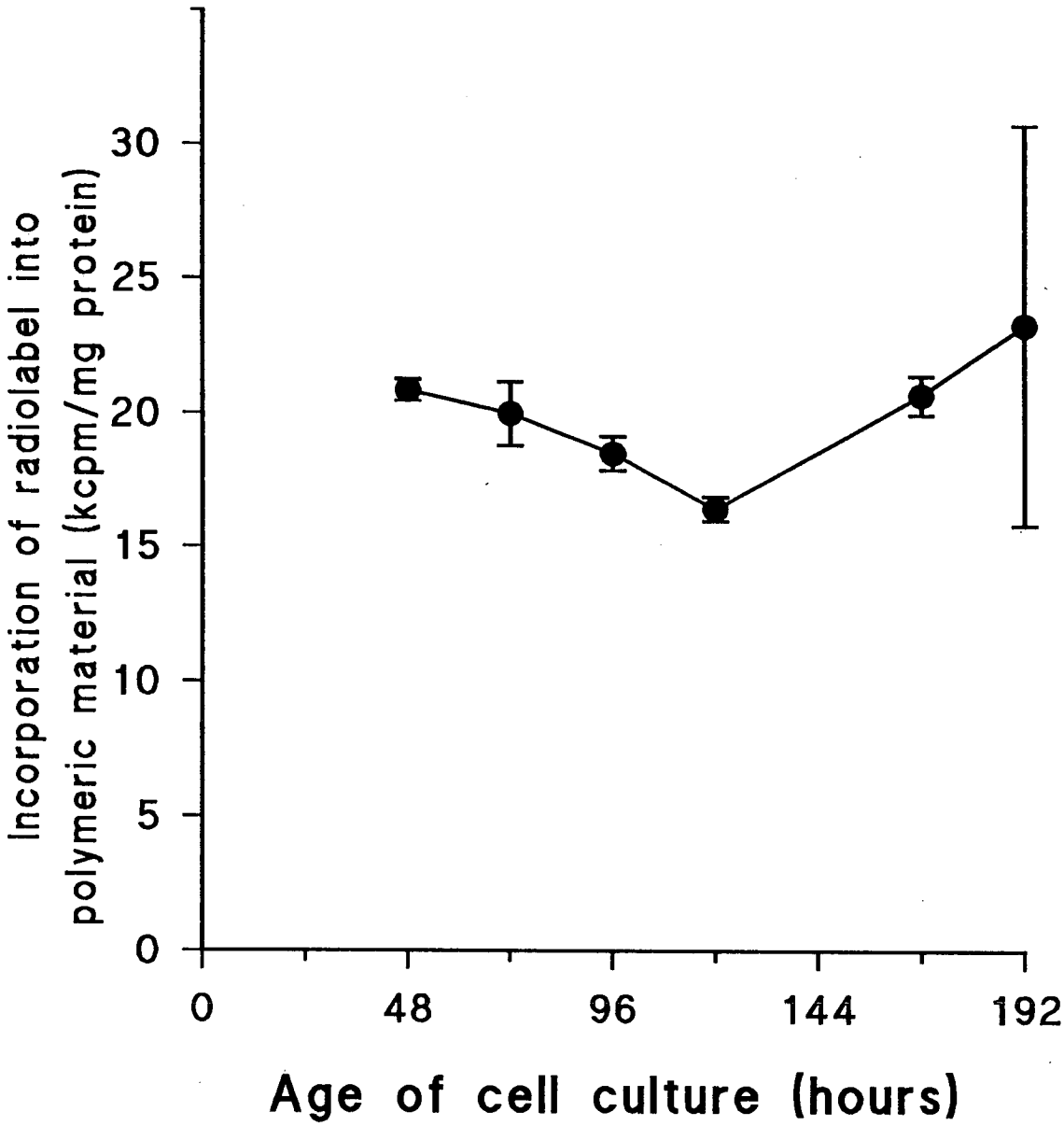


Fig 4.1.3. The incorporation of radiolabel from [methoxy-^{14}C]feruloyl-CoA into polymeric material by membrane preparations from cell cultures of different ages. Points are for stopped assays of 15 minutes, containing 0.5 kBq [methoxy-^{14}C]feruloyl-CoA, 2 mM $CoCl_2$, 70 µl membrane preparation, pH 7. Points represent means of four replicate assays from two membrane preparations

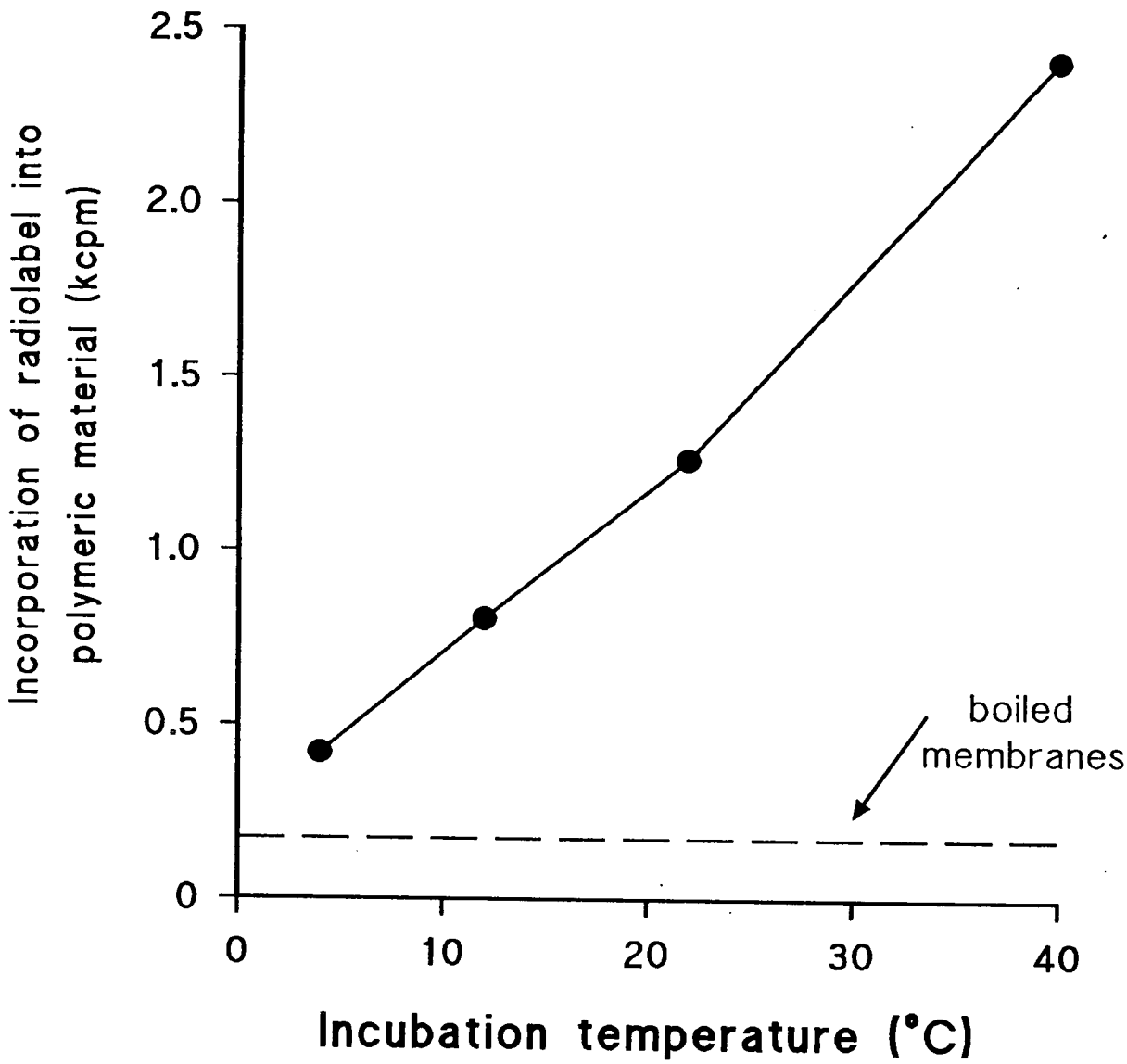


Fig. 4.1.4 The effect of increasing temperature on the rate of incorporation of radiolabel from [methoxy-^{14}C]feruloyl-CoA into polymeric material. Points shown are for stopped assays of 15 minutes, containing 0.5 kBq [methoxy-^{14}C]feruloyl-CoA, 2 mM $CoCl_2$, 75 µl membrane preparation, pH 7. The dotted line represents the radioactivity present in assays conducted with boiled membranes. Each point represents the mean of duplicate assays from two membrane preparations

4.1.5 The effect of changing pH on the transfer of radiolabel from [methoxy-^{14}C]feruloyl-CoA into polymeric material.

Endomembranes were prepared and re-suspended in buffer at a range of pH values (5.8, 6.5, 7.2, 8.0). Preparations were supplied with 2 mM $CoCl_2$ and 1 kBq [methoxy-^{14}C]feruloyl-CoA, and the assays incubated for 12 minutes before termination by the addition of formic acid. There was a pH optimum for activity of ≈7.2 with lower activity at pH 5.8 and 6.5. A sharp decrease in activity was seen at pH 8.0.(fig. 4.1.5).

4.1.6 The effect of the addition of catalase on the incorporation of radiolabel from [methoxy-^{14}C]feruloyl-CoA into polymeric material.

Peroxidase activity has been reported in membrane preparations from a variety of tissues (Keifer *et al.* 1985, Ros Barceló *et al.* 1988). The binding of these peroxidases to membranes is partly due to a soluble molecule (a 'binding factor') and partly due to the membranes and is increased by the presence of calcium but partly inhibited by EDTA (Penel 1991). In the presence of hydrogen peroxide these membrane associated peroxidases show activity against guaiacol and phenylpropanoids e.g. ferulic acid and caffeic acid (Rodgers *et al.* 1993).

Such peroxidases may be expected to act upon feruloyl-CoA by oxidising the phenol moiety resulting in free radical formation and subsequent polymerisation . In the assays reported here radiolabel had been incorporated from feruloyl-CoA into insoluble material and it was necessary to determine whether this (presumably polymeric) product was formed through the action of a peroxidase rather than a feruloyltransferase.

In order to ascertain the presence and possible significance of peroxidase in the membrane preparations, catalase was added to reaction

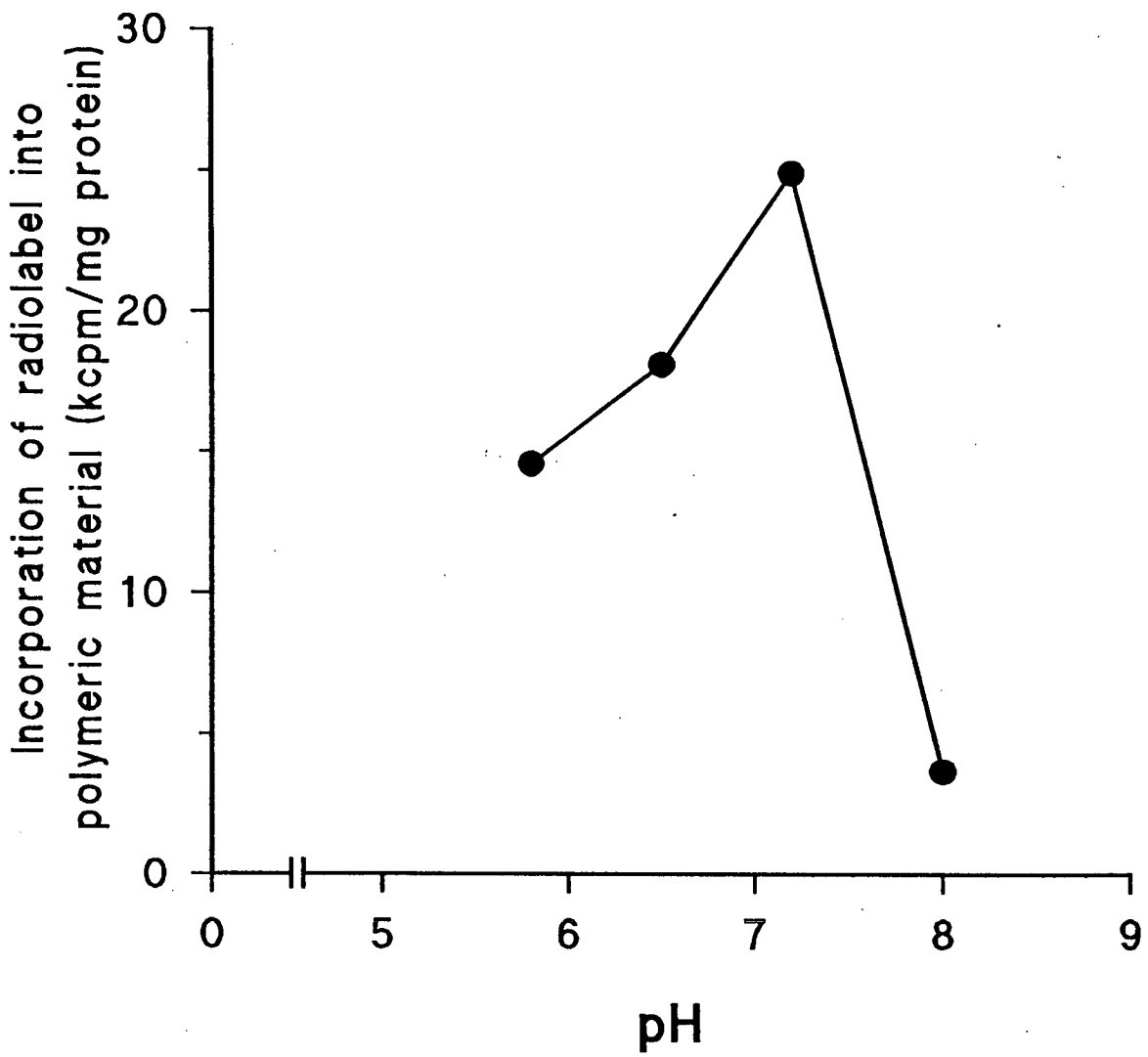


Fig 4.1.5 The transfer of radiolabel from [^{14}C]feruloyl-CoA into polymeric material at different pH. Time points are for stopped assays of 15 minutes and represent means of triplicate assays of a single membrane preparation. Assays contained 0.5 kBq [^{14}C]feruloyl-CoA, 2 mM $CoCl_2$, 50 µl membrane preparation.

mixtures. Catalase will degrade H_2O_2 which is required as an electron acceptor for peroxidase and thus its addition to assays should minimise peroxidase activity.

The addition of catalase to the enzyme assays completely suppressed transfer of radiolabel into polymeric material in the short term (fig 4.1.6.1) although over a longer period of time (up to 1 hour, fig. 4.1.6.2) there was a slight incorporation of radiolabel into polymeric material, despite the presence of catalase.

The activity of these membrane preparations against ferulic acid in the presence or absence of catalase was also monitored. Radiolabel from [methoxy-^{14}C]ferulic acid was incorporated into polymeric material at a greater rate than that from [methoxy-^{14}C]feruloyl-CoA (fig 4.1.6.3). However, the presence of catalase in the assay mixture almost completely suppressed this activity, again suggesting that a peroxidase was responsible.

4.1.7 Effect of the addition of sodium azide on the transfer of radiolabel from [methoxy-^{14}C]feruloyl-CoA into polymeric material.

Sodium azide (NaN_3) is reported as a specific inhibitor of haem proteins and would thus be expected to inhibit peroxidase (Dawson *et al.* 1986). Its presence in assay mixtures at a concentration of 10 mM resulted in a slight reduction of the total amount of radiolabel incorporated into polymeric material and an decrease in the initial rate of reaction (fig. 4.1.7).

4.2 Analysis of the reaction product of long term assays containing catalase.

Although catalase completely inhibited the short term incorporation of radiolabel into polymeric material, longer term (up to an hour) experiments

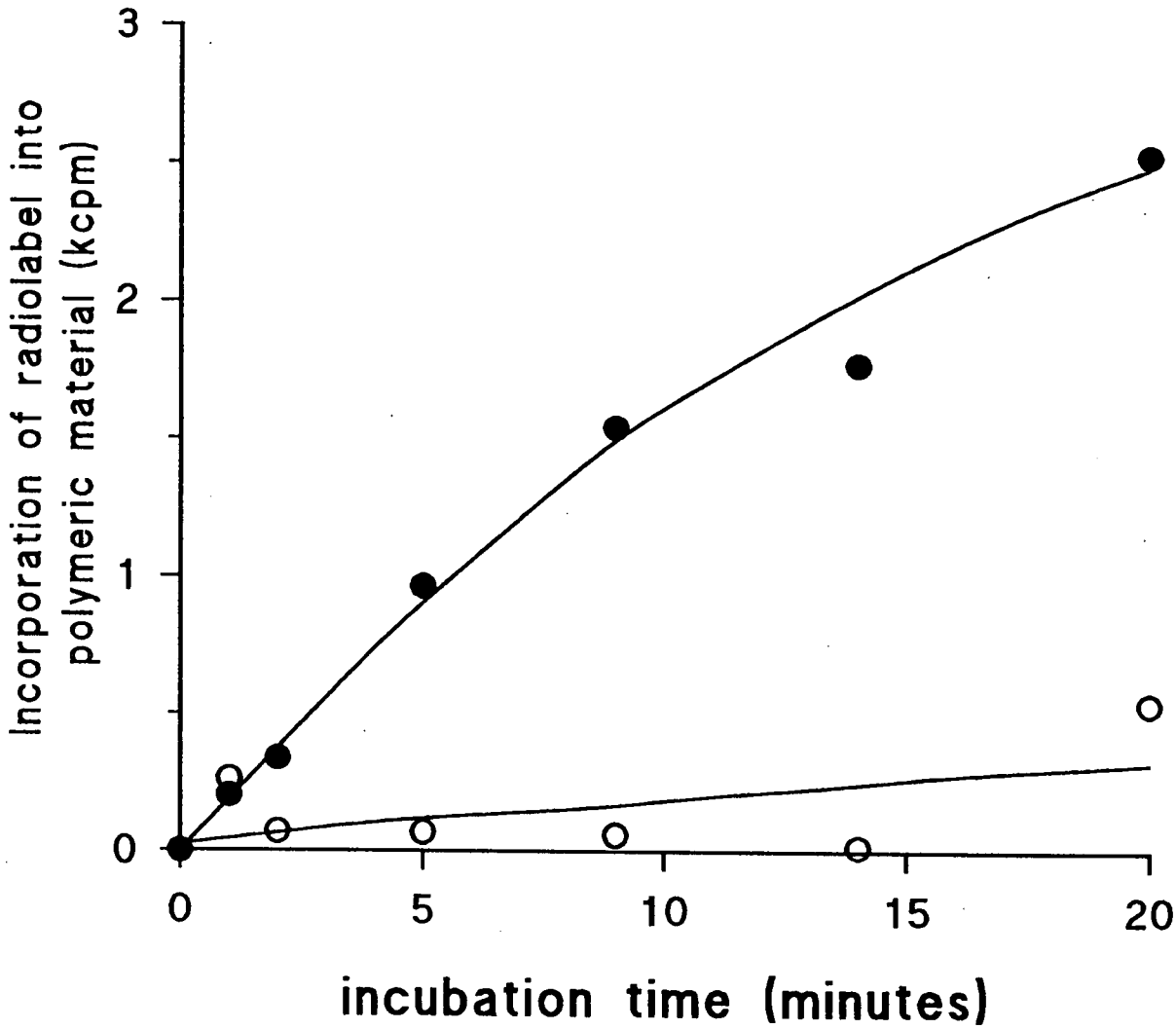


Fig. 4.1.6.1 The effect of catalase on the incorporation of radiolabel from [methoxy-^{14}C]feruloyl-CoA into polymeric material.
Assays were conducted using 0.5 kBq [^{14}C]feruloyl-CoA, 2 mM $CoCl_2$, 75 µl membrane preparation, pH 7 and contained either 55 units of catalase (O) or an equivalent volume of buffer (●). Points represent the mean of duplicate assays from two membrane preparations.

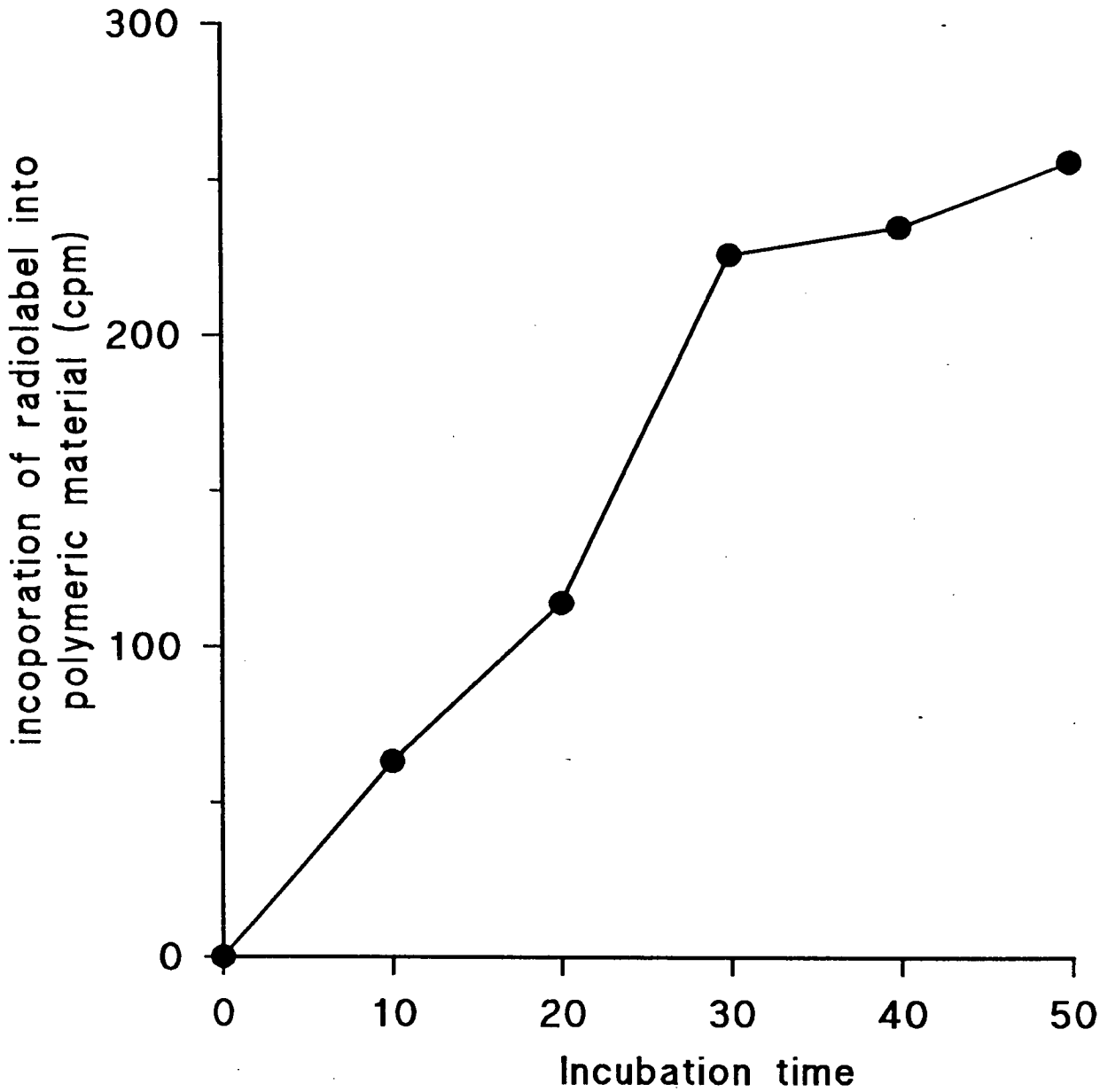


Fig 4.1.6.2 Longer term incorporation of radiolabel from [methoxy-^{14}C]feruloyl-CoA into polymeric material in the presence of catalase. Assays were conducted with 0.5 kBq [methoxy-^{14}C]feruloyl-CoA, 2 mM $CoCl_2$, 75 µl membrane preparation, pH 7 and contained 55 units of catalase.

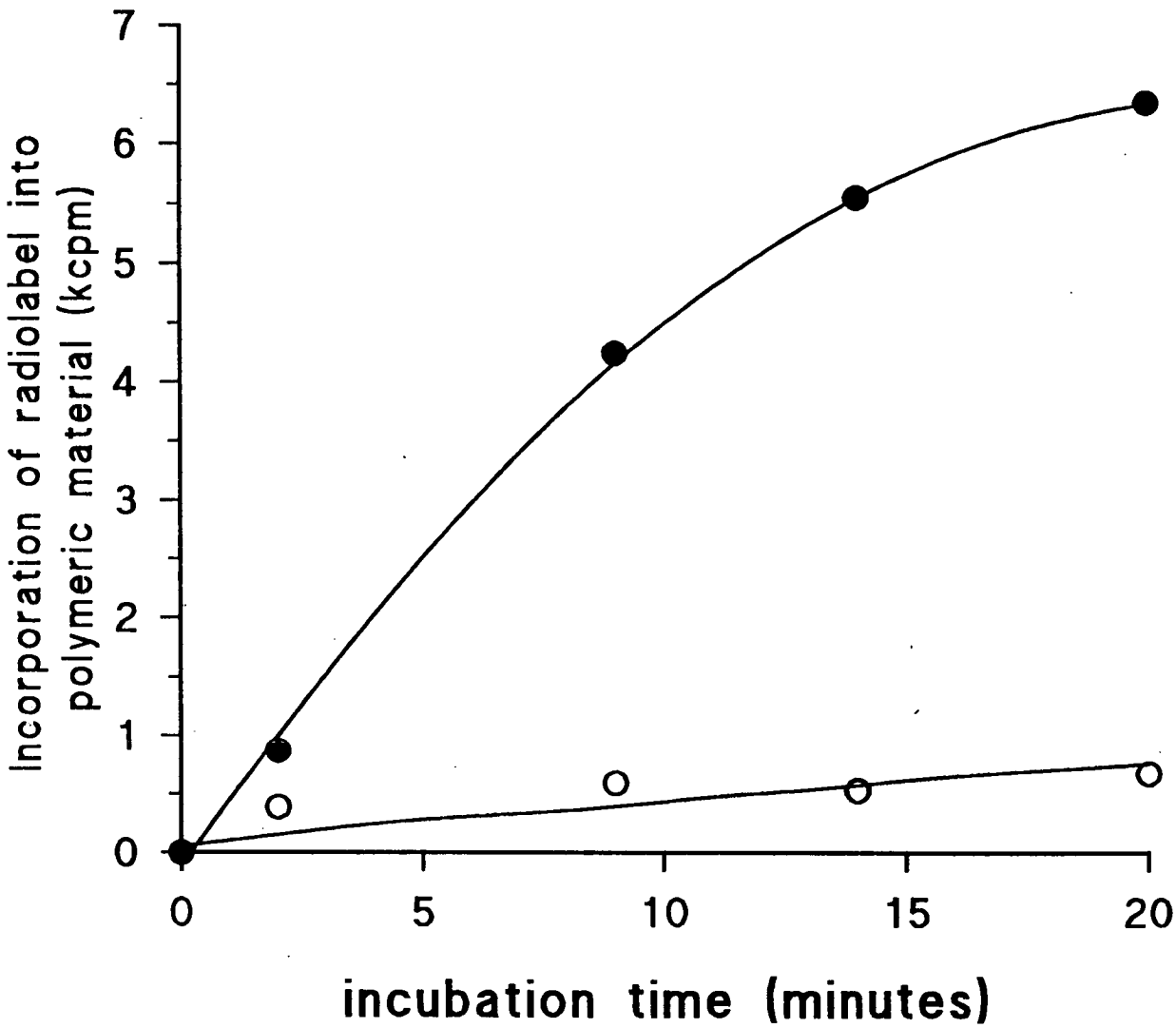


Fig 4.1.6.3 The incorporation of radiolabel from [^{14}C]ferulic acid into polymeric material. Assays were conducted using 0.5 kBq [^{14}C]ferulic acid, 2 mM $CoCl_2$, 75 µl membrane preparation, pH 7 and contained either 55 units of catalase (O) or an equivalent volume of buffer (●). Points represent triplicate assays from two membrane preparations.

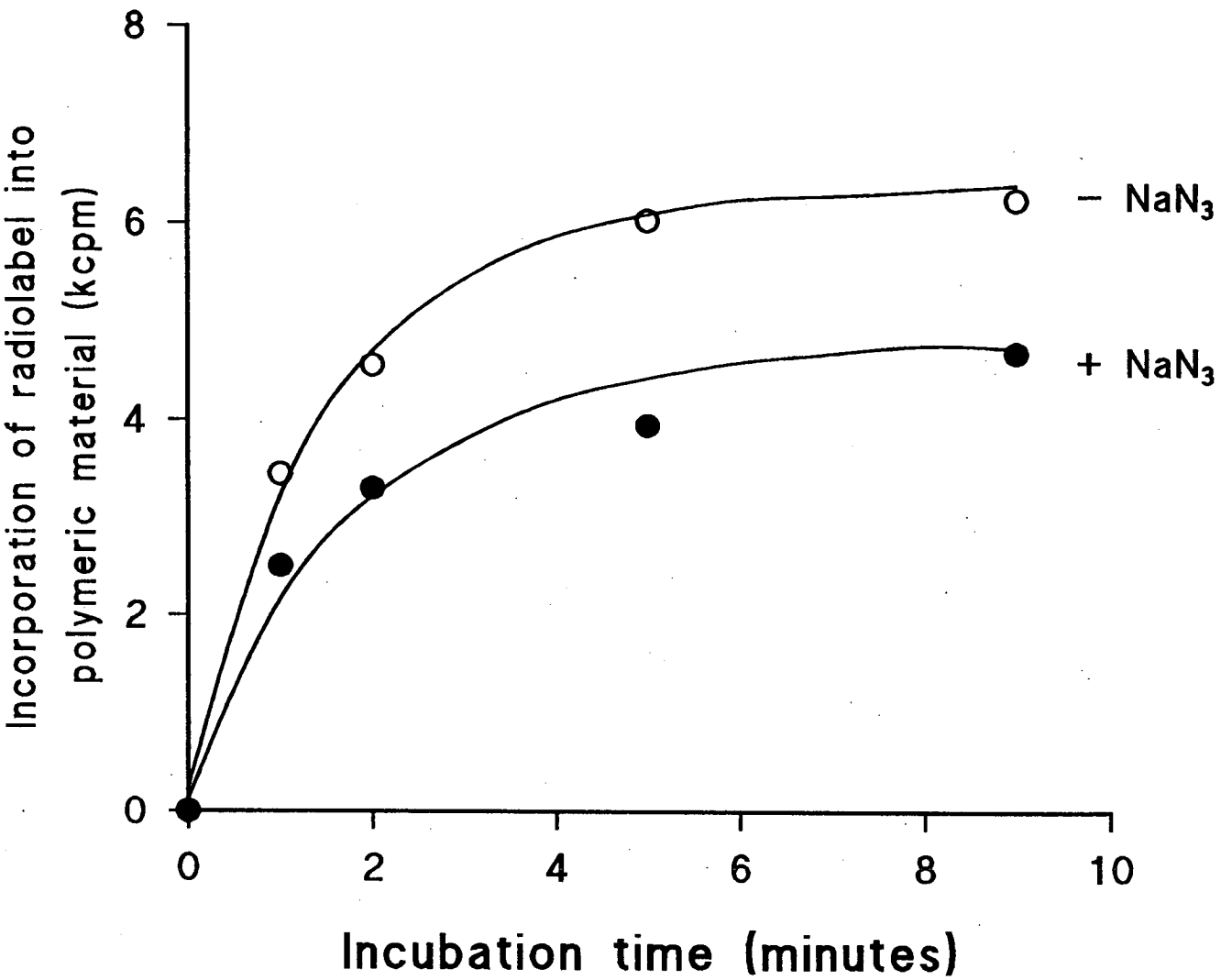


Fig. 4.1.7 The effect of sodium azide on the incorporation of radiolabel from [methoxy-^{14}C]feruloyl-CoA into polymeric material. Assays were conducted with 0.5 kBq [^{14}C-methoxy]feruloyl-CoA, 2 mM $CoCl_2$, pH 7 and either (●)10 mM NaN_3 or (O) an equivalent volume of buffer. Points represent the mean of duplicate assays from two membrane preparations.

suggested that there was some non-H_2O_2 dependent activity towards feruloyl-CoA. It is possible that this long term activity was the result of a feruloyltransferase and the product may be a feruloylated polysaccharide. In order to provide some preliminary indication of the nature of the product, assays, containing catalase, were conducted over an hour and the polymeric product was precipitated with EtOH:HOAc (6:4), washed and then treated with 0.1 M TFA. This treatment would be expected to release [^{14}C]feruloyl-arabinose from the expected product of a maize feruloyl-CoA: polysaccharide feruloyltransferase reaction, feruloylated arabinoxylan.

Paper chromatography of the products of TFA hydrolysis in three solvent systems (BAW, EPW, EAW, section 2.2.3) resulted in the detection of a radiolabelled compound that exactly co-chromatographed with authentic internal marker feruloyl [^{3}H]arabinose (fig 4.2). The percentage of the supplied [methoxy-^{14}C]feruloyl-CoA incorporated into this compound was very small, up to 1% of the supplied [methoxy-^{14}C]feruloyl-CoA. Upon saponification this product released a radiolabelled substance with the same Rf. value as ferulic acid. It is possible that polysaccharide feruloyltransferase is active in these membrane preparations, but at a very low activity, and although these results are not inconsistent with the presence of a feruloyltransferase, neither are they strong evidence for its activity in membranes from maize.

4.3 Preliminary investigation of the peroxidase activity of membrane preparations.

Membrane preparations exhibit catalase-sensitive incorporation of feruloyl-CoA and ferulic acid into insoluble material, and thus apparently have peroxidase activity towards feruloyl-CoA and ferulic acid. This would imply that the results presented previously in this chapter represent peroxidase activity

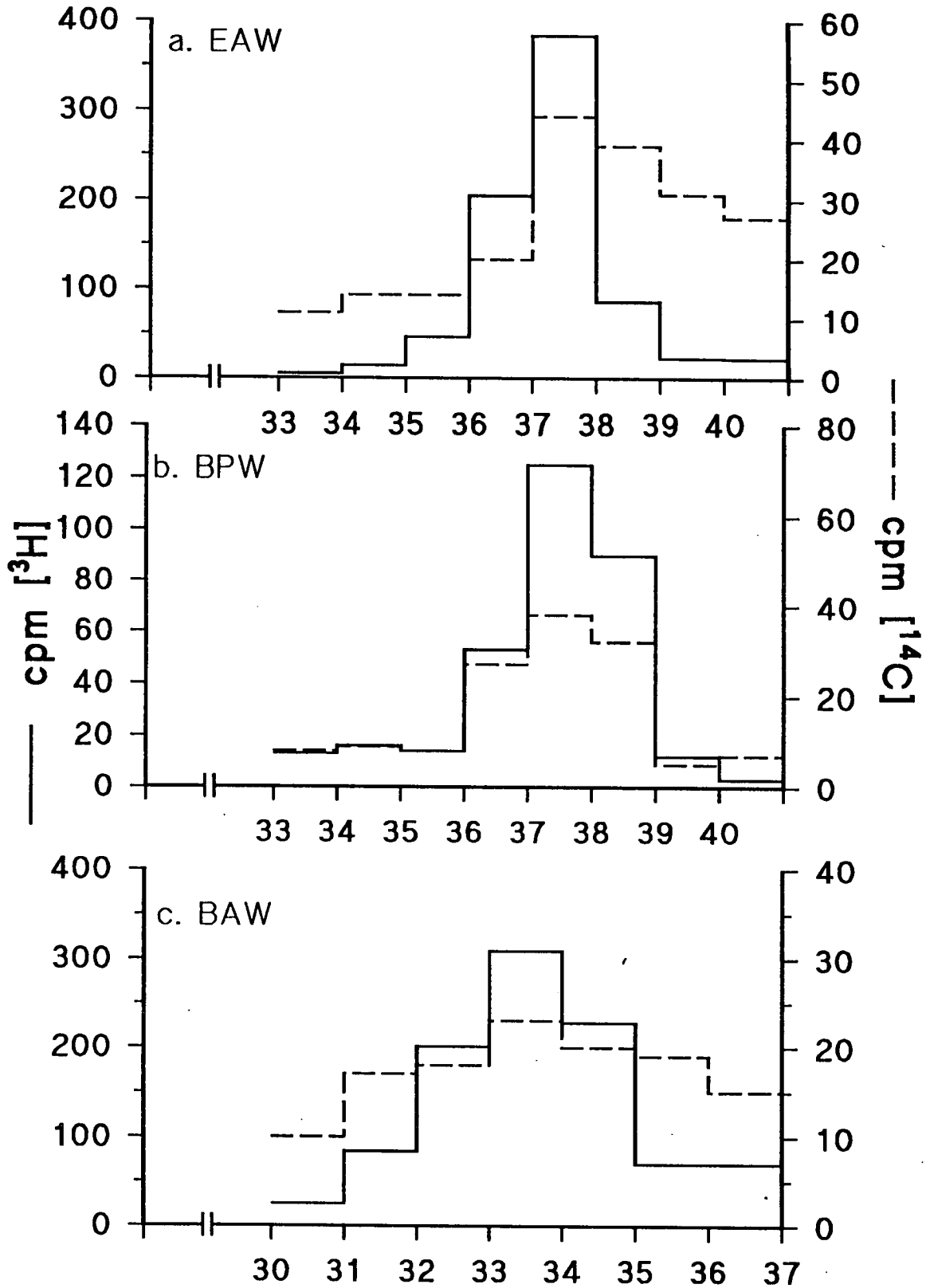


Fig. 4.2 p.c. of acid hydrolysate of the product of membrane assays. Assays contained 100 units of catalase and were incubated for 45 minutes. The polymeric product was washed and acid hydrolysed with 0.1 M TFA, 1 hr 100°C and the solubilised product (+ fer[^{3}H]-ara) chromatographed in three solvents.

rather than the effects of a feruloyltransferase. Some experiments were conducted to investigate some of the apparently anomalous properties of this peroxidase activity.

4.3.1 Experiments to investigate the peroxidase activity of membrane preparations against guaiacol.

Guaiacol is frequently used as a standard substrate to determine peroxidase activity. Therefore, to confirm the presence of peroxidase activity, membrane preparations were assayed for activity against guaiacol (section 2.4.4.3).

Membrane preparations were capable of oxidising guaiacol, resulting in a rapid increase of absorbance at 470 nm but only if H_2O_2 was supplied exogenously (fig. 4.3.1). Membrane preparations alone did not appear capable of oxidising guaiacol. This result confirms the presence of a peroxidase in membrane preparations.

4.3.2. The effect of dithiothreitol on the peroxidase activity of membrane preparations.

It has been shown that some plant peroxidases possess a thiol oxidase function (Pichorner *et al.* 1992) and will use O_2 to oxidise thiol groups via reactive thiyl radicals. The reaction of the thiyl radical with O_2 results in the formation of the superoxide radical and this further reacts to form hydrogen peroxide and this last step is stimulated by the presence of super oxide dismutase. In this way thiol groups can contribute to the generation of hydrogen peroxide by peroxidases (for proposed mechanism of this reaction, see section 4.4.2)

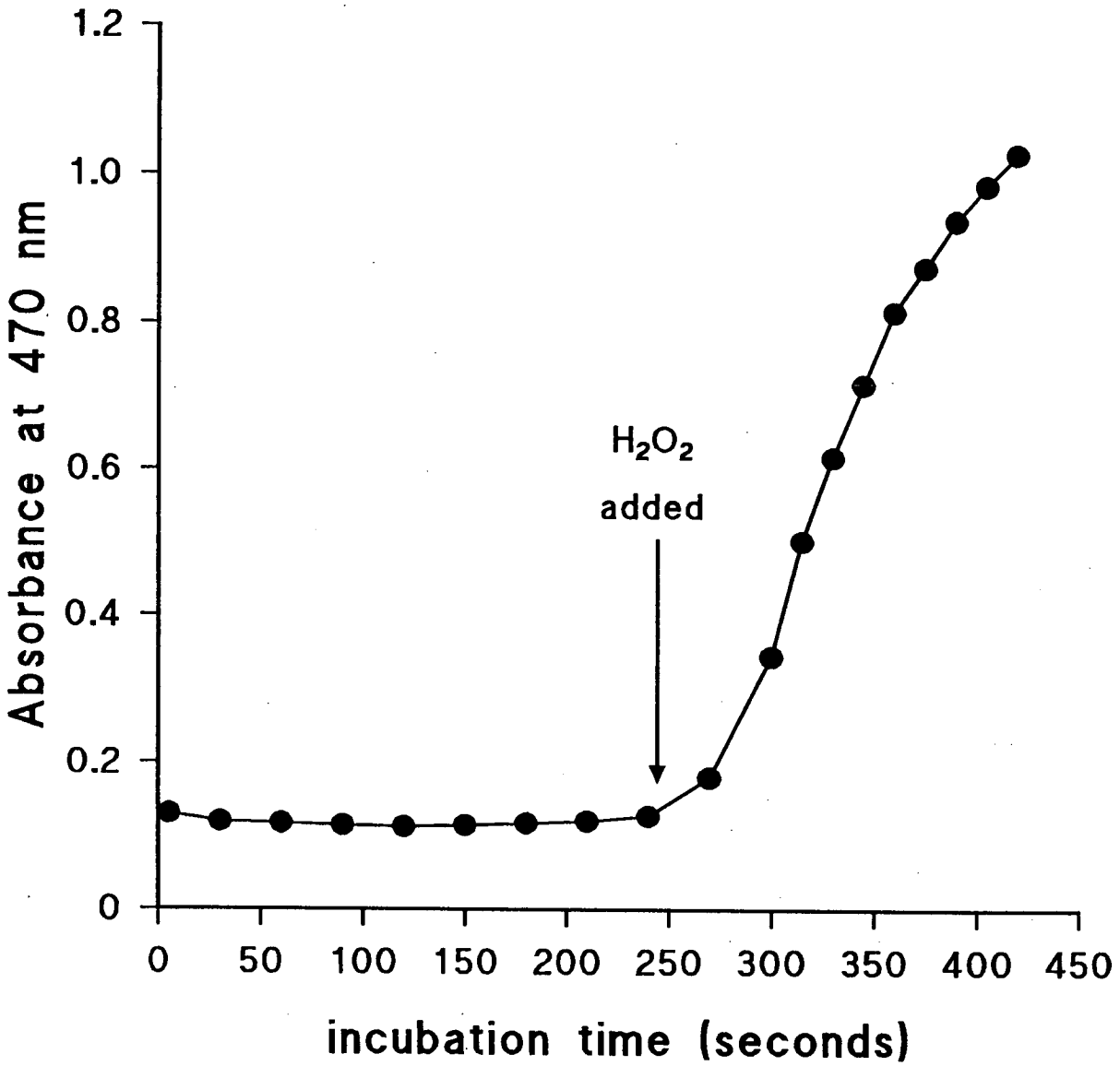


Fig. 4.3.1 The oxidation of guaiacol by membranes. Assays were conducted with 4 mM guiaicol in phosphate buffer, pH 7.5. 100 µl membrane preparation was assayed in a total volume of 1 ml. 2 mM H_2O_2 was added where indicated. The assay did not contain $CoCl_2$ or DDT.

The presence of thiols will temporarily inhibit the action of peroxidase against a model substrate such as guaiacol, since thiol groups, being strong reducing agents, will be preferentially oxidised. Dithiothreitol was routinely added to the membrane re-suspension medium as a protein protectant (Cleland 1964) and it is possible that its presence may have affected the results obtained from previous assays demonstrating the apparent requirement of membranes for cobalt ions (section 4.1.7).

Membrane preparations in the presence or absence of 1 mM DTT were incubated with guaiacol, with the addition of 2 mM H_2O_2 (Fig. 4.3.2). Where thiol groups were not present there was rapid oxidation of guaiacol, but this activity was temporarily suppressed in the presence of thiol groups for several minutes before oxidation occurred. These data are very similar to those obtained by Pichorner *et al.* (1992) when investigating the horseradish peroxidase-catalysed oxidation of cysteine.

4.3.3 The activity of membranes against [methoxy-^{14}C]feruloyl-CoA in the absence of DTT and cobalt ions.

As DTT had been added routinely to the membrane re-suspension buffer it was possible that the lack of incorporation of radiolabel from [methoxy-^{14}C]feruloyl-CoA into insoluble material in assays not containing cobalt (section 4.1.2) was due to DTT acting as a competing electron donor substrate for peroxidase. The addition of cobalt ions to assay mixtures stimulated the incorporation of radiolabel into polymeric material, possibly by preventing DTT from competing with other electron donors e.g. feruloyl CoA.

To test this idea membranes were assayed for their activity against [^{14}C]feruloyl-CoA without the addition of H_2O_2, DTT or cobalt.

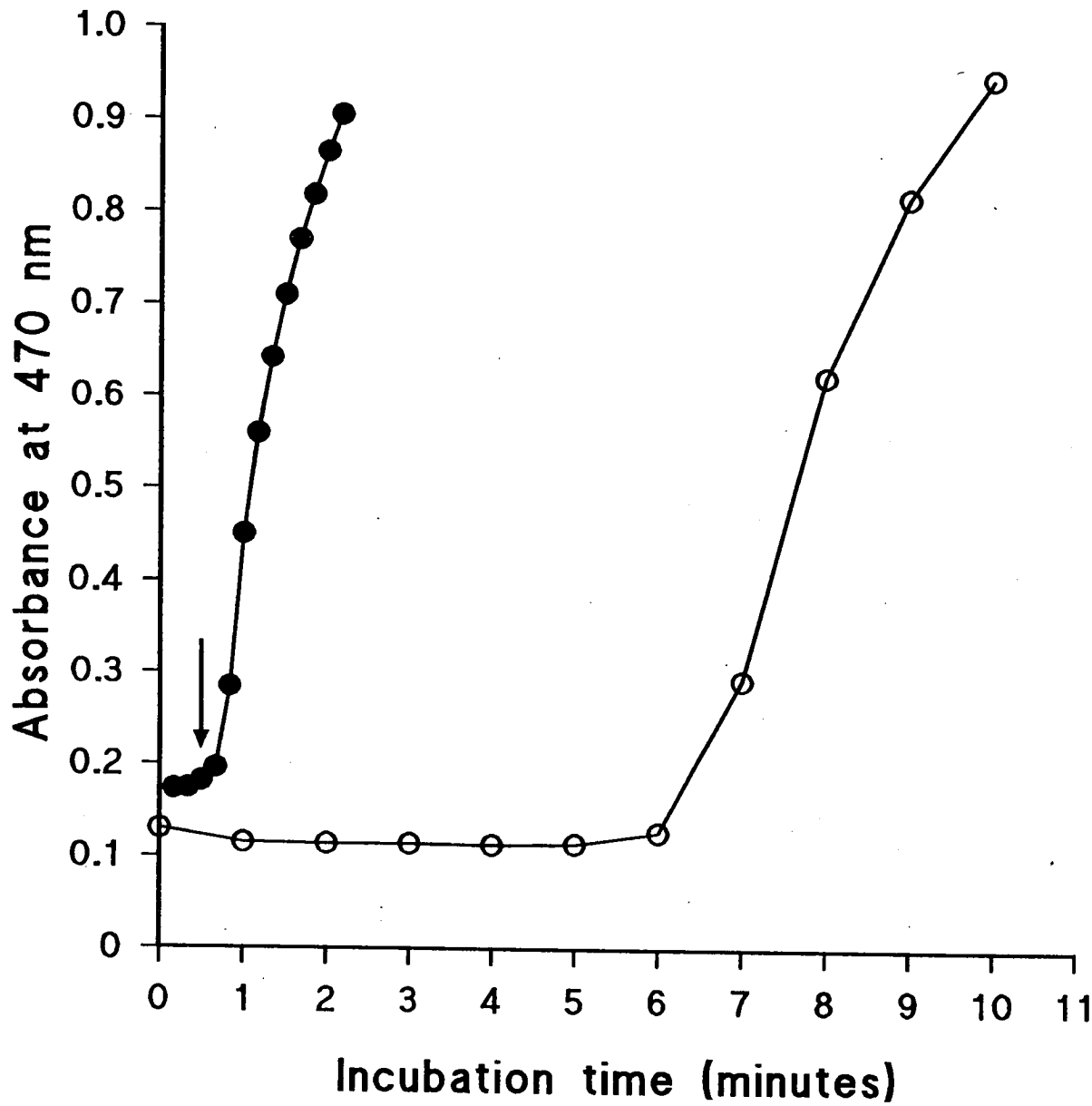


Fig. 4.3.2. The effect of DTT on the oxidation of guaiacol by membrane preparations. Assays were conducted with 4 mM guaiacol, 2 mM H_2O_2 in phosphate buffer, pH 7.5 with the addition (O) or exclusion (●) of DTT. The arrow indicates the time of addition of 100 µl membrane preparation (total volume 1 ml).

Assays containing membranes and [^{14}C]feruloyl-CoA in the absence of DTT and exogenous H_2O_2 showed incorporation of radiolabel into polymeric material, but this activity was inhibited by the addition of 1 mM DTT to the reaction mixture (fig 4.3.3). However, when cobalt was added to the assay at 2 mM the activity was only slightly less than in assays lacking both DTT and Co^{2+}.

Cobalt will interact with thiols and form an insoluble precipitate (Capozzi and Modena 1974). Thus apparent stimulation of enzyme activity by cobalt ions is presumably due to the removal of DTT, which would have been preferentially oxidised by peroxidase, from the re suspension buffer. This fact would explain the apparent lack of efficacy of other divalent ions as co-factors, since only certain ions e.g. Co^{2+} and Ni^{2+} will form insoluble precipitates with thiol groups.

4.4 Discussion of results.

4.4.1 Assays for a putative feruloyltransferase.

In the experiments described above I attempted to demonstrate the activity of a feruloyltransferase responsible for the feruloylation of cell wall polysaccharides.

Initial results suggested that endomembrane preparations from maize cell cultures possessed feruloyltransferase activity since they were capable of catalysing the incorporation of radiolabel from [^{14}C]feruloyl-CoA into an insoluble (presumably polymeric) product. This activity was similar to that reported by Meyer *et al.* (1991).

However, this activity was almost completely suppressed by the addition of catalase to the assay mixture, thus indicating that the incorporation of radiolabel into insoluble material was dependant on H_2O_2 - contrary to expectation for a feruloyltransferase activity, but indicative of a peroxidase activity.

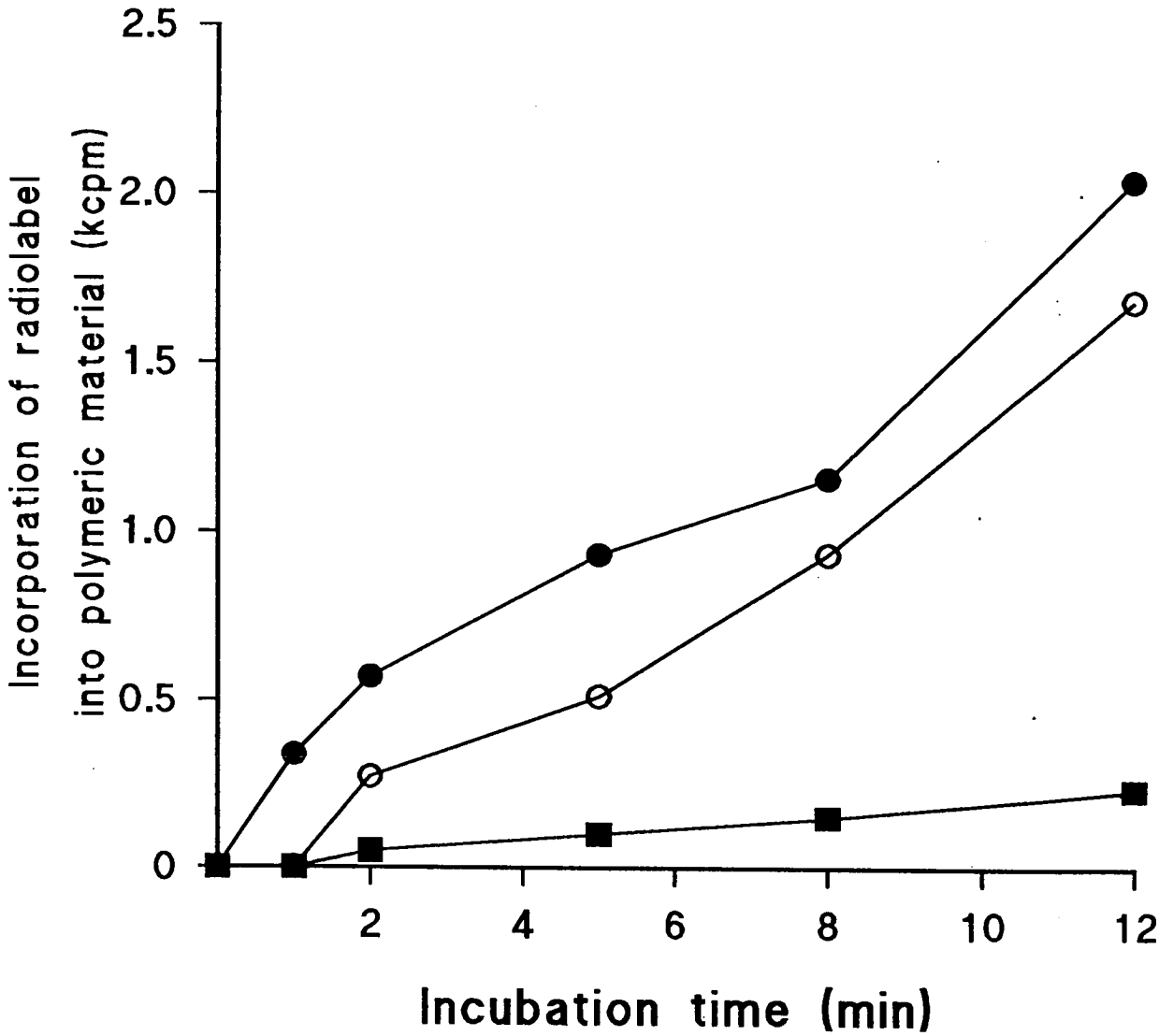


Fig. 4.3.3 The interactive effect of cobalt and DTT. Membranes preparations were assayed for their ability to transfer radiolabel from [methoxy-14C]feruloyl-CoA into polymeric material in the presence and absence of DTT and Cobalt. (●) No additions, (■) + 1 mM DTT, (O) + 1 mM DTT and 2 mM $CoCl_2$

Initial results had been highly suggestive of a feruloyltransferase activity, with a number of results, e.g. divalent cation dependency, enhancement by exogenous polysaccharide substrate, consistent with those obtained in other polysaccharide synthase studies

4.4.2 Interactions of DTT and Cobalt.

The transfer of radiolabel from [methoxy-^{14}C]feruloyl-CoA appeared to be dependent on the presence of cobalt ions in the assay mixture. It is likely that the absolute requirement for cobalt in this system was not due to cobalt acting as a co-factor or stabilising ion within the enzyme, but rather to its tendency to interact with thiol groups to form precipitates.

Many heavy metal ions, e.g. Ni^{2+}, Cu^{2+}, Pd^{2+}, Fe^{3+} will catalyse the oxidation of thiols by molecular oxygen, and this phenomenon has been quite thoroughly investigated (Capozzi and Modena 1974, Cullis *et al.* 1968). However, metal ions in an aqueous solution will undergo hydrolysis to produce slightly soluble hydroxides according to the reaction:

$$M^{2+} \leftrightarrow MOH^{+} + H^{+} \leftrightarrow M(OH)_2 + H^{+}$$

The addition of thiols to these slightly soluble hydroxides causes the formation of conjugates of metal and thiol which often precipitate. This precipitation is often accompanied by a change in colour, particularly if the metal species is a transition metal. It was noted that membrane assays containing cobalt developed a red/brown colouring at pH 6.8 and above. This change in colour was not evident at pH 5.5 and the depth of colour increased as the pH rose. This could be due to an increase in the concentration of $Co(OH)_2$ in suspension since its formation will be favoured at higher pH (the pKa for hydrolysis of cobalt to the hydroxide ion, $CoOH^{+}$, is pH 8.9 and onset of precipitation occurs at 2-3 pH units lower than the pKa). This precipitate has

been characterised in the reaction of ethanthiol with Co^{2+}, Ni^{2+} and Pd^{2+} ions as $Co(SC_2H_5)_3$, $(C_2H_5S)_3NiOH$ and $Pd(SC_2H_5)$ respectively (Hopton *et al.* 1968).It is possible that the changing interaction of cobalt and DTT with increasing pH may have affected peroxidase activity resulting in a sharp decline in activity at pH 8.

In the assays presented here it is probable that cobalt will react with the dithiothreitol present in the resuspension medium, thus forming a precipitate and removing DTT from solution. If cobalt is not present the DTT will remain in solution. This is an important factor in these assays where a peroxidase is present, because thiol groups will act as electron donors for peroxidase.

Pichorner *et al.* (1992) have demonstrated the thiol oxidase activity of horseradish peroxidase. The peroxidase will oxidise a thiol group to yield a reactive thiyl radical thus reducing the haem group from Fe^{3+} to Fe^{2+}.

i.e. (i) $\mathbf{E\text{-}Fe^{3+} + RSH \rightarrow E\text{-}Fe^{2+} + RS^{\bullet} + H^{+}}$

The reduced enzyme may then combine with molecular oxygen to form compound 3:

i.e. (ii) $\mathbf{E\text{-}Fe^{2+} + O_2 \rightarrow E - Fe^{3+}O_2^{\bullet -}}$

Compound 3 can then react further with a thiol group to produce another thiyl radical, H_2O_2 and the regenerated enzyme.

i.e. (iii) $\mathbf{E - Fe^{3+}O_2^{\bullet -} + RSH \rightarrow E\text{-}Fe^{3+} + HO_2^{-} + RS^{\bullet}}$

In this way thiols will compete with other compounds, e.g. phenylpropanoids, as electron donor substrates of peroxidase. After their production the thiyl radicals can react with each other to form the disulphide, and this reaction is favoured as the concentration of thiyl radicals increases.

The thiyl radical may also react with molecular oxygen leading to the production of the superoxide radical:

i.e. (iv) $RS^{\bullet} + O_2 \rightarrow RSOO^{\bullet} \rightarrow RS^{+} + O_2^{\bullet -}$

(v) $RS^{\bullet} + RS^{-} \rightarrow RSS^{\bullet -}R$

(vi) $RSS^{\bullet -}R + O_2 \rightarrow RSSR + O_2^{\bullet -}$

The superoxide radical then is reduced to produce peroxide:

i.e. (vii) $2H^{+} + 2O_2^{\bullet -} \rightarrow H_2O_2 + O_2$

This last step is stimulated by superoxide dismutase, which has been reported as being bound to plant membranes (Hayakawa *et al.* 1985).

In the work presented, DTT was routinely added to membrane preparations and may have been acted on by any peroxidase present. Since thiols are strong reducing agents they are more likely to be oxidised by peroxidase and therefore will 'out compete' other possible substrates. The peroxidase will oxidise the DTT in preference to the [methoxy-^{14}C]feruloyl-CoA and thus there will not be an incorporation of radiolabel into polymeric material, resulting in an apparent lack of 'feruloyltransferase' activity in assays that contain DTT. However, if cobalt is added to the assay mixture, the Co^{2+} ions and DTT will form an insoluble complex which has the effect of removing DTT from solution. Since DTT is no longer available as an electron donor, peroxidase will oxidise the supplied substrate, [^{14}C]feruloyl-CoA, leading to a transfer of radiolabel from feruloyl-CoA into polymeric material. This is clearly demonstrated in figure 4.3.3.

In previous work on feruloyl-CoA : polysaccharide feruloytransferase (Meyer et al. 1991) cobalt was found to be the most stimulatory divalent cation, although calcium and magnesium ions were also effective as co-factors. The

efficacy of cobalt in this report could be due to its precipitation with DTT resulting in *peroxidase* activity against feruloyl-CoA

The possibility that cobalt was acting as a redox couple in the thiol mediated oxidation of feruloyl-CoA was considered. Manganese dependent peroxidases (MnPOD) have been isolated from the white rot fungi that are primarily responsible for the degradation of wood (Wariishi *et al.* 1989). These MnPOD are capable of acting on compounds with high redox potentials that are otherwise resistant to oxidation by peroxidases such as horseradish peroxidase. The first step is the oxidation of Mn^{2+} to Mn^{3+}, which occurs via a typical peroxidase catalytic cycle (Wariishi *et al.* 1988). The Mn^{3+} in turn oxidises thiol groups to reactive thiyl radicals, which then are capable of oxidising a variety of non-phenolic lignin model compounds (Wariishi *et al.* 1989) to produce aldehydes and coupled dimers.

Manganese and thiol dependent peroxidase activity has also been demonstrated with horseradish peroxidase, whereby HRP, if thiols and Mn^{2+} are supplied can oxidise substrates that are not usually utilised (McEldoon and Dordick 1991). Cobalt can exist as either Co^{2+} or Co^{3+} and is therefore capable of acting as a redox couple. However the utilisation of manganese in thiol mediated oxidation requires the presence of an organic acid e.g. lactate or malonate, to chelate the Mn^{3+} ion and stabilise it at a high redox potential. In the 'feruloyltransferase' assays described above no such organic acid was added. Also membrane preparations were able to catalyse the transfer radiolabel from feruloyl-CoA into polymeric material in the absence of both DTT and cobalt (fig 4.3.3), indicating that neither were necessary for peroxidase activity.

Since membranes possess peroxidase activity, and this activity is suppressed by the addition of catalase which removes H_2O_2, the membrane

preparations used in these assays must also contain H_2O_2. This H_2O_2 may be generated by the production of thiyl radicals as described above. The addition of cobalt to assays will remove DTT from solution and therefore prevent its oxidation by peroxidase and the subsequent production of H_2O_2 during the time course of the assay. However, DTT was present throughout the preparation of the membranes and they are resuspended in medium containing DTT prior to their use. It is suggested that oxidation of DTT in the membrane preparation before use in cobalt containing assays will allow the production of sufficient H_2O_2 for the peroxidase reaction. The preparation of membranes without any thiols added during homogenisation or preparation resulted in a total lack of incorporation of radiolabel into polymeric material.

Since peroxide is not produced during the time course of the assays (owing to the presence of cobalt), the decline in peroxidase activity seen over the time course of assays may be due to depletion of H_2O_2. This decrease did not appear to be as a result of depletion of the feruloyl-CoA since there appeared to be little degradation of the substrate, either through saponification to ferulic acid or through phosphohydrolysis (Negrel and Smith 1984) during assays (Data not shown).

Peroxidases themselves can generate H_2O_2 at the expense of NAD(P)H and this process is stimulated by Mn^{2+} and monophenols (Gross *et al.* 1977, Madder and Amberg-Fischer 1982). However in the 'feruloyltransferase' assays NAD(P)H was not added and thus the contribution of H_2O_2 via this pathway would be negligible.

Although the activity of membrane preparations against [^{14}C]feruloyl-CoA is almost completely suppressed by the addition of catalase to the assay mixture, there appears to be little inhibition of the reaction by sodium azide. NaN_3 is reputed to be a specific haem enzyme through its chelation of iron.

However the efficacy of NaN_3 as an inhibitor of peroxidase, at least in mammalian systems, has not been totally verified with reports of only partial inhibition of peroxidase at mM concentrations of NaN_3 (Straus 1972). Thus it would appear that if the transfer of radiolabel form feruloyl-CoA into polymeric material *is* due to peroxidase activity, this activity was partially resistant to inhibition by sodium azide, or the enzyme responsible for this activity is not a haem protein.

4.4.3 The effect of arabinoxylan on peroxidase activity.

Incorporation of radiolabel from feruloyl-CoA into insoluble material was stimulated and prolonged by the addition of arabinoxylan to the medium in which the cells were homogenised. This appeared to suggest that exogenous polysaccharides were becoming encapsulated into membrane vesicles and were being utilised as an acceptor substrate in a feruloyltransferase reaction . Meyer *et al.* (1991) suggested that the decline in feruloyltransferase activity observed in endomembrane preparations from parsley was due to the exhaustion of endogenous nascent polysaccharide acceptors, and the results presented here appeared, at first, to support the hypothesis that a feruloyltransferase was utilising polysaccharide as a feruloyl acceptor. However, if the activity present in the membrane preparations from maize was due to a peroxidase, the addition of arabinoxylan to the homogenisation medium would appear to increase the activity of peroxidase against feruloyl-CoA.

It is possible that the products of peroxidative oxidation (radiolabelled feruloyl radicals) are becoming linked to polysaccharides. It has been suggested that carbohydrate can enhance polymerisation reactions during the production of dehydrogenation polymers of phenolics, with certain

polysaccharides being more active in the polymerisation process than others (Siegel 1968, Stafford 1964).

Alternatively the polysaccharide molecules could act as a framework which may non-covalently interact with the feruloyl-CoA, perhaps optimising the orientation of the molecules for oxidative polymerisation.

4.4.4 The product of the peroxidase reaction.

The product of this membrane associated peroxidase is a polymer or oligomer (Rf 0.0 on p.c. in BAW) containing radiolabelled feruloyl groups, or derivatives of ferulic acid, which appears able to bind to cellulose. This product has not been characterised but it is possible to consider the nature of the product.

In a typical peroxidase reaction, the peroxidase would oxidise the (phenolic) substrate using H_2O_2, to form a number of free radicals. These free radicals can then spontaneously react with one another to form a wide range of products and ultimately dehydrogenation polymers. It is possible that the phenolic moiety of feruloyl-CoA is being oxidised and then randomly polymerising to form a product similar in nature to the dehydrogenation polymers produced through the action of peroxidase on monolignols (Ralph *et al.*1992, Robert and Brunow 1984, Ohnishi *et al.* 1992).

Proteins, particularly those containing hydroxyproline residues have been demonstrated to be susceptible to reaction with peroxidase generated dehydrogenation polymers of coniferyl alcohol (Whitmore 1978, 1982) and are often found closely associated with lignin-carbohydrate complexes (Ford 1986). It has been hypothesised that aromatic residues of wall proteins (e.g. tyrosine residues of extensin) may act as priming sites for lignification *in vivo* (Whitmore 1978) with peroxidase (or laccase) oxidatively linking these residues to

monolignols and phenolic acids. In the experiments described above it is feasible that the proteins present in the membranes are participating in the formation of a 'dehydrogenation polymer' of aromatic residues from feruloyl-CoA in a similar manner. This would result in the formation of radioactive material of high molecular weight.

The addition of arabinoxylan appears to stimulate the reaction, suggesting that it is being utilised as a co-substrate along with feruloyl-CoA. It has been shown that peroxidase generated dehydrogenation products of ferulic acid can become linked, via an alkali stable bond, to carboxymethylcellulose, microcrystalline cellulose and filter paper (Stafford 1964, Whitmore 1976). It is possible that the peroxidase in membrane preparations is oxidatively coupling ferulate (from feruloyl-CoA) and that the dimers and polymers thereof are being bonded to nascent polysaccharides or exogenously supplied arabinoxylan. There have been reports of dimers of ferulic acid being formed by membrane associated peroxidases (Gröger *et al.* 1993, Rodgers *et al.* 1993) and it is possible that such dimers may subsequently become further polymerised or attached to polysaccharides or proteins.

In vitro dehydrogenation polymers of coniferyl alcohol become covalently linked to carbohydrate when present in the reaction medium (Ohnishi *et al.* 1992) and this reaction appears to occur through the formation of quinone methide intermediates. Such reactive quinone methides appear to be produced by the action of horseradish peroxidase with feruloyl-esters (G. Wallace and S.C. Fry, University of Edinburgh, unpublished results), and it is likely that membrane associated peroxidase can produce similar intermediates from feruloyl-CoA which then become attached to carbohydrate through nucleophilic substitution. The addition of arabinoxylan *during resuspension* of the membrane pellet also increased the incorporation of radiolabel into polymeric material, but

was less effective than arabinoxylan added during the homogenisation of cell cultures (data not shown). Possibly polysaccharides may have to be closely associated with membranes in order for the them to be available as reactants with the quinone methide intermediates produced by the membrane associated peroxidase. Since the reaction of quinone methides with hydroxyl groups of sugar residues is a nucleophilic substitution reaction there is considerable competition for reaction sites from water. Close association of the polysaccharide and the products of peroxidatic oxidation (reactive quinone methides) through their binding to membranes may enhance the likelyhood of the reaction with a sugar moiety, rather than with water.

4.4.5 Possible roles of membrane bound peroxidases.

The homogenisation of tissue to produce an membrane preparation will disrupt sub-cellular organisation and compartmentalisation, resulting in the release of numerous previously separated enzymes into the homogenate. During this process it is feasible that peroxidases previously localised within the vacuole (Takahama and Egashira 1991) or chloroplast stroma (Asada *et al.* 1993, Nakano and Asada 1981) become associated with the membrane fraction through non-specific binding, thus creating an important experimental artifact.

However, investigations into membrane associated peroxidases suggest that their presence in membrane fractions of cells is a reflection of their in vivo localisation, rather than an artifact of the membrane isolation procedure.

The occurrence of peroxidases associated with membrane fractions of the cell has been reported in several species (Penel and Greppin 1979, Penel *et al.* 1979, Askerlund *et al.* 1987, Ros Barcelo *et al.* 1988, Rogers and Bolwell 1993). Generally they are thought to be only loosely bound to membranes although an integral membrane bound peroxidase has been localised in the

plasma membrane (Askerlund *et al.* 1987). The binding of peroxidases to membranes is enhanced by the presence of Ca^{2+} and Mn^{2+} and the removal of peroxidase activity from the microsomal fraction by treatment with concentrated salt solutions suggest that the enzyme is ionically associated with the membranes (Kiefer *et al.* 1985, Ros Barcelo *et al.* 1988). Peroxidases are glycoproteins and inhibition of binding of both basic and anionic peroxidases to membranes by chitosan fragments and tunicamycin suggests that the carbohydrate moiety of the enzyme is involved in the binding process (Penel 1991, Kiefer *et al* 1985). The involvement of a protein 'binding factor' has also been suggested (Penel 1991). The secretion of peroxidases from cell cultures is stimulated by the addition of calcium (Sticher *et al.* 1981) and this may be due to enhanced exocytosis.

Peroxidase is synthesised on the rough endoplasmic reticulum and then presumably secreted through the endomembrane system. Differential centrifugation studies suggest that peroxidases are associated with membranes during their transport to the wall in secretory vesicles, whence they are released into the wall (Ros Barceló *et al.* 1988). Anionic and cationic peroxidases have been found both associated with membranes and involved in wall metabolism (Zheng and van Huystee 1992, Ferrer *et al.* 1992) and it is likely that some of the peroxidases associated with membranes are in the process of being transported.

However, although peroxidases are likely to be transported whilst associated with membranes it as also possible that they have a specific physiological role within the endomembrane system.

Although ferulic and diferulic acid content of cell walls is correlated to a decrease in wall extensibility in oat and rice coleoptiles (Kamisaka *et al.* 1990, Tan *et al.* 1991), the ratio of ferulic to diferulic acid remains relatively constant

regardless of the growth rate or the extensibility of the wall. This suggests that the rate of formation of diferulate bridges is controlled not by the rate of oxidative coupling of feruloyl residues but by the rate of feruloylation of the polysaccharides. Data presented previously indicates that feruloylation is occurring post- or co-synthetically to nascent polysaccharides and it is feasible that peroxidases present in the Golgi system are responsible for the dimerisation of newly esterified phenolic residues. The localisation of two membrane associated peroxidases, one with high affinity for hydroxycinnamic acids, capable of dimerising ferulic acid to 5,5'-bisferulic acid (fig 1.2) has recently been reported (Rodgers *et al.* 1993, Bolwell 1993). Although the activity was subsequently localised also in the cell wall fraction (Zimmerlin and Bolwell 1993) it may also be involved in ferulate dimerisation within the EM system.

Since the formation of cross-links between wall polysacharides has implications for cell growth, their formation might be expected to be stricty regulated. Tight control of this process could be acheived if it occurred within the endomembrane system, possibly through peroxide availabilty or through peroxidase only being active in Golgi cisternae where mature polysacharides occur i.e., the *trans* Golgi network. Thus, a constant ratio of feruloyl to diferuloyl residues could be maintained although the rate of feruloylation may increase during the cessation of growth.

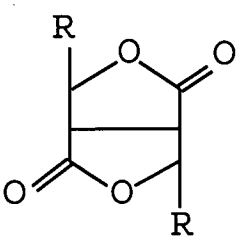
The formation of crosslinks via peroxidase generated quinone methide intermediates have been hypothesised (see fig I.5). However, models of such cross-links have proved difficult to synthesise *in vitro* (Wallace and Fry, unpublished results) owing to water acting as a strong nucleophile in competition with hydroxyl groups on sugars. If the reaction between quinone methides and polysaccharides were to occur in the endomembrane system

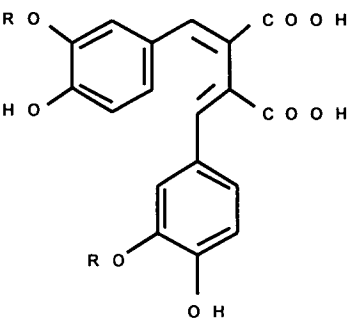
(rather than in the wall, as assumed), the *in vivo* reaction conditions could be altered to favour the formation of phenolic-carbohydrate bond above the reaction with water.

Earlier work (Stafford and Brown 1976) identified an enzyme, called ferulic acid dimerase, which dehydrogenatively coupled ferulic acid to form the 8,8' linked dilactone dimer (fig 4.4.5.1a). The reaction was light sensitive, stimulated by the addition of H_2O_2, inhibited by the presence of ascorbate and required a supply of air (presumably O_2). Evidence for it being a distinct enzyme from peroxidase was the lack of inhibition of the reaction by KCN and the fact that ferulic acid dimerase had a different distribution within the tissues from peroxidase. The majority of the ferulic acid dimerase activity was found associated with a 37,000g pellet, whereas the majority of peroxidase activity was soluble and precipitated with $(NH_4)_2SO_4$.

Gröger *et al.* (1993) recently reported a membrane bound peroxidase from maize capable of utilising hydroxyphenylpropanoids as a substrate. This enzyme was stimulated by the presence of $CaCl_2$ and $MgCl_2$ and was inhibited by the presence of cyanide and catalase. The majority of the product of the reaction with ferulic acid was identified as 8,8'-bis ferulic acid (fig 4.4.5.1b) with only minor amounts of other products. Similarly, a soluble peroxidase also producing 8,8'-dimers has recently been purified and named caffeate peroxidase. This peroxidase utilises only caffeic and ferulic acid as substrates (Frías *et al.* 1991) and the product has been identified as 8,8'-bis(caffeic) or 8,8'-bis(ferulic) acid, respectively (fig 4.4.5.1). It has been sugested that these peroxidases are involved in the formation of lignans, dimers of phenylpropanoids found widely distributed in plants and believed to have pharmacological activity (for a review see MacRae and Towers 1984). Little is known about their subcellular distribution or site of synthesis and it is plausible that enzymes

involved in lignan formation could be membrane associated, possibly with the tonoplast.





(a) where R= 3 methoxy phenol (b) R= CH_3 (feruloyl) or H (caffeoyl)

Fig. 4.4.5.1 Reaction products of (a) ferulic acid dimerase (Stafford and Brown 1976) and (b) caffeate peroxidase (Frias *et al.* 1991)

Thylakoid associated peroxidases are present in the chloroplasts (Miyake and Asada 1992) and it is possible that thylakoid membranes present in a crude membrane fraction will retain their peroxidase activity and oxidise exogenously supplied substrates.

The problems arising from presence of peroxidases in microsomal preparation have been addressed by Rodgers *et al.* (1993) with respect to the isolation of plant cytochrome P450, specifically the cinnamate-4-hydroxylase involved in phenylpropanoid biosynthesis. The polymerisation of feruloyl-CoA by microsmal fractions from maize appears to be due to a peroxidase rather than a feruloyltransferase. Previous work on the feruloyltransferase responsible for the feruloylation of polysaccharides (Meyer *et al.* 1991) did not investigate the possibility of peroxidase activity in the endomembrane preparations used in assays. It is possible that at least some of the activity attributed to a polysaccharide feruloyltransferase may in fact be due to peroxidatic oxidation activity. The fact that a donor: polysaccharide feruloyltransferase exists is not

debatable but it is prudent in future work on this enzyme to be aware of the possible artifact caused by the presence of peroxidases.

CHAPTER 5
ENZYME ACTIVITIES ALONG THE NEWLY EXTENDED MAIZE INTERNODE

5. Changes in Enzyme Activity along the Maize Internode.

The internodes of graminaceous monocots offer a model system in which to study the biosynthesis and deposition of secondary cell wall components. Owing to the presence of an intercalary meristem at the base of the internode there is a gradient of maturity along the internode, enabling the investigation of sequential events that occur during lignification and maturation. In order to assess whether the internode could provide a model system in which to study phenylpropanoid metabolism and the supply of lignin precursors during tissue maturation, enzyme activities were monitored along the internode.

The inbred lines Co125 and W401 have been extensively investigated by a number of collaborators working within the Zeneca Seeds Forage Maize Project because of their striking differences in cell wall digestibility (Dolstra 1992) and thus these two lines were used for enzymatic analysis.

5.1 Harvest and Protein Extraction.

At harvest plants had been silking for approximately five days. Co125 plants were taller and more robust than W401 plants, with longer and thicker internodes.

Internodes 2 and 4 were divided into five sections and individual segments of internode were ground in liquid nitrogen by a pestle and mortar. The soluble proteins were extracted, partially purified by precipitation with ammonium sulphate and desalted. Protein concentrations for individual preparations ranged from 0.117 mg ml^{-1} to 2.94 mg ml-1 in the W401 lines and 0.34 mg ml^{-1} to 2.32 mg ml^{-1} in the Co125 line. However, the vast majority of samples were in the range of 0.5 -1.5 mg ml^{-1}.

Extractable protein contents per gram fresh weight are reported in (Figs 5.1.1 and 5.1.2). There is was no consistent trend or difference in the

extractable protein content of tissue along the internode, although the apical and basal sections appear to have a higher extractable protein content than those in the middle of the internode. This may well be due to contamination of these sections with nodal meristematic tissue, which would have a high protein content. Previous work on these cultivars (Scobbie *et al.* 1993,) reported that protein content was highest in the youngest sections of the internode (base) and rapidly declined in older tissue. However these figures were calculated directly from the fresh tissue and so accounts for total protein as opposed to extractable protein as presented in Figs 5.1.1 and 5.1.2.

The extractable protein content of fresh tissue will vary because experimental procedures will introduce certain errors. Firstly the tissue was ground manually by a pestle and mortar and so individual segments of tissues may not be uniformally homogenized, especially as certain segments are more fibrous than others. Secondly, loss of protein during the extraction, precipitation and desalting procedure may not be at a constant percentage across all samples.

5.2 Establishing the Kinetics of the Enzyme Assays.

In order to ensure that radiometric assays were stopped and the products extracted whilst the enzyme mediated reaction was proceeding in a linear manner, the reaction velocities of PAL and OMT were measured with respect to substrate concentration, protein concentration and time, using protein extracts prepared from tissue from Co125 and W401 and from all parts of the internode. The spectrophotometric CAD assay was monitored on a chart plotter connected to the spectrophotometer. In order to calculate reaction velocity for CAD a tangent was taken to the initial *linear* portion of the trace of ΔA_{400}.

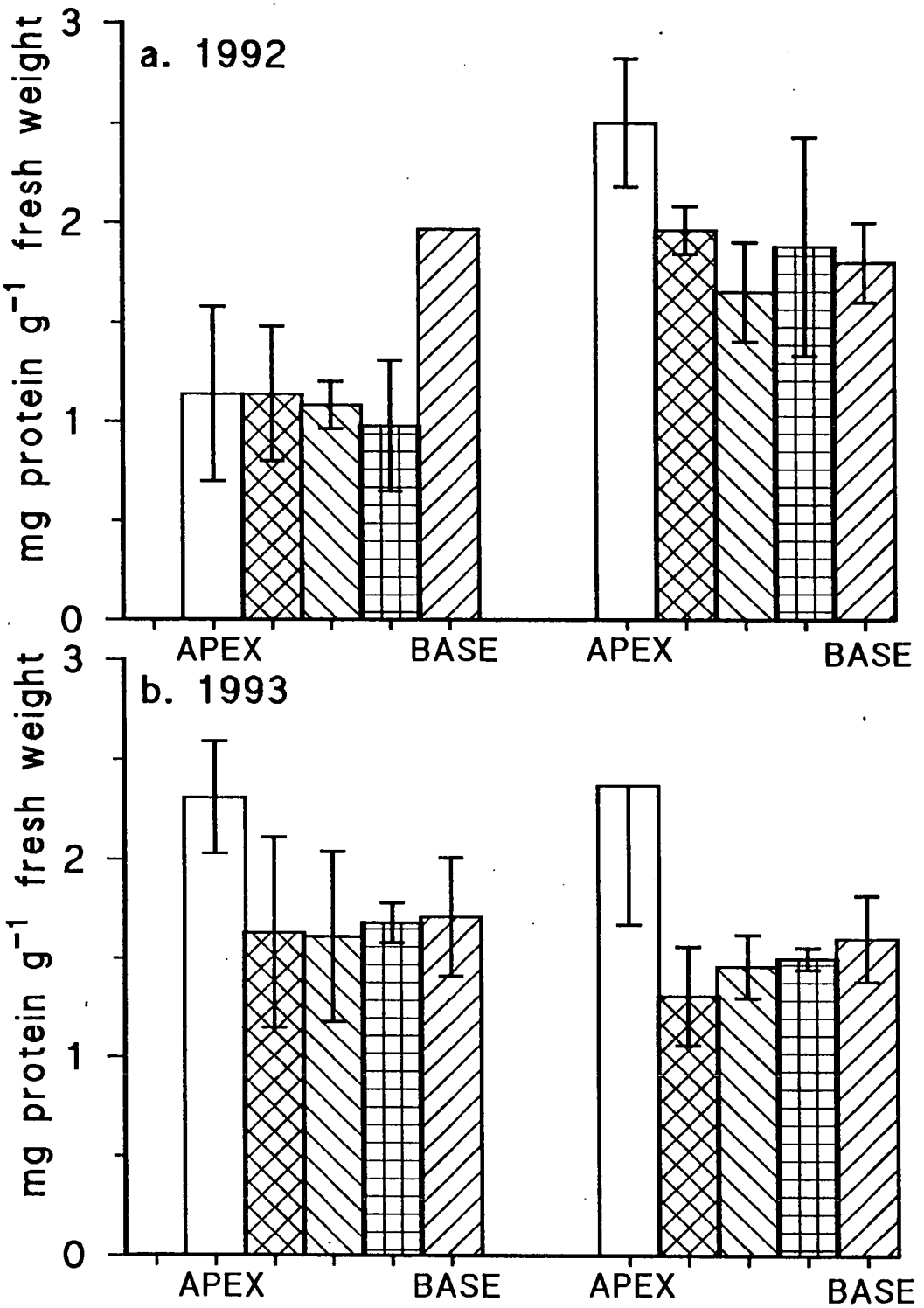


Fig 5.1.1 Extractable protein content of tissue from line Co125. Data is for segments along the internode for plants harvested in a) 1992 and b) 1993. Error bars represent standard errors of the mean of 4 plants.

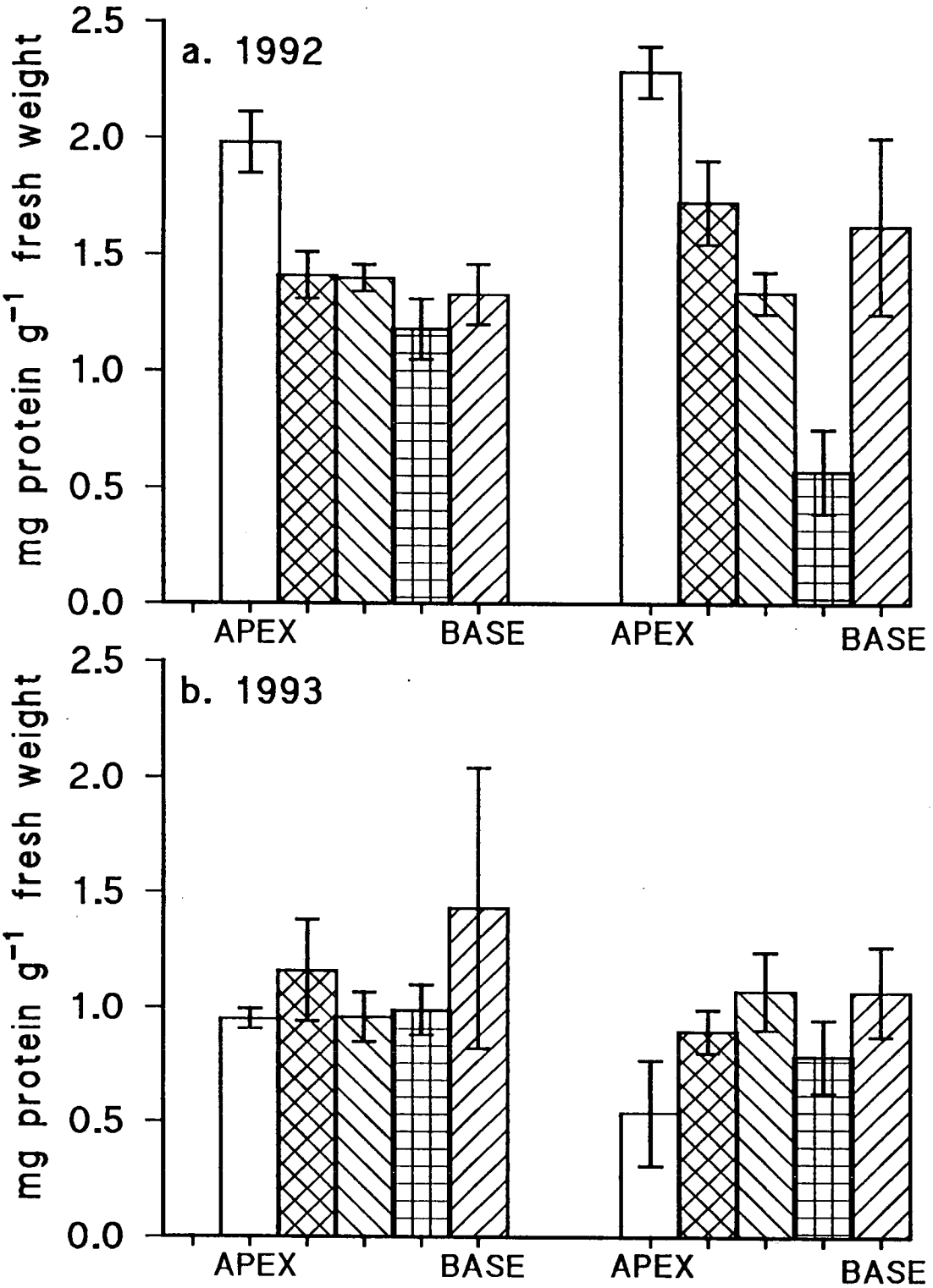


Fig. 5.1.2 Extractable protein content of tissue from line W401. Data is for segments along the internode for plants harvested in a) 1992 and b) 1993. Error bars represent standard errors of the mean of 4 or 5 plants respectively.

5.2.1 The Determination of the Km for PAL.

In order to determine the Km, i.e. the concentration of substrate required to result in half the maximal intial velocity in an enzyme mediated reaction, PAL activity was monitored over a range of concentrations of phenylalanine. Radiolabelled phenylalanine was added to retain the specific activity at 1 kBq $nmol^{-1}$. The reciprocals of substrate and intial reaction velocity were plotted on a Lineweaver-Burke plot and linear regression applied (fig 5.2.1). The reciprocal of Km for reaction is the intersect of the fitted curve with the X-axis. The Km was calculated as 288 μM, using the linear equation y=mx + b, where the constant terms m and b were calculated as 0.151 pkat mol^{-1} and 0.523 pkat respectively. When the data were plotted in the form [substrate] against [substrate] ÷ velocity (Hanes plot) the Km was calculated as 310 μM (data not shown). To ensure that substrate concentration was not limiting, routine assays were therefore conducted with 1 mM phenylalanine.

5.2.2 The Determination of Km for Caffeate O-Methyl Transferase.

Caffeate O-methyltransferase activity was monitored over a range of concentrations, with [^{14}C]SAM added to ensure a constant specific activity of 1 kBq $nmol^{-1}$. Intial reaction velocities were calculated, the reciprocals plotted and linear regression was applied (fig 5.2.2). Km was calculated as 247 μM, using the equation y=mx + b, where the constant terms m and b were calculated as 0.0372 pkat $mol^{-1}$and 0.15 pkat respectively. When data were plotted in the form [substrate] against [substrate] ÷ velocity (Hanes plot), the Km was calculated as 225 μM. Test assays were therefore conducted with 1 mM SAM.

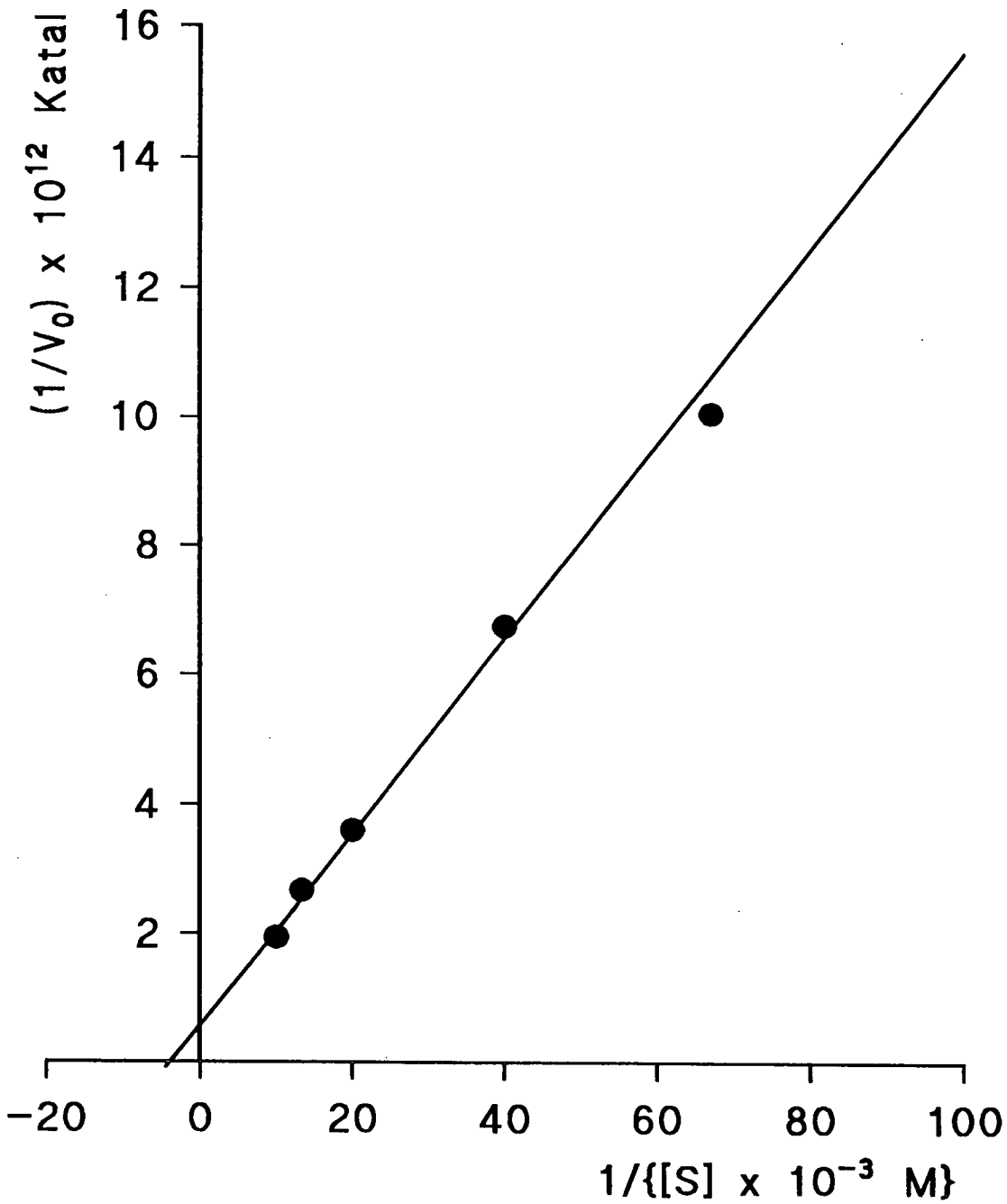


Fig 5.2.1 Lineweaver-Burke double reciprocal plot of PAL activity with increasing substrate concentration. Assays were incubated for 1 hour with a range of substrate concentrations. Points represent the average of triplicate assays and are adjusted for background radioactivity. The intersect of the fitted line with the x-axis represents -1/Km.

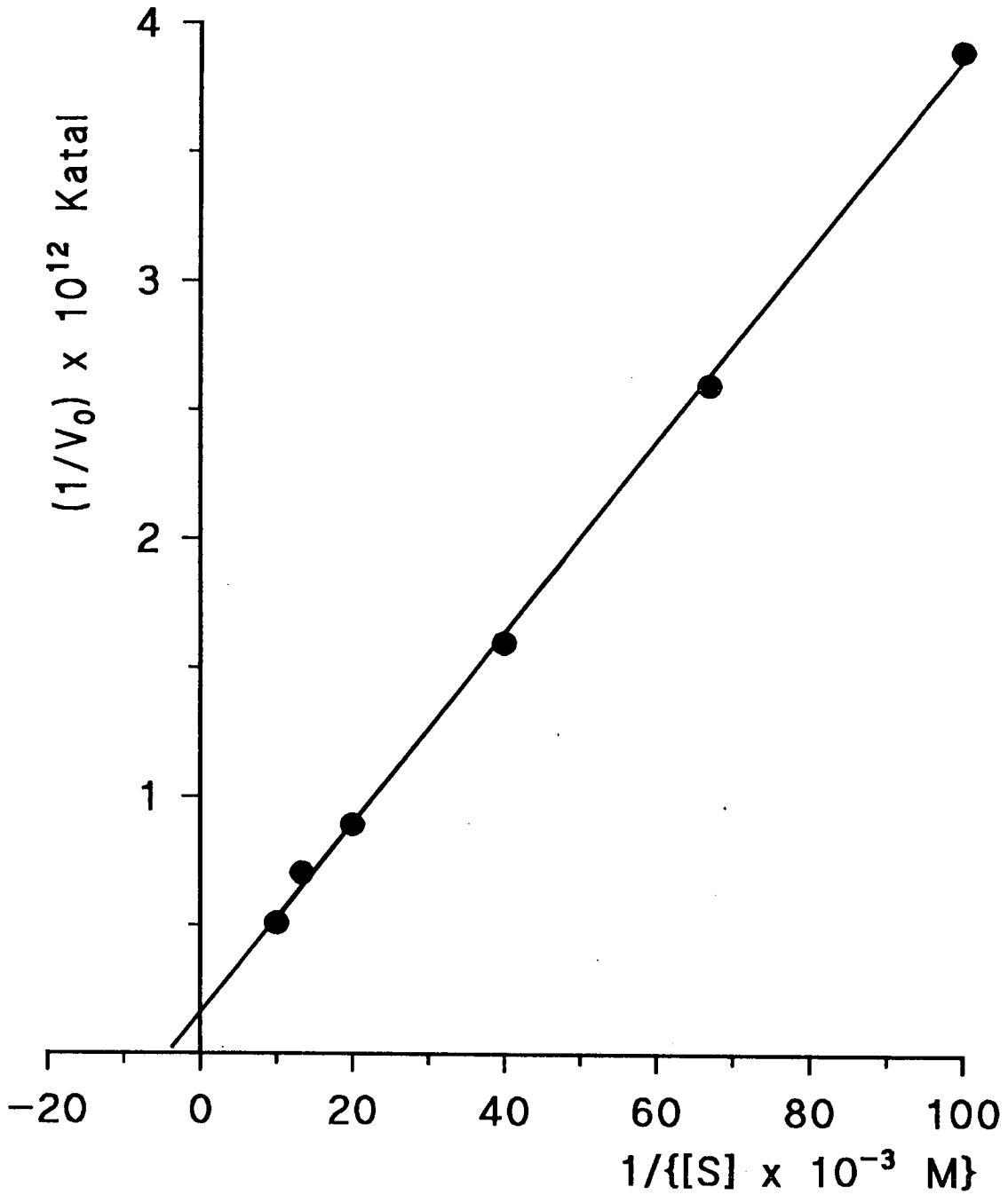


Fig 5.2.2 Lineweaver-Burke double reciprocal plot of OMT activity with increasing substrate concentration. Assays were incubated for 30 min with a range of initial substrate concentrations. Points represent the average of triplicate assays and are adjusted for background radioactivity. The intersect of the fitted line with the x-axis represents -1/Km.

5.2.3 The Calculation of Km for Coniferyl Alcohol Dehydrogenase.

CAD ativity was monitored by the increase in absorbtion at 400 nm at alkaline pH during the conversion of coniferyl alcohol to coniferaldehyde . Reaction assays were conducted using varying concentrations of coniferyl alcohol, and the initial reaction velocities calculated by taking tangents to the trace produced by a chart plotter during the initial linear period. The reciprocals of the initial velocity and [substrate] were plotted on a Lineweaver-Burke plot and linear regression applied (fig 5.2.3). The Km was calculated as 34 μM using the equation y=mx + b. where the constant terms m and b were calculated as 0.513 pkat mol^{-1} and 15.41 pkat respectively. When data was plotted in the form [substrate] versus substrate÷velocity (Hanes plot) the Km was calculated as 38 μM. Test assays were therefore conducted using 200 μM coniferyl alcohol.

5.2.4 PAL Activity with Respect to Time.

In order to ensure that tests assays would be terminated whilst the enzyme reaction was proceeding in a linear fashion, radiometric PAL assays were conducted over a four hour time course. The initial substrate concentration was 1 mM and assays were terminated after 1,2,3 and 4 hours incubation. Accumulation of product was linear over 4 hours (Fig 5.2.4). There was no decline in activity with time as might be expected if [substrate] was a limiting factor or end product inhibition was occurring. Thus it was assumed that routine assays conducted over two hours would proceed linearly.

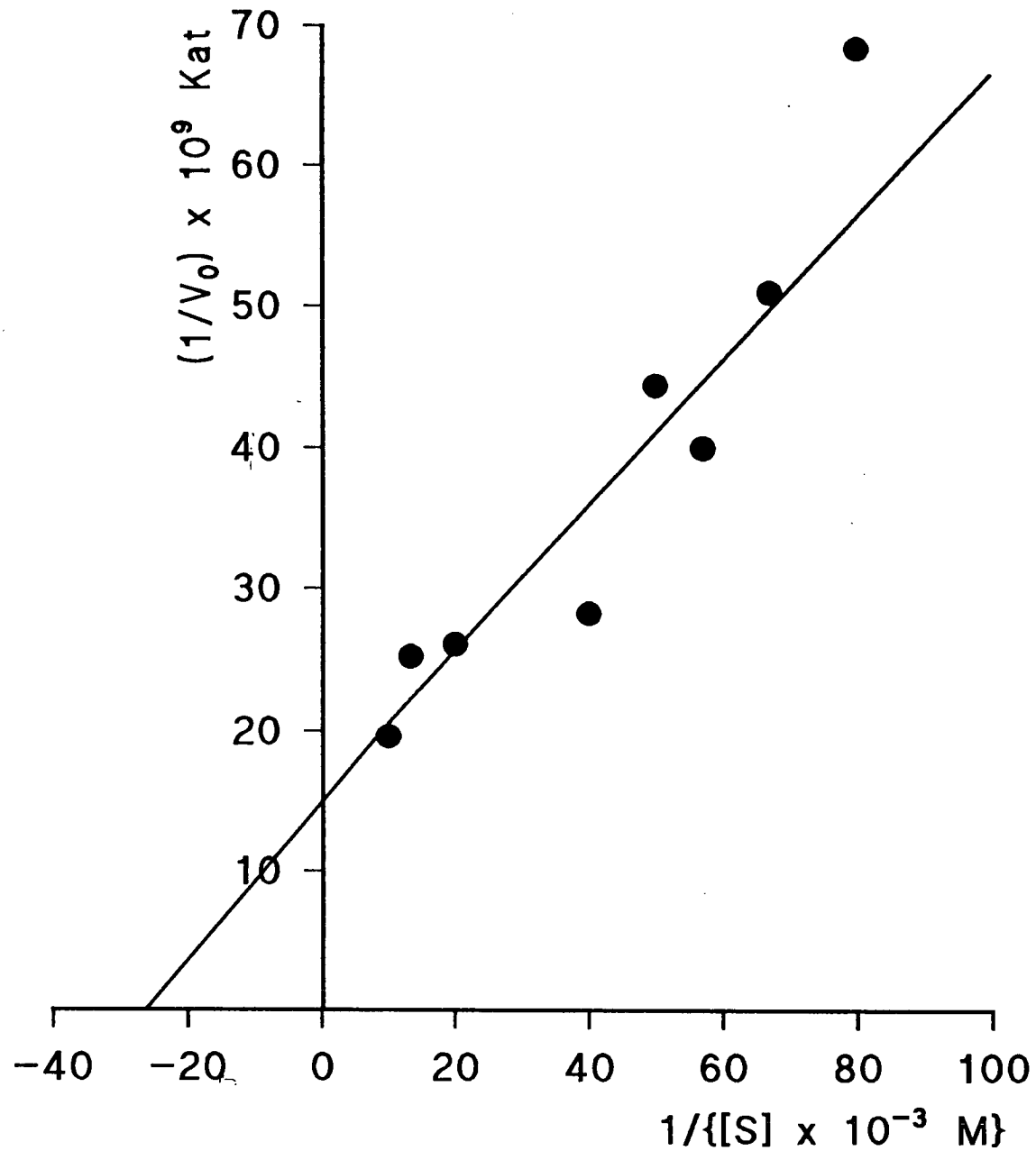


Fig 5.2.3 Lineweaver-Burke double reciprocal plot of CAD activity with increasing substrate concentration. Assays were incubated for several minutes with a range of initial substrate concentrations and the ΔA_{400} monitored. Points represent the average of triplicate assays. The intersect of the fitted line with the Y axis represents -1/Km.

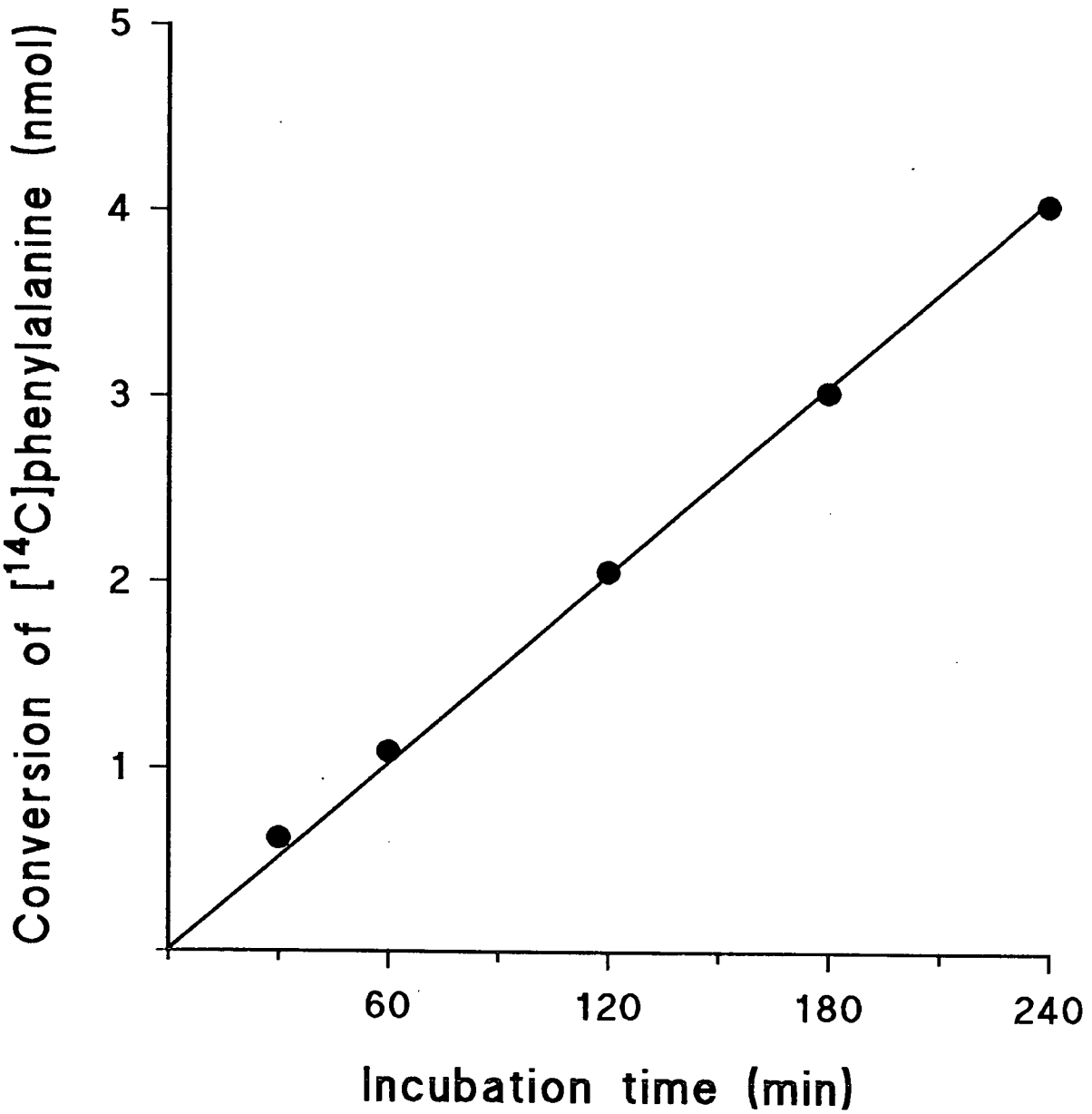


fig 5.2.4 PAL activity over time. Triplicate assays were incubated for 1,2,3 and 4 hours. Initial [^{14}C]phenyalanine concentration was 1 mM. The y axis represents accumulation of radioactivity into toluene extractable material.

5.2.5 Rate of conversion of phenylalanine by PAL with increasing protein concentrations.

The activity of PAL was measured with respect to increasing protein concentration. Assays were conducted using 1 mM phenylalanine in borate buffer and incubated for 2 hours with increasing volumes of enzyme extract. The rate of conversion of product per μg protein was constant with increasing protein concentration, upto ≈450 μg protein/assay, but decreased slightly at 800 μg (fig. 5.2.5).

The protein concentration of enzyme extracts tested ranged from 0.117 - 2.94 mg ml-1. This corresponds to a concentration of 8.78 - 220.5 μg/assay (assuming 75 μl preparation is used for each assay). At these protein concentrations the reaction can therefore be assumed to be obeying first order kinetics.

5.2.6 Rate of conversion of S-Adenosyl Methionine by OMT with Increasing Protein Concentrations.

Assays were conducted to measure caffeic acid O-methyltransferase activity with respect to the concentration of protein. Standard assays (section 2.5.3) were performed with increasing amounts of enzyme preparation. The rate of conversion of *S*-adenosyl[^{14}C]methionine increased approximately in proportion to protein concentration, up to 140 μg protein per assay (fig 5.2.6).

The majority of sample extracts had protein contents within this range (assuming 65 μl preparation is used for each assay) and thus reactions were assumed to be proceeding linearly during sample assays. When assayed, those few samples with a higher protein content gave reaction rates comparable to replicate samples.

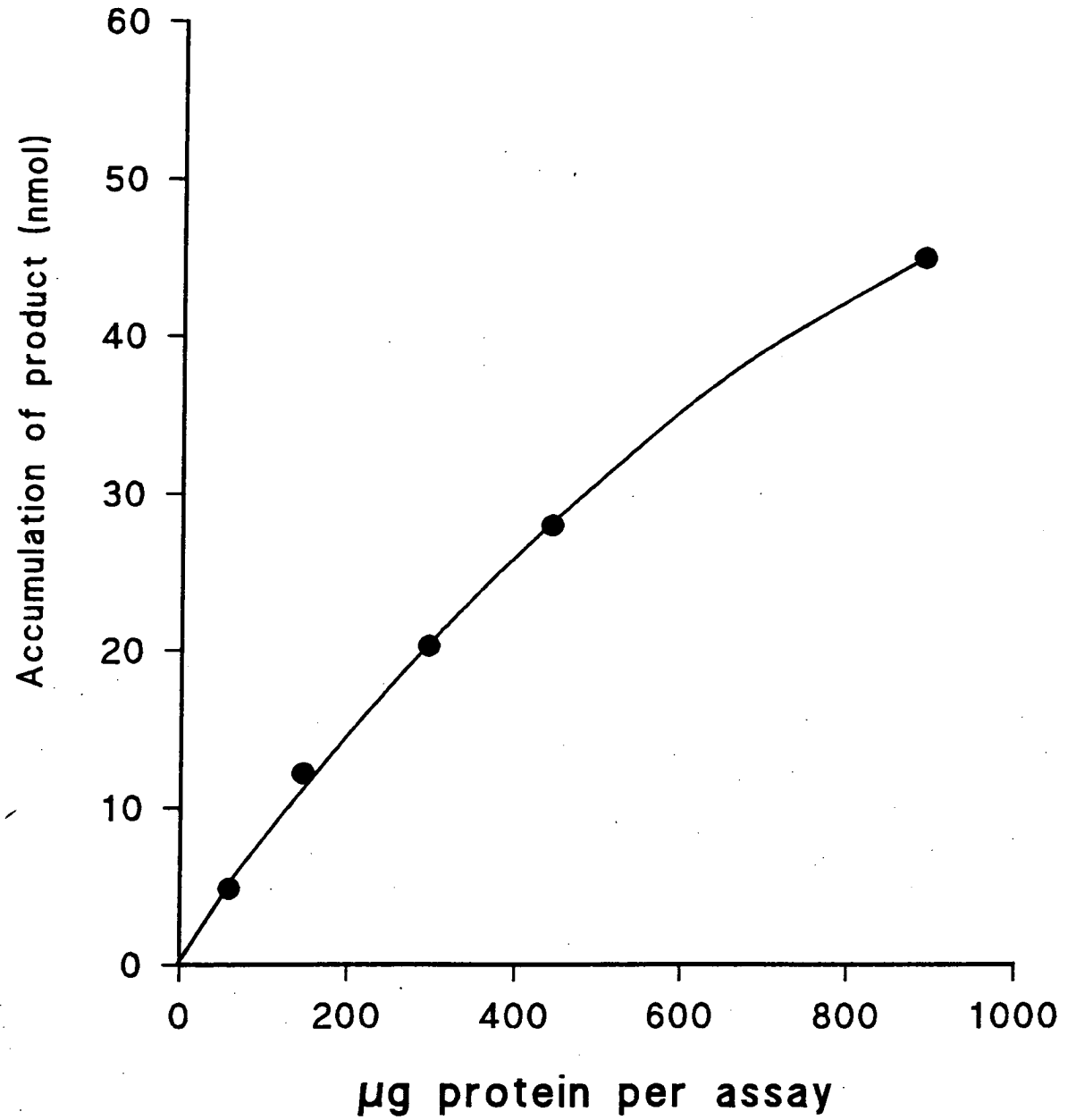


Fig. 5.2.5 PAL activity with respect to increasing protein concentration. Varying amoumts of enzyme preparation were incubated 1 mM [^{14}C]phenylalanine for two hours. Points represent the mean of triplicate assays adjusted for background radioactivity. The y axis represents the accumulation of radiolabel into toluene extractable material.

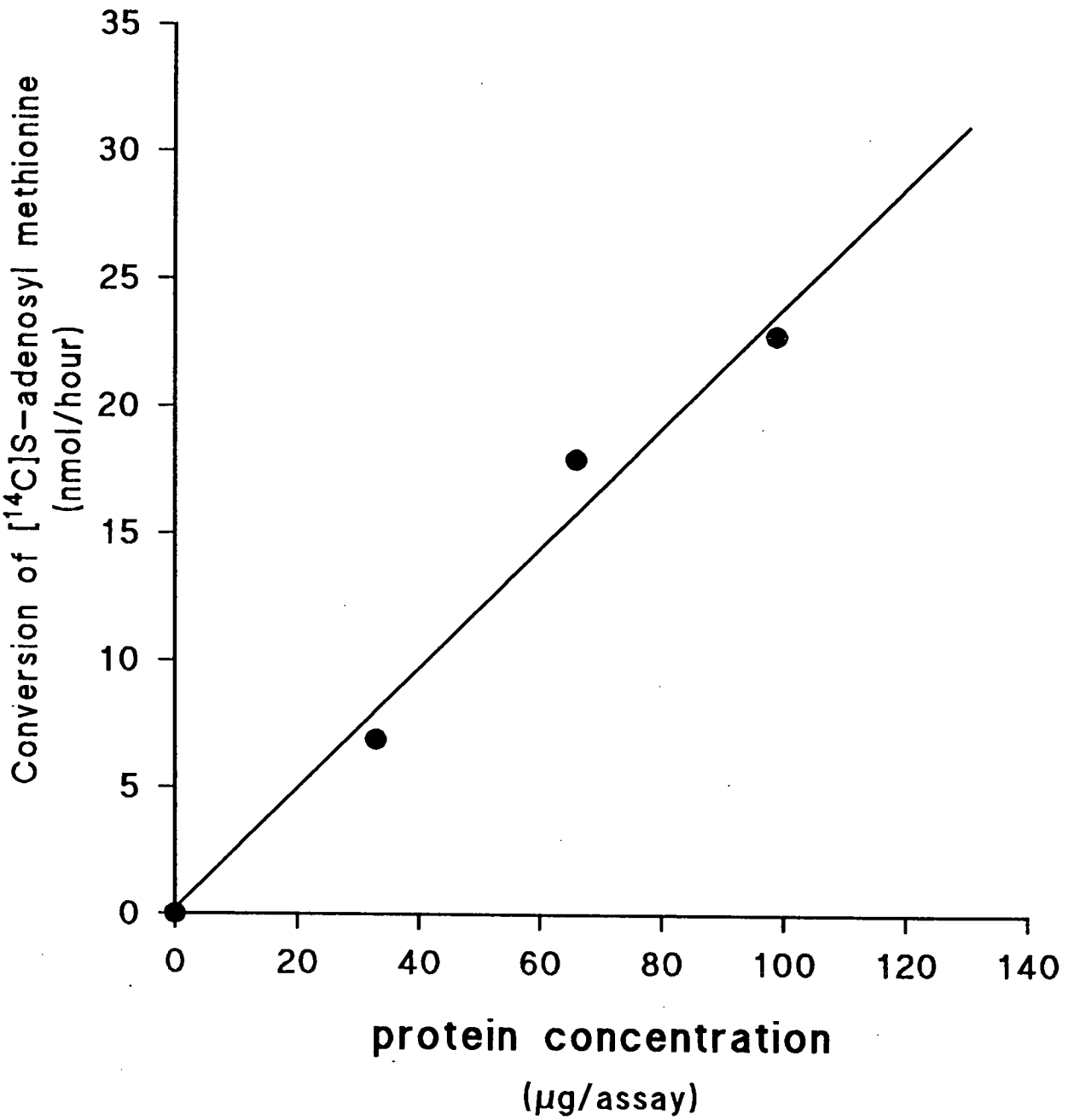


Fig. 5.2.6 Caffeate O-methyltransferase activity with respect to increasing protein concentration. Varying amounts of enzyme preparation were incubated with 1mM [^{14}C]SAM for 1 hour. Points represent means of triplicate assays. The y axis represents the accumulation of radiolabel into ethyl acetate extractable material.

5.3 The Activity of Enzymes along the Maize Internode.

Internode 2 and 4 from plants of the inbred lines Co125 and W401 were harvested and dissected into 5 approximately equal portions from A (apex) to E (base). Extractable PAL, OMT and CAD activity were assayed. Assays were duplicated for each segment and 4 plants from each line were assayed, unless otherwise stated.

5.3.1 Changes in PAL activity along the internode.

Line W401. In these plants there was a clear trend in PAL activity which declined from the base of the internode to the apex. This trend was particularly notable in plants grown in the 1991-1992 season, in both internodes 2 and 4 (Fig. 5.3.1.1). The trend was apparent whether activity was expressed as a specific activity or with respect to fresh weight. The pattern of activity in plants harvested in 1993 is less clear in internode 4, with large standard errors occurring between samples (Fig. 5.3.1.2). There is, however a decline in PAL activity from the base to the top of internode 2. The average activity of plants harvested in 1993 was approx. 1.6 times that in plants harvested in 1992.

Line Co125. The results from line C0125 were far more variable than those for W401. Plants harvested in 1992 had a very low activity (1-5 pkat/mg protein), and the standard error within sample populations was large (up to 40%)(fig. 5.3.1.3). However, the older internode (4) showed a decrease in PAL activity from base to apex in a similar manner to line W401. Plants harvested in 1993 had a higher PAL activity, which also showed considerable variability (fig 5.3.1.4).

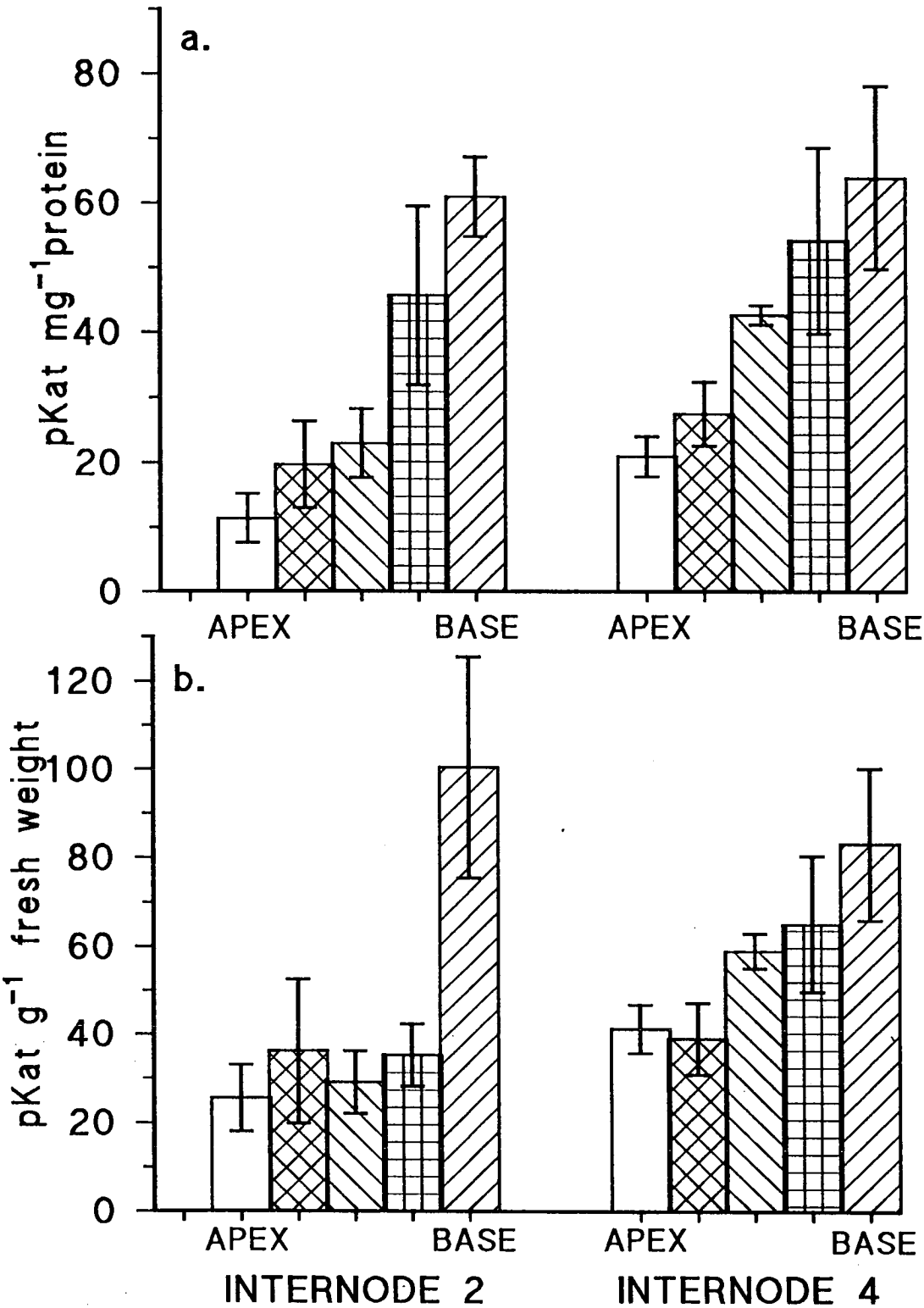


Fig.5.3.1.1 PAL activity in line W401 harvested in 1992. The histograms show a) specific PAL activity, and b) activity on a fresh weight basis, for segments along the internode. Error bars represent standard error of 4 replicate plants

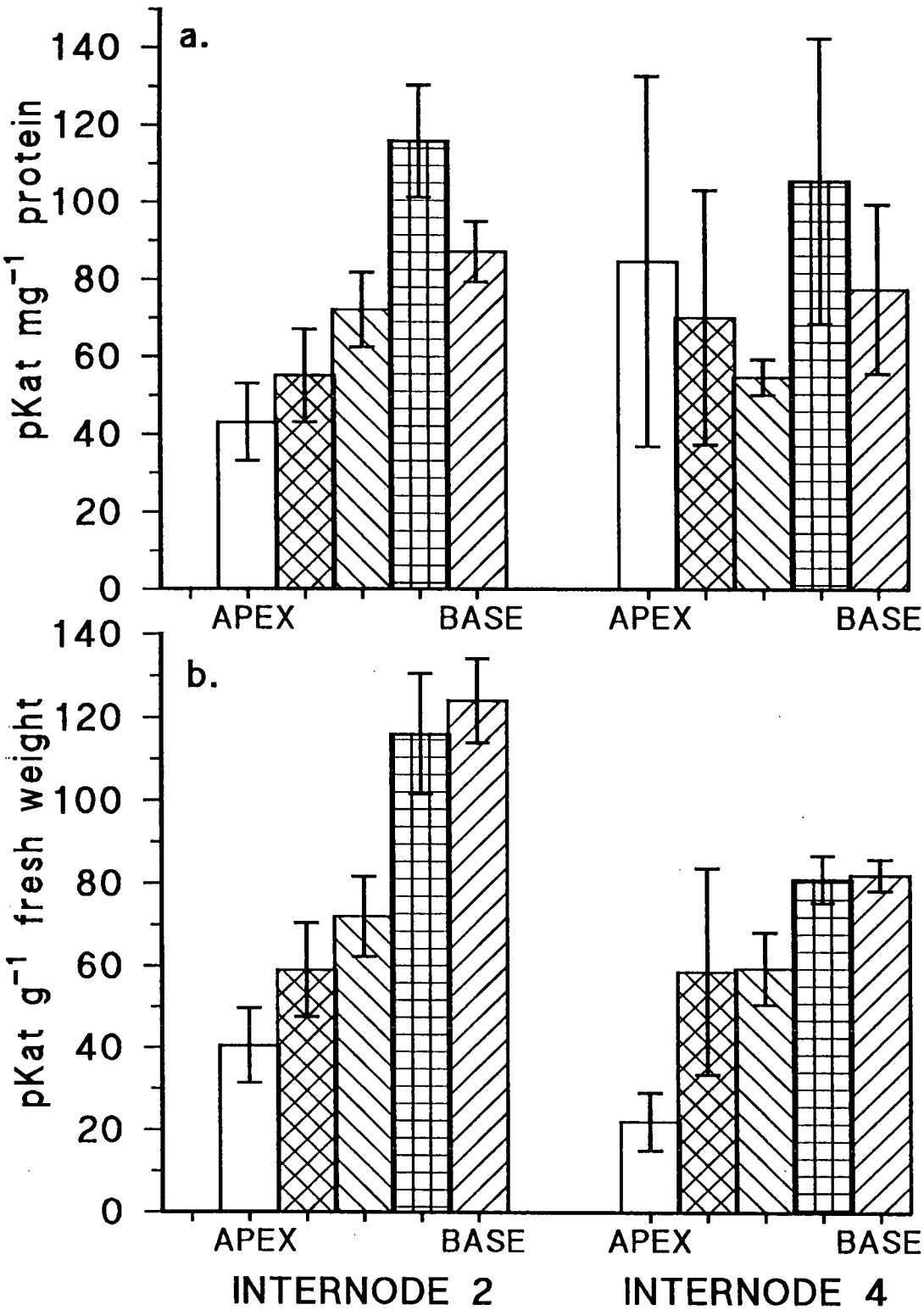


Fig. 5.3.1.2 PAL activity in line W401 harvested in 1993. The histograms show a) specific PAL activity, and b) activity on a fresh weight basis, for segments along the internode. Error bars represent standard error of 5 replicate plants

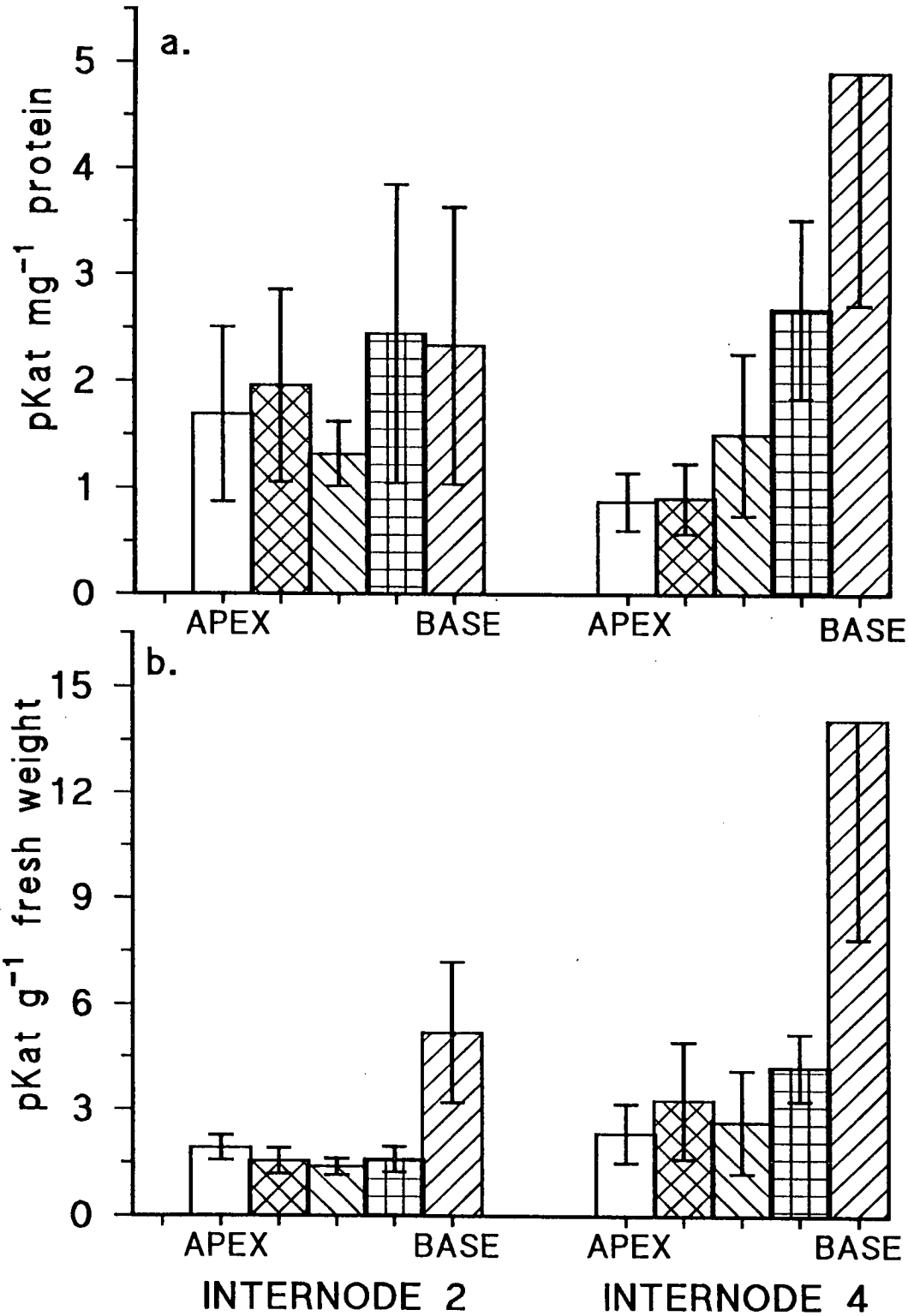


Fig. 5.3.1.3 PAL activity in line Co125 harvested in 1992. The histograms show a) specific activity, and b) activity on a fresh weight basis, for segments along the internode. Error bars represent standard error of 4 replicate plants

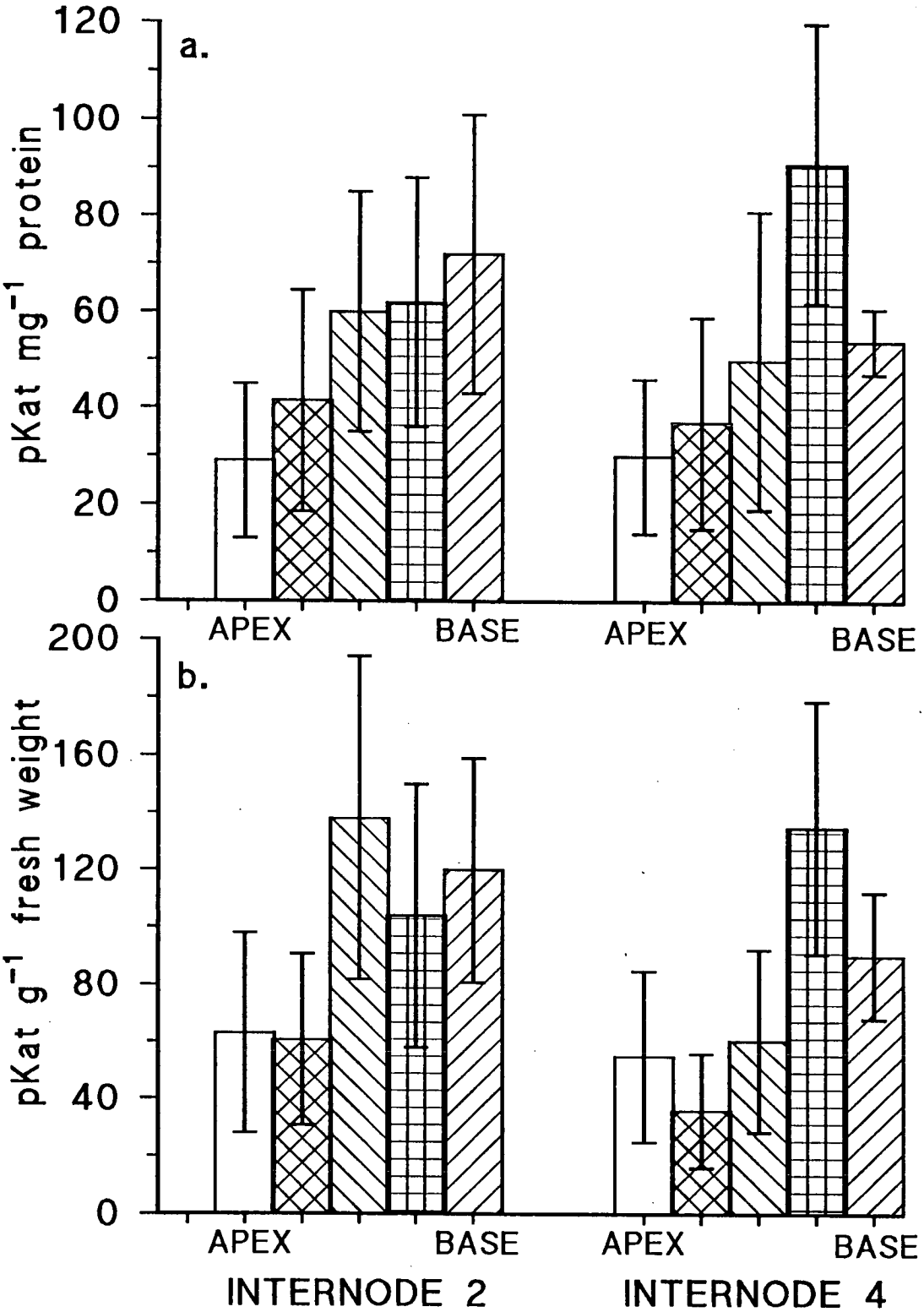


Fig. 5.3.1.4 PAL activity in line Co125 harvested in 1993. The histograms show a) specific activity, and b) activity on a fresh weight basis, for segments along the internode. Error bars represent standard error of 4 replicate plants

Of the 4 plants of the Co125 line assayed in 1993, two had relatively low PAL activities, although higher than the previous year's crop. The other plants had greatly elevated PAL activities. Data for the second internode of individual plants are presented in table 5.1

section	1993	1992	1993	1992	1993	1992	1993	1992	1993	1992
plant #	5	1	6	2	7	3	8	4	Average for 4 plants	
A	56.2	.8	50.1	1.26	2.29	3.0	8.84	n/a	29±16	1.7±0.8
B	79.8	.57	72.1	4.28	8.9	0.71	5.04	2.3	41±23	1.9±0.9
C	101.3	.95	95.7	2.02	29.3	1.27	n/a	1.04	60±36	1.3±0.3
D	67.4	1.52	123.6	1.23	42.8	6.11	15.4	0.94	62±26	2.4±1.4
E	147.8	2.1	43.5	3.28	60.7	n/a	40.8	1.4	73±29	2.3±0.7

Table 5.1 PAL activity in individual plants of the Co125 line. Figures are shown for each internode segment from apex (A) to base (E) for plants harvested in 1992 (plants 1-4) and 1993 (plants 5-8). Data for internode 2 is presented and a similar trend was repeated in internode 4.

It can be seen in the 1993 harvested plants that PAL activity is greatly elevated in plants no. 5 and 6 when compared to plants no.7 and 8 and this phenomenon was apparent also in the fourth internode. This results in a considerable standard error. However, within each internode the trend is for PAL activity to increase from base to apex. It appears that individual plants possess a low or high PAL activity, suggesting that an external environmental effect is influencing PAL activity in specific plants.

The most likely factor that is known to influence PAL activity is infection by fungal pathogens or feeding insects (Friend 1985, Buendgen 1990). There was a small infestation of blackfly on plants grown in 1993 but this was limited and confined to the tassle. Nor was there any obvious fungal infection on the plants.

Plants have been shown to respond to tactile stimulii such as wind, rain and touch. In general plants subjected to a mechanical stress tend to be shorter than control plants with thicker stem diameters and smaller leaves (Telewski and Jaffe 1986a, Jaffe 1973). Responses are swift with certain genes induced within 30 minutes of the stimulus being applied (Braam and Davis 1990). Among the enzymes induced by such tactile stimuli are PAL and covalently bound wall peroxidase (de Jaegher *et al.* 1985). Mechanical perturbation results in an increase in lignin and monolignol content of tissue and a concomitant decrease in cellulose content. The syringyl/vanillyl ratio also increased dramatically after stimulation, indicating an accelerated ageing of tissue, with increased levels of sinapyl and coniferyl alcohol. It has been hypothesised that touch responses may be mediated through the action of ethylene (Telewski and Jaffe, 1986b, de Jaegher *et al.* 1987), which is known to induce PAL and peroxidase (Rhodes and Wooltorton 1973, Miller *et al.* 1985).

Glasshouse grown plants may be particularly sensitive to mechanical perturbation since they exist in a controlled and uniform environment and so have not previously been exposed to environmental stress. It is not inconceivable that mechanical perturbation e.g. movement, direct physical contact, or a change in their position within the greenhouse prior to harvest could have resulted in erratic results and high PAL activities from single plants and such a phenomenon has been noted previously (A.J. Travis, Rowett Research Institute, personal communication).

Average PAL activity was higher in plants harvested in 1993 than in 1992. Those plants assayed in 1992 had activities in the range 0.8 - 5.0 pkat mg-1 protein (1.5 - 13.5 pkat g-1 fresh weight). Plants harvested in 1993 had a much higher range of activity of 30-90 pkat mg protein (35-130 pkat g-1 fresh weight). This variation in activity suggests that an environmental

difference from one year to the next may be influencing enzyme activity or extractabilty. In order for a change in the protein *extractibility* to influence the *specific* activity of PAL, which varies from year to year and from plant to plant, proteins would have to exhibit differential extractabilities. If the extractability of total proteins increased then the specific activity of PAL would remain constant. If however, PAL extractability increased without a concomitant increase in the extractability of total protein, the specific activity of PAL would increase.

5.3.2 Changes in Caffeate O-Methyltransferase along the Internode.

Line W401. There was no obvious pattern in the activity of OMT along the internode in plants harvested in 1992, except a slight increase in activity towards the apex of internode 2 (fig. 5.3.2.1). Neither was there a strong trend in material harvested in 1993 although activity was very low in the basal segment of both internode 2 and 4 when expressed as specific activity (pKat mg-1 protein). This reduction was less apparent when activity was expressed on a fresh weight basis (fig. 5.3.2.2)

Line Co125. Again material harvested in 1992 showed little variation in the OMT activity during maturation of the internode, although in internode 2 there was a slight increase in activity towards the apex (fig. 5.3.2.3). However, specific activity in the most apical segment was very low and this may be as a result of contamination of these samples with nodal tissue, resulting in extracts with artificially high protein contents.

In 1993 activity appeared to increase towards the middle of the internode in both internodes, although the standard error on several points is quite large and samples were quite variable (fig.5.3.2.4)

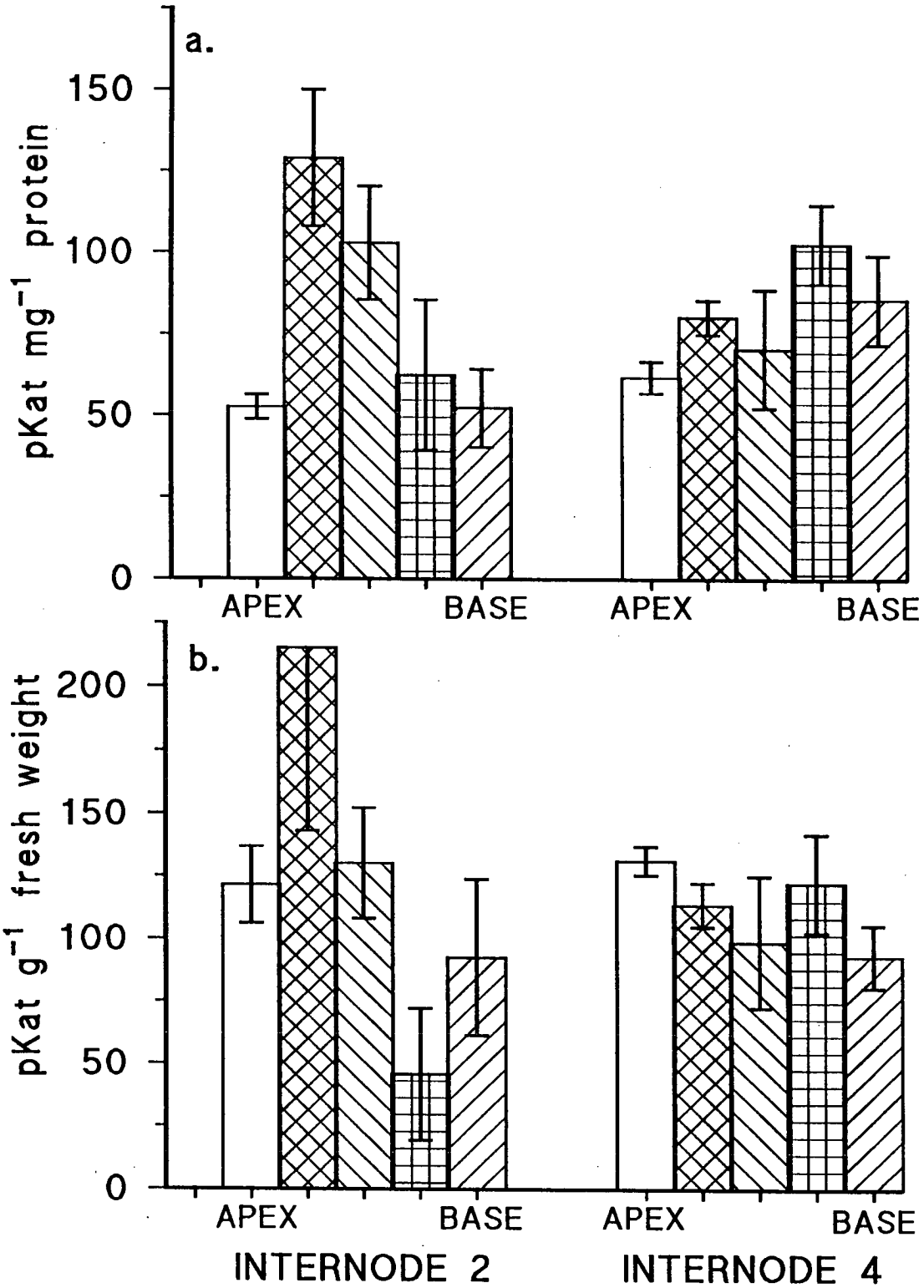


fig. 5.3.2.1 OMT.W92. OMT activity in line W401 harvested in 1992. The histograms show a) specific activity, and b) activity on a fresh weight basis, for segments along the internode. Error bars represent standard error of 4 replicate plants

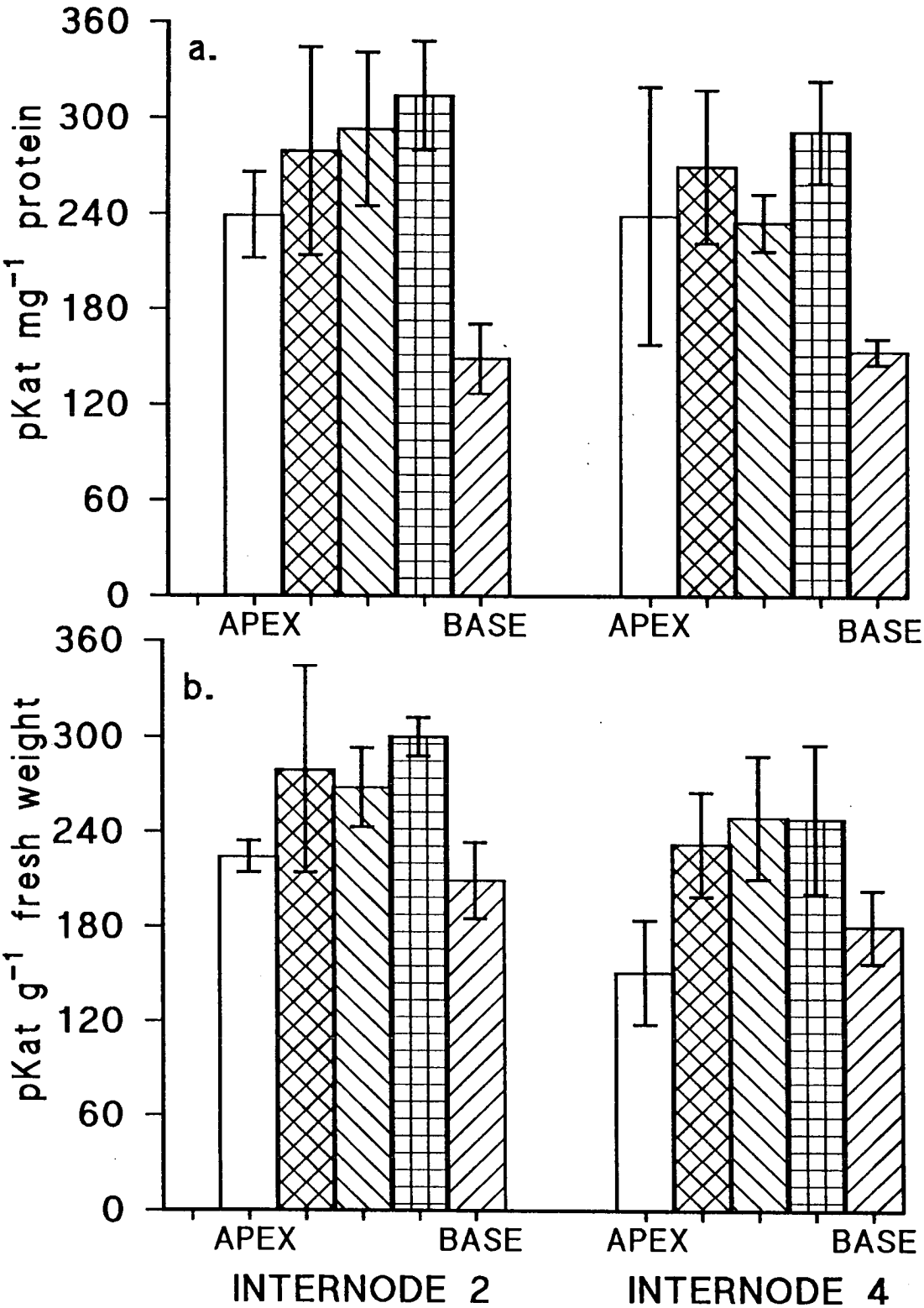


Fig. 5.3.2.2. OMT activity in line W401 harvested in 1993. The histograms show a) specific activity, and b) activity on a fresh weight basis, for segments along the internode. Error bars represent standard error of 5 replicate plants

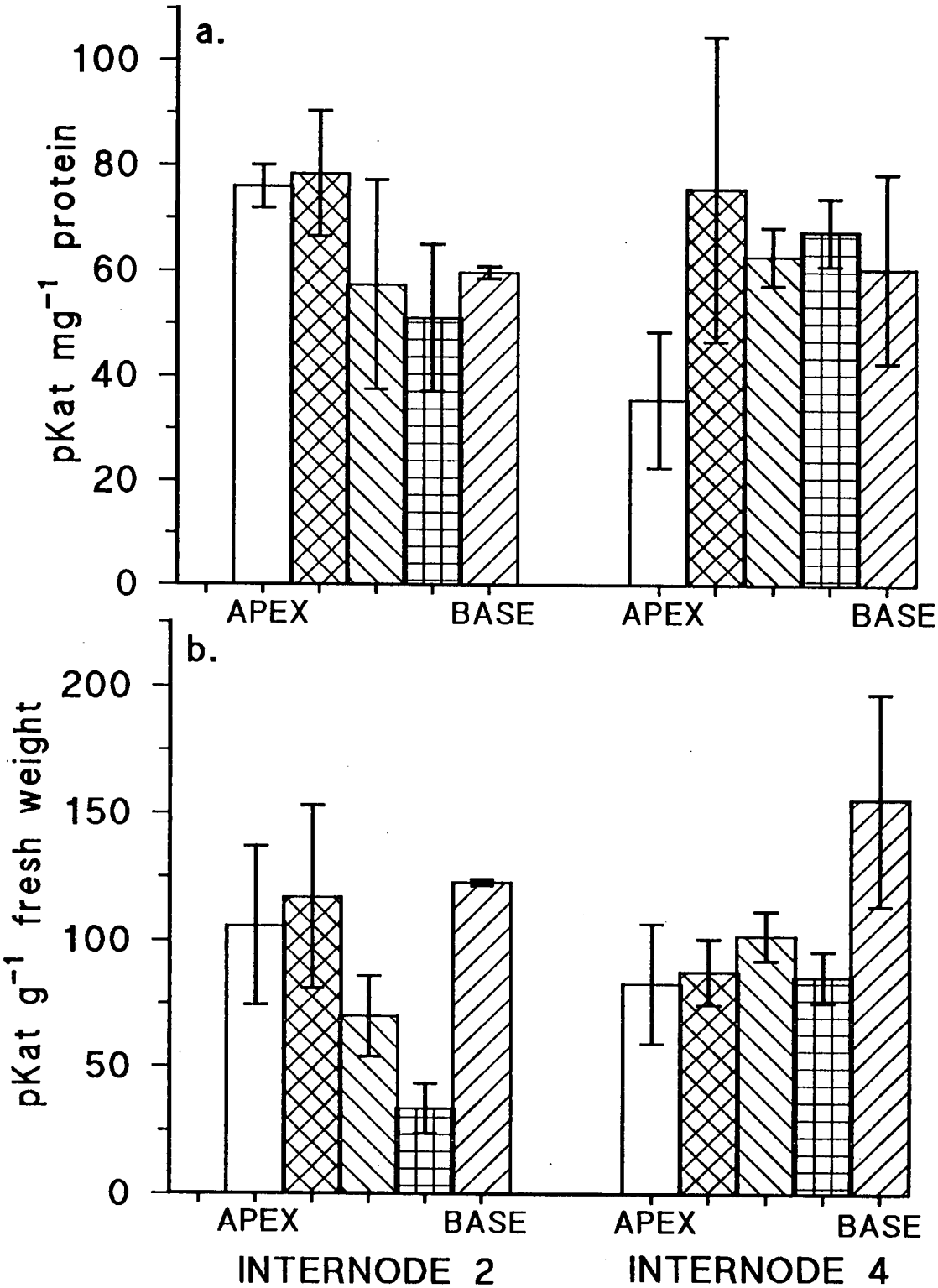


Fig. 5.3.2.3. OMT activity in line Co125 harvested in 1992. The histograms show a) specific activity, and b) activity on a fresh weight basis, for segments along the internode. Error bars represent standard error of 4 replicate plants

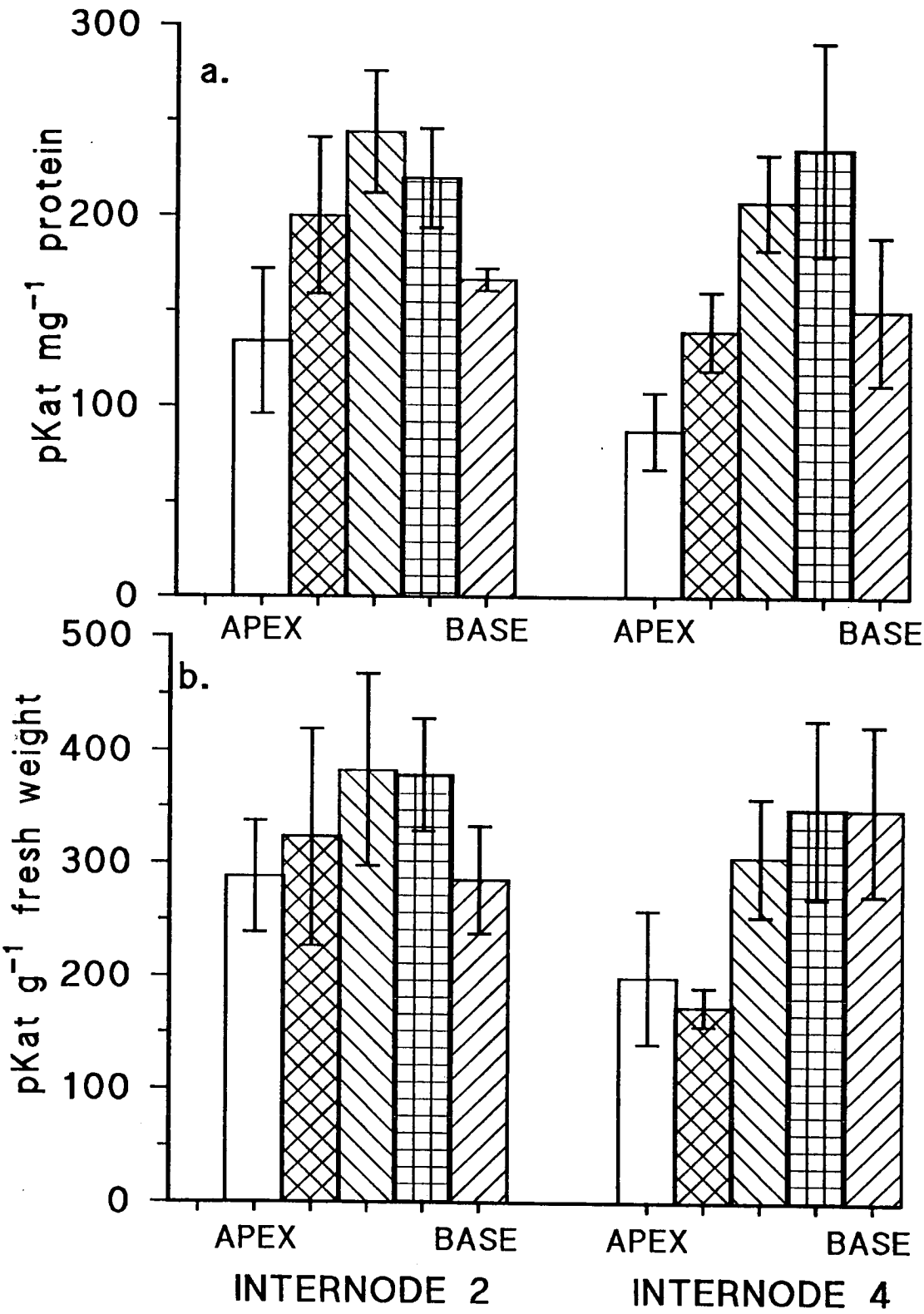


Fig. 5.3.2.4 OMT activity in line Co125 harvested in 1993. The histograms show a) specific activity, and b) activity on a fresh weight basis, for segments along the internode. Error bars represent standard error of 4 replicate plants

5.3.3 Changes in activty of CAD along the Internode.

In both lines W401 and Co125, CAD activity was highly variable along the internode and there were no trends in activity during maturation (figs 5.3.3.1-4). CAD activity was higher than PAL and OMT. The lack of variation in CAD activity and its high activity is unsurprising. CAD is the last enzyme in the pathway for production of monolignols and it may be present in excess to utilise all of the available substrate, it is not competing with other enzymes so would not be expected to control the flux of intermediates through separate branches of the pathway. Although it has been hypothesised as a controlling enzyme in lignification (Grand *et al.* 1985), in plants transformed with antisense to CAD mRNA, activity has to be reduced to <5% of control plants to have a significant effect on lignin quality (Halpin *et al.* 1993).

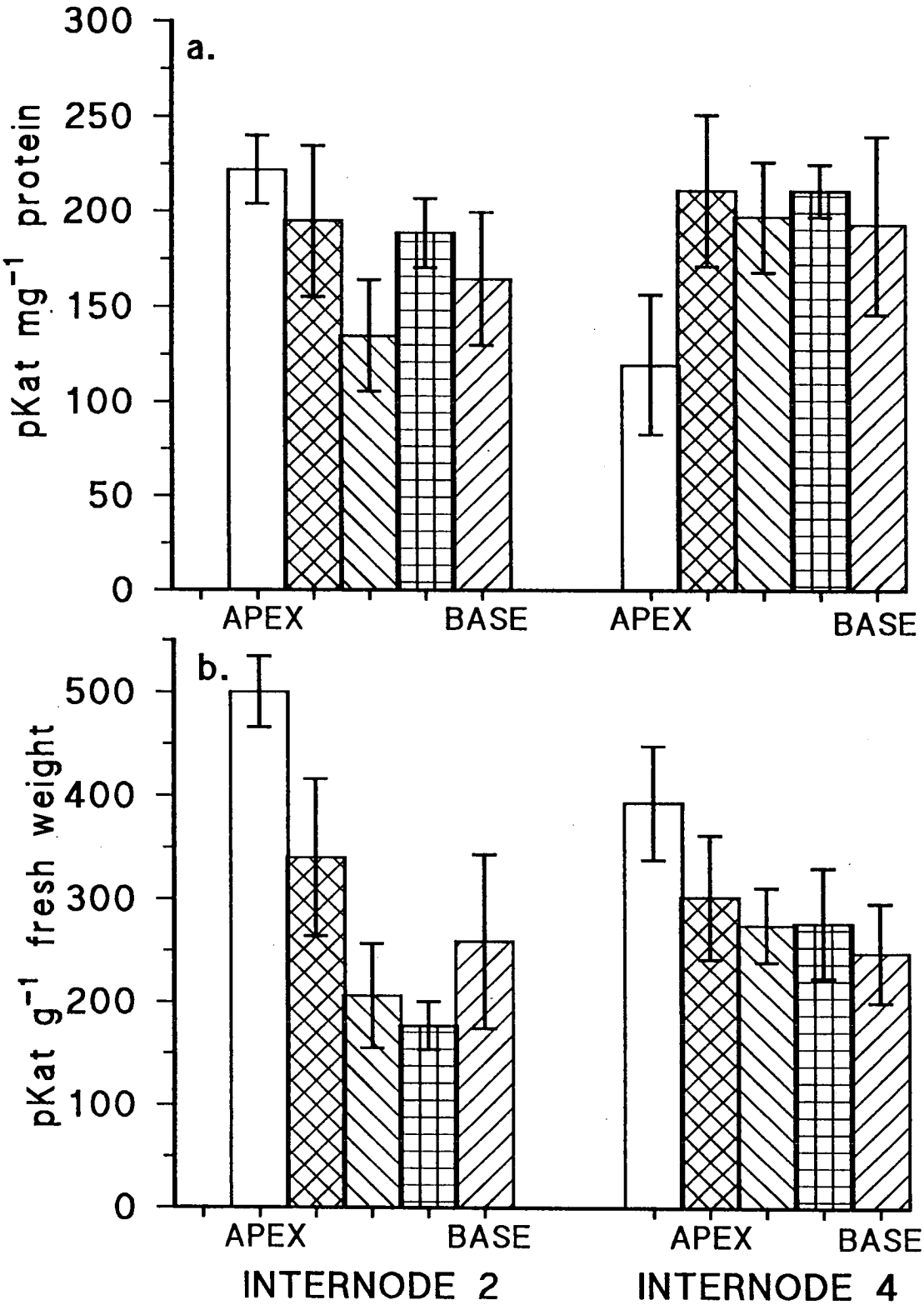


fig. 5.3.3.1. CAD activity in line W401 harvested in 1992. The histograms show a) specific activity, and b) activity on a fresh weight basis, for segments along the internode. Error bars represent standard error of 4 replicate plants

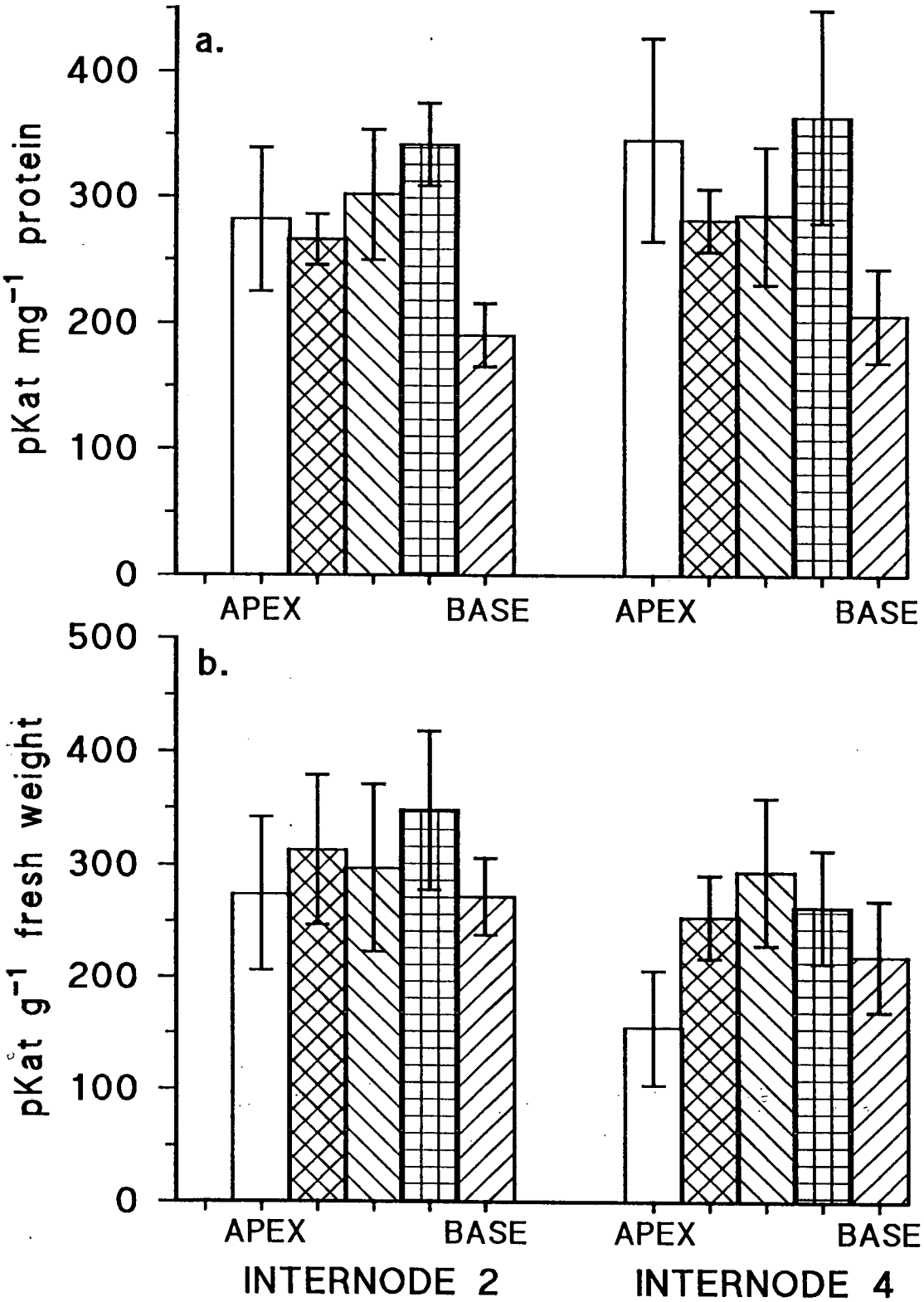


Fig. 5.3.3.2 CAD activity in line W401 harvested in 1993. The histograms show a) specific activity, and b) activity on a fresh weight basis, for segments along the internode. Error bars represent standard error of 5 replicate plants

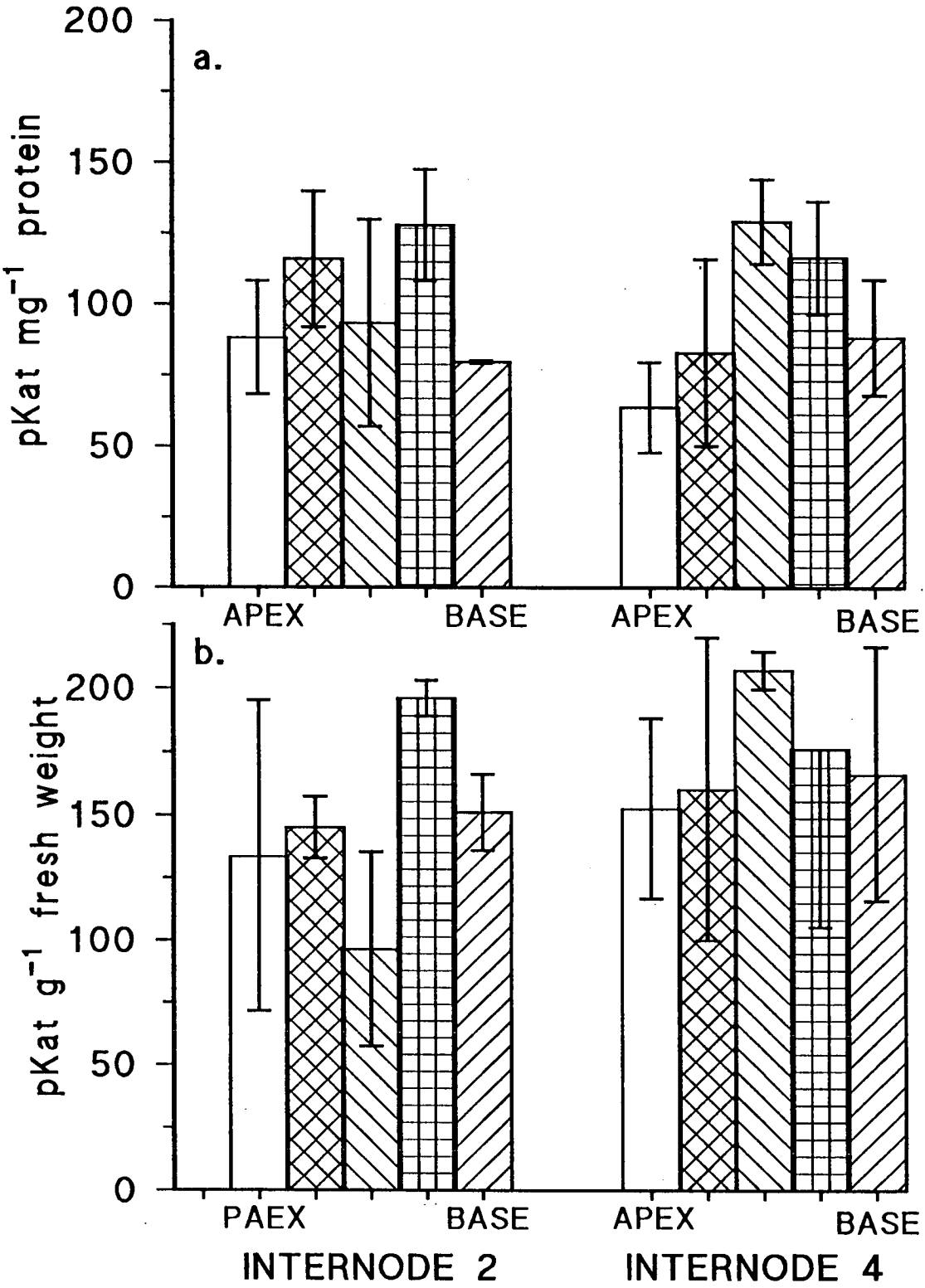


Fig 5.3.3.3 CAD activity in line Co125 harvested in 1992. The histograms show a) specific activity, and b) activity on a fresh weight basis, for segments along the internode. Error bars represent standard error of 4 replicate plants

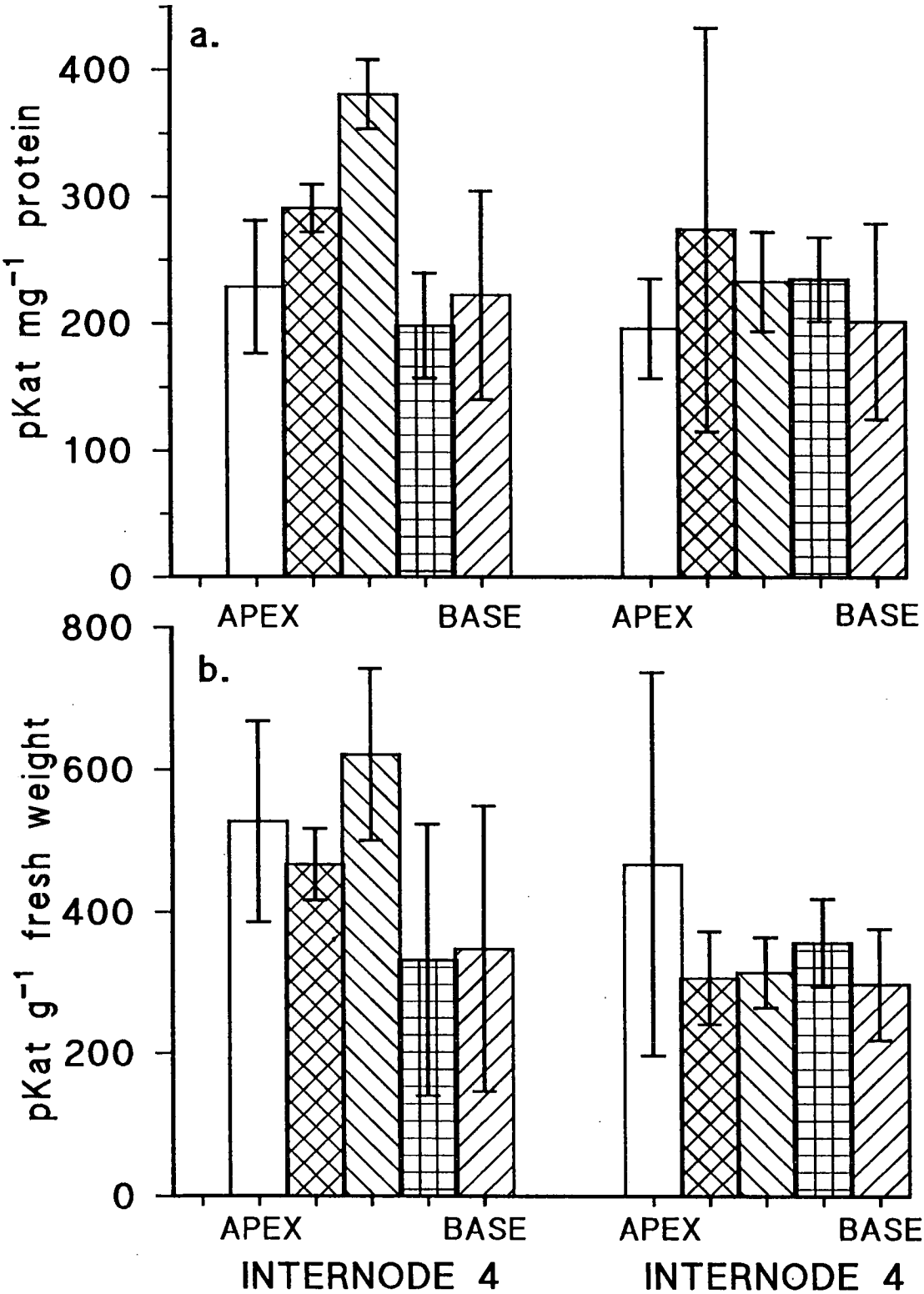


Fig 5.3.3.4 CAD activity in line Co125 harvested in 1993. The histograms show a) specific activity, and b) activity on a fresh weight basis, for segments along the internode. Error bars represent standard error of 4 replicate plants

5.4 DISCUSSION OF RESULTS.

5.4.1 Enzyme activity in comparison to lignin and phenolic content.

Of the three enzymes assayed along the maize internode only PAL showed a clear reproducible alteration in extractable activity during the maturation of tissue. PAL activity was consistently greater at or near the base of the internode, with activity declining towards the apex.

It has been reported that the lignin and phenolic acid content of internodes taken from these lines increases rapidly from the base up, on a gram kg^{-1} cell wall dry matter basis, in the basal half of the internode. Phenolic content then remains fairly constant in the apical third (Scobbie *et al.* 1993). This is illustrated in fig. 5.4.1.1. Although only data from internode 2 of line Co125 is presented in fig. 5.4.1.1 the trend of increasing phenolic content from base to apex occurs in both internodes 2 and 4 and similarly in line W401. This increase in phenolic material is indicative of maturation during internode development and elongation. The rate of phenolic production is given by the gradient of the curve in fig. 5.4.1.1. It is clear from these data that the rate of production of wall bound phenolic material is at a maximum in the basal third of the internode and this part of the internode is actively lignifying, with a decline and cessation of lignin production in the apical two thirds of the internode.

During this period of active lignification it is likely that there is a large demand on lignin precursors and phenylpropanoid and it is at this stage of development that PAL activity is at a maximum in the internode. Thus there appears to be a coreelation between the rate of lignification along the internode and the PAL activity..We can gain a very approximate estimation of the rate of lignification and demand on precursor supply from the above data (Scobbie *et al.* 1993) by calculating the increase in lignin (or total phenolics)

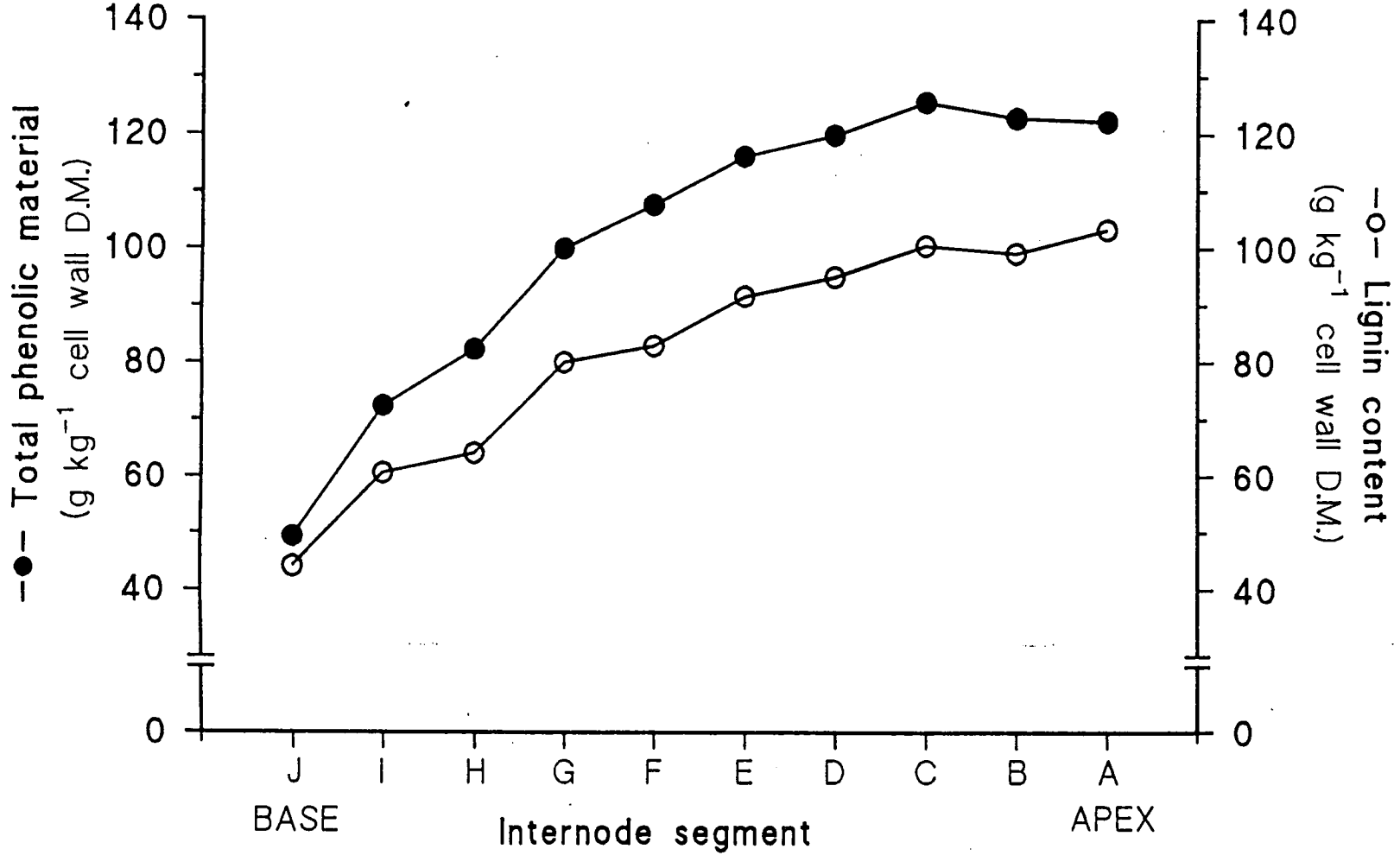


Fig. 5.4.1.1 The increase in total phenolic material and lignin along the maize internode. Data is from analysis of internode 2 of line Co125 (taken from Scobbie *et al.* 1993) and similar patterns of lignin and phenolic distribution were apparent in internode 4 and in line W401. Total phenolics were determined by the acetyl bromide method and lignin calculated as total phenolic minus saponifiable phenolics.

from one segment of the internode to the next. When compared a positive correlation is seen between PAL activity and the rate of increase in total phenolic content (fig 5.4.1.2).

There must be caution when drawing a direct comparison between the data presented by Scobbie and colleagues and the data for enzyme activity since the material used in the determination of cell wall structural and chemical characteristics was field grown. There are known to be considerable differences between field and glasshouse grown material from these two cultivars in several agromonic and physiological aspects (Patrick Balis, SES-Europe, Tienen, Personal Communication) and so samples from these two studies will not be strictly comparable.

However these figures do demonstrate, albeit as crude estimates, the relationship between PAL activity and active lignin deposition.

5.4.2 Calculation of specific rates of lignification.

The lack of relevant growth data available for this internode system makes full interpretation of the enzymological data difficult. As yet no studies within the Forage Maize Project have measured elongation rates within the internode and so, although there is a spatial resolution of events occurring during secondary development we are unable to assign a temporal scale to the internode. The assumption has been that since maize internodes take 5-7 days to extend fully (Morrison and Buxton 1991) each segment of the internode (of which there are 10) represents 12 hours of growth (A. Chesson, Rowett Research Institute, personal communication). However, it is unlikely that the internode would represent a linear time scale along its length since cell division and subsequent elongation is confined to a small area from the meristem (Sauer and Kende 1992, Padu *et al.* 1993), after which there is no increase in cell length. This growth profile would result in the basal area of

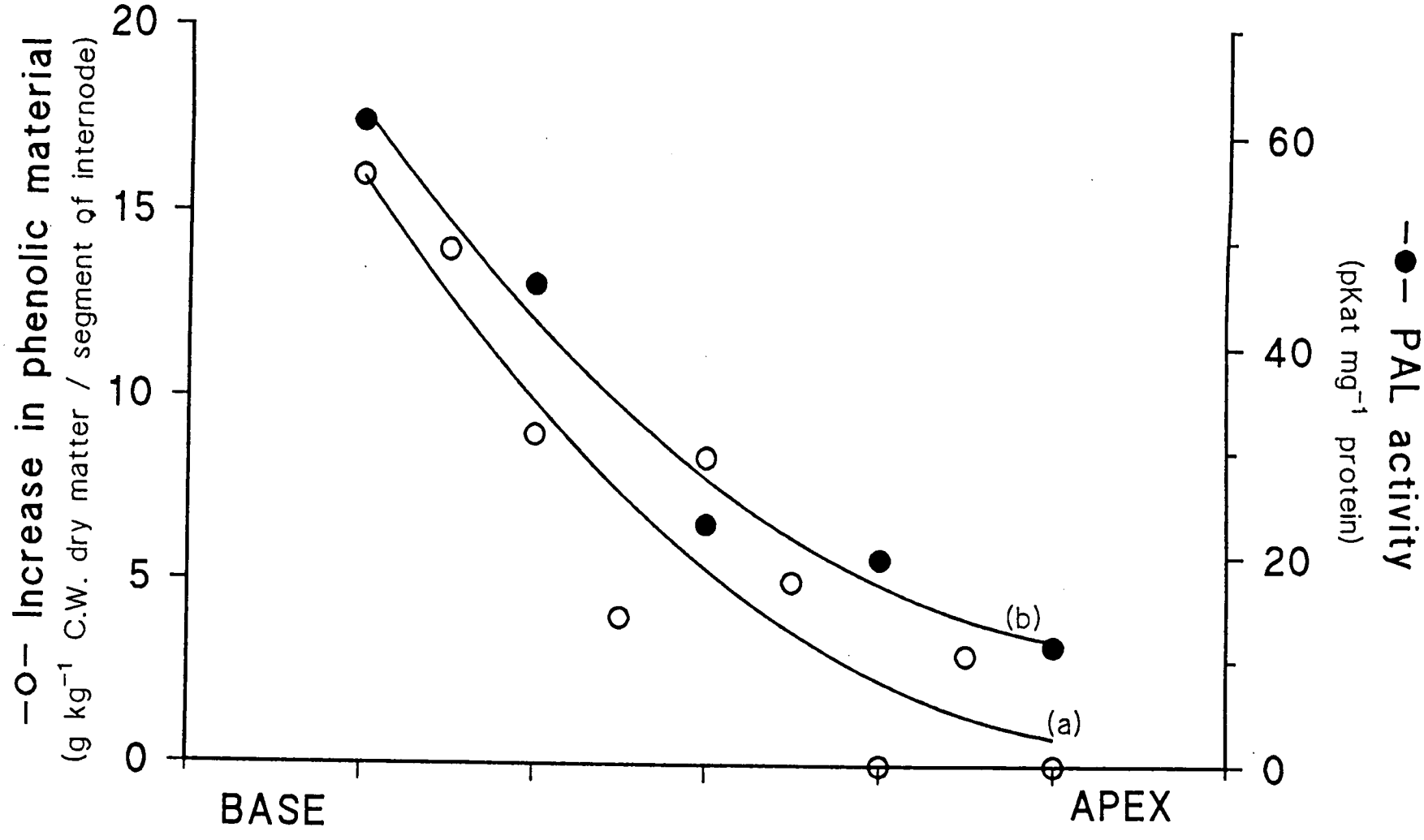


Fig. 5.4.1.2 Relationship between rate of increase of phenolic material and PAL activity. Increase of phenolic material between neighbouring tissue segments were calculated from the data of Scobbie *et al.* (1993) by subtraction, and expressed on a per segment basis. Curve (a) represents the rate of increase in phenolic material and curve (b) PAL activity. A positive correlation between PAL activity and a high rate of production of phenolic material is apparent.

the internode representing a comparatively shorter length of time.

Ideally, growth measurements should be taken during the elongation and maturation of the internode thus allowing the expression of the spatial separation along the internode in terms of specific time periods. If this information were known the specific rates of lignification (g [kg $^{-1}$ cell wall] hour^{-1}) could be calculated and a direct comparison to enzyme activity and precursor supply made. The rates of increase in total phenolic content shown in fig 5.4.1.3 are by necessity approximations. However, they do enable an illustration of the relationship between PAL activity and lignification.

5.4.3 The maize internode as a model system.

The usefulness of the graminaceous monocot internode as a model system has been established in the analysis of the gross physical and chemical changes that occur during secondary development. The phenolic content and constitution of the internode has been established (Scobbie *et al.*1993, Mulder 1992) as has the size and thickness of the cell walls (Travis *et al.* 1993). It has also proved useful in assessing the impact that these changes have on the digestibility and thus nutritive value of forage material (Dolstra 1992).

The assessment of this system as a model in which to study enzyme regulation during secondary development has been hampered by the lack of available growth data. Although clear trends were seen in the PAL activity along the internode, the internode at this stage of growth may be too mature for the detection of the earliest events in lignification. More informative data may be obtained by assaying internodes at a slightly earlier growth stage. An analogous model utilising rice internodes that have been induced to elongate rapidly by submergence in water has been used to analyse lignin and β-

glucan content of cell walls. In rapidly elongating tissues PAL and CAD activities were suppressed and this correlated to a low lignin content. In tissues that were not undergoing rapid elongation both PAL and CAD activities were elevated and this correlated to a higher lignification rate.

During internodal maturation there is presumably a transition period during which elongation slows and ceases, and cell wall production switches from primary to secondary and lignification.

In order to monitor this change internodes harvested during the final stages of elongation could be investigated and such internodes would be likely to contain tissue at the transition stage. Monitoring the activities of enzymes along internodes of this age may yield data from, during and after the onset of tissue lignification, thus providing information on the control of enzyme activities.

CHAPTER 6
CONCLUSIONS AND GENERAL DISCUSSION.

This thesis has examined some aspects of phenolic metabolism in relation to cell wall polysaccharide biosynthesis.

The presence of phenolic material in the cell wall affects properties such as extensibility, resistance to degradation (either through fungal pathogenesis or by gut micro-organisms) and the extractability of fibres and wall polymers. Thus an understanding the mechanism by which these phenolic moieties become integrated into the wall and the control of these processes is of fundamental importance to our efficient exploitation of plant material. Studying such mechanisms will also increase our understanding of the control of plant growth and metabolism and of the exacting organisation that exists within the growing plant.

Phenolic acids, predominantly feruloyl residues, are found esterified to specific sugar moieties of primary cell wall polysaccharides and the precision with which feruloyl residues are attached to sugars suggests that the process of feruloylation is effected by an enzyme. There has been some debate about where the feruloylation of polysaccharides occurs and evidence had been presented supporting intraprotoplasmic and *in muro* feruloylation. I have presented data showing that in graminaceous monocot cell cultures the kinetics of arabinosylation and feruloylation of xylan are similar and that feruloylation must be occurring intraprotoplasmically, presumably to nascent polysaccharides. This conclusion is contradictory to previous work on graminaceous monocots (Yamamoto and Towers 1985, Yamamoto *et al.*1989).

It is suggested that a feruloyltransferase present in the protoplasm of the cell is responsible for the feruloylation of cell wall polysaccharides. The continued increase in wall bound feruloyl groups (on a per mg of carbohydrate basis) that is observed after net carbohydrate deposition has ceased (Yamamoto and Towers 1985, Nishitani and Nevins 1990) must either

be as a result of increased co-synthetic feruloylation of the few new polysaccharides that are still being made, or through turnover involving selective degredation of the less highly feruloylated polysaccharides. It is suggested that non-feruloylated regions of polysaccharides may be more susceptible to degradation during turnover than those with attached feruloyl groups, resulting in an increase in the proportion of feruloylated sugar residues.

An increased degree of feruloylation may occur through an increase in acyldonor : polysaccharide feruloyltransferase activity, perhaps as a control point in the partitioning of activated feruloyl intermediates between wall bioynthesis and other phenylpropanoid metabolism, or possibly through increased substrate availability. Polysaccharides from different plant species are also feruloylated to different degrees and there is no information about the control of the process of feruloylation. Further investigation into the kinetics of feruloylation with respect to polysaccharide synthesis and in response to fungal disease may increase our knowledge of this possible regulatory step in cell wall biosynthesis.

Since feruloylation is occurring intraprotoplasmically and the synthesis of polysaccharides occurs in the Golgi bodies it is reasonable to suggest that feruloylation occurs co- or post-synthetically to nascent polysaccharides in these organelles. Endomembrane preparations from maize were capable of transferring radiolabelled feruloyl groups from feruloyl-CoA into polymeric material. However, this was completely suppressed by the removal of peroxide from the assay mixture, suggesting that transfer of radiolabel into polymeric material was due to peroxidase activity, rather than feruloyltransferase activity. Thus the data in this thesis do not support the work of Meyer *et al.* (1992) which was asserted to show that feruloyl groups were being esterified to pectic polysaccharides from feruloyl-CoA by

endomembranes of parsley. Meyer and colleagues did not investigate the possibility of peroxidase activity in the endomembrane preparations used in their feruloyltransferase assays and it is possible that at some or all of the activity attributed to the feruloyl-CoA : polysaccharide feruloyltransferase was due to peroxidatic oxidation of the feruloyl-CoA. The putative feruloyl-CoA : polysaccharide feruloyltransferase activity did not co-purify with IDPase activity (a general marker of Golgi derived microsomal vesicles) upon centrifugation in a continuous sucrose gradient. However, the product of the feruloyltransferase activity was partially characterised and had some properties similar to a feruloylated pectin i.e., solubilisation in ammonium oxalate, precipitation in 80% ethanol, release of free radiolabelled ferulic acid (verified by HPLC) upon saponification. Radiolabelled material was digested with polysaccharide hydrolase to yield oligomers which were suggested to be feruloyl-arabinobiose and feruloyl-galactobiose. However, in order to establish unequivocally the activity of a feruloyl-CoA : polysaccharide feruloyltransferase, the product of the reaction, or, as it will be a feruloylated polysaccharide, its digestion product, must be sought and characterised by a number of methods and its identity firmly established.

This activity must be demonstrated not to be peroxide dependent and to be distinct from membrane associated peroxidase activity, before further characterisation can proceed. The present thesis clearly shows that peroxidases present in endomembrane preparations can create a serious artifact in assays conducted to determine feruloyltransferase activity.

The problems arising from presence of peroxidases in microsomal preparations have been addressed by Rodgers *et al.* (1993) with respect to the isolation of plant cytochrome P450, specifically the cinnamate-4-hydroxylase involved in phenylpropanoid biosynthesis. It is possible that a number of other studies into the metabolism of phenylpropanoid and related

compounds may also suffer from artifactual results produced by peroxidase activity.

The possible roles of membrane associated peroxidases have been briefly discussed and it is feasible that although some will be associated with membranes for the purposes of transport, other isoenzymes may have specific physiological functions within the endomembrane system itself. At the subcellular level it is becoming apparent that the organisation of the plant endomembrane system is sophisticated, highly organised and strictly regulated (Moore *et al.* 1991, Zhang and Staehelin 1992). Peroxidase, if active in membranes *in vivo,* would be expected to be precisely controlled, possibly through segregation from potential substrates by compartmentalisation within organelles. Informative data on the roles of membrane bound peroxidases may be gained through the use of immuno- and histolocalisation of the enzyme and potential substrates.

Routine assays for peroxidase utilise guaiacol or o-dianisidine as substrates, but the determination of the activity of membrane peroxidases towards other (phenylpropanoid) substrates and the specificities of various isoperoxidases would be more useful in determining the reason for their presence in membrane preparations.

In the study of the control of cell wall biosynthesis and metabolism it is desirable to use model systems in which growth and differentiation are occurring. Several model systems have been developed and in this thesis I have presented data on the enzyme activity in the maize internode, a potential system in which to study secondary cell wall development.

This system has proved useful in determining the gross physical and chemical changes that occur in the wall during maturation. However it is likely that it lacks the discrimination required to monitor changes in enzyme

activity and intermediate utilisation during phenylpropanoid metabolism. The pattern of enzyme activities in the internode may be a result of their persistance in tissue rather than an indicator of continued *de novo* synthesis. However, an investigation of enzyme activity in younger internodes may prove more enlightening.

Adequate growth data have proved to be required for full interpretation of the data presented in chapter 5, and this could be relatively easily obtained through measurement of internode dimensions, cell number and length and elongation rate. Such information could subsequently be applied to the data presented in this thesis.

CHAPTER 7
APPENDIX

A.1 Synthesis of feruloyl-CoA

Feruloyl CoA was synthesised according to the methods of Stöckigt and Zenk (1979) via a feruloyl-hydroxysuccinimide ester intermediate. The products of this synthesis were separated by TLC on silica gel in acetonitrile:water 9:1. The synthesised feruloyl-CoA was identified by its turquoise fluorescence under long wave UV light and had an Rf of 0.85, comparable with authentic feruloyl-CoA (E. Savins, Zeneca Agrochemicals, Bracknell, personal communication). On paper chromatography in BAW the Rf was comparable to that reported by Stöckigt and Zenk (1979). This compound was eluted in H_2O and had an intense yellow colour at pH > 8.

The compound had a UV spectrum (at pH 7) typical of hydroxycinnamic acid thiol esters (Stöckigt and Zenk 1979, Negrel and Smith 1984) with absorption maxima at 258 nm (adenine) and 347 nm (feruloyl thioester). These are comparable to previously reported spectra (257 nm and 346 nm, Stöckigt and Zenk 1979, 257 nm and 346 nm, G. Foxon, Zeneca Agrochemicals, Bracknell, personal communication).

Treatment with mild alkali followed by TLC resulted in the disappearance of the characteristic turquoise fluorescence of feruloyl ester and the appearance of a compound co-chromatographing with ferulic acid.

Nuclear magnetic resonance spectroscopy of Co-A and Feruloyl-CoA

NMR spectroscopy was performed by G. Provan, Rowett Research Institute, Aberdeen on a Bücher 300 MHz machine, calibrated to tetramethyl silane. Pure samples of CoA (Sigma Chemicals Ltd, Poole, U.K.) and synthesised feruloyl-CoA were dissolved in deuterated methanol for analysis. The spectra were interpreted by G. Wallace.

The major change that will occur in the spectrum of Co-enzyme A upon esterification to another moiety is a downfield shift of the signal generated by proton no. 2 (see fig A.1 for numbering of protons).

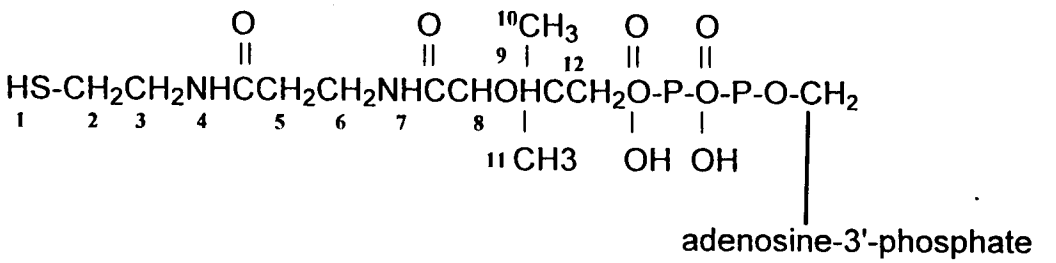


Fig A.1 The structure of Co-A. Protons are numbered sequentially from the thiol group.

The NMR spectrum of CoA exhibits clear signals at δ 1.3, 2.4, 2.55, 3.3 and 3.45 ppm which were assigned to protons 1,2,5,6 and 3 respectively (fig A.2 spectrum of CoA). These assignments were further confirmed by Correlation Spectroscopy (COSY-NMR, Fig A.3).

The spectrum of the synthesised feruloyl-CoA (fig A.4) clearly shows signals for aromatic protons between δ 6.5-7.1 ppm and for the α and β protons at 7.25 (α) and 6.5 (β) ppm. This area of the spectrum is identical to that for authentic ferulic acid. The signal corresponding to H-2 of the CoA moiety is still present but greatly reduced at 2.1 ppm, suggesting that there has been some hydrolysis of the feruloyl-CoA. This is not unexpected as feruloyl-CoA is relatively unstable and degrades in solution at room temperature. The signal from the corresponding proton in the esterified product would be expected further downfield. However, it is obscured by the complexity of the spectrum and the considerable interference in the 1-3 ppm

region of the spectrum. This is most probably as a result of turbidity caused by the viscosity of the feruloyl-CoA solution

These spectra cannot positively identify the synthesised compound, but they do strongly support its identity as feruloyl-CoA particularly when considered in conjunction with spectrophometric and chromatographic data.

A.2 Media for Cell Suspension Cultures.

Maize cell cultures

Murashige and Skoog* basal salts	4.4 g^{-1}
6-γ,γ-dimethylallylaminopurine	2.0 mg l^{-1}
MES	100 mg l^{-1}
α Naphthaleneacetic acid	0.5 mg l^{-1}
Pyridoxine HCl	0.5 mg l^{-1}
Thiamine	0.1 mg l^{-1}
2-4-Dichlorophenoxyacetic acid	2.0 mg l^{-1}
Sucrose	2.0 %
pH 5.8.	

In cultures used for [U-^{14}C]glucose radiolabelling, the sucrose was replaced with either 1% glycerol + 1% glucose or 1% glycerol

Festuca cell cultures.

Murashige and Skoog*basal salts.	4.4 g l^{-1}
Nicotinic acid	0.5 mg l^{-1}
Aneurine-HCl	0.1 mg l^{-1}
Pyridoxine-HCl	0.5 mg l^{-1}
Glycine	2 mg l^{-1}
2,4-Dichlorophenoxyacetic acid	2 mg l^{-1}
Glucose	20 g l^{-1}
pH 5.7	

*Murashige and Skoog basal salts mixture (Sigma Chemica Company).

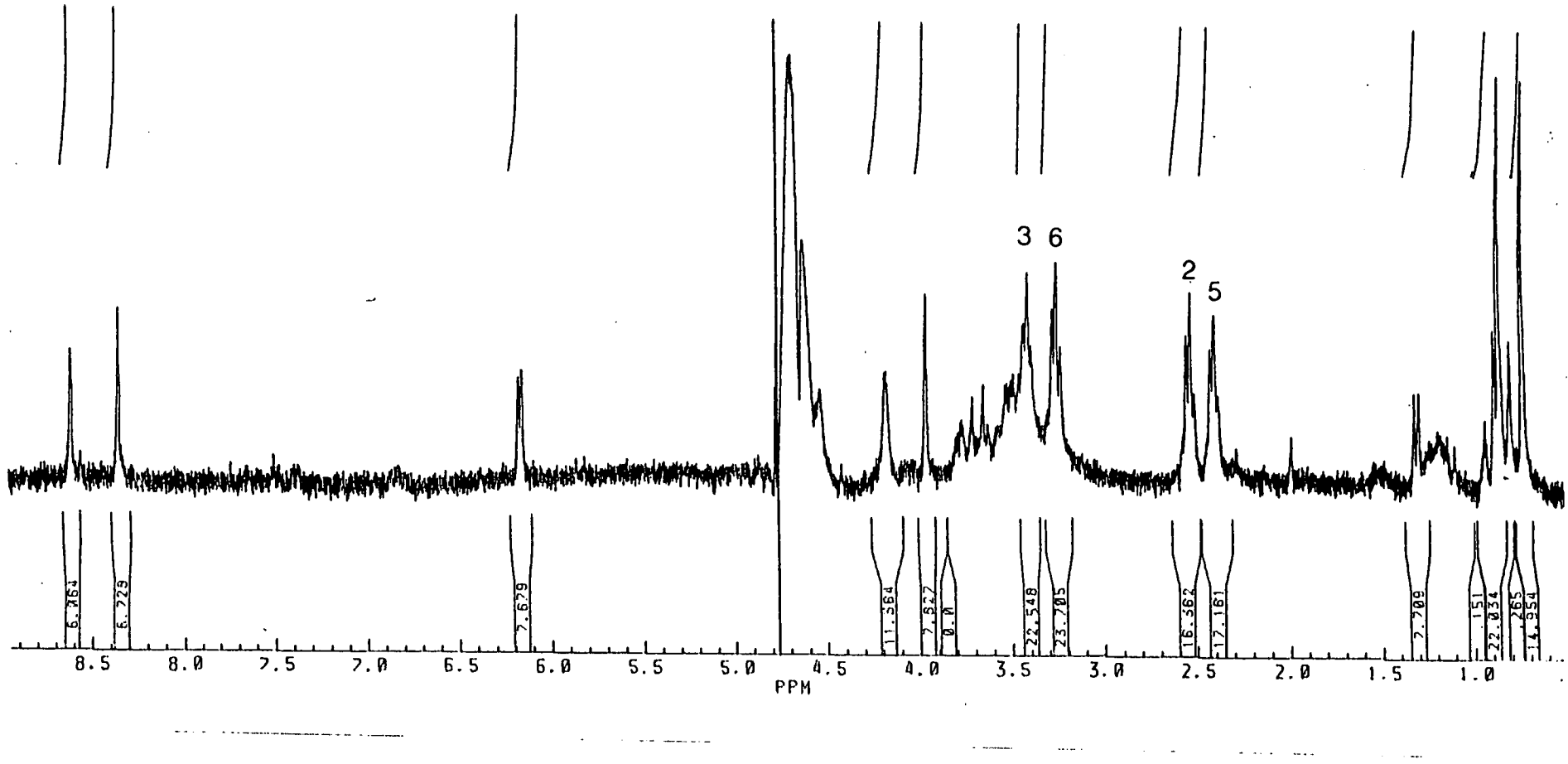


Fig. A.2 NMR spectrum of Co-enzyme A. Signals assigned to specific protons are marked with the appropriate number according to diagram A.1.

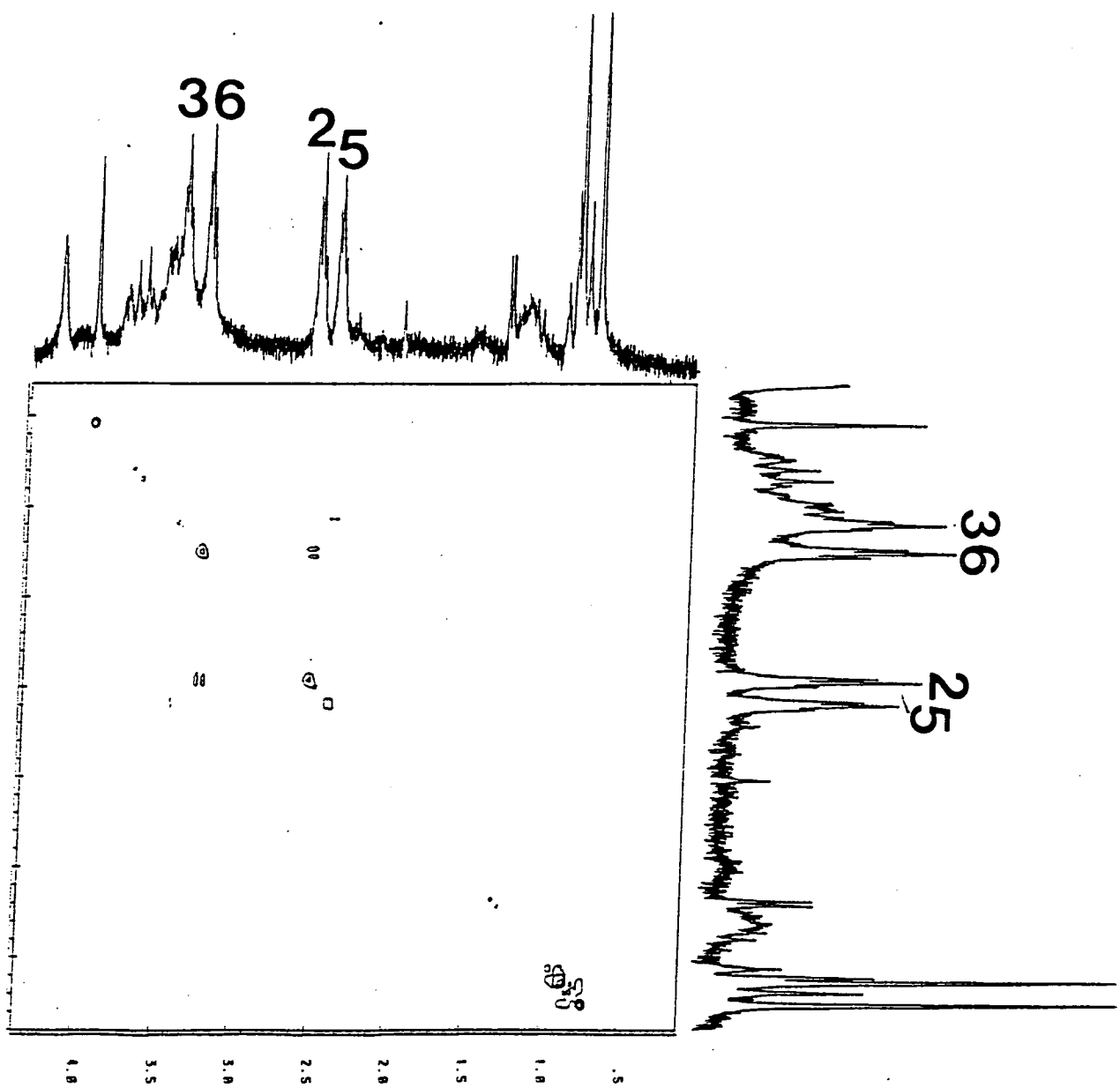


Fig A.3 COSY-NMR spectrum of Co-Enzyme A. Positive correlation can be seen between signals assigned to protons 2 and 3, and also between 5 and 6. This correlation confirms their attachment to adjacent carbon atoms.

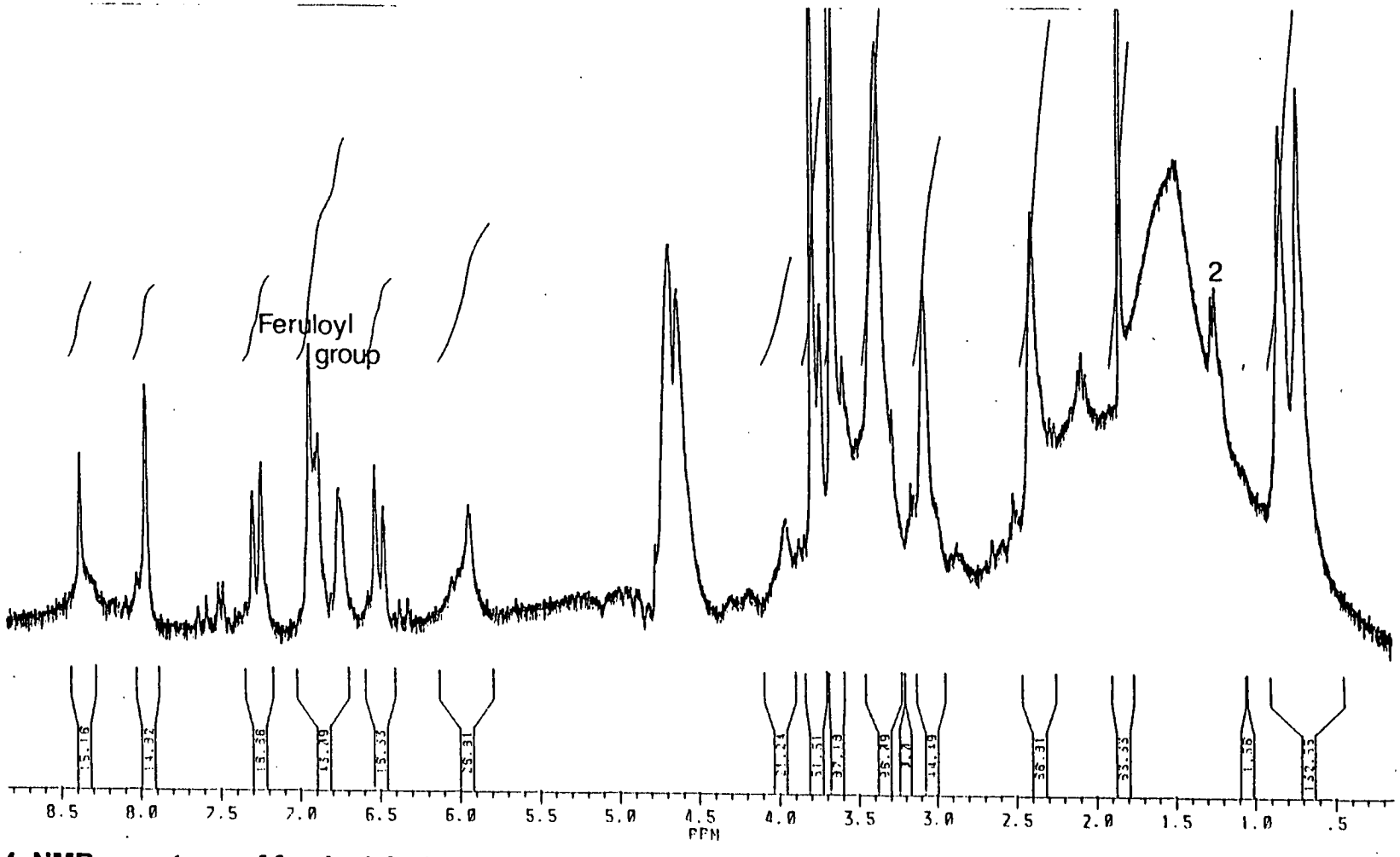


Fig A.4 NMR spectrum of feruloyl-CoA. The aromatic, α and β proton signals of the feruloyl moiety are clear at δ 6.5-7.3 ppm. The signal from proton no. 2 of the CoA moiety is much reduced at 2.1 ppm, implicating its invovlement in an ester bond. The complexity of the spectrum between 1-3 ppm obscures the signal from the corresponding proton in the esterified product.

CHAPTER 8
REFERENCES

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PUBLICATIONS

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Intraprotoplasmic feruloylation of arabinoxylans in *Festuca arundinacea* cell cultures

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Abstract. Graminaceous primary cell walls contain polysaccharides to which are esterified feruloyl residues. Ester biosynthesis is highly specific and the present experiments were performed to ascertain the likely site of feruloylation in living grass cell cultures. Cell cultures of tall fescue grass (*Festuca arundinacea* Schreber) incorporated exogenous L-[1-^{3}H]arabinose into polymers at a linear rate after a short lag of approx. 1--3 min. Radiolabelled polymers did not start to accumulate in the culture medium until 20--35 min. after [^{3}H]arabinose was supplied. However, polymer-bound feruloyl-arabinose residues began to accumulate ^{3}H after a lag of 1--3 min. Assuming that the onset of secretion of radiolabelled polymers into the medium indicates the time before which essentially all the radiolabel was internal to the plasma membrane, the results show that the polysaccharide-bound [^{3}H]arabinose residues must have been feruloylated within the protoplast.

Key words: Arabinoxylan -- Cell Wall -- Ferulic acid -- *Festuca.*

Introduction

Growing plant cell walls contain polysaccharides which bear a small proportion of phenolic side

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Abbreviations: BAW=butan-1-ol/acetic acid/water (12:3:5 by volume); BEW=butan-1-ol/ethanol/water (20:5:11 by volume); EPW=ethyl acetate/pyridine/water (8:2:1 by volume); *f*= furanose; nd, not determined; *p*=pyranose; POPOP= 1,4-bis(5-phenyloxazole)benzene; PPO=2,5-diphenyloxazole; R_{Ara}= chromatographic mobility relative to that of L-arabinose; TFA=trifluoroacetic acid.

Running title: Feruloylation of arabinoxylans in a grass.

chains (Hartley 1973). Certain dicotyledonous plants have ferulate and *p*-coumarate esterified to arabinose and galactose residues of pectic arabinogalactans (Fry 1982; Rombouts and Thibault 1986) whilst in graminaceous monocots they are esterified to terminal arabinofuranose residues of arabinoxylans (Kato and Nevins 1985) and terminal xylose residues of xyloglucans (Ishii et al. Thomas 1990). These wall-bound phenolics may be important because they provide opportunities for cross- linking between polysaccharides by oxidative coupling to form diferulate (Markwalder and Neukom 1976; Ishii 1991) or through photodimerization to form derivatives of truxillate (Hartley et al. 1990). Such cross-linking could mechanically regulate cell growth by restricting cell wall extensibility (Fry 1979).

Recent work (Iiyama et al. 1990) indicates that in lignified tissue the majority of ester-linked ferulate is also ether linked, suggesting that the phenol groups are acting as a link between lignin and the matrix polysaccharides. It is possible that the phenols in the primary cell wall act as a template for subsequent lignin deposition.

The precision with which feruloyl groups are attached to wall polysaccharides suggests that an enzyme system is involved and Meyer et al. (1992) recently reported the ability of parsley endomembranes to transfer ferulate from its CoA derivative on to endogenous acceptors. This *in vitro* activity appeared to be localized in the Golgi derived membrane fraction and was stimulated by divalent cations and spermine. Earlier work (Fry 1987) had demonstrated *in vivo* intraprotoplasmic feruloylation of pectic polysaccharides in cell cultures of spinach. However, in barley coleoptiles, Yamamoto and Towers (1985) suggested that feruloylation of polysaccharides occurred after their secretion into the cell wall. It appeared possible that dicots and graminaceous monocots employ different biosynthetic pathways to attach feruloyl groups to their wall polysaccharides and it was therefore our aim to investigate the kinetics of feruloylation of nascent polysaccharides in order to determine for the first time the sub-celluar site of this process in cell cultures of a graminaceous monocot.

Materials and methods

General. Festuca arundinacea Schreber (tall fescue grass) cell cultures were maintained in a Murashige and Skoog (1962) basal salts medium supplemented with vitamins, 2,4-D (2 $\mu g \cdot l^{-1}$) and 2% glucose. Lines were sub-cultured every 14 d and maintained on a rotary shaker at 24°C under continuous dim light.

Chromatography was on Whatman 3MM paper using the descending method with the following as solvents: butan-1-ol/ acetic acid/ water (12:3:5 by vol.; BAW); ethyl acetate/ pyridine/ water (8:2:1 by vol.; EPW); and butan-1-ol/ ethanol/ water (20:5:11 by vol.; BEW).

Paper electrophoresis was conducted using Whatman no. 1 paper in 2% $Na_2B_4O_7.10H_2O$ buffer, pH 9.4, at 2000 V for approx. 4 h.

Scintillation counting of paper strips was by immersion of the dry paper in 2 ml of scintillant A (0.5% 2,5-diphenyloxazole (PPO) +0.05% 1,4-bis(5-phenyloxazole)benzene (POPOP) in toluene). Counting efficiency was improved in some samples by the addition of 0.5 ml water to the dry paper followed by 5 ml of scintillant B (0.33% PPO + 0.033% POPOP in toluene/Triton X-100, 2:1 v/v).

The kinetics of feruloylation. Cell cultures of the required age were adjusted to a settled cell volume of approx. 20% and incubated under standard conditions for 2 h. L-[1-^{3}H]Arabinose (2.0 $MBq \cdot ml^{-1}$; 92 $TBq \cdot mol^{-1}$) was supplied to the cultures; samples (750 µl) were removed at intervals and the cells were rapidly filtered off and killed in 5 ml ethanol. An aliquot of the aqueous culture filtrate was chromatographed in BAW (see abbreviations) and material of R_f 0.00 assayed for radioactivity to monitor the incorporation of radiolabel into soluble extracellular polymers. The cells were washed in 80% ethanol until the washings were no longer radioactive, dried, and then treated with 0.1 M trifluoroacetic acid (TFA) for 1 h at 100°C. This treatment preferentially cleaves furanose bonds whilst leaving most pyranose and feruloyl ester bonds intact, thus removing arabinose side chains. The solubilized material was passed through a C18 solid phase extraction cartridge (BondElut, 1 ml, Varian, Analytichem, Harbor City, Cal., USA) which was rinsed with 3 ml

of water; retained (aromatic) compounds were then eluted with 3 ml methanol. The two fractions were dried *in vacuo* and then resuspended in 100 µl of water. The methanolic eluate was chromatographed in BAW for 16 h to resolve the various feruloylated compounds whilst the water eluate was chromatographed in EPW (see abbreviations) for 24 h to resolve mono and disaccharides, which were assayed for radioactivity. Two fluorescent feruloyl esters (R_f 0.75 and 0.61; feruloylated compounds A and B respectively) identified by their turquoise fluorescence under 366 nm UV light in the presence of ammonia vapour (Fry 1982) were present in the chromatographed methanolic fraction. These were assayed for radioactivity, eluted from the paper in 50% methanol and dried in two halves. One half was treated with 0.5 M NaOH for 1 hour at 24°C whilst the other was retained as a control. Both halves were re-chromatographed in BAW for 16 h. The control showed the majority of the radiolabel remaining at the original R_f value, whereas the majority of the radiolabel from the saponified sample had been converted to products with lower R_f values.

Partial characterisation of feruloyl esters A and B. To provide further evidence for the identity of these products, a suspension culture of *Festuca* cells (250 ml; 4 d old, packed cell volume approx. 10%) was incubated with 47 MBq of L-[1-^{3}H]arabinose under standard conditions for 5 h. The cells were then percolated overnight with 2 litres 80% ethanol to produce an alcohol insoluble residue (AIR) which was dried *in vacuo* (yield 220 mg). One portion (40 mg) was treated with 2 ml 0.1 M TFA at 100°C for 1 h and the cooled hydrolysate subjected to preparative paper chromatography on Whatman 3MM in BAW. Compounds A and B were identified and prepared as previously and further purified by paper chromatography in BEW (see abbreviations). They were eluted by the method of Eshdat and Mirelman (1972) and subjected to alkaline hydrolysis in 0.5 M NaOH at 25°C for 90 min to yield the de-feruloylated, ^{3}H-labelled sugar moieties (A_s and B_s from compounds A and B respectively). Portions of A_s and B_s were re-chromatographed in BAW and EPW. A sample of B_s was subjected to hydrolysis in 2 M TFA at 120° C for 1 h, and the resulting monosaccharides were analysed by paper chromatography in EPW. In addition, a portion of B_s

was passed through a column of Bio-Gel P-2 (in acetic acid/pyridine/H_2O,1:1:23 by vol., pH approx. 4.7) along with non-radioactive internal markers (2 mg each of xylose, xylobiose, glucose, maltose and maltotriose). A portion of each fraction was chromatographed in BAW and the marker sugars were located by staining with aniline hydrogen phthalate. Compounds A_s and B_s were treated with sodium borohydride to convert reducing termini to the corresponding alditols. After destruction of the excess borohydride with acetic acid, and removal of borate by co-evaporation with methanol, the samples were treated with 2 M TFA for 1 hr at 120°C. The resulting monosaccharides and alditols were chromatographed on paper in EPW to separate xylose from a lower-R_f zone containing arabinose, arabinitol and xylitol. Xylose (identified by comparison to authentic markers) was assayed for radioactivity and components from the second zone were eluted and analysed by paper electrophoresis in borate buffer. Sugars and alditols were identified by comparison with authentic markers and assayed for radioactivity.

In order to determine the specific radioactivity of the [^{3}H]arabinose and [^{3}H]xylose residues in the AIR from which A_s and B_s had been derived, we subjected a further portion of AIR (110 mg) to hydrolysis in 2 M TFA at 120°C for 1 h and separated the products in EPW. The zones co-migrating with external marker arabinose and xylose were eluted with water, and portions were assayed (i) for ^{3}H by scintillation counting in scintillant B, and (ii) for reducing sugar by the *p*-hydroxybenzoic acid hydrazide test (Lever 1972), using L-arabinose and D-xylose dried in a vacuum desiccator as standards; hence the specific radioactivity was calculated.

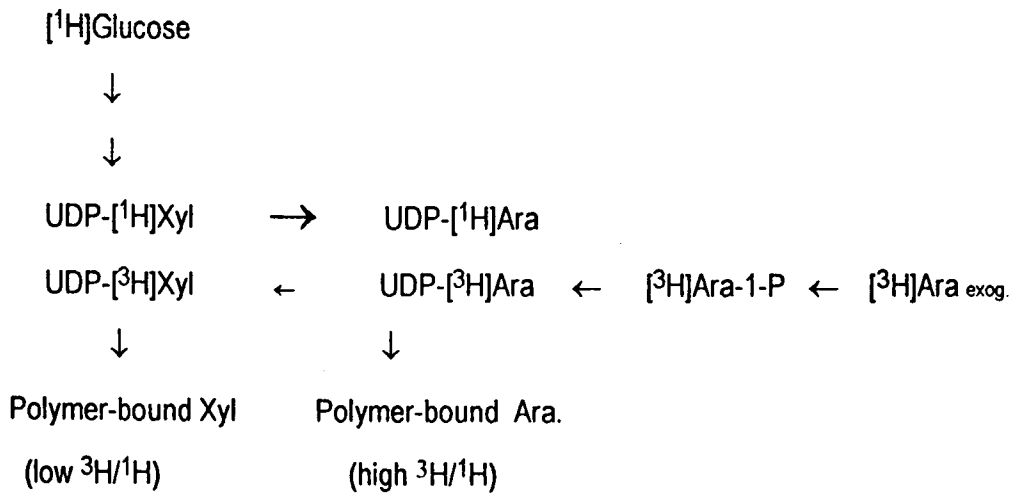
Results

The kinetics of feruloylation. Soluble radiolabelled polymers appeared in the medium after a lag of approx. 30-35 min in 2-d-old cells and approx. 20-30 min in 5-d-old cells (Fig. 1). Cellular polymer-bound arabinofuranose residues began accumulating ^{3}H after a lag of approx. 1-3 (Figs. 2a and 3a), indicating a rapid uptake of [^{3}H]arabinose and incorporation of the ^{3}H into polymer-bound pentose residues. Hydrolysis of the radiolabelled alcohol-insoluble residues with mild acid released two fluorescent compounds with R_f of 0.75 (compound A -- subsequently identified as feruloyl-

arabinose; see below) and 0.61 (feruloylated compound B). These feruloyl esters started to become radioactive after a short lag of approx. 1-3 minutes (Figs. 2b,c and 3b,c). These data indicate that within approx. 3 min [^{3}H]arabinose residues had been incorporated into polysaccharides and were beginning to be feruloylated. This is well before any radiolabelled polymer had been liberated into the medium.

The total rate of incorporation of [^{3}H]arabinose was higher in day-2 cultures than in day-5 cultures, but this is impossible to interpret in terms of arabinoxylan synthesis rates (either absolute for relative) because the specific activities of the UDP-pentose pools were not measured. These specific activities will have been considerably lower than that of the exogenous [^{3}H]arabinose, which was supplied to the cells at a concentration (22 μM) well below the apparent k_m of the arabinose-scavenging system (approx. 0.1 mM; unpublished observations), and they may have varied between day-2 and day-5. However, it is valuable to compare the ratio of feruloyl-[^{3}H]arabinose residues to [^{3}H]arabinose residues in the two different ages of cell culture. In day-5 cultures, the ratio was relatively constant at approx. 0.03. There was a similar value for feruloylated compound B but the figures were more variable. The ratios in 2-d-old cell cultures were about 10-fold lower for feruloyl-[^{3}H]arabinose (approx. 0.0029) and about 5-fold lower for feruloylated compound B (approx. 0.008). These data indicate that a 10-fold lower proportion of the newly-incorporated arabinose residues were being feruloylated in day-2 cultures than in day-5 cultures.

Partial characterisation of feruloyl esters A and B. The alcohol-insoluble residue from cells that had been fed [^{3}H]arabinose for 5 h was heavily labelled in the pentose residues. The arabinose and xylose from AIR hydrolysates were separated and estimated to have specific activities of 5.136 and 2.379 TBq·mol^{-1}, respectively. This indicates that the ^{3}H from exogenous [^{3}H]arabinose, after entering the pool of UDP-[^{3}H]arabinose via a scavenger pathway (Fry and Northcote, 1983), did not fully equilibrate with the pool of UDP-xylose that is formed from the major carbon source, non-radioactive glucose:



^{3}H-Labelled compounds A and B exhibited blue fluorescence under a 366-nm UV lamp, intensifying and turning blue-green on exposure to ammonia vapour. This behaviour is highly diagnostic of feruloyl esters. Chromatographic data for compounds A, B and their alkaline hydrolysis products are given in Table 1.

Alkaline hydrolysis of A yielded a single radioactive product (A_S) that co-chromatographed with internal marker non-radioactive arabinose. Sodium borohydride reduction of A_S yielded only [^{3}H]arabinitol, indicating that A was an *O*-feruloyl-L-arabinose derivative -- possibly 5-*O*-feruloyl-L-arabinose, which has been reported from many graminaceous cell walls (Gubler et al; 1985, Kato and Nevins 1985; Ahluwalia and Fry 1986; Mueller-Harvey et al. 1986).

Alkaline hydrolysis of B gave a single radioactive product (B_S) which migrated much slower that authentic arabinose, indicating that it was an oligosaccharide. On complete acid hydrolysis, B_S yielded [^{3}H]arabinose and [^{3}H]xylose in the ratio 2.29:1 on a ^{3}H basis, which corresponds to 1.06:1 on a molar basis if the specific radioactivities of the pentose moieties in B_S are the same as in bulk cell walls. This suggests that B_S is a disaccharide of D-xylose and L-arabinose, although the labelling data alone do not exclude the possibility of an additional non-pentose (and therefore non-radioactive) residue being present. However, gel-permeation chromatography on Bio-Gel P-2 was consistent with B_S being a disaccharide (Fig. 4).

Since compound B was released by mild acid hydrolysis, it is likely that its reducing terminal sugar moiety had been furanosidically linked within the parent polymer. Since arabinofuranose residues are abundant whereas xylofuranose is unknown in plant cell walls, the constitution of B_S is concluded to be D-xylopyranosyl-L-arabinose. Borohydride reduction of B_S followed by acid hydrolysis yielded xylose and arabinitol, but no xylitol or arabinose. This observation confirms the assumed constitution of B_S. The detailed structure of B_S, and the position of the feruloyl group in compound B will be investigated in future work. However, the origin, constitution and inter-relationships of compounds A, A_S, B and B_S as currently understood are summarised in Fig. 5.

Discussion

By supplying a radiolabelled sugar to cell cultures and analysing its incorporation into cell wall material we can estimate the time required for a sugar to be taken into the cell and incorporated into polymers and for those polymers to be subsequently feruloylated. In cell cultures, non-cellulosic polysaccharides can fail to be retained by the wall and be released directly into the medium. The kinetics of appearance of ^3H in these soluble extracellular polymers can be used as an estimate of the time required for newly synthesised polysaccharides to be secreted out of the protoplast. The data presented here show clearly that radiolabelled sugar residues were being incorporated into nascent polysaccharides and had started to have feruloyl groups attached to them at least 17 min before the appearance of extracellular radiolabelled polysaccharides. Although it is possible that polysaccharides may be secreted through the plasma membrane into the wall some minutes before their appearance in the medium and spend an appreciable time in transit through the matrix, this time is likely to be very much less than 17 min. Indeed, the Golgi vesicle transit time for wall polymer secretion alone is often greater than 17 min (Robinson et al. 1976). Thus it seems clear from the kinetics of these experiments that feruloylation was occurring in the protoplasm. This evidence is in agreement with that from dicots (Fry 1987; Meyer et al. 1992).

There was no appreciable increase in the rate of incorporation of ^{3}H into feruloylated products after 20-35 minutes (the time taken for labelled polymers to be secreted) and this would therefore indicate that little or no additional feruloylation occurs in the wall. This conclusion is in contradiction to Yamamoto and Towers' (1985) suggestion that feruloylation of arabinoxylans in the Gramineae occurs mainly in the wall. The increases in amount of ferulate that occur in barley coleoptiles (Yamamoto and Towers 1985) and in maize coleoptiles (Nishitani and Nevins 1990) after *net* deposition of wall polysaccharides has ceased could be due to a number of factors. Firstly it is feasible that the de novo rate of synthesis of heavily feruloylated polysaccharides increases as cells age and the rate of synthesis of total polysaccharides decreases. Secondly wall polysaccharides undergo enzyme-mediated degradation (Labavitch 1981) and it is feasible that feruloyl groups may protect the polysaccharides to which they are esterified from this process. This could give rise to an increasing degree of feruloylation, since feruloylated polysaccharides would be left intact in situ.

We are very grateful to Mr. Gundolf Wende for assistance with the characterisation of the feruloyl esters. K.E.M. is funded by a studentship from the Science and Engineering Research Council in collaboration with Zeneca Agrochemicals.

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Table 1. Chromatographic data for compounds A,B and their alkaline hydrolysis products.

nd, = not determined.

Compound	R_{Ara} in BAW	R_f in BEW	R_{Ara} in EPW
A	2.21	0.74	nd
A_S	1.00	nd	1.00
B	1.86	0.57	nd
B_S	0.7	nd	0.27

Figure legends.

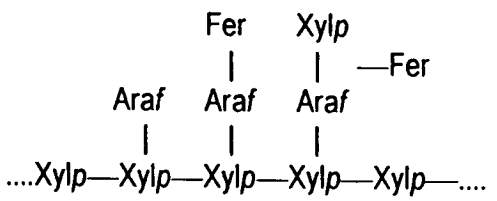
Fig. 1 a,b. Incorporation of ^{3}H from [^{3}H]arabinose into soluble extracellular *Festuca* polymers in 5-d-old cell cultures (a) and 2-d-old cell cultures (b)

Fig. 2 a--c. Incorporation of ^{3}H from [^{3}H]arabinose into cell wall components of 2-d-old *Festuca* cell cultures: a arabinofuranose residues, b feruloyl-arabinofuranose residues, c feruloylated compound B

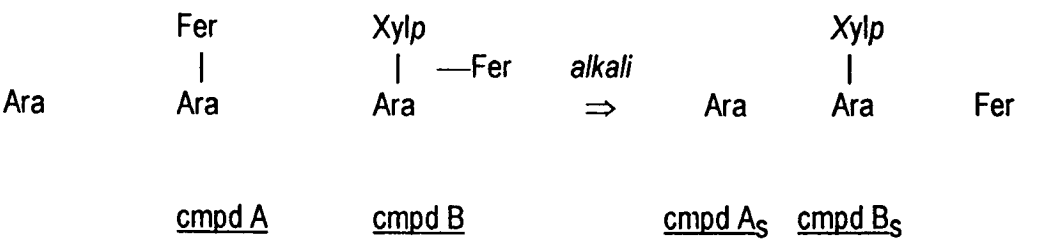
Fig. 3 a--c. Incorporation of ^{3}H from [^{3}H]arabinose into wall components of 5-d-old *Festuca* cultures; a arabinofuranose residues, b feruloyl-arabinofuranose residues, c feruloylated compound B

Fig. 4. Gel-permeation chromatography of compound B_S on Bio-Gel P-2. The histogram shows the distribution of radioactivity between the Bio-Gel fractions, and the stippled spots show the location of non-radioactive internal markers (xylose to maltotriose) after subsequent paper chromatography (PC)

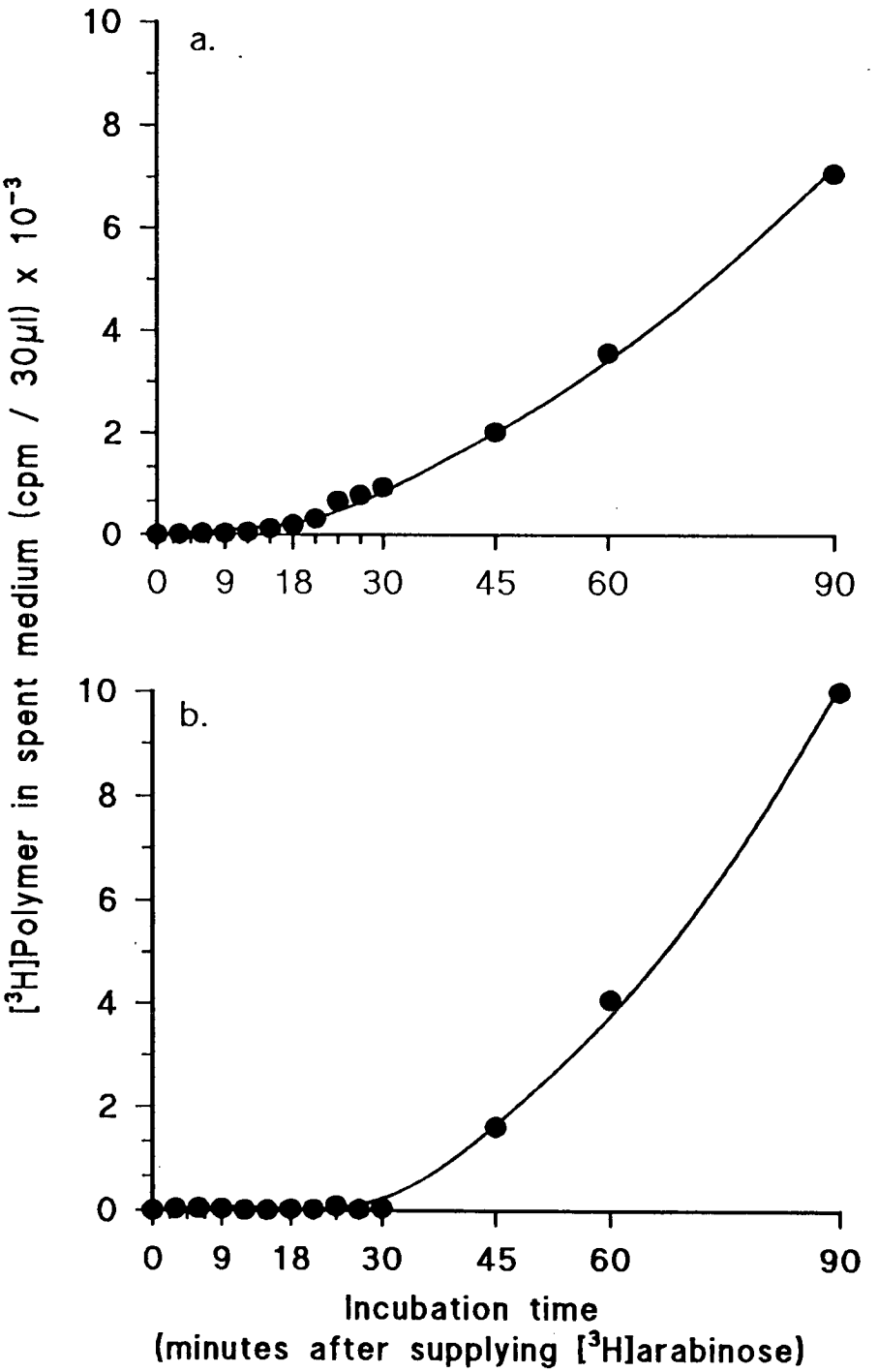
Fig. 5. Proposed effect of mild acid hydrolysis on *Festuca* cell wall arabinoxylan, and the effect of mild alkaline hydrolysis on compounds A and B. Ara, arabinose; Fer, ferulate; Xyl, xylose; *f*, furanose; *p*, pyranose

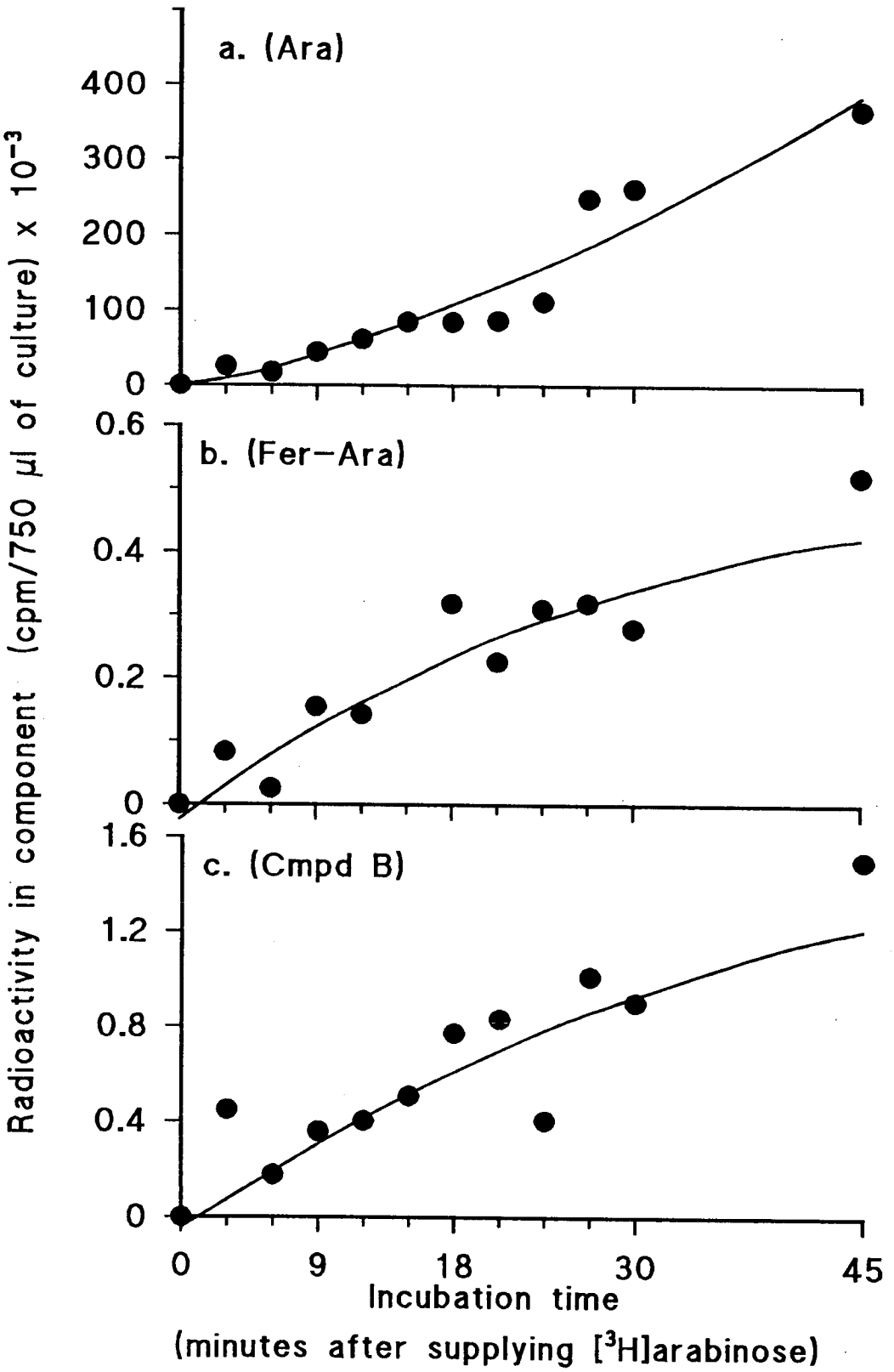


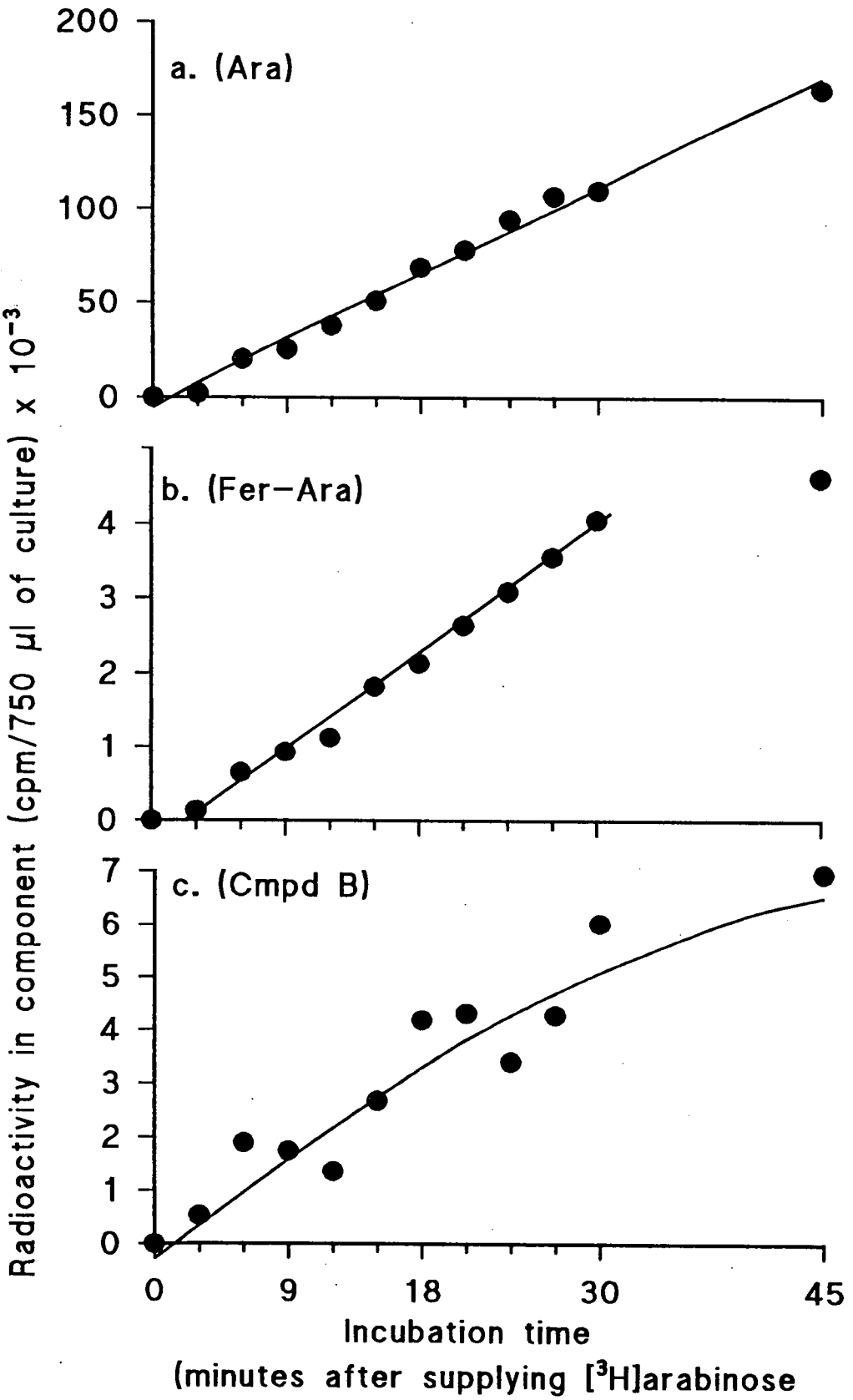
acid ⇓

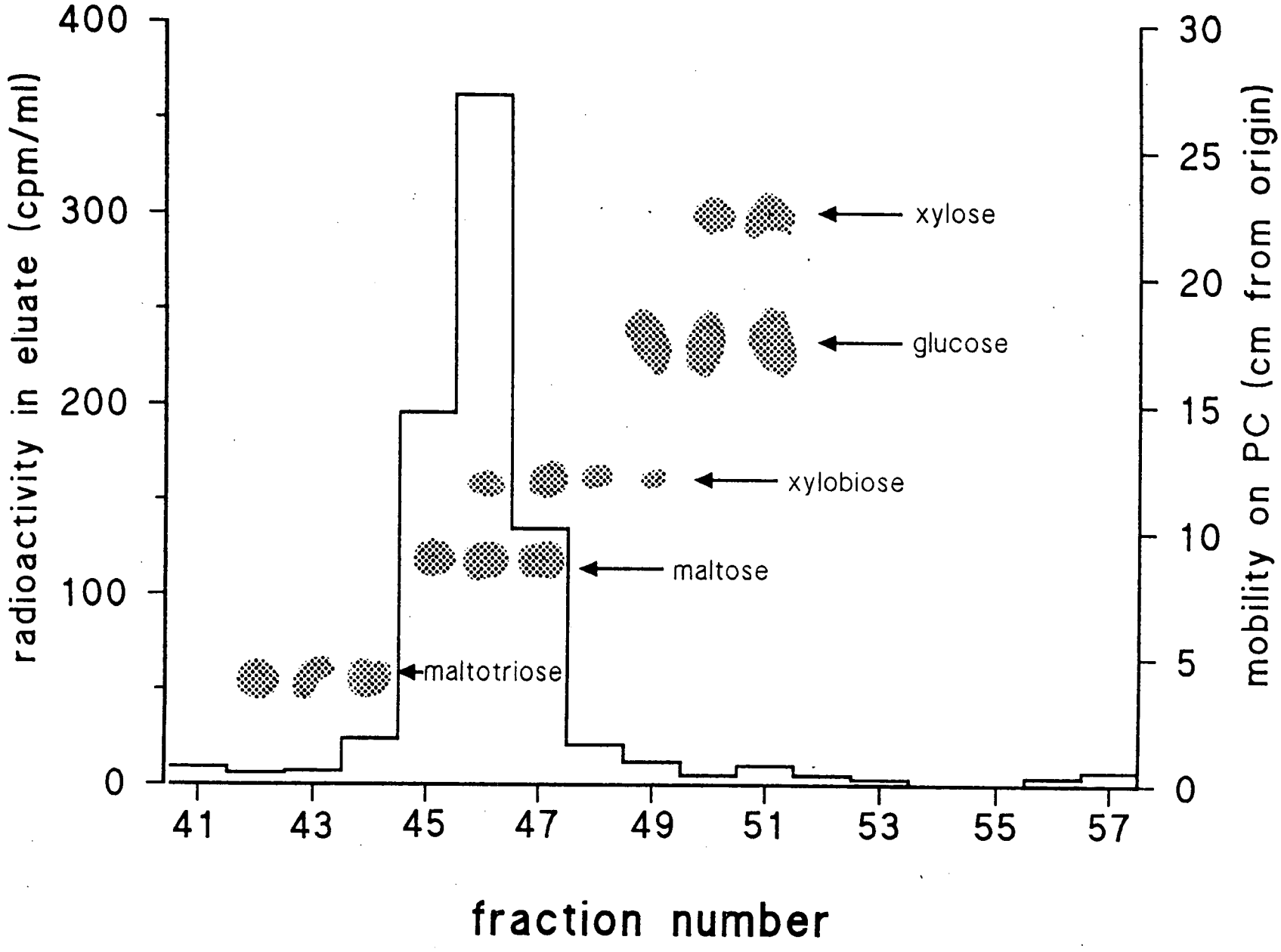


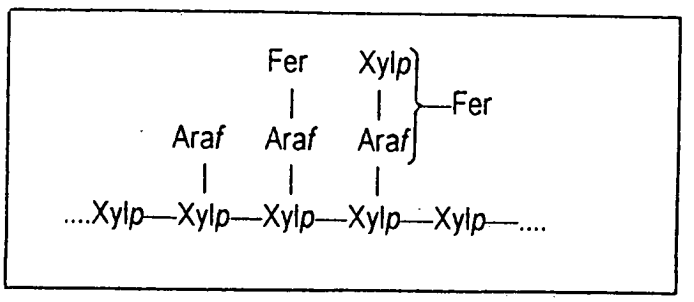
....Xylp— Xylp — Xylp — Xylp — Xylp —....


a.
b.
10
8
6
4
2
0
0 9 18 30 45 60 90
[3H]Polymer in spent medium (cpm / 30µl) x 10-3
Incubation time
(minutes after supplying [3H]arabinose)

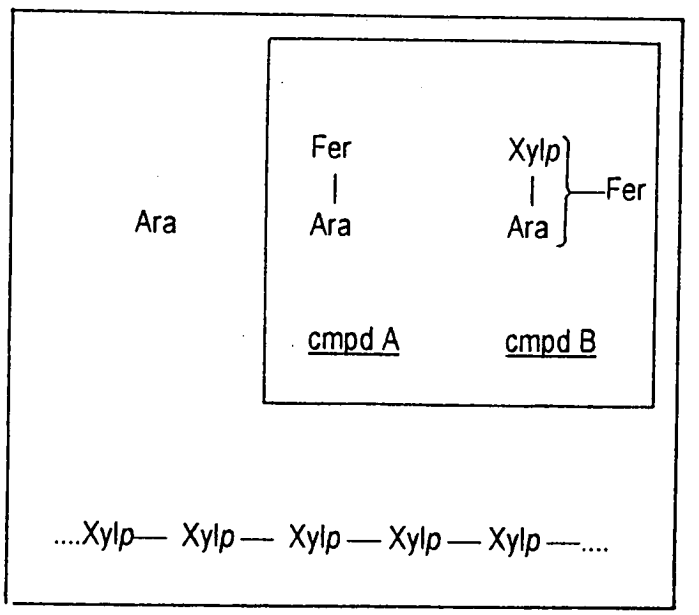

a. (Ara)
400
300
200
100
0
0.6
b. (Fer−Ara)
0.4
0.2
0
1.6
c. (Cmpd B)
1.2
0.8
0.4
0
0
9
18
30
45
Incubation time
(minutes after supplying [3H]arabinose)
Radioactivity in component (cpm/750 µl of culture) x 10−3


a. (Ara)
200
150
100
50
0
b. (Fer−Ara)
4
3
2
1
0
c. (Cmpd B)
7
6
5
4
3
2
1
0
0
9
18
30
45
Radioactivity in component (cpm/750 µl of culture) x 10^{-3}
Incubation time
(minutes after supplying [^{3}H]arabinose

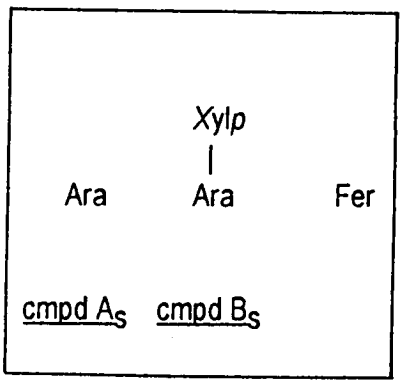

radioactivity in eluate (cpm/ml)
400
300
200
100
0
mobility on PC (cm from origin)
30
25
20
15
10
5
0
41
43
45
47
49
51
53
55
57
fraction number
xylose
glucose
xylobiose
maltose
maltotriose


Fer
Xylp
Fer
Araf
Araf
Araf
....Xylp—Xylp—Xylp—Xylp—Xylp—....

acid ⇓


Fer
Xylp
Fer
Ara
Ara
Ara
cmpd A
cmpd B
....Xylp— Xylp — Xylp — Xylp — Xylp —....

alkali ⇒


Xylp
Ara
Ara
Fer
cmpd A_S
cmpd B_S

Poster presented at the 1st Scottish Cell Wall Group Meeting, Edinburgh. (1991)

Abstract Number 9.

FERULOYLTRANSFERASES INVOLVED IN CELL WALL BIOSYNTHESIS IN MAIZE.

9

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Background to the project.

Ferulic (4-hydroxy, 3-methoxycinnamic) and coumaric (4-hydroxycinnamic) acids are covalently bound to the cell walls of plants from several families. These phenolic acids are thought to be esterified to cell wall polysaccharides[1] eg. pectin in spinach and arabinoxylan in maize, barley and *Lolium multiflorum*.

There are several suggestions regarding a role for these bound phenolic acids - the control of cell expansion through the formation of cross links between cell wall polysaccharides, due to oxidative coupling or photodimerization[2], as a precursor of lignin biosynthesis, as protectants against pathogen invasion. Phenolic acids have also been shown to affect digestibility in the bovine and ovine rumen.

The precision with which ferulic acid is bound to wall polysaccharides suggests that an enzyme system is responsible, though little is known about the highly discriminating machinery which must exist to attach ferulic acid to the correct hydroxyl group of specific sugar residues. Cell free enzymic feruloylation of polysaccharides has never been demonstrated and this project involves an attempt to discover and characterize this enzyme. Work to be conducted at ICI Jealotts Hill Research Station will involve the characterization of the major saponifiable phenolics in maize cell walls.

The experiment reported here was designed to show the fate of cinnamic acid when fed to cell cultures and to identify the major phenolic containing compounds.

Materials and Methods.

Cell cultures of maize were fed (^{14}C) cinnamic acid at various times after sub culturing and left for 24 hours. Cells were then harvested, immediately washed in 70% Ethanol until washings were no longer radioactive and then treated with 200µl 1% 'Driselase' solution (24 hours at 18°C). The Driselase digestion products were seperated by 2-D paper chromatography in first butan-1-ol: acetic acid: water then either butan-1-ol: ethanol: water or butan-1-ol: pyridine: water. The chromatograms were autoradiographed and radiolabelled compounds eluted with 50% methanol. The eluate was dried, treated with 0.1M NaOH acidified and then partitioned against butan-1-ol. These free phenolic acids were run on silica gel T.L.C. in benzene: acetic acid. The plates were then assayed for radioactivity.

Results.

Three distinct compounds were identified by autoradiography and the major phenolic in all these was ferulic acid. with very small amounts of coumaric acid. Considerable quantities of labelled compound remained at the origin and on saponification was identified as ferulic acid. It is possible that the compounds that remain at the origin are dimers or polymers of ferulic acid.

Conclusion.

Maize cell cultures actively incorporate cinnamic acid and produce large quantities of feruloylated sugars ie. FAXX, FAX.[3] This is therefore a useful system in which to study feruloyl transferase.

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Abstract Number A15.

THE SUB-CELLULAR SITE OF ARABINOXYLAN FERULOYLATION IN CULTURED CELLS OF *ZEA MAYS* AND *FESTUCA ARUNDINACEA*.

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Introduction. Ferulic acid is esterified to the *O*-5 of the L-arabinofuranose side chains of arabinoxylan in maize cell walls (Smith and Hartley 1983). Such bound ferulic acid may form cross links between cell wall polymers (Fry 1983, Hartley *et al.*, 1990), protect plants from pathogen attack and affect forage digestibility. The precision with which ferulic acid is attached to cell wall polysaccharides indicates that an enzyme is responsible. Cell free feruloylation of polysaccharides has never been demonstrated and it is the aim of this work to discover and characterise the enzyme responsible for this process, arabinoxylan feruloyltransferase. Towards this end experiments have been conducted to determine wether feruloylation occurs apoplastically or symplastically, using an approach similar to that of Fry (1987).

Methods. [^{14}C]Glucose and [^{3}H]arabinose were fed to maize and *Festuca* cell cultures respectively. Samples were removed at 3 minute intervals, rapidly filtered and washed in 80% ethanol until washings were no longer radioactive. The incorporation of radiolabelled arabinose and xylose residues into soluble extracellular polysaccharides was determined using acid hydrolysis followed by descending paper chromatography and scintillation counting. The lag time after which these radiolabelled residues started to accumulate in extracellular polymers was taken as the time in which all radiolabelled arabinose and xylose residues were symplastic. The cell wall residue was subjected to driselase digestion (a fungal enzyme preparation that lacks feruloyl esterase activity) and then passed through C18 Bondelut. This process effectively separates phenolic containing compounds from the other products of driselase digestion, mainly monosaccharides. Both fractions were chromatographed on paper to separate monosaccharides and feruloyl esters. Both chromatograms were scintillation counted and the incorporation of radiolabel into sugar residues and feruloyl esters was determined.

The results from this experiment will be presented and thus evidence for the subcellular site of feruloylation.

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Abstract Number 4.

I. Kinetics and Subcellular site of Feruloylation in *Festica arundiacea* cell cultures.

II. Changes in enzyme activity along the Maize Interode.

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Ferulic acid is esterified to cell wall polysaccharides in plants from several families and is thought to affect cell wall extensibility through the formation of cross-links between these polysaccharides (1). Recent work (2) has shown that ferulic and coumaric acid can also be etherified to lignin moieties, suggesting a rôle in the linking of lignin to matrix polysaccharides and phenolic acids also affect forage digestibility in the rumen.

Earlier work (3) indicated that the feruloylation of polysaccharides occurred intracellularly in spinach and more recently Meyer et al (4) have demonstrated feruloyl transferase activity in isolated golgi membranes from parsley cells. In a series of experiments broadly following the methods of Fry (1987), ^{3}H-arabinose was fed to *Festuca* cell cultures and samples removed at intervals. Analysis showed that radiolabel was incorporated into arabinofuranose residues of polysaccharides rapidly after a lag of c. 7·5 minutes and into polysaccharide bound feruloyl-arabinofuranosl after a lag of c. 11 minutes. However, radiolabel did not appear in excreted extracellular polysaccharides until c.38 minutes, the time before which essentially all radiolabel is within the plasmamembrane.

In view of the role feruloyl residues may play in the linking of lignin to the cell wall matrix it was decided to investigate the activity of some of the enzymes involved in lignification with a view to correlating this with feruloyl transferase activity in the future. Two inbred lines of maize with varying digestibilities were used - W401 and C0125. The activities of phanylalanine ammonia lyase (PAL), O-methyltransferase (OMT) and coniferyl alcohol dehydrogenase (CAD) were monitored along the length of a stem internode. Two internodes were used; that directly below the one being the tassel (internode 2) and the 4th from the top of the plant (internode 4). These were cut into 5 sections of approximately equal length.

In line W401 PAL activity increased from the top to bottom of internodes 2 and 4, whilst OMT activity appeared to peak towards the upper end of internode 2. In line C0125 a similar trend was seen though less clearly and to a lesser extent in intemode 2. No change in CAD activity along the intemode was detected in either line. The PAL activity in line C0125 was c. 30 fold less than in line W401.

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Abstract Number 36.

THE SUBCELLULAR SITE OF FERULOYLATION OF ARABINOXYLAN IN FESTUCA ARUNDINACEA CELL CULTURES.

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INTRODUCTION: Growing plant cell walls contain polysaccharides that bear a small proportion of phenolic side chains (1). The precision with which these phenolic residues are attached to wall polysaccharides suggests that an enzyme system is involved and Meyer *et al* (2) recently reported the *in vitro* ability of endomembrane preparations from parsley cell cultures to transfer ferulic acid from its CoA derivative onto a polysaccharide acceptor. Earlier work (3) had demonstrated *in vivo* intraprotoplasmic feruloylation of pectic polysaccharides by cell cultures of spinach. We are therefore interested in investigating this process in the monocots *Festuca* and maize. The experiment reported here was designed to investigate at which point in its career an arabinoxylan polymer gains it s feruloyl groups.

METHODS: *Festuca* cell cultures were fed [^{3}H]arabinose and samples removed at intervals and washed in 80% EtOH until the washings were no longer radioactive. The residue was subjected to mild acid hydrolysis with 0.1M trifluoroacetic acid 100°C, 1 hour, and solubilized products seperated by paper chromatography. This treatment specifically cleaves furanose bonds and so releases arabinose and feruloyl-arabinose. The incorporation of radiolabel into these two components was monitored.

RESULTS: Radiolabel was incorporated into arabinofuranose residues after a short lag of c.4.5 min, and into feruloyl-arabinose residues after a lag of c.6 min. In cell cultures non-cellulosic polysaccharides can fail to be retained by the wall and pass into the medium. Radiolabel did not appear in these extracellular polymers until after 21 min, the time before which essentially all the radiolabel can be said to be within the plasmamembrane.

DISCUSSION: The incorporation of radiolabelled arabinose into polysaccharides and its subsequent feruloylation occurs well before radiolabel appears in soluble extracellular polysaccharides. Taking that this point is the time before which essentially all radiolabel is within the protoplast, and allowing for a short period of time for the polysaccharide to move through the wall these results support the theory that feruloylation is an intraprotoplasmic process in monocots. This evidence is consistent with that from dicots.

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Abstract Number 10.

FERULOYL TRANSFERASE INVOLVED IN MAIZE CELL WALL BIOSYNTHESIS.

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Introduction. Growing cell walls contain polysaccharides to which are esterified ferulic acid residues. The precision with which these feruloyl groups are attached to sugar residues suggests that an enzyme system is involved and we have been investigating this possible enzyme action in cell cultures of *Zea mays*.

Materials and Methods. Particulate membrane preparations from *Zea* cultures were prepared using differential centrifugation and were supplied with [^{14}C]feruloyl CoA and divalent cations. Radiolabelled polymeric material was either precipitated by the addition of HOAc/EtOH and washed thoroughly or spotted onto Whatmann 3MM paper and washed in running water for 2 hours to remove unreacted [^{14}C]feruloyl CoA. The incorporation of radiolabel into polymeric material was monitored under a number of different experimental conditions. In one experiment the polymeric material was treated with 0.1 M TFA (1 hour at 100°C) and solublized material separated by paper chromatography. Low molecular weight radiolabelled compounds released by this treatment were recovered and treated with 0.5M NaOH (30 mins at 25°C).

Results. Endomembrane preparations were capable of transfering [^{14}C]ferulic acid from its CoA derivative onto polymeric material. This reaction was enhanced by the addition of arabinoxylan to the initial homogenization medium. The addition of EDTA to the preparations resulted in a decreased reaction rate and cobalt was found to be the only effective co-factor with an optimum concentration of c.5mM. Activity increased with temperature but was destroyed by boiling and reduced by freezing. Specific activity apparently changed with age of culture and activity with changing pH was variable. Acid hydrolysis of polymeric material resulted in the release of two radiolabelled products with R_f values identical to authentic feruloylated sugars. Treatment of these products with NaOH resulted in the majority of radiolabel being released as free ferulic acid.