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TRANSPORT OF SUBSTRATE WITHIN THE WHEAT GRAIN

by

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Thesis submitted for degree of

Doctor of Philosophy

1987

Awarded 19/8/87

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SUMMARY

In wheat, both yield and grain quality are determined to a large extent by factors intrinsic to the grain. The work reported in this thesis studies the transport of substrate within the grain and examines whether or not factors associated with this transport may influence yield and quality.

The first part of the experimental programme (sections 3.1, 3.2 and 3.3) used microsectioning, HPLC, and radiotracer techniques to (1) identify the solutes that form the principal substrate for starch and protein deposition in the grain, (2) describe the concentration gradients of these solutes throughout the endosperm and (3) test whether or not the regional patterns of deposition of the polymeric products within the endosperm could be due to the patterns of substrate supply.

Carbohydrate entered the grain as sucrose and most of the carbohydrate transported through the endosperm was transported as sucrose, even though sucrose accounted for only 40-50% (w/w) the total soluble carbohydrates. On the other hand, a wide range of amino acids formed the complement of amino-substrate. Sucrose, and indeed soluble carbohydrate as a whole, and amino acids did not conform to the same distribution pattern throughout the endosperm. But these differences in the distribution of substrate did not account entirely for the regional differences in the deposition of starch and protein. Also there were regional differences in the kinetics of conversion.

Overall, the rate of turn-over of sucrose within the endosperm was nearly 10 times that of amino acids.

The second part (sections 3.4, 3.5 and 3.6) described the route of solute movement throughout the grain and tested whether or not the observed concentration patterns of substrate within the endosperm could be due to differences in the direction of substrate supply. Both carbohydrates and amino acids destined for the endosperm moved by the same route; i.e. longitudinally in the vascular tissue at the base of the crease, then

radially through the endosperm cavity and endosperm. There was no detectable movement of solutes into the endosperm in an inward radial direction from the inner pericarp or any other tissue surrounding the endosperm. Differences in the distribution of carbohydrate-substrate and of amino-substrate throughout the endosperm were due to kinetic factors within the endosperm itself.

The results are discussed in relation to the distribution of dry matter within the endosperm and to the source-sink interactions for carbohydrates and for nitrogenous compounds between the plant and grain as a whole.

STATEMENT

I hereby declare that the work reported in this thesis contains no material previously published or written by another person except where due reference is made in the text, and that it contains no material previously accepted for the award of any other degree or diploma. This thesis is available for photocopying and loan if it is accepted for the award of the degree.

Trelawney David Ugalde

ACKNOWLEDGEMENTS

First, I would like to acknowledge the encouragement given to me by my parents during the early years of my education by creating in the home the environment conducive to study, and to express my gratitude to Glenice, Anna and Sarah who now provide the support I need.

I am grateful also to Prof. David Chalmers who through his advice, support and encouragement has guided the development of my career, and to the Victorian Department of Agriculture and Rural Affairs for allowing this candidature to be a part of my employment responsibilities.

I would like to thank the supervisor of this project, Dr. Colin Jenner, for his advice and assistance. His incessant curiosity towards the workings of the wheat grain has been a constant source of encouragement and fun.

I am grateful to Brian Walker and Mike Dowling of Millipore-Waters who coached me in the art of HPLC usage.

I would like to acknowledge the assistance given to me by Pieter Faber; together we fell into, but fortunately climbed out of many of the traps that await beginners in the field of HPLC.

Part of this thesis was typed competently and quickly by Julie Noonan and for this contribution I am most grateful.

Finally, I would like to thank Alison Knowles. Although she was not involved directly in my project, it was a joy indeed to work along side some-one so cheerful.

ABBREVIATIONS, SYMBOLS AND DESCRIPTIVE NOMENCLATURE USED IN THIS THESIS

sec	second
min	minute
h	hour
ng	nanogram
ug	microgram
mg	milligram
g	gram
kg	kilogram
nm	nanometer
um	micrometer, microns
mm	millimeter
cm	centimeter
m	meter
ul	microlitre, 1 x 10^{-6}l
ml or cm^3	millilitre, 1 x 10^{-3}l, cubic centimeter
l	litre
m^3	cubic meter
^{o}C	degrees celsius
kPa	kilopascal
uE	microEinsteins
xg	force times gravity
>	greater than
<	less than
cf.	compare
cv.	cultivar
cvs.	cultivars
diam	diameter
EDTA	ethylenediaminetetraacetic acid
ha	hectare

HPLC	high performance liquid chromatography
i.d.	internal diameter
p.a.	post anthesis
*	registered trade mark
RF	response factor, i.e. Area units per amount of solute
RT	retention time
S.E.	standard error of mean
viz.	videlicet
w.r.t.	with respect to
w/v	weight per volume
w/w	weight per weight

Nomenclature:

Dorsal side of the grain - the side of the grain opposite the crease, adjacent to the lemma

Ventral side of the grain - the crease side of the grain, adjacent to the palea

Distal end of the grain - the end situated away from the point of attachment

Proximal end of the grain - the stalk end of the grain

SECTION 1

GENERAL INTRODUCTION

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1.1 WHEAT PRODUCTION IN AUSTRALIA AND THE AIMS OF THIS REVIEW

Annual production of wheat in Australia over the past 10 years (1975-76 to 1984-85) has averaged 13.6 x 10^6 tonnes. Seventy-five percent of this was exported, mainly to USSR, Egypt, China, Japan, Iran and Iraq. Together these six countries accounted for 70% of Australia's wheat exports (Australian Wheat Board Annual Report 1984-85; Love 1985a). Export from Australia comprises 16% of world trade (Fig. 1-1).

Wheat is Australia's second largest export earner; in 1985/86 it brought in $3.7 billion which is 15% of all export earnings (National Farmers' Federation, Australian Agricultural Year Book, 1986). The principle regions within Australia where wheat is grown and the types of wheat produced in each are shown in figure 1-2.

Over the past 6 years (1979-1986), the world price for wheat has declined in real terms, falling short of inflation by 2% annually. This reflects increased competition between exporting countries brought about mainly by increased production from Asia and Western Europe. A succession of 3 record years in China has elevated that country to the position of the world's largest producer of wheat. In 1984, China produced 83 x 10^6 tonnes compared to 76 x 10^6 tonnes by the EEC and 70 x 10^6 tonnes by the United States (Love, 1985a).

The export price for Australia's wheat is set by the Australian Wheat Board and is based on the United States export price. The U.S. price is taken as the standard because (1) the U.S. is the largest exporter (Fig. 1-1), (2) the U.S. price takes into account transitory fluctuations in supply and demand, and (3) Australia competes directly with the U.S.. Deviations from the U.S. price reflect decisions by the Australian Wheat Board to price at a premium or to discount Australia's wheat on the basis of quality or local supply and demand (Australian Wheat Board, Annual Report 1983-84, Love, 1985a).

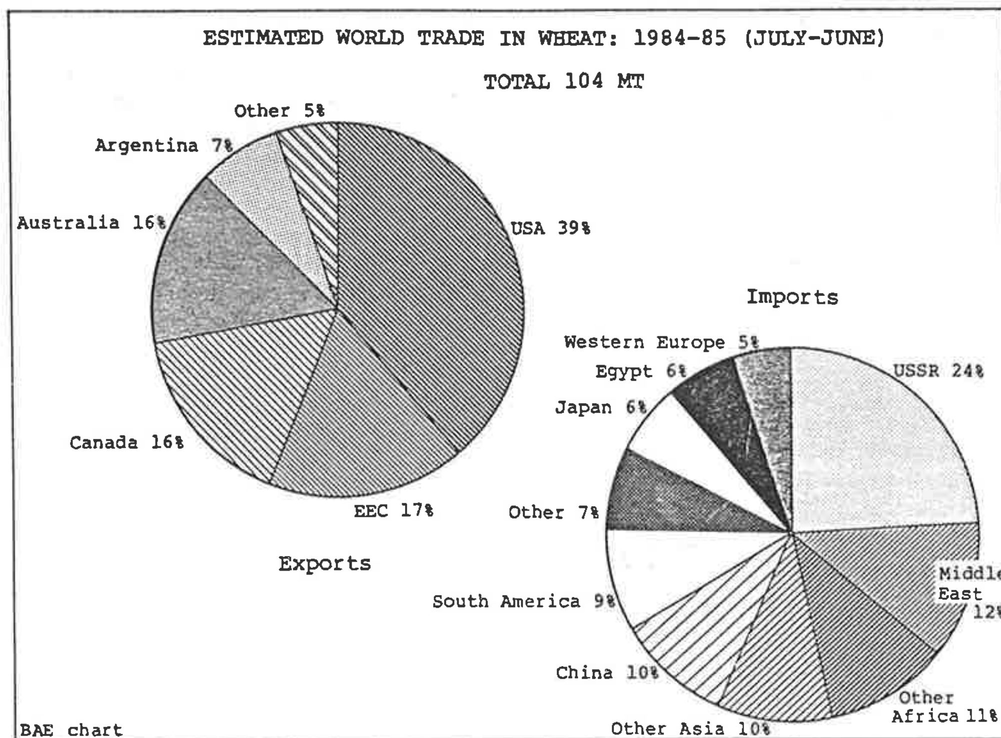


Figure 1-1. World trade in wheat, 1984-85. Reproduced from Love (1985a).

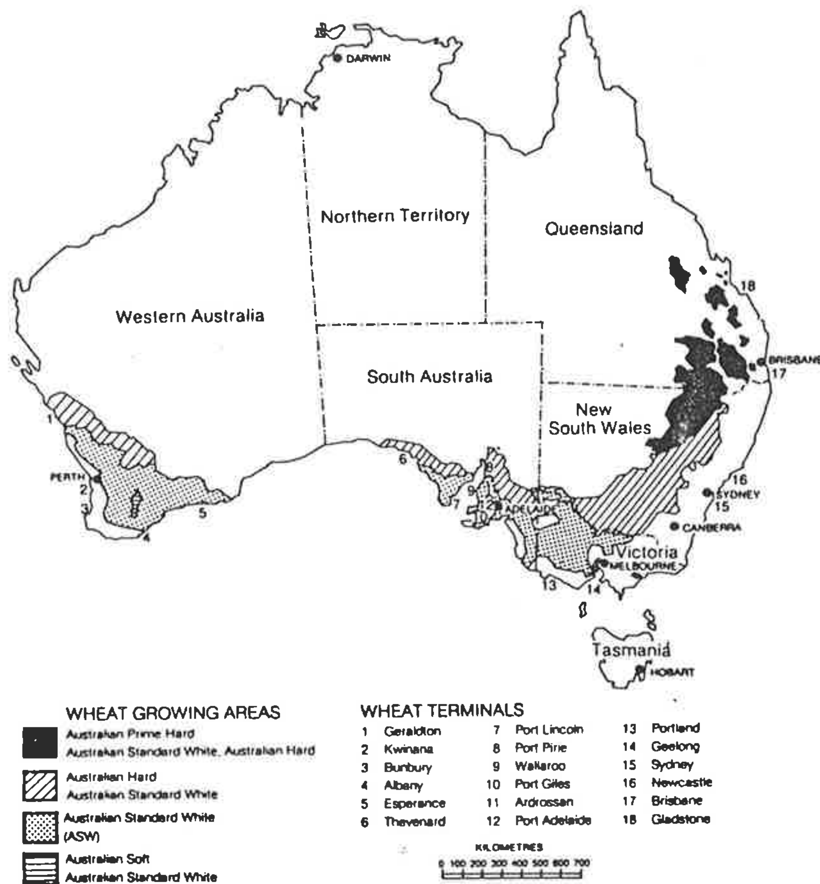


Figure 1-2. Wheat growing areas within Australia and the types of wheat produced. Reproduced from Australian Wheat Board, Crop Report, 1985-86.

The EEC subsidizes heavily its sales of wheat on the world market. In December 1985 the U.S. government passed a bill called the United States Food and Security Act 1985 which effectively does the same thing in an attempt to reduce the stockpile of grain in that country. The overall effect of this competition has been, and will be, to decrease the price of wheat on world markets still further. The Australian Wheat Board has stated on a number of occasions that in this climate the only avenue open to the Australian Wheat Industry is to improve the efficiency of production while maintaining or increasing grain quality (e.g. Sir Leslie Price, Chairman of the Australian Wheat Board as quoted in Wheat Australia Vol 19, No 1, Feb. 1986 and by Thornhill (1985); Clinton Condon, Chairman Designate of the Australian Wheat Board at the Agricultural Outlook Conference 1986).

The aims of this General Introduction are as follows.

1. To define the parameters of grain quality and yield and to define the factors that influence them (section 1.2).
2. To study the role of irrigated agriculture in the wheat industry with special reference to quality and yield (section 1.3).
3. To describe the anatomical development of the wheat grain, essential in any study relating structure and physiological function to grain composition (section 1.4).
4. To describe some of the mechanisms of grain growth and to outline the research work undertaken during the course of this candidature (sections 1.5 to 1.10).

1.2 GRAIN QUALITY

Wheat is the greatest single source of nourishment for the world's population (Inglett and Anderson, 1974). However, in the raw state it is neither digestible nor palatable (Payne and Rhodes, 1982). Techniques to prepare wheat for consumption have been developing over 6 millenia (Jacob,

1944) and so today there is a range of sophisticated products (section 1.2.3.2.1). Wheat quality, the principle factor determining the saleability of Australia's wheat (section 1.1) is not an absolute property, but rather is judged according to the suitability of the grain for producing one of these consumable products.

First, wheat must be free of disease, contamination, kernel damage and sprouting. These factors are not considered further. The other principle criteria of grain quality are discussed below, together with discussion of the major effectors of these quality characteristics.

1.2.1 Texture of the Endosperm

The texture of the endosperm determines the way the endosperm breaks down during milling. The endosperm may be 'hard' or 'soft'. The endosperm of hard wheats tend to fracture along cell walls whereas the endosperm of soft wheats fracture in a random way. Hardness is related to the degree of adhesion within the cell. Strong adhesion makes the cell wall, relatively, a zone of weakness.

Several explanations of the biochemical basis of hardness have been proposed. First, Symes (1969) suggested that hardness was determined by the type of protein deposited in the endosperm. However, no difference in protein characteristics could be found within isogenic lines differing in hardness (Simmonds et al., 1973). It now appears that hardness is determined by the nature and strength of adhesion between starch granules and the protein matrix. (Barlow et al., 1973). In the mature grain, starch granules are surrounded by lipid and protein components that are the residue of the amyloplast membrane. This material is appressed to the membrane remnants of the protein bodies and rough endoplasmic reticulum. These substances, combined, cement together the starch granule and the protein matrix.

The characteristic of grain hardness is due to a single major gene. There is little or no influence of environment or cultural practice on grain hardness (Symes, 1965).

Hard wheats produce a course gritty flour, free flowing and easily sifted. It consists of regular shaped particles, many of which are whole endosperm cells. Soft wheats produce a fine flour consisting of irregular shaped cell fragments and individual whole starch granules. These fragments tend to stick together making sieving difficult.

There is a greater degree of mechanical damage to starch granules of hard wheats during milling. This is due partly to the heavier rolling needed to reduce the grain to flour and partly to the strong bonding between the granule and the protein matrix. Individual starch grains can be sheared when, occasionally, the endosperm of hard grains fractures intracellularly. The degree of granule damage influences the rate of water absorption and the amount of water needed to obtain a standard dough consistency (Kent, 1978).

Amylases can attack starch in damaged granules more readily than starch in whole granules. So, during bread-making it is the starch from damaged granules that provide most of the substrate for fermentation. It is essential, therefore, that flour contains an adequate amount of damaged starch. Excessive starch damage, however, has an adverse effect on bread quality (Kent, 1978).

The texture of the endosperm can also be described as vitreous (steely, flinty) or mealy (starchy, chalky). Vitreous kernels are translucent and appear bright against a strong light, whereas mealy kernels are opaque and appear dark under similar circumstances.

The opaque appearance of mealy kernels is due to minute air-filled fissures between and maybe within the endosperm cells. These fissures are absent from vitreous kernels (Kent, 1978). The specific gravity of vitreous grains is higher (1.42) than that of mealy grains (1.40, Bailey, 1916).

The vitreous or mealy characteristic is largely genetic. The more vitreous types are _T. dicoccoides_, _T. monococcum_ and _T. durum_, while the more mealy types are _T. turgidum_, _T. compactum_ and _T. aestivum_ (Percival, 1921). This characteristic is modified by environment, however. Dried immature grains of all wheat types are vitreous. Vitreousness is carried through to the mature grain when the grain develops and ripens quickly, when yield is low, and when the grain protein percentage is high (Percival, 1921). Mealiness is favoured, therefore, in varieties with slow growth or long maturation period, or in conditions of growth that induce the same, e.g. good nutrition, low water stress or cool temperatures.

Wheat is graded according to the vitreousness of the kernel, but this characteristic in itself is not a major determinant of milling or baking quality. Vitreousness is used to estimate grain hardness and protein content (Stenvert and Kingswood, 1977). It is not a good measure of grain quality, however. Vitreousness is influenced by environment, hardness is not (see above); some soft wheats may be vitreous (Simmonds, 1974a), and some hard wheats may be mealy (Berg, 1947).

1.2.2 Physical and Chemical Attributes of Grain Carbohydrate

The physical and chemical properties of grain carbohydrate, in themselves, are largely invariant, influenced only to a small degree by genotype or environment (Moss and Miskelly, 1984). There may be small changes in the amylose/amylopectin ratio or in the proportional content of the fibrous pentosan-rich material from the cell wall (Duffus and Murdoch, 1979; Moss and Kirby, 1976; Moss and Miskelly, 1984; Yamazaki, 1955). While these, in turn, may influence paste viscosity, they are not sufficiently important to warrant a rating on world markets for the purpose of suitability of the grain for end-product use.

The amount of starch is important in so far as the ratio of starch to

other components, particularly protein, is concerned. The starch to protein ratio is discussed later (section 1.2.3.2).

1.2.3 Attributes of Grain Protein

The quantity of protein and the chemical attributes of that protein do not greatly influence the milling performance of the grain. However, they have a major effect on the baking performance of the milled flour.

Baking quality is described in terms of 'strength' or 'weakness'. Flour of strong wheat produces bread of large loaf volume, good crust structure and good keeping properties. Flour of weak wheats is ideal for biscuits and cakes (section 1.2.3.2.1). Strong wheats also have the ability to produce bread of large loaf volume and good crumb structure when blended with a proportion of weak flour (Kent 1978).

In general, the stronger wheats have a higher grain protein percentage. But equally important the protein must be of suitable quality. Durum wheats, for example, are high in grain protein percentage, but poor in bread-making quality.

1.2.3.1 Protein Quality

Protein quality is a function of the chemical attributes of the protein and is determined by the way in which the protein creates the visco-elastic (rheological) properties of the dough. It has been recognized for a long time that the quality characteristic of protein is a genetic trait largely independent of protein content (see Mullaly and Moss, 1961; Wrigley et al., 1982a). Today, we understand more fully the nature of the properties that combine to form these quality characteristics and the genetic inheritence of those properties.

Wheat proteins can be classified into various groups depending on their solubility. This scheme was first proposed by Osborne (1907). Since then, however, there have been many proposals to change this classification

(for listing see Konzak, 1977) due mainly to differences between the solubility of a protein and the degree to which it can be extracted from the kernel in a specific solvent (Payne and Rhodes, 1982; Shewry and Miflin, 1985; Wrigley, 1982). As a result, there is no standard classification for wheat proteins and this makes comparisons difficult (Wrigley, 1982). Nevertheless, basic features of Osborne's classification still apply and these are discussed below.

1.2.3.1.1 Soluble Proteins

The soluble proteins are the albumins and the globulins. Albumins are soluble in water, their molecular weights are between 12,000 and 16,000, and they comprise between 3% and 10% of total grain protein. Globulins are soluble in salt solutions (usually 10% NaCl), have molecular weights between 20,000 and 200,000, and comprise 5-10% of grain protein (Konzak, 1977; Payne and Rhodes, 1982; Porceddu et al., 1983).

The soluble proteins are located mainly in the embryo and the aleurone layer, and they comprise mostly the enzymes of metabolism and the hydrolytic enzymes for germination (Ching, 1972). The higher the overall protein content, the lower is the percentage of albumins and globulins (Feillet, 1976). The bulk of evidence now suggests that these proteins do not affect dough development or baking quality in any significant way (Kasarda et al., 1971; Wall, 1979).

1.2.3.1.2 Gluten Proteins

Protein quality appears to be determined entirely by the gluten proteins. These are the water-insoluble ones and are the storage proteins of the endosperm. Gluten proteins are unusually high in glutamine and proline; these amino acids account for 30% (w/w) and 14% (w/w) of the gluten respectively (MacRitchie, 1980).

The protein of gluten is divided into two classes; glutenin and gliadin. Glutenin is soluble in alkali and comprises 30-50% of total grain protein; gliadin is soluble in alcohol (usually 70% ethanol) and comprises 40-50% (Konzak, 1977; Payne and Rhodes, 1982; Proceddu et al., 1983). Gliadin is the wheat protein equivalent of the broad class of proteins called prolamines.

Glutenin and gliadin have different visco-elastic properties (see Wall, 1979, for photographic representation). Glutenin imparts resilience and elasticity to the dough, while gliadin imparts extensibility. Dough strength is related more to the properties of glutenin than gliadin (Payne and Rhodes, 1982; Wall, 1979), although the final visco-elastic properties of the dough are due to complex interactions between the two protein types (MacRitchie, 1980; Miflin et al., 1983; Wall, 1979).

1.2.3.1.3 Structure, Role and Heritability of Glutenin Proteins

Glutenin consists of very large aggregates of protein. The molecular weight of these proteins is usually between 1×10^6 and 4×10^6 (Wall, 1979), although it can be as high as 20×10^6 (Payne et al., 1983). These aggregates are held together by both covalent and non-covalent linkages (Bietz and Huebner, 1980). When these bonds are disrupted, the aggregate breaks down into a number (15-20) of specific subunit types (Lawrence and Shepherd, 1981). These subunits can be separated by sodium dodecyl sulphate, polyacrylamide-gel electrophoresis (SDS-PAGE) into bands; each band represents a different protein.

These protein subunits have molecular weights ranging from 11,000 to 133,000 (Bietz and Wall, 1972). Four to eight of these are distinctly heavier than the others (>95,000). It is these high molecular weight subunits, comprising about 20% (w/w) of the total glutenin, that are the major effectors of bread-making quality. Quality is related (1) to the number of discrete high molecular weight subunits, (2) to the presence or

absence of specific bands that individually impart a large change to quality (Bietz et al., 1975; Konzak, 1977; Moonen and Zeven, 1985; Payne and Rhodes, 1982; Payne et al., 1980, 1981; Wall, 1979), and possibly (3) to interaction of specific high molecular weight subunits with smaller ones (Payne and Corfield, 1979).

The presence or absence of any one particular band is heritable with single gene characteristics (Garcia-Olmedo et al., 1981; Moonen and Zeven, 1985; Payne et al., 1981, 1983).

Ordinary bread wheats (T. aestivum) are hexaploid; they have 3 sets of genomes, the A, B and D. Each genome consists of 7 pairs of chromosomes. For a detailed description of the hexaploid genome and its origin see Konzak (1977) or Porceddu et al. (1983). Durum wheats which lack the D genome also lack four of the high molecular weight glutenin subunits (Joppa et al., 1975). This was a first step in mapping the gene location of the proteins specific for good bread-making quality.

Orth and Bushuk (1973) and Bietz et al. (1975) compared the SDS-PAGE pattens of reduced glutenins from two hard red spring bread wheats to those of tetraploid wheats derived from the hexaploids, but devoid of the D genome. Like the durum wheats these derived tetraploids lacked four of the high molecular weight subunits.

Because of the multiplicity of genomes, loss of a chromosome pair will not be lethal, although the functions encoded by that pair will be absent. Sears (1954) prepared a complete series of wheats from which he selectively eliminated each chromosome pair in turn and substituted a homologous pair from another genome. Using this series, it has been shown that the genes that code for storage proteins occur at 9 complex loci on the homologous group 1 and group 6 chromosomes. Furthermore, the genes that code for the high molecular weight glutenin subunits are located in a proximal position of the long arm of the 1A, 1B and 1D chromosomes (Garcia-Olmedo et al.,

1981; Konarev et al., 1979; Moonen and Zeven, 1985; Payne et al., 1982, 1983; Wrigley, 1982).

1.2.3.1.4 Structure, Role and Heritability of Gliadin Proteins

The gliadin group of proteins can be separated by SDS-PAGE into about 50 individual protein types, each with a molecular weight below 100,000. Possibly however, there are hundreds of protein types, but because of very small changes in the protein structure many may not be separable with electrophoretic techniques (Bietz and Wall, 1972; Kasarda, 1980; Kasarda et al., 1974). The protein types have been classified into α, β, γ and ω groups according to their mobility on gel electrophoresis (Woychik et al., 1961).

In a similar way to that described for the glutenin proteins (section 1.2.3.1.3), attempts have been made to associate specific gliadins with protein quality. Early attempts were not successful (Doekes, 1968; Huebner and Rothfus, 1968; Orth and Bushuk, 1972). However more recently, associations have been reported (du Cros and Hare, 1985; Sozinov and Peperelya, 1982; Wrigley et al., 1982). The question remains, however, whether associations between specific gliadins and quality is a result of other associations between those gliadins and some other quality-inducing factor(s) (e.g. specific glutenins), or alternatively, is a result of an independent role of those gliadins, themselves, forming the visco-elastic properties of the dough.

The occurrence of specific proteins in the gliadin complex seems to be determined entirely by genotype (Lee and Ronalds, 1967; Wrigley, 1970; Zillman and Bushuk, 1979). Because of this, the gel-electrophoretic pattern of the gliadin proteins is used extensively for determining cultivar identity of grain samples (Anderson et al., 1985; Ellis, 1984; Wrigley, et al., 1982a). The genes controlling gliadin proteins are located on the same chromosomes as the genes controlling the glutenin proteins (i.e. group 1

and group 6 chromosomes), although they are situated on different arms (Kasarda et al., 1974, 1976; Lafiandra, unpublished-reported in Porceddu, 1983; Lawrence and Shepherd, 1981; Payne, et al., 1982, 1984; Shewry and Miflin, 1985; Wrigley, 1982).

1.2.3.1.5 Environmental Influence on Protein Quality

Although protein quality is genetically determined, the question of whether it may be modified by environment or cultural practice is a difficult one to answer. In extreme cases, environment can influence the proportional content of amino acids in the storage proteins. For instance, the proportion of sulphur containing amino acids decreases when the plants are grown in sulphur-deficient conditions (Beyers and Bolton, 1979; Wrigley, et al., 1980). Even so, it is not known whether a change such as this could produce a recognizable change to baking quality.

The amount of protein (both in absolute terms and in grain protein percentage) is influenced substantially by environment. Any change within a variety to the visco-elastic properties of the dough or to its baking quality is usually ascribed to this (section 1.2.3.2).

The ratio of albumins and globulins to gluten can change in response to environment (Feillet, 1976, Finney, et al., 1957; Orth and Bushuk, 1972) or the duration of grain development (section 1.7). Hence the baking quality per unit of grain protein can change also; but this effect is small (Bushuk and Wrigley, 1972; Finney et al., 1957; McNeal, et al., 1971; Mesdag, 1964). There is no effect of environment on protein quality as measured by the electrophoretic pattern of the reduced glutenin and gliadin fractions (Doekes, 1968; Elton and Ewart, 1966; Lee and Wrigley, 1963; Tanaka and Bushuk, 1972). But this test is qualitative. There are no studies examining whether there are quantitative changes in key proteins in response to environmental change.

1.2.3.2 Protein Quantity

1.2.3.2.1 Role of Protein Quantity in Determining End-Product Use

Grain protein percentage, often referred to as protein content, is the weight of protein per unit weight of grain or flour. When expressed per unit of grain weight, it usually is converted to a standard 14% moisture content of the grain. It essentially measures the ratio of protein to starch. While grain hardness and protein quality are largely independent of environment and cultural practice (sections 1.2.1 and 1.2.3.1), the grain protein percentage is affected a great deal. Vogel et al. (1973) measured the grain protein percentage on 12,613 common wheats. Grain protein percentage ranged from 7% to 25%, but only 5 percentage points of this variation could be attributed to differences in genotype.

The quality criterion is not simply grain protein percentage but rather the interaction of protein percentage and grain hardness (Fig. 1-3). Some examples of this interaction in determining end-product use are described below.

1. Biscuits and Cakes

A soft wheat with low protein percentage is required for biscuits and cakes. Low protein percentage produces a dough that has low elasticity but high extensibility. Soft texture is required because the flour is less granular and it needs less water to make the dough. Less water means (1) less energy needed to dry the biscuit during cooking and (2) shallower water gradients during cooking reduces cracking. For a detailed review of the products of soft wheats see Yamazaki and Lord (1971).

2. Breakfast Cereals

Breakfast cereals can be made a number of ways (Kent, 1978). For instance, shredded wheat is made from soft wheats of low protein content. The grain is boiled to gelatinize the starch, then rolled. With low elasticity the

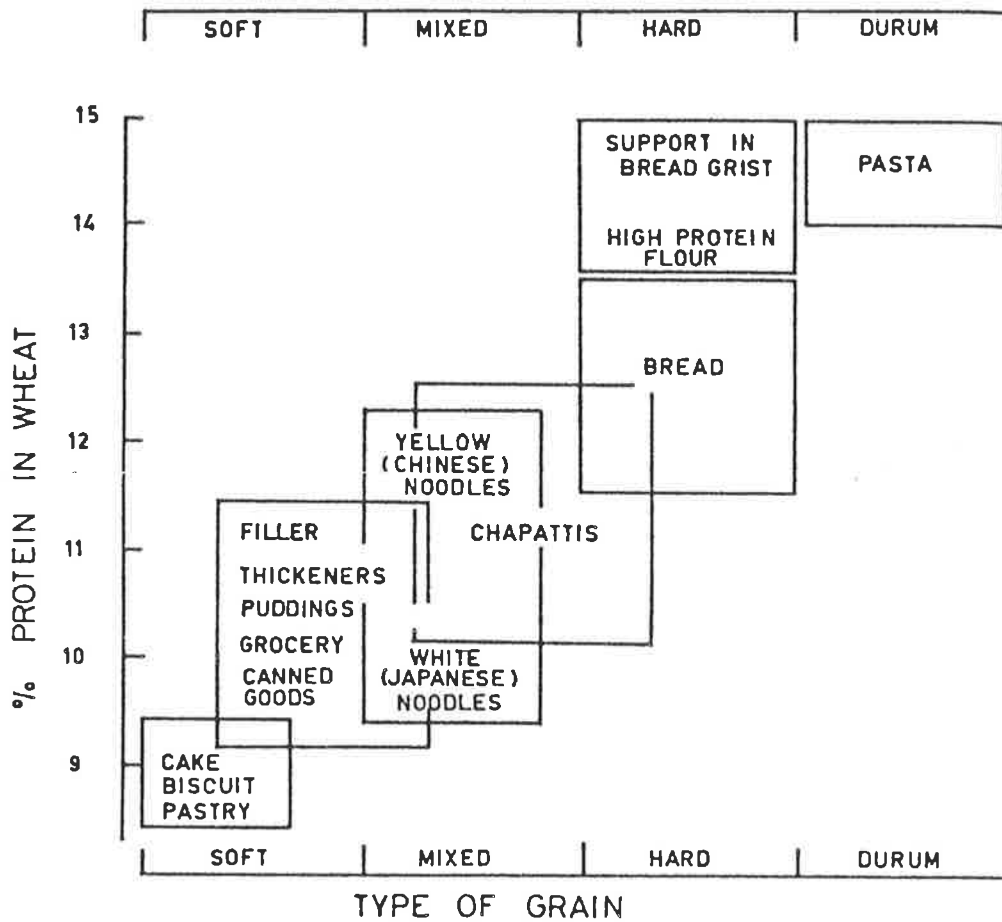


Figure 1-3. Requirements of certain wheat products in terms of protein percentage and grain hardness. Reproduced from Moss (1973).

imprint of the roller stays on the flake. In contrast, puffed wheat is made from hard wheat of high protein content. The grain is placed into a superheated pressure chamber, the pressure is suddenly released causing the grain to increase in volume to several times its original. For satisfactory puffing the material at the moment before expansion requires cohesion to prevent shattering (soft wheats would shatter) and elasticity from glutenin to permit controlled expansion.

3. Chapattis

Chapattis are a kind of unleavened bread that are the major form in which wheat is consumed in India, Pakistan and parts of Tibet and China. They are made from dough which is rested before baking. Water adsorption of the flour should be high, hence hard wheat. A strong gluten is not required, but it should be stronger than the gluten required for biscuits (Payne and Rhodes, 1982).

4. Bread

Both hard and soft wheats can be used for bread making. Usually hard wheats are preferred because this flour absorbs and retains more water making the dough and bread 'lighter'. The grain protein percentage needs to be high so that the dough is resilient, elastic and extensible. During the baking of bread, the gas must be retained until the starch and protein have coagulated. There are many books and articles on the art of breadmaking, e.g. 'Bread' (Spicer, A., ed., 1975) and Ponte (1971).

5. Pasta

Pasta products can only be made from extremely hard wheats. The protein percentage should be 13 to 14.5% but the protein should be weak. These protein/hardness characteristics contribute to the flour hydrating more evenly, the dough becoming stiff, stable, and flowing easily under pressure, and to the dough having low elasticity. Upon cooking the product

becomes pliable but remains firm (see Irvine, 1971).

In order to satisfy these diverse requirements the Australian Wheat Board markets wheat under a number of categories (Fig. 1-4), most of which reflect this protein percentage hardness interaction. The price a grower receives for his wheat depends on its classification; generally a higher price for higher protein percentage (Table 1-1).

1.2.3.2.2 Breeding for Increased Protein Quantity

Because of the importance of protein quantity (see above) there have been many attempts to produce improved varieties. Nearly always, genotypic performance has been assessed directly on grain percentage (see Konzak, 1977) because (1) this is the criterion used by trade and industry and (2) the heritability of this characteristic is high (e.g. Davis et al., 1961).

However, it was pointed out long ago (Kraybill, 1932) that an increase in grain protein percentage can be achieved by increasing the amount of protein, decreasing the amount of carbohydrate, or both. Factors that appear to alter grain protein percentage (e.g. genotype, temperature and water availability during maturation, nitrogenous or phosphatic fertilizers) may do so through their effect on carbohydrate deposition. In the vast majority of cases where genotypic performance is assessed on the basis of grain protein percentage, an improvement is attainable only at the expense of yield (e.g. Benzian et al., 1983; McNeal et al., 1972; Rhodes and Jenkins, 1978; Rietz, 1964; Schlehuber and Tucker, 1959; Terman et al., 1969; Whitehouse, 1973).

Over recent years, a number of alternative strategies have been used in attempts to increase grain protein percentage without a decrease in yield. These are discussed below.

1. Recurrent selection is a technique widely practised in breeding

Australia's Wheat Classes

Australian Wheat is marketed in eight general classes:

Australian Prime Hard

A high quality class of wheat comprising selected hard grained wheat varieties with excellent milling characteristics and well balanced dough properties. This class is marketed at guaranteed minimum protein levels of 13%, 14% and 15%. Major uses are for blending with lower protein wheats and for the production of high protein breads and noodles.

Australian Hard

Wheat in this class contains specified hard grained varieties of proven bread making quality. It is marketed at guaranteed minimum protein levels of 11.5%, 12%, 13% and 14%. This wheat is used for the production of a wide range of volume breads, Middle Eastern style flat breads and Chinese style noodles.

Australian Standard White

This is a multi-purpose class of wheat of intermediate grain hardness with protein levels in the range of 9% to 11.5%. Australian Standard White (ASW) is suitable for a wide variety of flour products including European style loaf breads, Middle Eastern and Indian style flat breads, steamed bread and most types of noodles.

Australian Soft

A low protein wheat of specified soft grained varieties, is segregated in the southern part of Western Australia. Australian Soft is suited to the production of sweet biscuits, cakes and pastry goods.

Australian General Purpose No. 1 and No. 2

These two wheat classes relate to wheat of the same varieties as the above classes but which fail to conform to the receival standards of any of the above classes, usually in terms of test weight, weather damage, unmillage material or contamination with foreign matter or seeds. General Purpose No. 1 comprises milling wheats with mild quality defects and is marketed essentially as a second grade milling wheat. General Purpose No. 2 comprises lower grade milling wheats and may include mildly sprouted grain.

Australian Feed

This class consists of wheat with physical defects such as low test weight, high levels of screenings, foreign material, excessive weed seeds and high levels of sprouting. This class is only suited for feed purposes.

Australian Durum

Wheat received into this class is limited to durum wheat varieties. Australian Durum is suited to the production of semolina for pasta products.

Australian Wheat

Class	Grade	
PRIME HARD	15% PROTEIN 14% PROTEIN 13% PROTEIN	QLD NSW
HARD	14% PROTEIN 13% PROTEIN No. 1 12% PROTEIN No. 2* 11.5% PROTEIN	QLD* NSW* VIC* SA WA
ASW	STANDARD WHITE	QLD NSW VIC SA WA
SOFT	SOFT	NSW VIC SA WA
DURUM	No. 1 No. 2	NSW
GENERAL PURPOSE No. 1	LIGHTWEIGHT SCREENINGS WEED SEEDS	QLD NSW VIC SA WA
GENERAL PURPOSE No. 2	LIGHTWEIGHT SCREENINGS WEED SEEDS MINOR SPROUTING	QLD NSW VIC SA WA
FEED		

Figure 1-4. Classification of Australia's wheat. Reproduced from the Australian Wheat Board's Annual Report, 1985-86.

Table 1-1. Guaranteed minimum price (to growers) for wheat in accordance with classification; Season 1984-85.

Classification	Price per tonne
Category A1, Australian prime hard and durum wheat	$160
Category A2, Australian hard, number 1	$150
Category B, Australian Standard White	$145
Category C, Australian General Purpose	$135
Category D, Australian Feed Wheat	$100

programmes for cross-pollinating species, only recently has it been applied to self-pollinating ones. As an example of recurrent selection, Loffler et al. (1983) selected 10 hard red spring wheats with medium to high grain protein percentage and other desirable agronomic characteristics. These were crossed to form 5 F1 groups, each group with a unique pair of parents. The groups were crossed in diallele fashion to produce 10 double-cross combinations. Using this sort of programme, grain protein percentage may increase, by up to 2.5 percentage points (Loffler et al., 1983; McNeal et al., 1978), but again only at the expense of yield. There was no noticable increase in the amount of protein per grain.

2. Jain and coworkers (Jain et al., 1976; Singhal and Jain, 1981) selected first for large grains in an attempt to counteract the possibility of losing yield, and then they selected for grain protein percentage. However, they still observed a strong negative correlation between grain protein percentage and yield.

3. Mutant varieties have been used. Narahari et al. (1976) screened several thousand mutant lines, but found none that gave high protein percentage without a sacrifice of yield. Similar results were obtained by Parodi and Nebrada (1979), Bhagwat et al. (1979) and Favret (1979). Kushnir and Halloran (1984) crossed a high protein content/large kernel weight line of wild tetraploid wheat with Chinese Spring and its two homeologous pairing mutants. The three pentaploids were then crossed with two commercial hexaploid wheats. In most of the F2 and F3 lines, higher protein percentage was not accompanied by higher protein per grain. In a few cases where grain protein percentage and grain weight both were high, the number of tillers per plant was low. There was no estimate of grain yield per area given.

4. Vogel et al. (1973) examined over 20,000 entries of the World Wheat Collection for grain protein percentage. One of the 'high-protein-

percentage' varieties was Atlas 66, a soft-milling wheat. It was crossed with a good bread-making, hard milling wheat, Comanche, and the F1 progeny was crossed to another good-quality wheat, Lancer. After several generations of inbreeding a final selection was made; line NE701132, later to be known as Lancota (Johnson et al., 1973, 1975, 1979). In a small trial comparing Lancota with two commercial varieties Scout 66 and Centurk, it appeared that the new variety had elevated protein percentage (1-2 points) without losing yield (Johnson 1975). Further field testing continued to show promising results (Johnson and Mattern, 1978). However, Brunori et al. (1982) argued that the characteristic of high protein percentage displayed by Atlas 66 was due entirely to low carbohydrate yield, and that on a per grain basis the yield of protein for Atlas 66 was indeed also quite low. This challenge by Brunori et al. (1982) may not be valid as they failed to consider grain number per hectare (see #5 below).

5. Favret et al. (1970) and Nelson (1970) proposed that the amount of nitrogen per grain is influenced less by environment than is grain protein percentage, and therefore it may be a better criterion for selecting high protein yielding varieties. Recently, Brunori et al. (1980) found that certain varieties (e.g. Pusa 5-3, F26-70) accumulate more nitrogen per grain than others (e.g. Atlas 66). Furthermore, the amount of nitrogen per grain was constant when these genotypes were tested for several years, even though seed weight varied substantially (Brunori et al., 1982; see also Favret et al., 1970). These results have led Brunori et al. (1982) to propose that by selecting for high nitrogen per grain, it should be possible to obtain a genetic combination that will express high grain yield per spike, large grains, high protein percentage and high nitrogen per grain. However, these authors failed to consider grain number and hence they have no assessment of the total yield of protein per hectare.

In conclusion, it seems that wheat breeders have to reassess their

views as to what constitutes a high-protein wheat variety, and to design breeding progammes accordingly. Underlying all considerations should be the fact that in commercial agriculture, wheat is grown nearly always in conditions of nitrogen shortage (Frey 1973, 1977). A crop rarely (if ever) is able to realize its capacity for deposition of protein in the endosperm. The selection criteria should be two-fold.

1. High uptake efficiency of soil nitrogen. Efficiency of uptake involves both the rate of uptake (see Baer and Collet, 1981; Huffaker and Rains, 1978; Johnson and Mattern, 1978; Singh and Anderson, 1980) and the affinity for uptake (see Schrader and Thomas, 1981).

2. High nitrogen harvest index; i.e. that the proportion of nitrogen lost from the plant or remaining in any tissue other than the grain (viz. endosperm) at harvest is low. It is encouraging to see that just recently some workers have proposed that nitrogen harvest index may be a useful criterion for selection of high protein wheats (Day et al., 1985).

Nitrate reductase which catalyses the conversion of nitrate to nitrite (section 1.6) may limit the conversion of nitrate to grain protein (Hageman, 1979; Klepper, 1976). If so, nitrogen harvest index may be related to the activity of this enzyme. Certainly there are genotypic differences in the activity of nitrate reductase (Abrol et al., 1984), and correlations between in vitro nitrate reductase activity and the yield of grain protein have been reported (Abrol and Nair, 1978; Croy and Hageman, 1970; Deckard et al., 1977; Eilrich and Hageman, 1973; Hernandez et al., 1974; Johnson and Mattern, 1978; Nair and Abrol, 1982; Reilly, 1976).

Pushman and Bingham (1976, Fig. 3) present correlative data between weight of grain nitrogen per area and grain yield per area taken from the performance of 10 varieties at 3 different levels of nitrogenous fertilizer. One variety, TJB 54/224 produced more protein per area at all

fertilizer levels than the other varieties. Maybe this variety, for whatever reason, is a high protein one. Lancota and other 'high-protein' varieties developed by Johnson and colleagues should be tested in this way.

1.2.3.2.3 Environmental Influence on Grain Protein Percentage

Nitrogenous fertilizers affect the yield/protein percentage balance in different ways depending on the rate and timing of the application (see also section 1.3). Generally nitrogen applied to a young crop increases yield; the grain protein percentage at harvest may decrease, increase, or remain the same depending on the amount of nitrogen applied and the level of soil nitrogen at sowing. The later the nitrogen is applied, the greater is its influence on protein percentage and the less is its influence on yield (e.g. Davidson, 1922; Davidson and LeClerc, 1917, 1923; Finney et al., 1957; Gericke, 1920, 1922, 1927; Johnson and Lay, 1974; Kraybill, 1937; Neidig and Snyder, 1922; Pushman and Bingham, 1976; Spiertz and de Vos, 1983; Terman, 1979). The most striking example of this reponse is reported by Finney et al. (1957). A single spraying of urea, 7 weeks before flowering doubled the yield, while spraying post anthesis increased the grain protein percentage from 10.8% to 21%.

Elevated temperature (>30°C) post anthesis may cause premature cessation of starch deposition in the endosperm. While the rate of deposition may be faster under the higher temperature, it does not compensate adequately, so total amount of starch deposited is less. Deposition of protein is largely unaffected by elevated temperature however, hence an increase in grain protein percentage may result (Bhullar and Jenner, 1985).

Empirically, the response to mild water stress is similar to the response to elevated temperature. The deposition of starch may be less while the deposition of protein remains unchanged (Brooks et al., 1982;

Morgan and Riggs, 1981).

1.2.3.3 Nutritional Quality of Wheat Protein

The nutritional quality of wheat protein is limited primarily by the small amount and poor balance of four essential amino acids; lysine, threonine, isoleucine and methionine. The amount of lysine is especially low (see reviews by Kasarda et al., 1971; Konzak, 1977; Payne and Rhodes, 1982). Frequently, the argument is heard that scientists should take up the challenge of improving the nutritional quality of wheat, in particular by increasing the lysine content, so to improve the diet of people for whom wheat is the main source of dietary protein (e.g. Konzak, 1977; Micke, 1983; Porceddu et al., 1983; Swaminathan, 1983). Sadly at present this view is somewhat esoteric; there is no economic advantage on the world market for high-lysine wheat. High-lysine wheat is considered only briefly here. For a more detailed review see Porceddu et al. (1983).

The percentage of lysine per unit of protein is inversely related to grain protein percentage. This relationship is most pronounced between 7% and 13% protein. Above 15% protein the ratio of lysine to grain protein percentage remains constant (Gunthardt and McGinnis, 1957; Hepburn and Bradley, 1965; Johnson and Mattern, 1975; Lawrence et al., 1958). Due to this relationship, there is very little difference between wheat cultivars on a nutritional basis (Report by the Miller's National Federation, Chicago, 1967).

Johnson et al. (1975) calculated that the genetic component of total lysine varied in the World Wheat Collection by no more than 0.5% (as a percentage of protein). This is only one-third the amount required to bring lysine into reasonable balance with the other essential amino acids in wheat protein (Johnson and Mattern, 1978).

The whole wheat kernel has a higher percentage of lysine (per unit protein) than does the endosperm. This is because the albumins and the

globulins that are concentrated in the non-endosperm fraction have a higher percentage of lysine than do the glutenins and gliadins (Folkes and Yemen, 1956; Kasarda et al., 1971; Konzak, 1977). High-lysine wheat varieties, and most of the high-lysine varieties of other cereals have shrunken endosperms or low amounts of storage protein in the endosperm (Boulter, 1977; Konzak, 1977). In barley, some attempts to increase the lysine content of the storage protein seem promising (Doll, 1976; Hagberg et al., 1979).

1.3 QUALITY AND YIELD OF IRRIGATED WHEAT

The yields of wheat grown under irrigation are high and fluctuate less from year-to-year than do yields from wheat grown in rain-fed areas. The main areas of irrigated wheat in Australia are north-central Victoria and the Murrumbidgee Irrigation Area of southern New South Wales. These areas produce about 70,000 tonnes from 20,000 ha and 130,000 tonnes from 40,000 ha respectively (estimated from Australian Bureau of Statistics publication; Land Utilization and Crops - Victoria, Season 1980-81 and Crook et al., 1980; Woodroofe, pers. comm.). Although in normal years, this amount represents only 3% the total production from these states, irrigated wheat is an important industry for the regions concerned, having a gross value of $30 million annually. In 1983, a year of widespread drought in south-eastern Australia, the irrigated wheat industry remained viable, accounting for 15% of the total production in Victoria and for 10% in NSW (from Australian Wheat Board, Annual Report 1984-85).

In a recent report commissioned by the Victorian Department of Agriculture (Crook et al., 1980), it was proposed that the irrigated wheat industry has a great potential for expansion. This is because of (1) decreased viability of existing animal and horticultural industries in the irrigation areas, (2) the stability of yields from year to year, and (3)

the long-term stability of the wheat industry as a whole. However, the trend to this more intensive form of wheat production is restricted mainly because of the inability of growers to achieve routinely high yields (>5 $tonnes.ha^{-1}$) and by the poor quality of the grain produced. In this regard, grain quality is judged almost entirely by grain protein percentage, the parameter that cannot be genotypically defined (section 1.2). The grain protein percentage of wheat grown under irrigation is low; 8.5% to 10.5% (Woodroofe, 1981) whereas a value greater than 11.5% is preferred (Fig. 1-4).

Two challenges face the irrigated wheat industry in Australia:

1. For scientists to define and farmers to create the agronomic conditions so that existing cultivars can be used for economic production of high yields of grain with a suitable grain protein percentage (e.g. 8 $tonnes.ha^{-1}$, 12% protein).

2. For scientists to identify, then genetically modify the limiting factors to harvest yield and protein yield, creating new varieties to allow (a) the 'acceptable' crop (e.g. 8 $tonnes.ha^{-1}$ at 12% protein) to be produced with less intensive and less expensive management inputs or (b) a further increase in harvest yield and/or protein percentage under the same cultural practice.

1.3.1 Factors Limiting Carbohydrate Deposition in the Grain

Most of the grain carbohydrate is derived from CO_2 fixed during the grain filling period (Evans et al., 1975). Hence the limit to yield will be either the capacity to produce substrate (source-limited) or the capacity to utilize it (sink-limited). Most of the wheat grown in Australia is grown in the arid or semi-arid zones and the major limiting factor to production in these zones is water. Productivity can be defined in terms of the amount of water available, the efficiency of water use for dry matter production

and the partitioning of dry matter to the economically important part. All attempts to increase productivity, either through breeding new cultivars or improving cultural practice aim to improve at least one of these parameters. Ultimately though, it is the drought-induced cessation of photosynthesis that applies the final constraint (see review by Fischer and Turner, 1978).

However, a different set of limitations to yield operate when crops are grown under conditions of abundant water availability. Generally, when water is not limiting, neither is photosynthetic capacity. In this case, the deposition of carbohydrate in the grain is sink-limited. A number of lines of evidence, leading to this conclusion are discussed below.

1. Source-limited growth and sink-limited growth can be distinguished one from the other by studying the correlation between grain yield and kernel number. A positive correlation implies that grains have grown during grain fill to a predetermined size, hence growth was sink-limited. On the other hand, a lack of association demonstrates the influence of source-limiting factors (Fischer et al., 1977). In detailed studies encompassing a wide range of locations, environmental conditions and cultural practice, Shanahan et al. (1984, 1985) found that there was indeed a close correlation between grain yield and kernel number per unit area. Similar results from less comprehensive studies have been obtained by other workers (Ellen and Spiertz, 1980; Evans, 1978; Koomanoff, 1981).

2. A well watered wheat crop has the photosynthetic capacity to produce in excess of 22 $tonnes.ha^{-1}$ of dry matter over a 50 day period of grain fill. This was calculated on the basis of light absorption and the demonstrated rates of net daily CO_2 exchange as a function of intercepted light (Table 1-2). However, the highest wheat yields obtained in crops with a period of grain fill of this duration is less than 10 $tonnes.ha^{-1}$. Furthermore, in

Table 1-2. Summary of study by T.R. Jones estimating the potential yield of wheat grown in central Victoria (unpublished).

1. 90% of grain carbohydrate is derived from photosynthesis post anthesis.
2. Duration from anthesis to maturity, 50 days.
3. Average irradiation during grain fill, 400 $cal.cm^{-2}.day^{-1}$
4. Photosynthetically active radiation, 45% solar radiation
5. Reflected light, 5.5% solar radiation
6. Inactive absorption by crop, 2%
7. Photochemical conversion, 26%
8. Light saturation of flag leaf and ear, 20%
9. Respiration, 1.5% of dry weight per day, plus 30% of photosynthesis
10. Dry matter conversion, 3900 $cal.g^{-1}$

From the above information, it can be shown that:

Energy fixed = 346,732 $cal.m^{-2}.day^{-1}$

Respiratory loss = 192,809 $cal.m^{-2}.day^{-1}$

Net energy fixed = 153,922 $cal.m^{-2}.day^{-1}$

*Potential grain yield at 12% moisture = 22.1 $t.ha^{-1}$

Under irrigation, the crop may remain photosynthetically active for 65 days p.a.

*Maximum yields obtained 8 $t.ha^{-1}$

controlled environment experiments, decreasing illuminance to half the normal value did not decrease the rate of grain growth, and indeed a six-fold decrease in illuminance imposed after anthesis decreased the rate of grain growth only 8% (Sofield et al., 1977).

3. The rates of photosynthesis per unit area of leaf decrease substantially (halve) in response to removing the ear, and increase substantially (double) in response to decreasing photosynthetic activity in other regions of the plant (King et al., 1967; Birecka and Dikic-Wlodkowska, 1963). Furthermore, photosynthetic activity of the ear and the blade of the flag leaf alone can provide all the photoassimilate required by the plant at all times (Evans and Rawson, 1970) and lastly, the rate of phososynthesis of the flag leaf varies throughout the season in response to assimilate demand by the developing ear (Evans and Rawson, 1970).

4. Spikeless shoots can be considered as an additional source of carbohydrate supply for other parts of the wheat plant, yet their presence has little effect on the reproductive development of spike-bearing shoots (Shanahan et al., 1985).

5. During grain fill, the rate of starch synthesis appears to be a function of the concentration of sucrose within the endosperm, yet, the concentration of endosperm sucrose seems tightly controlled. The rates of starch synthesis observed in ears cultured on a solution containing 40 $g.l^{-1}$ sucrose correspond to rates observed _in vivo_. At concentrations below 40 $g.l^{-1}$, both the concentration of endosperm sucrose and the rate of starch synthesis are lower than the values observed _in vivo_. Higher substrate concentrations have little promotive effect (Jenner, 1968; Jenner, 1970; Jenner and Rathjen, 1978). Trimming ears after anthesis causes an increase in sucrose in the rachis, indicating that this treatment increases the amount of substrate available for distribution to the grain.

However, likewise, the concentration of endosperm sucrose does not change greatly (Jenner, 1980).

6. Starch deposition in the endosperm terminates quite suddenly, but at this time leaf photosynthesis is still active and ample assimilates for growth are still available (Sofield et al., 1977; Jenner and Rathjen, 1975).

7. Carbohydrate deposition in the grain is not limited by the transport of assimilates to the grain. Wardlaw and Moncur (1976) found that grain growth was unaffected by cutting through half the vascular bundles of the peduncle. There appeared to be a compensating increase in the concentration and speed of movement through the vascular bundles that remained.

1.3.2 Factors Limiting Protein Deposition in the Grain

In contrast to sink-limited deposition of carbohydrate in the grain, the deposition of protein is source-limited. Increasing the external supply of nitrogenous precursors, by for instance, increased nitrogen nutrition (Giesse and Hejgaard, 1984), increased uptake and transport of nitrogenous compounds (Iqtidar and Rehman, 1984) or supplying precursors directly to the aerial parts (Blacklow, 1982; Finney et al., 1957; Pavlov, 1982; Pavlov et al., 1981; Pushman and Bingham, 1976) causes a direct increase in the deposition of endosperm protein (see also section 1.2.3.2.3).

Physiological studies also demonstrate that deposition of grain protein is limited by substrate supply. The rate of protein deposition in ears cultured on substrate solution is comparable to _in vivo_ rates when a solution containing 0.5 $g.l^{-1}$ N (as glutamine) is used. Increasing the concentration of glutamine in the culture solution to 2.0 $g.l^{-1}$ N doubles the deposition of grain protein (Barlow et al., 1983).

Trimming ears after anthesis causes an increase in soluble amino acids

in the rachis. The concentration of soluble amino nitrogen in the endosperm parallels this increase and a greater amount of protein accumulates. An increase in grain protein percentage results (Jenner, 1980; Konovalov, 1966; Peterson, 1983; Simmonds and Moss, 1978; Thorne, 1981).

1.3.3 Strategy for High Yields and High Protein Percentage

When photosynthetic capacity (in itself) is not the key determinant of yield, the most important limit to productivity becomes the extent to which the available substrate is utilized for grain development and growth (section 1.3.1).

Yield of wheat (in any circumstance) is the product of the number of grains per hectare and the weight per grain. For instance, 100 plants per square meter, each producing 5 ears of 40 grains per ear with a grain weight of 40 mg would produce a yield of 8 tonnes.ha^{-1}. Yields of this tonneage are achieved, although not regularly at present, in the irrigation areas of northern Victoria and southern New South Wales (Farmers' Newsletter Jan. 1982; Bacon and Cooper, 1985; Strapper et al., 1984; Woodroofe, M. and Gyles, O., unpublished results from Victorian Department of Agriculture trials).

Nitrogen application early in crop development promotes tillering, leaf growth, the number of fertile florets per spike and the subsequent grain set (Langer, 1980). After the boot stage, however, applied nitrogen has little, if any, affect on grain number, although it may increase total dry matter of the crop and grain weight (Spiertz, 1980).

The most comprehensive attempt to define the nitrogen required to produce high yields of high protein grain has been done by Spiertz and De Vos, (1983). Working with winter wheat in Europe, these authors defined the amount of crop dry matter both at tillering and anthesis required to give the crop the potential for high yields (500 ears.m^{-1}) and the nitrogen regime necessary to achieve this. Additional nitrogen was required after

anthesis to raise the level of grain protein to an acceptable level.

A nitrogen programme such as this needs to take into account the efficiency of uptake from the soil. Soil moisture plays a major role, influencing both the rate and affinity of nitrogen uptake, the degree of leaching and the extent of denitrification (Tinker, 1979). In conditions of low moisture stress, Ellen and Spiertz (1980) found that an application of 70 $kgN.ha^{-1}$ during grain fill produced a measurable increase in crop nitrogen of 60 $kg.ha^{-1}$. Efficiency values between 50% and 70% are more usual (Tinker, 1979).

Spiertz and De Vos (1983) applied nitrogen to a wheat crop at tillering, jointing and heading of 50, 60 and 70 $kgN.ha^{-1}$ respectively and achieved yields in excess of 8 $tonne.ha^{-1}$ with a grain protein percentage of 12.5%. Similar results are seen in the experiments of Benzian and Lane (1979, 1981), Benzian et al. (1983), Holmes (1977), Jonsson (1975) and Pearman et al. (1978). Indeed there is a linear or near linear relationship between grain nitrogen and applied nitrogen up to a value of applied nitrogen of between 150 and 210 $kgN.ha^{-1}$ (Benzian and Lane, 1981; Holmes, 1977; Jonsson, 1975; Pearman et al., 1978). Thereafter the response is curvilinear (Miflin, 1980). Johnstone (1981) obtained yields of 10 $tonne.ha^{-1}$ with a grain protein percentage in excess of 12% protein after applying 280 $kg.N.ha^{-1}$. To my knowledge, there are no similar studies showing the interaction between applied nitrogen, yield and protein percentage in crops grown in the irrigation areas of northern Victoria and southern New South Wales. There are a few reports on the interaction of nitrogen and yield alone, however (e.g. Bacon and Cooper, 1985a, 1985b).

1.4 GRAIN DEVELOPMENT

In wheat, especially when grown under irrigation, factors that operate within the grain influence strongly both the rate and duration of

carbohydrate deposition, whereas they influence far less the rate and duration of protein deposition (sections 1.2.3.2.3, 1.3.1, 1.3.2 and 1.3.3). Questions concerning the mechanism of this difference are fundamental to any attempt, be it cultural or genetic to improve simultaneously the harvest yield and grain protein percentage. In turn, any study on the mechanism of this difference must be integrated totally with an understanding of grain anatomy. This section (1.4) provides a description of grain anatomy.

1.4.1 The Flower and Early Development of the Ovary and Ovule

The following brief discription of the flower, the ovary and the ovule is collated mainly from the work of Alexandrov and Alexandrova (1939a, 1939b), Bennett et al. (1975), Brenchley (1909), Cobb (1905), Esau (1977), Frazier and Appalanaidu (1965), Morrison (1975), Morrison and O'Brien (1976), Percival (1921) and Simmonds and O'Brien (1981).

Although the ovary is bicarpellate only 1 ovule is produced. This ovule consists of the meristematic nucellus, two surrounding integuments and the funiculus (stalk). The region where these components merge is the chalaza.

During the early stages of ovule differentiation and growth, the nucellus appears as a uniform meristem 6 or 7 cells across. A cell immediately beneath the nucellar epidermis and positioned in the apex of the axial row grows to become a large wedge-shaped archesporum. After 2 divisions of this cell in a periclinal direction a tetrad row of megaspore mother cells is produced. Three of these cells collapse and degenerate, the remaining one becomes the megaspore.

After 3 generations of mitotic division, the megaspore becomes an 8-nucleate embryo sac. Subsequently this sac becomes organized into 7 cells; 3 cells at the micropylar end become the egg apparatus i.e. the egg cell and 2 synergids, 3 cells at the opposite end are known as the antipodals

and 2 cells unite to form a large diploid cell in the centre, called the secondary endosperm nucleus. The 3 antipodals divide to form a group of 6-30 cells.

Mitotic activity in the pollen grain gives rise to a 2-celled gametophyte; the vegetative and generative nucleii. Both nucleii move down the growing pollen tube during which time the generative nucleus divides to form 2 sperm nucleii.

The pollen tube enters the embryo-sac and penetrates one of the synergids. There it releases its contents. The vegetative nucleus and the nucleus of the penetrated synergid degenerate to form x-bodies. At the same time the other synergid degenerates also. The sperm nucleii become tightly coiled and in this shape one fertilizes the egg cell and the other fuses with the secondary endosperm nucleus to form the triploid primary endosperm.

At the same time as the ovum is fertilized, the outer integument begins to degenerate; the cellular contents progressively disappear and the cell walls shrivel. A resinous material forms in its place. The inner integument remains as a 2-celled layer, the cells increasing in length as the grain enlarges. Ultimately, the inner layer also collapses to a degree, but persists to become the testa of the seed. The testa or seed coat is covered by a cuticle on both sides. This cutinized seed coat is interupted in the furrow by a coloured band of thin-walled cells; the pigment strand.

After fertilization the nucellar tissue also becomes disorganized and is absorbed into the developing embryo and endosperm. However, the epidermis of the nucellus continues to divide and expand as the ovule grows. This single-celled layer persists until ripening.

The boundary between the nucellar tissue and the outer-most layer of the endosperm marks the division between parental and filial tissue. There are no symplastic connections between these tissues (Carr, 1976) so

transfer of solutes across this boundary must be apoplastic.

1.4.2 The Vascular System of the Grain

The rachis in wheat is rich in vascular tissue. The number and cross-sectional area of xylem vessels and sieve tubes declines acropetally as bundles diverge into spikelets accompanied sometimes with branching (Whingwiri et al., 1981). All parts of the spikelet share vascular connections to the system in the rachis (O'Brien et al., 1985). However, the vascular connection to the ovary is unusual (O'Brien et al., 1985; Zee and O'Brien, 1970a). The lumen of all tracheary elements in the base of the ovary neck is blocked. The tracheary elements of the ovary and those of the rachilla converge upon a core of thick walled cells. These cells lie close together forming a central cylinder around which lie numerous sieve elements and transfer cells. There are no studies on the ontogeny of this xylem block, but from anatomical studies it seems that during grain fill at least supply to the grain is phloem only.

During the early stages of floret development, four tracheids branch from a basal vascular bundle and extend into the ovary. One extends through the funiculus to the ovule (Simmonds 1974b). The other three extend through the ovary wall, one dorsal and one in each of the flanks (Alexandrov, 1937; Alexandrov and Alexandrova, 1939a; Frazier and Appalanaidu, 1965; Simmonds, 1974b).

The tracheids in the ovary wall are of uniform structure and have little or no diffentiation into distinct phloem and xylem. The lateral veins continue to the 2 stigmas of the flower while the dorsal one terminates blindly. Shortly after fertilization these three veins degenerate.

During the period 5-7 days after anthesis the tracheid that serves the ovule becomes extended the length of the grain in a process apparently unique to cereal caryopses (Fig. 1-5). Concurrent with the development of

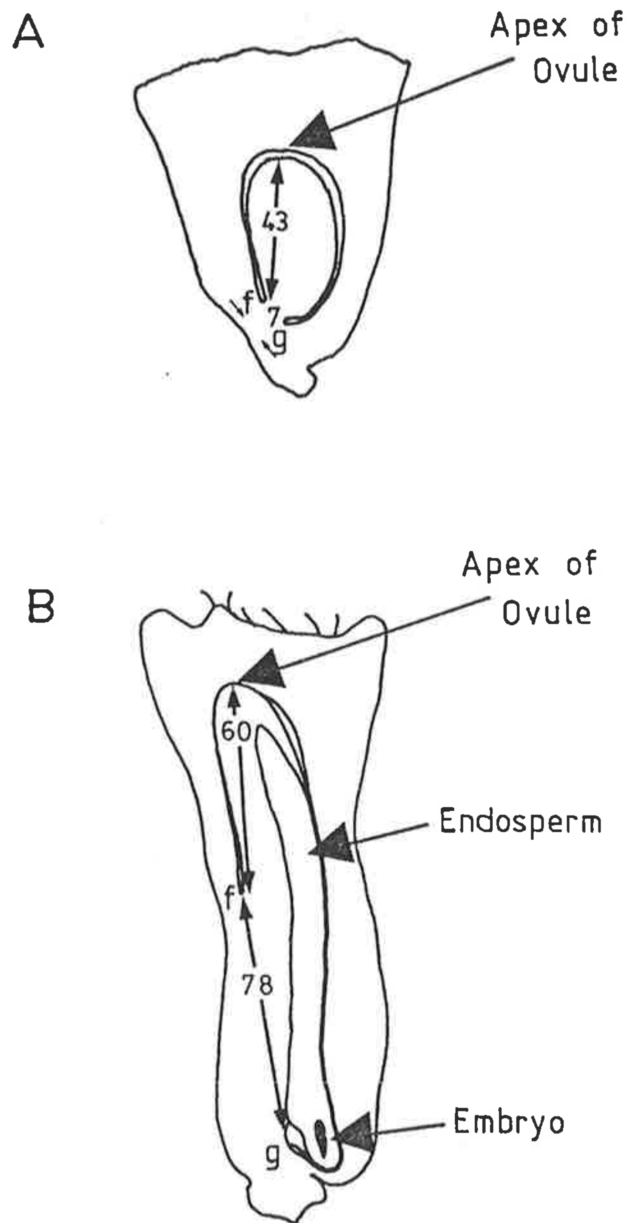


Figure 1-5. Longitudinal sections of developing wheat grains; (A), Prior to fertilization, the funicle (region f-g) is round with a diameter of 7 units, and (B), 5 days after anthesis, the ovule is now anatropic and the funicle has changed into an elongated structure 78 units long. Reproduced from Alexandrov (1937).

the embryo sac, the ovule changes from atropic to anatropic; i.e. the axis of the ovule turns through 90° so that the micropyle is turned towards the funicle. The funicle, previously short and round in cross-section elongates greatly in the direction of the lengthening young grain. The funiculus and the chalazal areas remain in close contact with one another and fuse. This fused tissue becomes what is presumed to be the main conducting mass at the base of the furrow (Alexandrov, 1937; Frazier and Appalanaidu, 1965).

1.4.3 Anatomy of the Tissues Surrounding the Grain During the Grain-fill Period of Development

The parts of the wheat kernel and their relationship to one another are shown in table 1-3 and figure 1-6, and are described below.

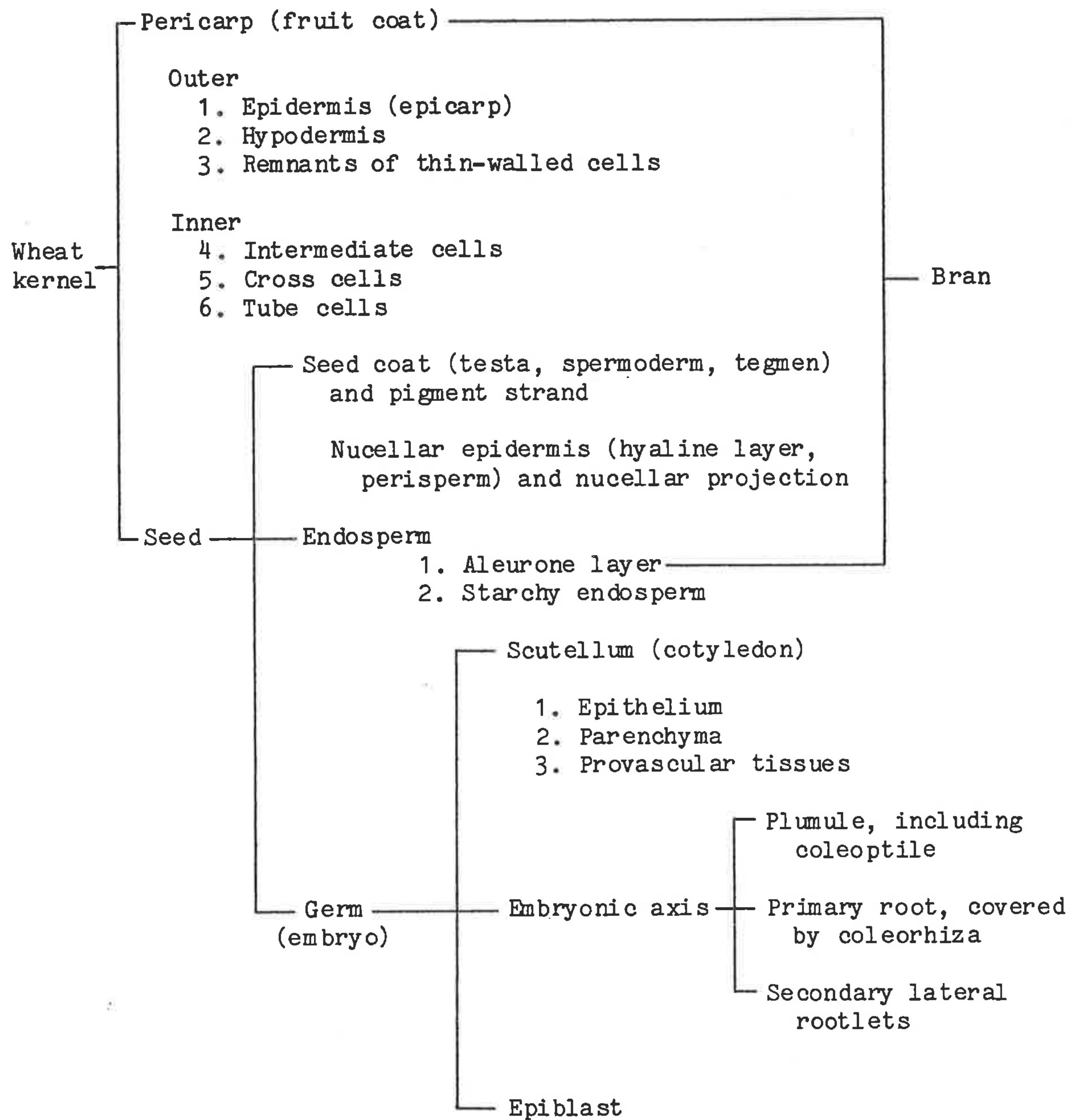
1.4.3.1 Outer Pericarp

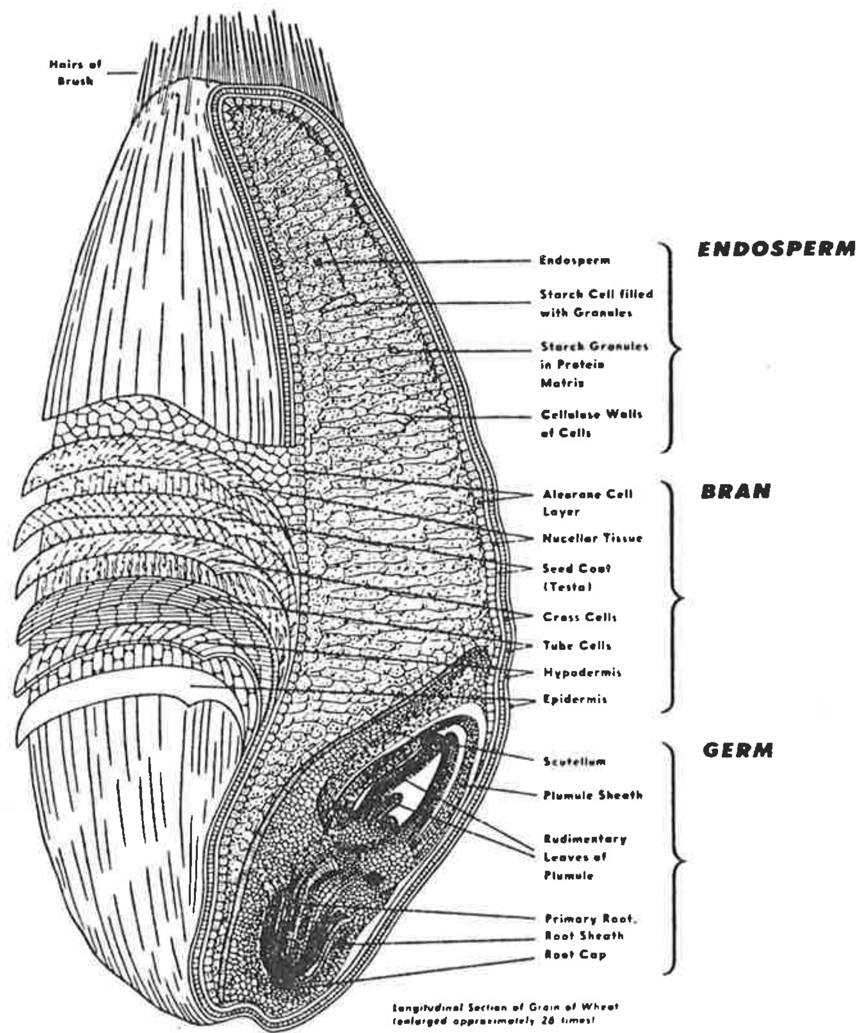
The outer pericarp comprises (a) the epidermis of the grain, a single layer of cells covered by a cuticle, and (b) the hypodermis which is a layer 1-2 cells thick. The walls of the cells in the outer pericarp are thickened. Immediately under the hypodermis is a layer of thin-wall cells which allows easy separation of the outer and inner pericarps.

1.4.3.2 Inner Pericarp

The outer integument of the ovule degenerates soon after anthesis. Cells from this tissue; tube cells in the dorsal region of the grain and cross cells in the cheek region become the inner pericarp. These cells contain chlorophyll and are photosynthetically active (Wirth et al., 1977). There are plasmodesmatal connections between them (Morrison, 1976). The cells of the inner pericarp contain large reserves of starch. Although the amount of starch decreases during development, the inner pericarp is never devoid of starch (Brenchley, 1909).

Table 1-3. Parts of the wheat kernel and their relation to each other. Reproduced from Bradbury et al., (1956a).





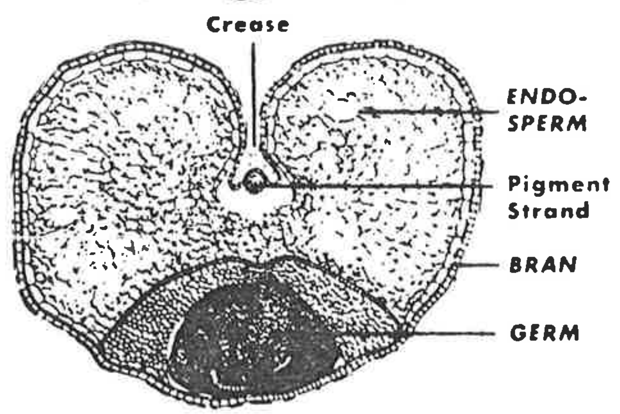


Figure 1-6. Diagramatic representation of the structure of the wheat kernel. Produced by the Wheat Flour Institute, Chicago, Illinois.

1.4.3.3 Seed Coat and Pigment Strand

The seed coat is derived from the inner integument. It is located between the inner pericarp and the nucellar epidermis, appearing firmly joined to both. The seed coat comprises 3 layers; a thick outer cuticle, a 'colour' layer and a thin inner cuticle.

The ultrastructure of the cuticles has been studied most thoroughly by Morrison (1975). By 7 days p.a. both layers are conspicuous, the outer one being 1.0 um to 1.5 um thick. They do not increase appreciably in thickness thereafter, although the outer one takes on a reticulate appearance. By 3-4 weeks p.a. the inner integument is no longer recognizable and the two cuticles are closely appressed.

The composition of the cuticles is not known. Despite their nomenclature, there is no evidence that cutin is present. The cuticle of Avena coleoptiles, which have similar visual and electron-dense characteristics to the seed coat in wheat (Morrison, 1975) are composed of a network of polyurides within pectinaceous material (O'Brien, 1967).

The seed coat is interrupted in the crease region by a strand of pigmented tissue, which presumably provides a channel for solute and water flux to the developing embryo and endosperm (see sections 1.8 and 1.10). When viewed in transverse section the pigment strand consists of 100-200 cells. During early grain development, these cells are thin walled and are interconnected with plasmodesmata (Cochrane, 1983; Zee and O'Brien, 1970b). At about 9 days p.a., lignin and adcrusting substances form between the cells. Insoluble material then forms within these lignified cells. Initially, this material appears confined to the vacuole, but later it appears throughout the whole cell. The nature of this substance seems to vary; sudanophilic bodies (Zee and O'Brien, 1970b), polyphenols and/or tannins (in barley, Cochrane, 1983), or cork (in barley and wheat, Krauss, 1933, cited from Cochrane, 1983). These deposits may restrict the movement

of solutes to, and possibly from, the embryo and endosperm (see section 1.8).

1.3.3.4 Nucellar Epidermis and Nucellar Projection

During development of the ovule, the embryo and endosperm grow within the nucellus and are nourished by it. Eventually only the epidermis of the nucellus and a band of cells adjacent to the pigment strand remain. This remaining band of tissue is called the nucellar projection.

During the grain-fill period of development, the nucellar epidermis is present over the entire kernel (Krauss, 1933, cited from Bradbury et al., 1965b), except perhaps for a break over the embryo (Bradbury et al., 1965b; Fairclough, 1947). As the grain matures, the nucellar epidermis may become compressed, losing its cellular structure (Bradbury et al., 1965b).

The nucellar projection contains transfer cells, suggesting a functional role of this tissue in the transfer of substrate from the maternal symplast to the apoplast (Cochrane and Duffus, 1980; Hughes, 1976, cited from Cochrane and Duffus, 1980). Recently, Smart and O'Brien (1983) also observed cells with the characteristic wall ingrowths of transfer cells in regions of the nucellar epidermis.

1.4.4 Development of the Endosperm

1.4.4.1 Cellular Development

Cellular development of wheat endosperm can be divided into 3 phases. The first commences with anthesis and covers the period of mitotic activity. For a time both cell multiplication and cell enlargement occur concurrently so some degree of cell enlargement takes place during this first phase. Evers (1974) divided this first phase further; (a) formation of the first endosperm triple-fusion nucleus, (b) synchronous free nuclear division and

(c) cellularization and cell division. The second phase commences with differentiation of the aleurone layer and is the main period of cell enlargement and dry matter accumulation. It is often termed grain filling or the linear phase of growth. The third phase is maturation. These phases are described now in more detail.

Phase I, Cell Formation.

The process of fertilization has been described elsewhere (section 1.4.1.). The triploid primary endosperm nucleus undergoes approximately 10 cycles of synchronous nuclear division without formation of intermediate cell walls (Percival, 1921; Bennett and Smith, 1973; Bennett et al., 1973). The nuclei then align adjacent to the embryo sac. Within this cytoplasmic mass is a large central vacuole (Evers, 1974).

Cellularization occurs at about 3 days post anthesis (p.a.). By this time the endosperm mother cell consists of about 1000 nuclei. The process of cellularization which takes 1-2 days has been studied extensively (Bennett et al., 1973; Brenchley, 1909; Buttrose, 1963a, 1963b; Evers, 1970; Gordon, 1922; Hughes, 1976; Mares et al., 1975, 1977; Morrison et al., 1975, 1976, 1978). The rudiments of cell walls form in the outer part of the peripheral layer of cytoplasm and develop to encase the nuclei. Once encased a nucleus divides. Cellular division then occurs and this process continues in an inward radial direction (see Mares et al., 1977 for diagramatic representation).

After cellularization is complete, nuclear division is no longer synchronous. The majority of cells divide by both radial and tangential divisions. However, cells in a double layer, on the ventral surface do not divide. They rapidly become thick-walled and granular, and many become oblong or wedge-like (Bradbury et al., 1965c). These cells collectively are called the modified aleurone. As this layer is a zone of restricted mitotic activity, a furrow forms the length of the grain on the ventral side as a

result of the other regions expanding and bending around it (Alexandrov, 1937; Evers, 1970). The modified aleurone eventually lines the endosperm cavity.

The rest of the aleurone layer forms as a single-celled layer around the endosperm after cell division in the peripheral zones has stopped, i.e. after about day 14 p.a.

Within the endosperm the rate of cell division decreases from about day 13 p.a. and has stopped completely by day 20 p.a. By this time there are about 10^5 cells (Briarty et al., 1979).

Phase II, Cell Expansion.

Fresh weight growth of the endosperm from day 14 p.a. to day 35 p.a. is due primarily to cell expansion. Commonly, cell size doubles from day 13 p.a. to day 20 p.a. and doubles again by day 35 p.a. (Briarty et al., 1979).

Endosperm cells, originally cuboid, differentiate during expansion into 3 types of cells; peripheral, prismatic and central (Bradbury et al., 1965c; Greer et al., 1951). These cell types differ in their shape, size and location.

The peripheral cells are a single row of cells adjacent to the aleurone layer. They are sometimes called subaleurone cells (Kent, 1966; Evers, 1970). They are isodiametric or slightly elongated in a radial direction, 50-60 um long.

Prismatic cells are radially elongated (128 um to 200 um by 40 um to 60 um) and form several rows inside the peripheral cells. From the dorsal side they extend nearly to the crease; elsewhere they extend part way to the centre of the cheeks. Prismatic cells occupy most of the endosperm volume. The central cells lie inward of the prismatic cells. In Pawnee wheat the central cells are in a band that extends from the central region of one cheek across the furrow into the central region of the other cheek (Bradbury et al., 1956c). In Manitoba wheat they occur only in the central

cheek region (Greer et al., 1951). These cells are irregular in shape and size; large cells in Manitoba wheat are 120 um to 144 um by 80 um to 120 um while small cells are 72 um to 104 um by 69 um to 96 um (Greer et al., 1951).

While there are large differences in cell size between cell types, positional gradients of size within any one cell type generally are small. Prismatic cells in the ventral region of the cheek are slightly wider than those in the dorsal region of the endosperm. Within the dorsal region itself, prismatic cells nearer the periphery are larger than cells nearer the crease (> 200 um by 40 um to 50 um cf. 128 um to 200 um by 40 um to 64 um) (Greer et al., 1951).

Plasmodesmata can be seen between cells of the endosperm (Duffus and Cochrane, 1982).

The aleurone layer (excluding the modified aleurone, see above) forms from about day 14 p.a. Small thin-walled cells in the outermost layer of endosperm enlarge slightly, maintaining their isodiametric shape. The wall thickens; to about 1 um at day 20 p.a. and to 4-5 um 10 days later. The nuclei enlarge and the cytoplasm becomes granular. They are rich in endoplasmic reticulum and mitochondria (Evers, 1970; Morrison et al., 1975, 1978). Plasmodesmata are abundant and clearly defined, connecting adjacent aleurone cells and aleurone cells to adjacent peripheral or prismatic cells (Guenther, 1927; O'Brien, 1895; Tschirch and Oesterle, 1900; Vogl, 1899, - all cited from MacMasters et al., 1971; Cochrane and Duffus, 1980; Morrison et al., 1975, 1978).

Membranous loops develop within the cytoplasm of cells of the modified aleurone. The general view is that these membranous features are not the same as the wall ingrowths of transfer cells (Cochrane and Duffus, 1980; Morrison et al., 1978; Zee and O'Brien, 1971. But see also Ayre and Angold, 1979). The thick walls of the modified aleurone fluoresce in the presence of aniline blue; characteristic of the presence of callose

(Cochrane and Duffus, 1980).

Phase III Maturation

During the final stage which commences at about 35 days p.a., the fresh weight of the kernel declines as water is lost. At about the same time deposition of dry matter stops. Over the next 20 days there is also a loss of dry matter. Although difficult to quantify, the loss of dry matter seems to amount to about 3% of the grain's dry weight (Donovan et al., 1977b). The grain has now reached harvest-ripeness.

1.4.4.2 Accumulation of the Storage Product

Starch is deposited as a granule within a cytoplasmic organelle, the amyloplast. Amyloplasts have two membranes, the inner one is invaginated (Buttrose, 1960, 1963b). It is rare for a wheat amyloplast to contain more than one starch granule (Buttrose, 1960), some amyloplast-like plastids are void (Briarty et al., 1979).

The origin of the first-formed amyloplast(s) is obscure. Suggestions include (1) by de novo synthesis (Muhlethaler and Frey-Wyssling, 1959), (2) by division of preexisting organelles associated with the polar nuclei (Buttrose 1963b), and (3) from precursors within the egg cell nuclei (Muhlethaler and Bell, 1962). Amyloplasts divide during the first 6-8 days p.a. forming similar large (20-45 um diam.) A-type amyloplasts. Since cell division continues until day 20 p.a. (section 1.4.4.1), the later dividing cells, those near the periphery (Gordon, 1922), contain fewer A-type amyloplasts (Briarty et al., 1979). For a while the outermost A-type granules grow faster than those nearer the centre, but later this trend is reversed. Briarty et al., (1979) suggested that this pattern of growth may reflect regional availability of substrate.

From about day 15 p.a. a second generation of plastids arise. These are smaller (< 10 um diam., Buttrose, 1963) and arise by evagination or

constriction of the envelope of existing plastids (Buttrose, 1960, 1963b; Hughes, 1976). Called the B-type, these plastids comprise about 97% of the total number of starch-bearing amyloplasts in mature endosperm, but because of their smaller size they account for only 25-50% of the total weight of starch (Evers, 1973; Evers and Lindley, 1977).

The starch granule in wheat endosperm has a layered appearance (Buttrose, 1960; Evers, 1971), apparently formed by successive irregular additions of starch onto the granule surface (Buttrose, 1960). These layers are not present in grains grown under constant light which has led Buttrose (1962) to propose that they result from diurnal fluctuations in substrate supply. On the other hand, the level of endosperm sucrose does not appear to fluctuate in a way that could account for this (Jenner and Rathjen, 1972).

The starch granule initially occupies a very small proportion of the plastid volume. By 20 days p.a., however, this rises to about 80% and by day 30 p.a. essentially all of the plastid volume is starch (Briarty et al., 1979).

Storage protein first appears in wheat endosperm at about 10 days p.a. and is located within membrane-bound spherical bodies. Typically, these bodies are 0.5 to 1.5 um in diameter and are closely associated with rough endoplasmic reticulum (Barlow et al., 1974; Graham et al., 1962; Jennings et al., 1963). These 'vacuoles' seem to arise as extensions of the lumen of the endoplasmic reticulum and either fuse with existing vacuoles (Briarty et al., 1979) or are budded off independently (Campbell et al., 1981).

At about 20 days p.a. only about 50% of the final amount of storage protein has been synthesized (Donovan et al., 1977b; Jennings and Morton, 1963a). Hereafter a second form of protein synthesis dominates. Protein is synthesized on the rough endoplasmic reticulum and transferred across the membrane into the lumen. The endoplasmic reticulum swells and becomes

distended as the protein is deposited (Campbell et al., 1981; Simmonds and O'Brien, 1981). During the final stages of grain fill, many protein bodies fuse, forming a continuous, highly compressed protein matrix in which the starch granules are firmly embedded (Adams et al., 1976; Betchel et al., 1980).

1.5 BIOCHEMICAL PATHWAYS OF STARCH SYNTHESIS

Sucrose is considered to be the major carbohydrate precursor for starch deposition in wheat endosperm, passing without modification from the site of photosynthesis to the cytoplasm of the endosperm cells (section 1.8). The pathway by which sucrose is converted to amylose and amylopectin cannot be described with certainty however. Alternative routes can be proposed based on the complement of enzymes already shown to be present. Ontogenetic change to the pathway cannot be ruled out.

Two recent papers (Duffus and Cochrane, 1982; Jenner, 1982a) discuss in detail biochemical pathways for the synthesis of starch. It seems sufficient for the present purpose to reproduce a figure from Duffus (1984) outlining the most plausible route for conversion (Fig. 1-7).

1.6 BIOCHEMICAL PATHWAYS OF PROTEIN SYNTHESIS

Nitrogen is available in soils primarily as nitrate (Pitman et al., 1976). It is absorbed by the roots both actively (Higinbotham, 1973) and passively Morgan et al., 1973). Small amounts of ammonia in the soil are also absorbed (Epstein, 1976).

Once absorbed, most of the nitrate is transported throughout the plant in the transpiration stream. Nitrate is converted to nitrite by nitrate reductase, a cytoplasmic enzyme that is synthesized in response to the presence of substrate (Hernandez et al., 1974). Nitrite is reduced further

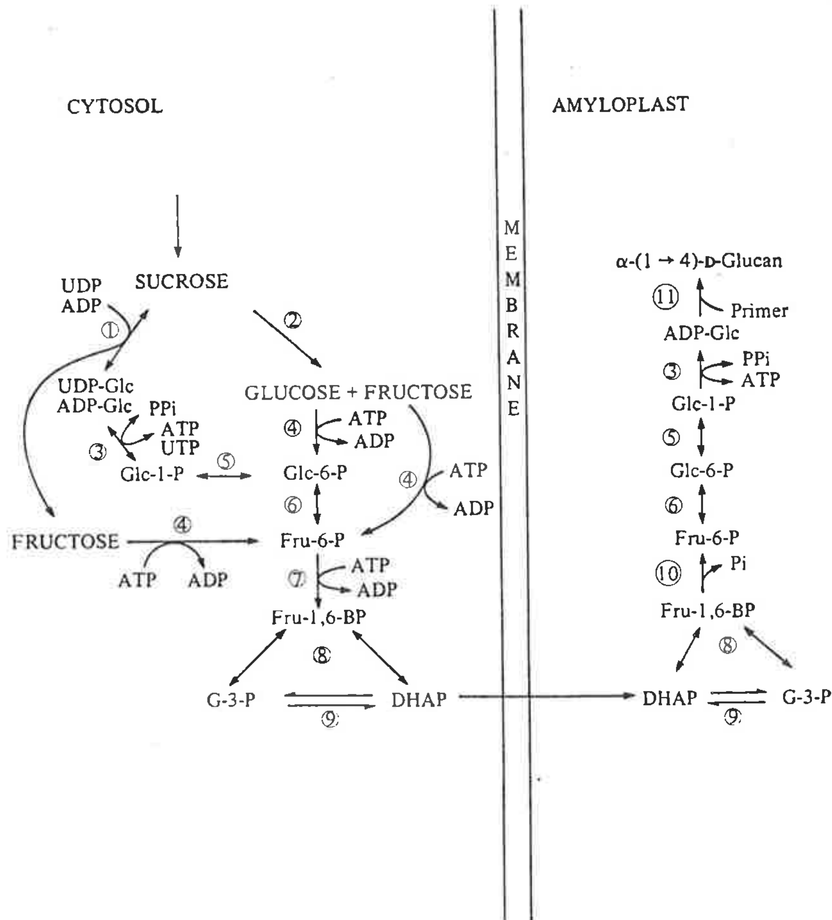


Figure 1-7. Hypothetical scheme for conversion of sucrose to starch. This scheme assumes selective permeability of the amyloplast inner membrane. Reproduced from Duffus (1984).

(1), sucrose synthase; (2), invertase; (3), ADP-glucose and UDP-glucose pyrophosphorylases; (4), hexokinase; (5), phosphoglucomutase; (6), phosphohexoisomerase; (7), phosphofructokinase; (8), aldolase; (9), triosephosphate isomerase; (10), fructose-1,6-bisphosphatase; (11), starch synthase.
UDP-Glc = UDP-glucose, ADP-Glc = ADP-glucose, Glc-6-P = glucose 6-phosphate, Fru-6-P = fructose 6-phosphate, Fru-1,6-BP = fructose 1,6-bisphosphate, Glc-1-P = glucose 1-phosphate, G-3-P = glyceraldehyde 3-phosphate, DHAP = dihydroxyacetone phosphate, PPi = pyrophosphate.

to ammonia by nitrite reductase, an enzyme mostly located in the chloroplast (Abrol et al., 1984; Huffaker and Rains, 1978; Porceddu et al., 1982).

Ammonia is combined with glutamate to form glutamine (glutamine synthetase) and then a second enzyme (glutamate synthetase, alternatively known as GOGAT) catalyses the reductive transfer of the amide group of glutamine to 2-oxoglutarate, forming two units of glutamate. Transaminases couple deamination of glutamate (back to 2-oxoglutarate) to the conversion of α-oxo acids to the α-amino acids for protein synthesis (Hageman, 1979; Miflin and Lea, 1980; Miflin, 1980).

A small amount (10-20%) of the absorbed nitrogen is reduced to ammonia in the roots (Beevers and Hageman, 1969). This ammonia, together with the ammonia absorbed from the soil, is converted to nitrate also in the roots (Miflin, 1978). Of the nitrate assimilated by the above-ground parts, 72% is reduced by the leaf laminae, 21% by the sheaths and 7% by the ear (Abrol et al., 1984).

Nitrogen absorbed and assimilated during the early stages of growth may be stored in all parts of the plant, either as vacuolar nitrate or as protein and remobilized later to provide nitrogen for deposition of protein in the grain (Austin and Nair, 1963; Miflin, 1978; Simpson et al., 1983; see section 1.3). Hydrolysis of stored protein to amino acids for remobilization involves the action of a wide range of enzymes (see Abrol et al., 1984).

Most of the nitrogen moving to the ear is transported in the xylem as glutamine (Kirkman and Miflin, 1979). However, Donovan and Lee (1978) have shown that detached and cultured wheat ears can utilize asparagine, glutamine, a spectrum of amino acids, or ammonium nitrate as their nitrogen source, apparently with equal proficiency. A summary of the pathways of transamination is presented in Miflin (1980). The composition of amino

acids within the phloem entering the grain (see section 1.4.2) has not been determined.

The mechanism for the conversion of amino acids to seed storage protein, and the regulation of this mechanism has recently been reviewed by Higgins (1984). A brief outline is as follows. Amino acids are esterified to their corresponding tRNA in the cytoplasm of endosperm cells and converted to a nascent peptide during polysomal translation of mRNA. This peptide contains a signal sequence at the start which 'directs' this peptide to rough endoplasmic reticulum. On the ER the signal sequence is removed and oligosaccharide side chains are affixed possibly to asparagine residues of the peptide. The oligosaccharide side chains comprise mainly mannose, glucosamine and neutral sugars such as fucose. These modified peptides are then polymerized into the large globules of storage protein.

1.7 ONTOGENETIC AND DISTRIBUTIONAL PATTERNS OF STARCH AND PROTEIN DEPOSITION IN THE ENDOSPERM

Starch is present in the developing grain shortly after anthesis, although the rate of synthesis at this time is low. Around day 10 p.a. there is a substantial increase in starch production and from day 12 p.a. to day 35 p.a. the rate of synthesis is relatively constant (Bilinski and McConnell, 1958; Donovan et al., 1977a, 1977b; Jennings and Morton, 1963a; McCalla, 1938).

Most of the protein extracted from wheat endosperm before day 12 p.a. is cytoplasmic. Synthesis of these soluble proteins continues until day 30 p.a. Storage protein is synthesized after day 12 p.a. which, like the synthesis of starch, is produced at a relatively constant rate until about day 35 p.a. The protein percentage of the endosperm decreases (from 18 to 13.8) from day 12 p.a. to day 20 p.a. but thereafter usually remains relatively constant (Jennings and Morton, 1963a). However, the protein

percentage may change during the later stages of development, being influenced by (a) the more abrupt cessation of starch synthesis at the end of grain fill or (b) the independent influence of environment or cultural practice on the rate and duration of both starch and protein deposition (sections 1.2.3.2.3, 1.3 and 1.4.4.2).

Within the endosperm itself, starch and protein are deposited in varying proportions depending on position. The first evidence for this was mostly indirect, based on experience in the milling industry. Flours from different mill-streams of the one batch of grain differed in their relative proportions of ash, protein and starch.

Cobb (1902- 1904) working with a soft wheat (purple straw type) dissected individual mature kernels by hand and demonstrated a gradient of protein percentage within the endosperm from the centre to the bran coat. Tissue from the centre had a protein percentage of 7.4 (w/w). This value increased serially (8.6, 9.5, 13.9) until in the peripheral zones the protein percentage was 16.5; overall a 2.2-fold increase. Hinton (1947) working with a soft English wheat also found a 2.2-fold gradient (5.7% to 12.5%, w/w at 12% moisture). Morris et al. (1945, 1946) reported that the protein percentage in the endosperm of a hard winter wheat ranged from 8.9 in the centre to 13.6 in the peripheral zones. Likewise in a soft red wheat the values ranged from 6.8 to 10.9. Normand et al. (1965) found that in two soft wheats the gradient was shallow (12% to 14%, w/w on a dry weight basis) whereas in two hard wheats the gradient was more steep (12% to 25%).

The studies cited so far show a continual increase in protein percentage from the centre of the endosperm to the periphery; there is no step-like increase at any point and the zone of higher protein content, although arbitarily defined, extends well into the endosperm. Indeed in the studies of Normand et al. (1965), where sections of the grain were abraded serially from the outside, the protein content of the removed particles

remained constant, maybe even rose slightly until about 25% of the grain's weight had been removed. Thereafter, it declined steadily.

Kent (1966) reported that after a bulked sample of red winter wheat was milled, the protein percentage of different flour fractions ranged from 8 to 43 (w/w at 14% moisture). He claimed that when compared to the results of Cobb (1902-1904) and Hinton (1947) (see above), his findings suggest that the steepness of the gradient is a real difference between hard and soft wheats. While this view is consistent with the results of Normand et al. (1965), it is contrary to those of Morris et al. (1945, 1946). In any case, this comment by Kent is misleading.

The very high values of protein content reported by Kent (33% to 54%) were in cells microscopically identified as peripheral cells (see section 1.4.4.1). As also reported by Kent in the same paper, the protein percentage in fractions taken from the inner endosperm itself (i.e. prismatic and central cells, see section 1.4.4.1) ranged from 8 to 15, and hence is similar to that reported by Cobb and Hinton.

Using data mainly of Kent (1966; Kent and Jones, 1952), Evers (1970) calculated that both the prismatic cells of the inner endosperm and the peripheral (subaleurone) cells had the same weight of protein per cell. He concluded that ... 'the gradient therefore results from the variation in dilution of this constituent (protein) by starch.' This calculation and the conclusion drawn from it must be taken carefully; they explain only the difference in protein percentage between two types of cells (prismatic and peripheral). No conclusions can be drawn as to the cause of the gradient of protein percentage within the bulk of inner endosperm, i.e. within the prismatic and central cells.

Pomeranz and Shellenberger (1961) showed clearly that in the mature grain there is more protein per endosperm volume in the peripheral zones. Cross-sections of the caryopsis were stained with protein-selective dyes in a way that gave some degree of quantitative assessment. The stain appeared

most intense around the periphery. This 'high-protein' region extended well into the endosperm, up to 50% its width. There was no sharp demarcation between the protein-rich zones and the protein-poor ones, and lastly, the zone adjacent to the cavity on the dorsal side was low in protein.

During maturation (day 35 p.a. to day 55 p.a.) the wheat grain shrinks by 35% its volume (Sofield et al., 1977). Since the pattern of shrinkage is not known, gradients of starch and protein in the mature seed cannot be used as a measure of deposition, on a volume basis, during development. The only information on the pattern of deposition during development is as follows.

Starch accumulates first in the centre of the cheeks near the tip of the grain, then extends both basipetally within the cheeks and around into the dorsal region. Within the dorsal region it becomes deposited first adjacent to the endosperm cavity and then extends outwards. Starch is present throughout the whole endosperm by about day 18 p.a. (Brenchley, 1909). The diameter of the A-type granules increases at about the same rate regardless of position (Briarty et al., 1979, see Fig. 6, diameter profile at 36 days p.a.). While this may suggest that after day 18 p.a. starch is deposited evenly throughout the profile, there is no information on the frequency distribution of A-type granules, the size and frequency distribution of B-type granules, or indeed, whether changes in granule diameter can be used as a measure of the rate of starch deposition.

1.8 TRANSPORT OF SUBSTRATE INTO AND WITHIN THE GRAIN

1.8.1 Route of Substrate Movement

Most of the carbohydrate (sucrose) and all of the amino acids and amides moving to the endosperm pass through the stalk. The xylem appears to be blocked at the base of the grain so it seems that solutes must move past

this point exclusively in the phloem (section 1.4.2). However, the phloem here also is abnormal. It comprises a conglomerate of sieve elements apparently running in all directions and numerous transfer cells (O'Brien et al., 1985). It is not possible to tell from anatomical studies whether there is lumen continuity between the phloem of the plant and the phloem of the grain. However, from physiological studies, Jenner (1985a, 1985b, 1985c) has proposed that the flow of sucrose into the grain is not accompanied by mass flow of water, hereby suggesting that the two phloem systems are not continuous. There are no studies that examine whether there is any selectivity of solute transfer from one system to the other.

Donovan et al. (1983) fed labelled sucrose and amino acids to cultured ears and studied the kinetics of longitudinal transport within grains. From this, there appears to be no difference in the transport of these solutes within the phloem of the grain. There are no studies that follow further, in a comparative way, the movement of these solutes.

The developing grain is surrounded entirely by the seed coat except for a break in the region of the crease. Here there is a strand of pigmented tissue (section 1.4.3.3). Although there are no direct measurements on the permeability of the seed coat to solute flux, it is expected that the seed coat would form a barrier of quite high resistance (Bradbury et al., 1956b, and references therein; Morrison, 1976; Zee and O'Brien, 1970b). In aged barley seeds, the seed coat does indeed show high resistance to inward flux of aqueous solutes (Briggs and MacDonald, 1983). Presumably therefore, most, if not all, the solutes and water moving to the developing endosperm and embryo move through the pigment strand (Cochrane, 1983; Zee and O'Brien, 1970b).

From about 9 days p.a. there are structural changes within the pigment strand (section 1.4.3.3). The significance of these changes has not been resolved. Zee and O'Brien (1970b) proposed that first there is a blockage to apoplastic flux, then a progressive restriction to symplastic flux

which, together, bring about cessation of dry matter deposition. Cochrane (1983) suggested that only symplastic transfer is restricted; water being lost through the conducting apoplast during maturation (see sections 1.4.4.1 and 1.7). On the other hand, maybe these structural changes have no influence on the termination of grain-fill at all. When the accumulation of starch stops, there is still an ample supply of sucrose in the endosperm (Jenner, 1982a).

In a shrunken-endosperm mutant of barley, premature cessation of grain growth has been linked both on a structural and physiological basis to early death of cells in the region of the pigment strand (Felker et al., 1983, 1984a, 1984b).

Fluorescein, a symplastic tracer, moves from the grain's phloem through the pigment strand. Then, it moves through the nucellar projection, the endosperm cavity and radially into the endosperm (Cook and Oparka, 1983). There is conflicting evidence, however, on whether sucrose moves through the nucellar projection and endosperm cavity in this way (detailed below). Furthermore, there are no studies on the route taken by amino acids.

The concentration of sucrose in the endosperm cavity is lower than it is in the vascular bundle, and it is lower again in the endosperm (Ho and Gifford, 1984; Jenner, 1974a). When Jenner (1974a) cultured wheat ears on a ^{14}C-sucrose solution, the specific activity of sucrose within both the vascular bundle, and the endosperm was always higher than the specific activity of sucrose in the composite layer, i.e. the peripheral layer comprising the inner pericarp, the seed coat, the nucellar epidermis and the aleurone layer. Since solutes destined for the endosperm must move either through the endosperm cavity or within the tissues of the composite layer (sections 1.4.3 and 1.4.4), Jenner concluded that these results support the hypothesis that sucrose moves to the endosperm via the

endosperm cavity. On the other hand, one could argue that if the composite layer comprised both conducting and non-conducting tissue, the measured specific activity in the composite layer as a whole may be well below the specific activity of sucrose in any tissue(s) conveying sucrose to the endosperm, and therefore this experiment is not definative.

Ho and Gifford (1984) measured the distribution of label within the grain after feeding a pulse of $^{14}CO_2$ to the flag leaf and although they did not convert their results into specific activities, this can be done easily from data given. Data from cv. Yandilla King form the basis of these calculations. Comparisons are made with cv. Sun 9E where possible.

Details of these calculations are given now together with reference to the relevant information in Ho and Gifford (1985). A summary is presented in Table 1-4.

In the endosperm the concentration of sucrose was 5.3 $mg.ml^{-1}$ (Table 6), the volume of water was 34.5 ul (2.28 x 100/6.6, Table 2; volume for Sun 9E was 35.7 ul), hence the amount of sucrose in the endosperm was 183 ug. The mass of liquid extracted from the cavity was 3.65 mg (Table 4), specific gravity of liquid was about 1 (Materials and Methods), concentration of sucrose was 13.8 $mg.ml^{-1}$ (Table 6), hence the amount of sucrose extracted was 50.4 ug. Dpm values in the tissues of the grain are given in Fig. 5. The proportion of label that is in sucrose is between 50% and 90% for both the endosperm cavity and the ethanol-soluble fraction of endosperm (p. 69). First of all, consider the amount of sucrose present in the endosperm cavity. If the cavity was a part of the principal supply route to the endosperm the turn-over of sucrose here is less than one hour. Therefore the specific activity of sucrose in the cavity should reflect quite closely the specific activity of the sucrose entering the endosperm. However first, in all cases, the specific activity of sucrose in the endosperm cavity is well below the specific activity of sucrose in the endosperm and secondly, after 200 minutes the specific activity of sucrose

Table 1-4. Specific activity of sucrose in the endosperm cavity and endosperm in wheat at various times after the flag leaf was fed with $^{14}CO_2$. This is calculated from data of Ho and Gifford (1984).

PART A. FLUID OF THE ENDOSPERM CAVITY

Time after $^{14}CO_2$ applied (mins)	dpm (from table 5a)	Specific Activity ($dpm.ug^{-1}$)	
		Assume 50% of soluble ^{14}C in sucrose	Assume 90% of soluble ^{14}C in sucrose
90	1	0.0	0.0
150	35	0.3	0.6
200	7080	70.2	126.4
270	5011	49.7	89.5
390	1778	17.6	31.8

PART B. ENDOSPERM

Time after $^{14}CO_2$ applied (mins)	dpm in whole grain (5a)	Portion of total ^{14}C in endos'm (5b)	Portion of endos'm ^{14}C in soluble form (5c)	Hence, Portion total ^{14}C in soluble form in endos'm	Assume 50% of soluble ^{14}C in sucrose		Assume 90% of soluble ^{14}C in sucrose	
					dpm in sucrose	specif. activ.	dpm in sucrose	specif. activ.
90	158	0.28	not given					
150	5,623	0.41	0.83	0.34	956	5.2	1,721	9.4
200	100,000	0.59	0.77	0.45	22,500	123.0	40,500	221.3
270	398,107	0.70	0.67	0.47	93,555	511.2	168,399	920.2
390	630,957	0.80	0.62	0.50	157,739	862.0	283,931	1,551.5

in the cavity declines while it continues to rise in the endosperm. Contrary to the conclusion drawn by Ho and Gifford, the conclusion from this study must be that sucrose can move to the endosperm in a route not involving the endosperm cavity.

Another question is whether solutes move through the endosperm in the apoplast or the symplast. Certainly plasmodesmatal connections are abundant between endosperm cells, and cells of the modified aleurone have membraneous loops which may assist solute transfer from the apoplast to the symplast (section 1.4.4.1). Physiological evidence on this question relies on washing solutes from the endosperm apoplast as a pool distinct from compartmented solutes.

The pattern of sucrose efflux from wheat endosperm (Gifford and Bremner, 1981) does not conform to the usual pattern of efflux used for compartmental analysis (e.g. see Walker and Pitman, 1976). A plot of (log rate of efflux) versus time shows an exponential decline which continues for 10 hours, rather than a series of discrete steps. There may be several reasons for this, for instance (1) coefficients of efflux change with time, (2) cells burst as a result of hypotonic shock (see Simon, 1977), or (3) the differences in the coefficients of efflux for separate compartments are small. Regardless of the reason, an efflux curve of this nature shows conclusively that a substantial amount of efflux is occurring from a membrane-bound compartment(s).

All experiments which have attempted to wash sucrose from the apoplast only of wheat endosperm have been done at low temperature; 1°- 4°C (Gifford and Bremner, 1981; Ho and Gifford, 1984; Jenner, 1974a; Jenner and Rathjen, 1975). However, sudden chilling of plant tissue can cause a sudden and rapid efflux of compartmented solute (Bange, 1979). In the experiments of Bange compartmented rubidium and potassium effluxed very slowly at 25°C but at 2°C the efflux was rapid with a half-time of about 10 minutes.

Usually efflux of solutes from the apoplast can be distinguished from efflux from other compartments only within the first 6-12 minutes (e.g. Patrick and McDonald, 1980; Walker and Pitman, 1976; Ugalde, 1976). The half-time of efflux is generally 2-3 minutes. Yet in the experiments with wheat endosperm, all sucrose that effluxed within the first 90 minutes was bulked together and classified as apoplastic.

The volume of the apoplast measured by efflux of pre-loaded sucrose has been compared to the measurement made by the influx of polyethylene glycol (PEG, MW 900 to 4000). Generally, the values obtained by the two methods are similar. However, the validity of this second method can be challenged also.

Ho and Gifford (1984) used the amount of PEG that had permeated the tissue after 6 hours to calculate apoplastic volume. At 6 hours, however, the amount of PEG in the tissue was still increasing. Indeed, Jenner and Rathjen (1975) found that at two stages of maturity (17 and 49 days) the amount of PEG that permeated endosperm doubled between 24 and 48 hours. Given that PEG can pass through plant membranes, albeit at a slow rate when observed previously (Lawlor, 1970), and that there are no studies on the permeability of the plasma membrane of wheat endosperm to PEG, these results cannot be used to corroborate measurements of apoplastic volume obtained by other methods.

Another problem with these influx or wash-out studies is that the intercellular region contains air space. As a result the volume of the water-saturated intercellular space is unlikely to equate with the volume of the apoplastic solution _in vivo_ (see for example Richter and Ehwald, 1983).

Jenner (1974a) measured the amount of sucrose that effluxed from wheat endosperm over a 90 minute period into an ice-cold bathing solution. The half-time for efflux was 6 to 10 minutes. Jenner presumed that most of the recovered sucrose originated in the apoplast, and on the basis of this

suggested that the apoplast was the major route for solute movement through the endosperm. In view of the comments above, the results of Jenner do not discount unequivocally the possibility that sucrose may move through the endosperm symplastically.

Peeled endosperm or endosperm slices take up solutes from a bathing solution. This is used as evidence for an apoplastic route of solute transfer (e.g. Rijven and Gifford, 1984). However, this also may not be valid. After all, solutes do leak from cells, therefore it is not unreasonable to propose the existence of a scavenging mechanism to reload solutes back into the symplast.

1.8.2 Role of Intercellular Invertase

For many tissues, hydrolysis of sucrose by intercellular invertase is an essential prerequisite to uptake from the apoplast. The most definitive way to test the role of intercellular invertase is to supply asymmetrically or uniformly labelled sucrose in the presence of non-radioactive hexose, and to examine the symmetry of label within sucrose after accumulation. The only work done in this way with wheat endosperm has been done by Jenner (1974b). From this, it is concluded that sucrose is absorbed without prior hydrolysis, a conclusion also reached by Ho and Gifford (1984), Sakri and Shannon (1975) and in barley by Felker et al. (1984b).

1.9 CARBON FIXATION BY THE INNER PERICARP AND TRANSPORT OF THE PHOTOSYNTHETIC PRODUCT TO THE ENDOSPERM

During most of grain fill, the inner pericarp is bright green and photosynthetically active (Carr and Wardlaw, 1965; Evans and Rawson, 1970). Strangely however, carbon fixation by this tissue has features characteristic of the C_4 pathway; (1) malate is an intermediate compound in

the formation of sucrose, (2) phosphoenolypruvate carboxylase and phosphoenolpyruvate synthetase are present, and (3) CO_2 can be fixed in the dark (Duffus and Rosie, 1973; Nutbeam and Duffus, 1976; Writh et al., 1977). In wheat, by contrast to barley, the proportional contribution of this C_4-like pathway to the total carbon fixed by the inner pericarp is small (Wirth et al., 1977; cf. Nutbeam and Duffus, 1976).

Edwards and Walker (1983) argue that these (above-mentioned) observations do not mean necessarily that the C_4 pathway of photosynthesis operates in the inner pericarp. As an alternative, label could be transferred from malate to sucrose in a way similar to that observed in castorbean endosperm, by malate dehydrogenase and PEP carboxykinase (Benedict and Beevers, 1961). The definitive test for the C_4 pathway is the transfer of label from the C-4 position of malate to the C-1 position of PGA.

Compared to other photosynthetic tissues, the pericarp has few stomata. These are located mainly around the brush end of the grain (Cochrane and Duffus, 1979; Kriedeman, 1966; Percival, 1921; Steiber, 1962). The low frequency of stomata has led to the view that photosynthetic activity in the inner pericarp has a role in minimizing respiratory loss of carbon from the grain (Kriedeman, 1966; Cochrane and Duffus, 1979) and supplying the grain with oxygen (Cochrane and Duffus, 1979).

The contribution of the inner pericarp to the overall carbon budget of developing grains has not been studied experimentally. However, isolated grains display sufficient capacity in vitro to supply 30% of the grain's dry matter (Nutbeam, 1978). Possibly therefore, the contribution of the inner pericarp is quite significant.

The route of solute movement from the inner pericarp to the endosperm also is not known, although several seem possible (Cochrane and Duffus, 1979).

1. Solutes could move symplastically within the inner pericarp either

circumferentially to the vascular bundle or to the base of the grain for transfer there to the vascular bundle. Plasmodesmatal connections are abundant in the inner pericarp (section 1.4.3.2).

2. Solutes could move apoplastically along a similar route to that described above (#1).

3. Solutes could move in an inward radial direction through the seed coat and nucellar epidermis.

1.10 CONCLUDING REMARKS AND OUTLINE OF RESEARCH PROJECT

The rates and timing by which different classes of solute are incorporated into insoluble products in the endosperm are influenced by factors within the grain. An empirical view is that the rate of starch deposition is 'sink limited' while the rate of protein deposition is 'source-limited' (sections 1.2.3.2.2, 1.2.3.2.3, 1.3, 1.4.4.2, 1.7 and 1.8). To put it another way, the normal levels of sucrose supply and the normal levels of amino acid supply appear to fall on different regions of an asymptotic curve for accumulation rate versus substrate supply (Fig. 1.8). An increase in sucrose supply has very little effect on the rate of starch accumulation, whereas an increase in amino acid supply, falling on the linear region of the curve, results in enhanced accumulation.

Yield of any product can be increased by increasing the rate of deposition, the duration of deposition, or both. Since the rate and duration of starch and protein synthesis are all essentially independent of one another (sections 1.2.3.2.2, 1.3.3.2.3, 1.3, 1.4.4.2, 1.7 and 1.8), they must be considered separately in any attempt to increase productivity through genetic or cultural means.

In the mature grain there is a decreasing gradient of protein

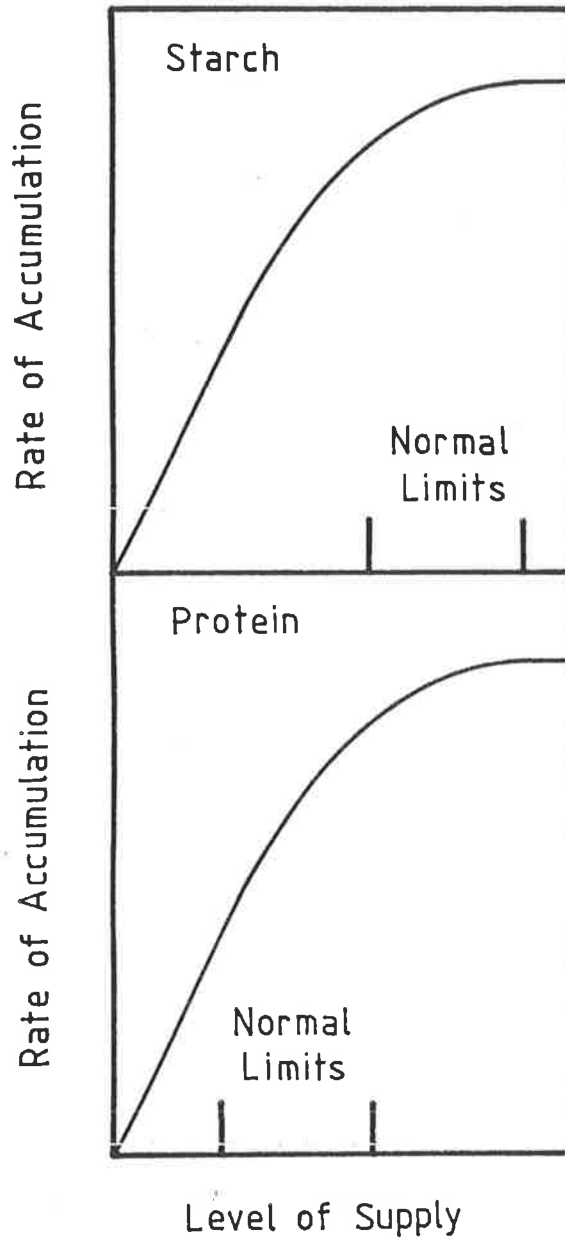


Figure 1-8. Dependence of the rate of starch and protein synthesis on the level of precursor supply. From Jenner, unpublished.

percentage from the outermost regions of endosperm to the regions nearer the cavity. This is due, probably, to the gradient of protein more so than starch (section 1.7). However there are no studies on (a) the distribution patterns of starch and protein within the endosperm on a unit volume basis during grain fill, and (b) whether any such gradients are due to supply gradients of the respective precursors.

Three questions for further consideration are listed below.

1. By what mechanism is the flux of sucrose into and within the grain controlled so that homeostatic concentrations are maintained within the endosperm despite fluctuations in external supply?

2. What is different about the movement of amino acids that allows these solutes to circumvent the process that controls sucrose flux?

3. What is the distribution pattern of starch and protein in the developing endosperm, and is it related to differential movement of sucrose and amino acids (Q 1 & 2) creating different supply gradients within the endosperm.

The differences in the deposition of starch and protein could arise a number of ways; by (1) alternative pathways within the grain for each class of solute and different kinetics therein, (2) differences in the kinetics of movement for each class of solute within the same pathway(s) and/or (3) regional differences in the kinetics of conversion of substrate to the insoluble product.

Figure 1-9 shows the sites within the wheat grain at which different solutes could be directed into alternative routes or could have different kinetics of movement. A brief explanation of each site follows. Considering each site separately does not imply the assumption that any difference in the overall movement of different solutes derives from an effect at one site only. The term 'directional differentiation' at a branch point is used to describe preferred channelling of each class of solute into a separate

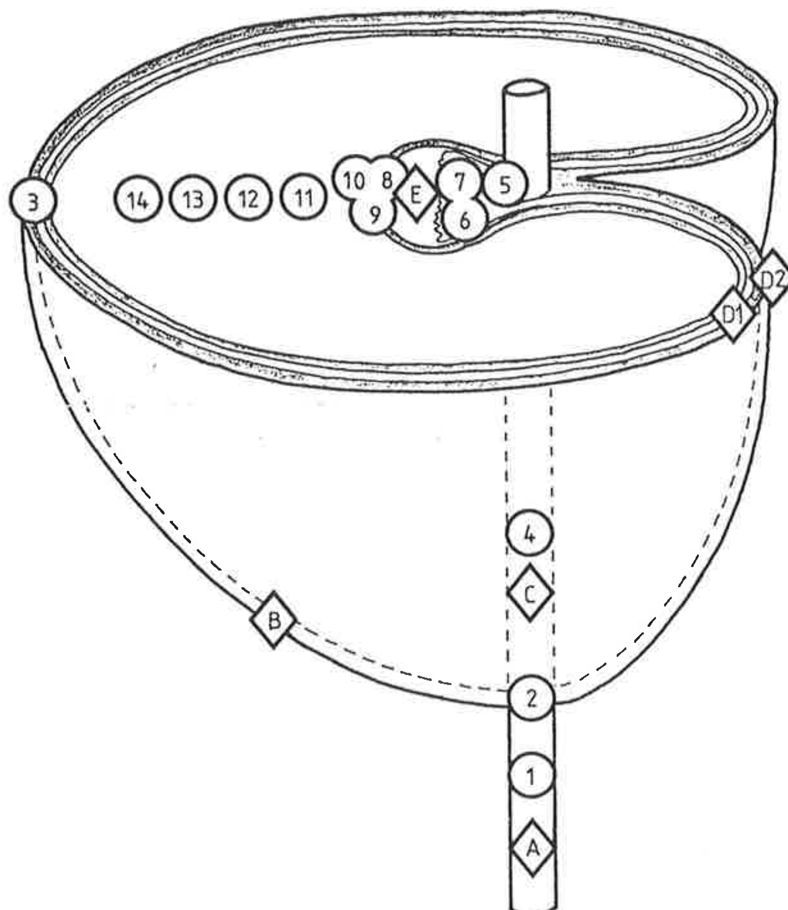


Figure 1-9. Sites within the wheat grain appearing to have the potential to direct different classes of solutes into alternative routes or to move these solutes with different kinetics. See text for details.

pathway, and 'selectivity of transfer' is used to describe preferred passage of one class of solute relative to another in the same pathway, i.e. differences in the kinetics of transfer.

Site 1. Selectivity of transfer within the vascular system of the plant or during transfer of solutes from the phloem of the plant to the phloem of the grain (section 1.4.2).

2. Directional differentiation into the inner pericarp or the vascular bundle of the grain (sections 1.4.2, 1.4.3.2 and 1.8).

3. Selectivity of transfer either within the inner pericarp or during passage of solutes from the inner pericarp to the endosperm in an inward radial direction (section 1.9).

4. Selectivity of transfer (transport) within the phloem of the grain (section 1.4.2 and 1.8).

5. Selectivity of transfer from the grain's phloem to the symplasm of the nucellar tissue (section 1.4.3.4).

6. Directional differentiation into the nucellar epidermis or into the endosperm cavity (section 1.4.3.4 and 1.4.4.1).

7. Selectivity of transfer (unloading) from the nucellar symplast to the endosperm cavity or to the apoplastic space between the nucellar epidermis and aleurone layer (section 1.4.3.4 and 1.4.4.1).

8. Selectivity of transfer (uptake) by the aleurone cells or of solute passage between them (section 1.4.4.1).

9. Selectivity of transfer around the endosperm in the symplasm of the aleurone layer (section 1.4.4.1).

10. Directional differentiation from the symplasm of the aleurone cells into the symplast or the apoplast of the inner endosperm (section 1.4.4.1 and 1.8).

11. Selectivity of transfer (transport) through the inner endosperm (whether in the symplast or apoplast) (section 1.4.4.1 and 1.8).

12. Selectivity of transfer (uptake) from the apoplast of the inner endosperm to the cytoplasm of the endosperm cells (section 1.8).

13. Differences in the kinetics of cytoplasmic functions, i.e. incorporation of amino acids into precursor peptides and transport of precursor peptides to the rough ER (section 1.6), or biochemical modification of sucrose and transport of carbohydrate into the amyloplast (section 1.5).

14. Differences in the kinetics of synthetic processes within the ER (section 1.6) or within the amyloplast (section 1.5).

Identifying the extent to which activity at each of the above sites contributes to the observed differences in starch and protein metabolism is a long term proposition. However, one of the first steps in such a programme must be to identify unequivocally the route(s) taken by each class of solute, and this indeed was one of the main aims of the project reported in this thesis. In addition regional patterns of substrate and insoluble material throughout the endosperm were measured to provide the link between route of substrate movement and the composition of the endosperm.

The approach was (1) to measure the positional gradients within the endosperm on a unit volume basis of starch, protein and their precursors (sections 3.2 and 3.3), and (2) to observe the pattern of movement of radioactive substrate into the endosperm and the effect on this pattern of

blocking transport in one or a number of putative pathways (sections 3.4, 3.5 and 3.6).

The distribution of substances within the endosperm was measured by extracting them from small sections taken serially along a radial axis using a cryotome, then separating them using chemical and chromatographic techniques. A grain suitable for such experiments is a large one with a large, well-rounded and accessible cavity (see section 3.1).

The positions at which transport can be blocked are noted also in Fig. 1-9 and described below.

A. Disrupting transport in the stalk by detaching the grain.

B. Disrupting longitudinal movement in the inner pericarp by cauterizing (ringing) the dorsal region of the grain.

C. Disrupting longitudinal movement within the vascular bundle at the base of the crease by heat or surgical treatment.

D1. Disrupting circumferential movement in the inner pericarp, nucellar epidermis and aleurone layer by surface cauterizing in a longitudinal direction on either side of the crease.

D2. Disrupting circumferential movement in the inner pericarp and the nucellar epidermis while leaving the aleurone layer intact by peeling away a strip of inner pericarp on either side of the crease.

E. Changing the resistance to movement across the cavity by flushing the cavity with liquid or with air. Alternatively, solutes can be introduced into the pathway at this point either by the same 'flush' technique or by removing the tissues of the crease and introducing solutes into the well that is formed when the grain is positioned ventral surface uppermost.

Not all these treatments were used in the course of this study.

The front of labelled solute moving into the grain needs to be as sharp as possible. Two techniques were used. In the first, label was introduced in a way that also preserved as much as possible the *in vivo* state of the plant, i.e. CO_2 was fed to an illuminated ear. In these series of experiments, primarily the movement of ^{14}C-sucrose was monitored. In the second technique, detached spikelets were cultured on a solution containing radiolabelled sucrose and amino acids.

SECTION 2

MATERIALS AND METHODS

2.1 SOURCE OF MATERIALS

2.1.1 Wheat Plants

Six varieties of wheat were used during the course of this study. Their genotype and agronomic habit are described in detail in section 3.1. Seeds of these varieties were obtained from the collection of Dr. Jenner (Plant Physiology Dept., Waite Agricultural Research Institute).

2.1.2 Soil Mix

Previously used experimental soil was aged for 2 years, steam sterilized (45 mins) cooled, and mixed with peatmoss (Detorf*, 5:1 parts volume). The following nutrients were added; blood meal (1 $kg.m^3$), potassium sulphate (400 $g.m^3$), superphosphate (200 $g.m^3$) and ground limestone (400 $g.m^3$). The mix was sieved (1 cm grid size). Soil from a single batch was used for all experiments.

2.1.3 Chemicals and Reagents

All chemicals and reagents were of analytical or laboratory grade and were obtained from local commercial suppliers. Radiochemicals were obtained from Amersham International plc; ^{14}C-sucrose was uniformly labelled and ^{3}H-glutamine was labelled at the 2, 3 and 4 carbon positions.

2.1.4 Water

Water for general laboratory use was either glass distilled or prepared by a Milli-RO* reverse osmosis system, followed by deionizing to resistivity $>$ 10 megohm-cm. Water for HPLC use (rinsing, solvent and sample preparation) was pretreated by Milli-RO* reverse osmosis then treated with Barnstead Nanopure* system (resistivity $>$ 17.5 megohm-cm). Just prior to use, this water was passed through the Millipore Norganic* system and filtered

through a disc filter with pore size 0.45 um. If required the water was degassed by sonication under vacuum for 2 minutes then boiled.

2.2 GROWING PLANTS

2.2.1 Environment

Plants were grown in pots in a controlled environment room. The growth room had a plant platform 2.9 m x 1.0 m with a light source comprising 8 Lu 400/EB high pressure sodium lamps (Lumalux, Sylvania) and 10, 65 watt white fluorescent tubes (Philips TL65/85W, 33RS) providing a photon flux density of about 560 $umol.m^{-2}.s^{-1}$ (400-700 nm) at ear level. Photoperiod was 14 h light and the day/night temperature was $21\pm1^{o}/16\pm1^{o}C$. There was no additional control of humidity.

2.2.2 Plant Culture

Each pot (25 cm diam.) was planted with 15 seeds and 2 weeks later the number of seedlings was reduced to 10, the most uniform and evenly spaced seedlings being retained. Once a week thereafter, each plant was trimmed to a single culm and the position of the pots on the plant platform was rotated.

At 2 weeks after planting, 3 weeks after planting and at 1 week before anthesis the plants received a dose (10 ml per pot) of fertilizer solution (266 $g.l^{-1}$ Aquasol*, Hortico (Aust.) Pty. Ltd.). The plants were watered daily, with sufficient water to ensure that the soil was always kept moist, but care was taken to ensure that no solutes leached from the pot. Plants were sprayed with appropriate pesticides as required.

2.2.3 Using Anthesis as the Reference Date

Anthesis was marked daily and was determined by the appearance of anthers

from at least 2 spikelets on both rows of the ear. This date became the reference date for all subsequent operations.

2.3 MANIPULATIVE TREATMENTS

2.3.1 Trimming the Ear (2 days after anthesis)

Ears were trimmed by removing the lowest 3 spikelets (whether or not appearing fertile) and by cutting through the rachis above spikelet position 9, (spikelets numbered acropetally).

2.3.2 Degraining Spikelets (2 days after anthesis)

First, florets distal to the b floret (labelled acropetally) were removed by cutting through the rachilla leading to the c (and other) grains. A pair of fine 'L' shaped forceps was used then to lift-out carefully the a grain while disturbing as little as possible its palea and lemma which were not removed.

2.3.3 Disrupting Putative Transport Pathways

Putative transport pathways in the grain were physically disrupted immediately prior to feeding radioactive substrate to the grain in experiments designed to elucidate the rate of solute movement (sections 3.4, 3.5 and 3.6). Only the b grain was used in these experiments. A diagram of the wheat floret has been included to assist with the description of the techniques used (Fig. 2-1).

2.3.3.1 Disrupting Transport in the Stalk

The tips of a pair of fine 'L' shaped forceps were placed on either side of the grain stalk and carefully the grain was raised, in a direction along its axis, by about 2 mm. A small piece of silicon vacuum grease (Dow Corning*) was injected into the region where the stalk was broken and the

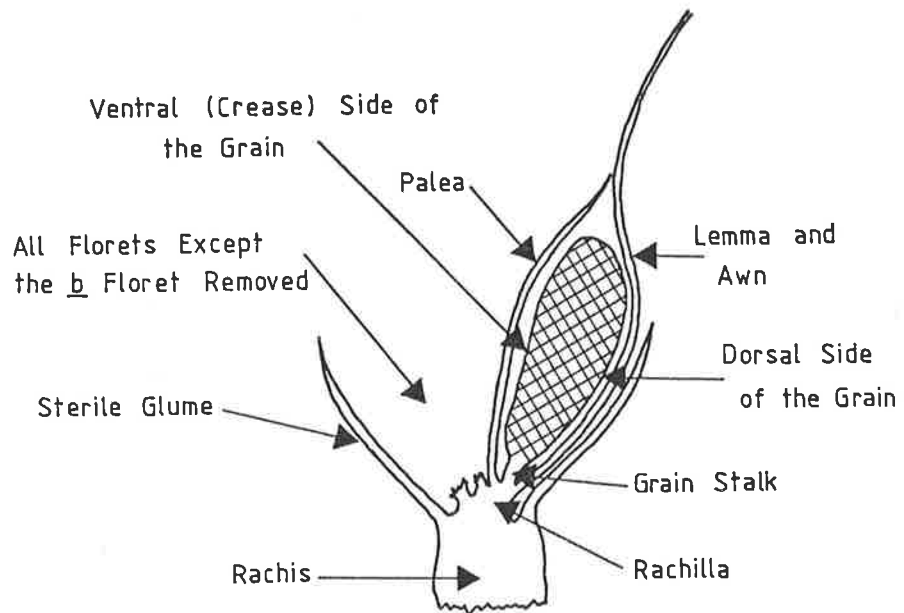


Figure 2-1. Sagittal view of a wheat floret.

grain was reseated. All parts of the floret re-enveloped the grain, the shape of the floret was unchanged by this treatment.

2.3.3.2 Disrupting Transport in the Vascular Bundle

In order to provide access to the grain for this treatment, all florets of the spikelet, apart from the one being used (the b floret) were removed. The palea of the b floret (the bract covering the crease region of the grain) was nipped at the distal end with micro-surgery scissors, and peeled back in two sections tearing it longitudinally in the process. Care was taken not to disturb the position of the grain relative to the rachilla or other parts of the floret. Also, care was taken to assure that the palea halves were not bent back too far, otherwise it was difficult to replace them in their original position. Two techniques were used to disrupt transport in the vascular bundle; both were applied at a spot about one-fifth of the length of the grain from the proximal end.

1. A hole (0.6 mm diam. x 1.5 mm deep) was drilled perpendicularly into the grain through the region of the vascular bundle using a high speed bit. A hot cauterizing tip (Concept* Cauterette* III, 0.6 mm diam.) was plunged briefly (approx. three-quarters of a second) into this hole. Vacuum grease was smeared into and over the hole and over the ventral surface of the grain before the palea-halves were returned to their original position. Microscopic examination of grains treated in this way showed that the hole penetrated through the region of the vascular bundle to the endosperm cavity, but the modified aleurone layer appeared unaffected. Tissue adjacent to the hole was superficially discoloured (burnt).

2. A transverse incision (1.5 mm wide x 1.5 mm deep) was made across the crease and a piece of thin acetate sheet (1.5 mm wide x 2.5 mm deep) was pushed into this cut. Vacuum grease was smeared over the area and the

palea-halves replaced as described above. Microscopic examination of these grains showed the tissues of the grain tightly pressed to the acetate sheet, with the sheet providing a physical barrier to longitudinal movement in the vascular bundle, the nucellar projection; the endosperm cavity and the modified aleurone.

2.3.3.3 Disrupting Lateral Transport in any Circumferential Pathway

The spikelet was reduced to the b floret and the palea of that floret peeled away to expose the ventral surface of the grain as described above (section 2.3.3.2). A hot canterizing tip (Concept* Canterette* III) was used to burn through the surface layers in a strip (0.3 mm wide x 0.5 mm deep) either side and adjacent to the crease, the entire length of the grain. Vacuum grease was smeared over the wounds and the floret reassembled (section 2.3.3.2). Microscopic examination showed that the trough formed by this treatment extended into the endosperm thereby blocking any movement of solutes in any tissue that appears anatomically to have the capacity to provide a circumferential transport route viz. inner pericarp, nucellar epidermis and aleurone layer (see sections 1.4.3.2, 1.4.3.4, 1.4.4.1 and 1.10). There was always a translucent zone adjacent to the trough within the endosperm itself which was due undoubtedly to heat damage. This zone was quite small (0.2-0.4 mm wide) when the cauterizing tip was very hot (bright red, power supply 40 V) and the operation was performed quickly. In all cases this translucent zone was well away from the vascular bundle, the endosperm cavity and the dorsal region of the grain, appearing only in the tissues of the ventral lobes.

2.3.3.4 Control Grains

Grains used as 'controls' to the grains treated as described above (sections 2.3.3.1, 2.3.3.2 and 2.3.3.3) were contained within intact florets, although the spikelets previously had been reduced to the b floret

only.

2.3.3.5 Prepared Grains

Any difference in the performance of treated grains (sections 2.3.3.1, 2.3.3.2 and 2.3.3.3) relative to that of control grains (section 2.3.3.4) could be due to the procedure of exposing the grain to apply treatments rather than to the treatments themselves. Accordingly, grains denoted as 'prepared' were used also as controls. The spikelet was reduced to the b floret and the palea of that floret was peeled away (see section 2.3.3.2). A thin strip (0.5 mm) of outer pericarp was peeled away using fine forceps on either side of the crease, the entire length of the grain so to mimic still further the effect on the grain of disrupting the putative circumferential route. Vacuum grease was smeared onto the ventral surface of the grain and the floret was reassembled (see section 2.3.3.2).

2.4 FEEDING RADIOACTIVE SUBSTRATE TO WHEAT GRAINS

2.4.1 $^{14}CO_2$ to Illuminated Ears

The most important requirement of the procedure for the first part of the study on the pathway of solute movement within the grain (sections 3.4 and 3.5) was that labelled substrate was supplied to the grain without modifying the normal processes of substrate supply, transport and utilization. However, to enable gradients of label within the endosperm to be measured at all, it was essential that the front of label moving into the grain be sharp and the specific activity of substrate be high. Accordingly, $^{14}CO_2$ of high specific activity was fed to an illuminated ear.

From the outset, it seemed that this technique would satisfactorily introduce sufficient label into the experimental system because of the high

rates of carbon exchange observed in wheat ears (e.g. Blum, 1985). However, it was imperative to ensure that while enclosed and illuminated the ear was not subject to unusual environmental conditions that may inadvertently cause abnormal behaviour in plant functions.

The purpose of the study reported here was (1) to measure the carbon exchange rate of a trimmed ear (see section 2.3.1) while enclosed in the illuminated chamber so to determine (a) the minimum time for substantial $^{14}CO_2$ to be fixed and (b) whether over a longer period of time the concentration of CO_2 would rise or fall to values largely different from atmospheric conditions, and (2) to monitor temperature within the chamber during the fixation period.

Experimental: A trimmed ear (see section 2.3.1) was enclosed in a perspex chamber (volume 2.5 l) within a controlled environment cabinet (1.0 m x 1.2 m, 21°C). It was illuminated from above by 4 LU 400/EB high pressure sodium lamps (Lumalux, Silvania) and 4, 80 watt white fluorescent tubes (Philips TL 65/80W, 33RS), and from the side by a ring of 8 lamps (Philips Prismatic* SL* 25). The air within the chamber was stirred constantly. Radioactive carbon dioxide was released from ^{14}C-sodium bicarbonate (1.8 umoles, 100 uCi) by the addition of 20% lactic acid.

The concentration of carbon dioxide in a sample (5 ml) of gas that was withdrawn from the chamber was measured by infra-red gas analysis (Uras 2).

Results: A trimmed ear (mass 2.86 gfwt) in the dark respired at a rate of 26.8 umoles CO_2.(mg.hour)$^{-1}$ (calculated from Fig. 2-2). With the lights turned on there was no net CO_2 exchange (Fig. 2-2), photosynthesis equalling respiration. From this it can be shown that there was a turn-over of CO_2 equivalent to the amount in the chamber every 30 minutes.

The temperature within the chamber rose from 21°C to 28°C during the first 15 minutes of illumination. Thereafter the rise in temperature was slow, increasing only another 4°C during the next 3 hours.

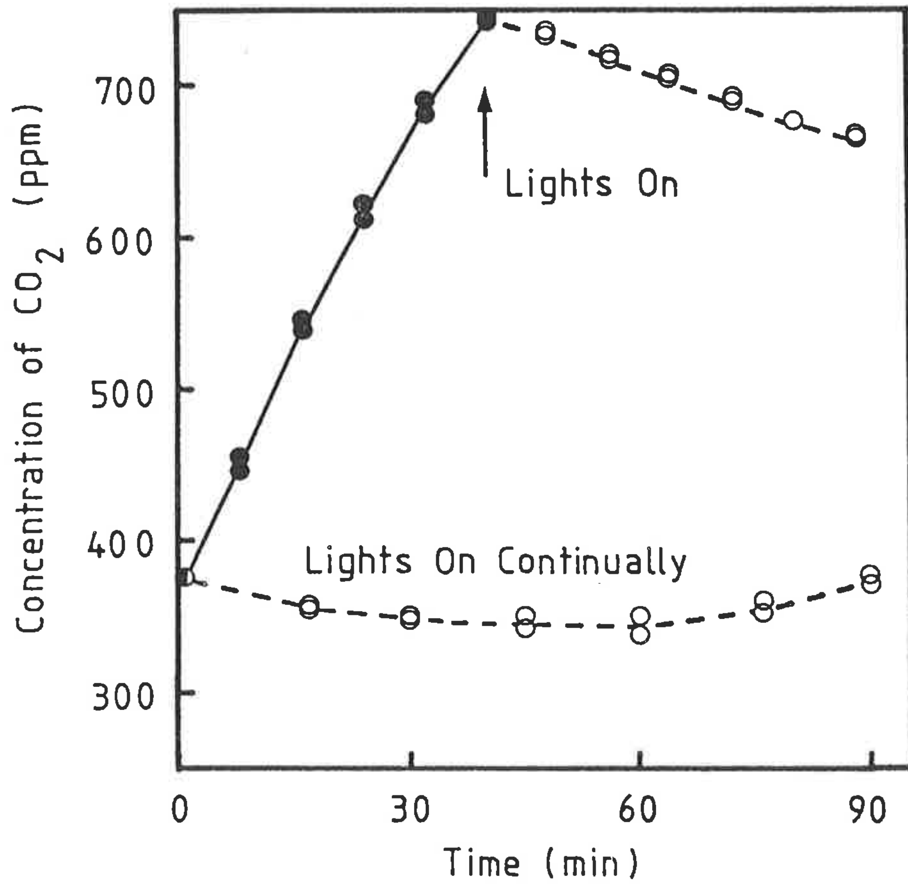


Figure 2-2. Change in the concentration of carbon dioxide within a chamber enclosing a trimmed ear of wheat in the dark (●) or in the light (○).

Conclusions: The chamber and lighting as described are suitable for feeding $^{14}CO_2$ to an illuminated ear. Half an hour seems ample time to introduce substantial amounts of ^{14}C into the ear, although the ear could remain safely enclosed for much longer periods.

2.4.2 ^3H-Glutamine and ^{14}C-Sucrose to Cultured Spikelets

Wheat plants were taken from the controlled environment facility (sections 2.2.1 and 2.2.2) to the laboratory (21°C) on the evening before the day of experimentation. Trimmed ears (section 2.3.1) were used and experimentation commenced always mid-morning.

The culm was cut under water several times; the first cut well below the penultimate node and the last cut 10 cm below the ear. The ear was 'cultured' in distilled water for 30 minutes.

Spikelets for experimentation came from the 4 central spikelet-positions and were selected only when they contained at least the a, b and c grains. Prior to experimentation the c and any distal florets were removed.

A spikelet was removed from the ear by cutting the rachis above and below with fine scissors and trimming the stub of the rachis with a sharp scalpel. All cuts were made under water (or EDTA solution, see section 3.6), but at no time was the spikelet, itself, submerged. Once detached, a spikelet was placed immediately into a small receptacle (the basal one-third of a 2.2 ml Eppendorf* tube) with the stub of the rachis immersed in culture solution (glutamine, 0.5 $g.l^{-1}$N, labelled with ^3H at the 2-, 3-, and 4-carbon positions, and sucrose, 40 $g.l^{-1}$ uniformly labelled with ^{14}C).

2.5 SECTIONING THE GRAIN

2.5.1 Dissecting the Grain into Component Tissues

Grains were dissected into component tissues for distribution analysis of radioactivity (section 3.6) and for the analysis of solutes in one type of tissue only (section 3.2). The transparent outer pericarp was removed and the embryo was lifted away from the endosperm with a pair of fine forceps. The fold of tissue at the base of the crease containing the main vascular bundle sometimes was removed separately by slicing through the inner pericarp on either side of this fold and lifting it out intact with fine forceps. Alternatively, the inner pericarp, including the main vascular bundle, was peeled away from the ventral side in one piece. The endosperm then was held vertically and 1 ml of water was flushed through the endosperm cavity over a 15 second period and collected.

The surface of these peeled endosperms (cv. Fransawi, 20 days p.a.) was usually smooth and shining, the aleurone layer still being intact.

2.5.2 Sectioning the Grain Radially from the Inner Pericarp to the Vascular Bundle

This is a description of the technique used to section the dorsal region of the wheat grain radially from the inner pericarp to the vascular bundle. This technique was used to determine the gradient profile either of radioactivity (sections 3.4, 3.5 and 3.6) or of substrate and insoluble product (section 3.3).

At harvest the outer pericarp was removed. The grains then were immersed immediately in liquid nitrogen and stored there until sectioning.

The frozen grains were placed on the stage of a cryotome with the ventral surface downwards. The stage of the cryotome had been pre-chilled to about -40°C by controlled release of pressurized CO_2 and it was held at this temperature throughout sectioning.

Unlike modern cryotomes, the one used did not have a surrounding

refrigerated chamber. However, the entire sectioning procedure was done in a cold room at $2^{\circ}C$, so that the surrounding air and all the instruments were held at this temperature. The grain and all sectioned pieces remained frozen throughout.

First, the grain was carved leaving a frozen block about 1 mm wide by 2 mm long in the midpoint of the grain that extended from the vascular bundle to the inner pericarp on the dorsal surface (Fig. 2-3). This block then was sectioned transversely.

The thickness of the first slice varied from 150 um to 300 um depending mainly on the shape of the grain on the dorsal surface. The thickness of this slice was the minimum required to remove nearly all of the chlorophyllous tissue. Thereafter, each slice was 150 um thick.

By examining the block carefully during sectioning, it was easy to tell which slice first contained sap (frozen) from the endosperm cavity. The block then was trimmed further to remove any endosperm tissue and a section of ice 300 um thick was taken from the endosperm cavity. Sometimes this section of endosperm cavity contained a very small amount of translucent tissue, presumably the nucellar projection. Usually one more slice (150 um) was taken and then the tissue surrounding and including the vascular bundle was carved out in the shape of a triangular rod (250 um x 250 um x 250 um x 2 mm long).

As each section was taken it was transferred quickly (still frozen) to a small preweighed dish (made from Alcan* Handifoil). The dish itself was within a refrigerated tray ($< -15^{\circ}C$). At the end of sectioning the tray was transferred to a freeze-dryer.

2.6 MEASURING DRY WEIGHT

2.6.1 Dry Weight of Whole Grains

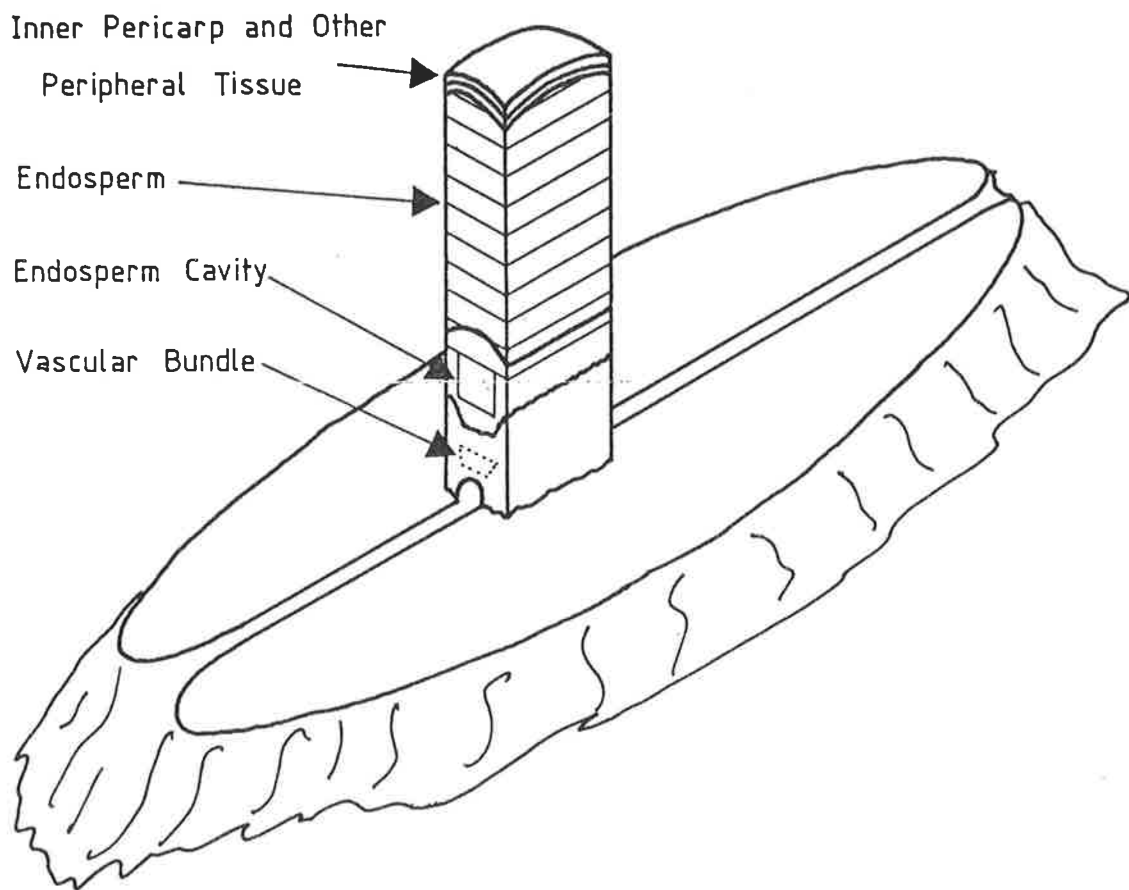


Figure 2-3. A frozen block (1mm wide x 2mm long) carved from the midpoint of the grain and extending from the crease to the inner pericarp on the dorsal surface. The way in which this block was then sectioned is shown also (thin lines).

Whole grains were weighed after oven drying to constant weight (24 hours at 105°C) and cooling in a desiccator over silica gel.

2.6.2 Dry Weight of Small Sections of Tissue

Small sections of the wheat grain after drying (section 2.5.2) rapidly absorb moisture from the air. Under laboratory conditions (controlled environment), they equilibrated to 5.5% moisture in less than 10 minutes. This was determined by continual weighing of representative pieces immediately after removing them from a hot (105°C) oven. For routine procedure, the pieces of tissue were allowed to equilibrate to air dryness (> 30 minutes) before weighing. This weight then was converted to a dry matter value (dry weight = air dry weight x 0.945).

2.7 MEASURING THE VOLUME OF SECTIONS TAKEN FROM THE DORSAL REGION OF THE GRAIN

A block of tissue was carved out of the dorsal region of a frozen grain and the first two slices were taken as described in section 2.5.2. This left a flat upper surface on the block. It was not possible to measure accurately the volume of those first two slices because of the irregular curvature of the dorsal surface.

The dimensions of the trimmed block were taken using needle-point microcallipers both at the flat upper surface and at a reference point further down (viz. level with the uppermost part of the endosperm cavity). The points of the callipers were pressed lightly onto a sheet of paper and the distance between the indentations was measured by calibrated microscopy. The block was sectioned transversely (section 2.5.2), the volume of each slice was calculated later using the measured dimensions of the block, the known thickness of each slice (150 um) and the relative

position of the slice in the block.

2.8 MEASURING THE VOLUME OF THE ENDOSPERM CAVITY AND THE AREA OF THE ENDOSPERM CAVITY - ENDOSPERM INTERFACE

2.8.1 Measuring the Volume by Planimetry

The grain was sliced transversely into 1 mm sections, the proximal (stalk) end of the grain was referred to as 'cut zero' and successive cuts were labelled C_1 to C_n, with C_n at or beyond the distal end of the grain. The cross-sectional area of the endosperm cavity at each cut position (C_1 to C_{n-1}) was determined using a planimetric measurement (Numonics* planimeter, model 1250-2) of a sketch drawn by camera lucida.

The volume of the cavity can be determined from the following equation

$$\text{Volume} = d_{C0,C1}\left(\frac{X_{C0} + X_{C1}}{2}\right) + d_{C1,C2}\left(\frac{X_{C1} + X_{C2}}{2}\right) + \ldots + d_{Cn-1,Cn}\left(\frac{X_{Cn-1} + X_{Cn}}{2}\right)$$

where $d_{Cx,Cy}$ is the distance from cut x to cut y

and X_{Cx} is the cross-sectional area of the endosperm cavity at cut x

Since $d_{Cx,Cy}$ is always 1 (mm) and X_{Co} and X_{Cn} are zero, the above equation simplifies to the following.

$$\text{Volume} = X_{C1} + X_{C2} + \ldots + X_{Cn-1}$$

Two assumptions are made in measuring the volume of the endosperm cavity in this way. These are now discussed.

Assumption 1 is that the interface between the endosperm cavity and the

endosperm within each segment is planar i.e. as a cylinder or as a section of a straight-sided cone. Since the cavity is convex, this assumption probably leads to an underestimate of volume. Any error becomes increasingly small as the number of cuts along the grain increases. One cut every 1 mm seemed sufficient to ensure that any error was small. In any case, the principle purpose of this technique was to allow comparisons of cavity volume between cultivar and/or cultural practice (section 3.1). Any bias resulting from the non-ideal shape of the endosperm cavity is likely to be consistent.

Assumption 2 is that the dimensions of the endosperm cavity do not change during slicing. The external dimensions of selected grains from each cultivar examined (section 3.1) were measured carefully using fine callipers. There was no change in the external dimensions of the grain during slicing and the measuring of cavity volume. Hence any change in cavity volume would have to be accompanied by a corresponding change in the density of other parts of the grain. One mm thick sections of the wheat grain, 20 days after anthesis (section 3.1) appear to have sufficient resilience to make unlikely any error caused by deformation.

2.8.2 Measuring Volume by Expressing and Weighing the Sap

Grains were sliced transversely at a point one-tenth of the grain's length from the distal end. Sap was expressed from the endosperm cavity by holding the grain between the thumb and forefinger and squeezing the grain with a peristaltic action from the proximal end. The sap was collected on a small piece of filter paper and weighed. A bulk density value of 1 (Bhuller, 1984; Ho and Gifford, 1984; or as measured for c.v. Fransawi 1.06 $\pm$ 0.08, see section 2.9) was used to calculate the volume of expressed fluid. Undoubtedly, this method underestimates cavity volume. However, it is a useful technique if differences in volume between grains are large and if a

high degree of accuracy is not required (see section 3.1).

2.8.3 Measuring the Area of the Endosperm Cavity - Endosperm Interface

The area of the endosperm cavity - endosperm interface was measured using the same procedure as, and concurrent with, that used to measure the volume of the endosperm cavity by planimetry (section 2.8.1).

Using the same principles and assumptions as described above (section 2.8.1) it can be shown that

Area of endosperm cavity - endosperm interface = $Y_{C1} + Y_{C2} + ... + Y_{cn-1}$

where Y is the length of the boundary between the cavity and the endosperm at the cut surface.

2.9 COLLECTING ENDOSPERM CAVITY LIQUID FOR COMPONENT ANALYSIS

The most important requirement in collecting cavity sap for component analysis (section 3.2) was that the sap should be free from contamination. Grains were placed on a stage with the dorsal side downwards and sliced transversely with a sharp scalpel at a point one-quarter of the length of the grain from the distal end. The end of a preweighed microcap (5 ul) was held adjacent to and abutting the cavity. As the grain was gently squeezed the exuding fluid flowed directly into the microcap without touching any other part of the grain or a cut surface. Callipers were used to measure the volume in the microcap and the microcap was weighed. The liquid was immediately expelled into hot (75°C) 90% ethanol and the microcap was rinsed. The bulk density of fluid from the endosperm cavity of grains of cv. Fransawi 20 days p.a. anthesis was 1.06 $\pm$ .08 g.ml^{-1} (mean $\pm$ S.E of 20 determinations).

2.10 EXTRACTING SOLUTES FROM TISSUES OF THE GRAIN AND FLORET

In experiments examining the movement of solutes within the wheat grain (sections 3.4, 3.5 and 3.6), simple sugars and amino acids were extracted from small pieces of tissue sectioned along a proposed route (section 2.5.2). So the principal requirement of the extracting solute is that in it, starch and protein are highly insoluble whereas the simple sugars and amino acids are soluble.

There are two solvent systems that appeared suitable for this purpose; methanol, chloroform and water in the ratio 4:2:2 (e.g. Bieleski and Turner, 1966; Laird et al., 1976) and the more frequently used ethanol and water mixture. The most suitable ratio of ethanol to water to extract solutes from tissues of the wheat grain is 9:1 because storage protein of the endosperm is least soluble at this ratio (J.W. Lee, pers. comm.).

From the outset, the ethanol-water mixture was favoured for the following reasons.

1. An ethanolic extract can be placed directly onto an ion-exchange column (section 2.11) obviating the need for the difficult (with small volumes) and time consuming operation of phase separation (A.C. Jennings, pers. comm.).

2. An ethanolic extract can be used directly in an enzymic (Jones et al., 1977) or chemical (Hodge and Hofreiter, 1965) assay of sugars, it can be injected directly onto some HPLC columns (e.g. DextroPak* and SugarPak*, see sections 2.13 and 3.2) and it can be used directly in a fluorometric assay of amino acids (results not shown).

Glutamine is the most abundant amino acid in the wheat plant (section 1.6) and was the form in which radioactive amino-substrate was supplied to cultured spikelets (section 3.6). However, glutamine is one of the least soluble amino acids in an ethanolic extract. The mole fraction of glutamine in a saturated solution, 5.38×10^{-3} in water decreases to 1.84×10^{-6} in

ethanol. Solubility values for glutamine in 90% ethanol could not be found but by comparing glutamine with glycylglycine which has similar solubility values in water and ethanol, it is expected that the mole fraction of glutamine in 90% ethanol will be about 2 x 10^{-5} (Greenstein and Winitz, 1961; Miester, 1957). This value represents a solubility of below 1 mM. Indeed, in an initial test, a precipate was observed when glutamine equivalent to 2 mM was placed in a solution of 90% ethanol.

For procedural reasons the solutes from the small pieces of wheat tissue (section 2.5.2) were extracted in 0.4 ml of hot 90% ethanol in a sealed vessel and after centrifuging, an aliquot of this first extract was used directly for the analysis.

The maximum expected concentration of glutamine in the extraction solution was 0.3 mM, assuming 0.4 mg of endosperm, 0.4 mls of 90% ethanol, 5% of dry matter in endosperm was non-protein nitrogen and that all of this NPN was glutamine. This concentration was sufficiently close to the expected solubility of glutamine to warrant a test on the effectiveness of extraction under the proposed conditions.

Segments sectioned from the dorsal region of a wheat grain (section 2.5.2) previously cultured as a detached spikelet on ^{14}C-sucrose and ^{3}H-glutamine (section 3.6) were extracted 4 times according to the procedure detailed above (and summarized below). Aliquots of the extracts were taken for the following analyses; (1) dpm ^{3}H (section 2.12.1), (2) dpm ^{14}C (section 2.12.1) and (3) amino acids using glutamine as standard by fluorometric assay (based on the method of Klein and Standaert, 1976, further development of this method not shown). In a separate study on the movement of ^{3}H-glutamine and ^{14}C-sucrose within detached spikelets (section 3.6), 85% of ^{3}H in the endosperm chromatographed by ion exchange (section 2.11) into the charged fraction and 85% of ^{14}C chromatographed into the neutral fraction. It is reasonable therefore, to use ^{3}H to determine the

extraction of the sparingly soluble amino-acids and ^{14}C to determine the extraction of the highly soluble neutral compounds. There was a small and, importantly, a uniform carry-over of ^3H, ^{14}C and amino acids from the first extraction to subsequent ones (Table 2-1). This carry-over was less in samples with smaller pieces of tissues showing that the carry-over was due to solution within and around the tissue. There was no evidence for the lesser soluble amino acids remaining insoluble in the first (or any other) extraction. The conclusion from this study, therefore, is that both the simple sugars and the amino acids are soluble in the first extraction.

SUMMARY OF EXTRACTION PROCEDURES

A. Extraction of Solutes from Small Pieces of Tissue.

Tissue (150-250 ug) sectioned from the wheat grain as described in section 2.5.2 was extracted in 0.4 ml of 90% ethanol at 75°C for 1 hour in a sealed vessel (Eppendorf* centrifuge tube). During this time the vessel was shaken frequently. After centrifugation (15,000 xg for 10 min.) an aliquot of this first extract was used for analytical procedures (section 2.11, 2.12 and 2.13). For analysis of solutes by HPLC, the Eppendorf* tubes could not be used due to interfering compounds released from the plastic (see sections 2.12.1 and 3.2). These extractions (section 3.2) were performed in sealed borosilicate glass culture tubes (Kimble 6 mm x 50 mm).

B. Washing the Insoluble Residue of Small Pieces of Tissue.

The extraction procedure (Part A, above) was repeated 3 more times to wash the insoluble residue free of solutes.

C. Extracting Solutes from Larger Samples of Tissue and Washing the Insoluble Residue.

Tissue sampled as described in sections 2.5.1, 2.9, 2.11, 3.2 and 3.6 was extracted essentially as described above (Parts A & B). However the tissue

Table 2-1. Effectiveness of extracting ^{3}H, ^{14}C and amino-acids from segments sectioned from dorsal region of wheat grain. Values in parenthesis are the proportion of ^{3}H, ^{14}C and amino acids of the initial extraction that are contained in the second extraction.

SEGMENT	EXTRACTION	^{3}H (dpm)	^{14}C (dpm)	Amino Acids (n moles)
ENDOSPERM	1st	500	445	10.21
(dwt:0.222mg)	2nd	20 (0.04)	22 (0.05)	0.74 (0.07)
	3rd	a	a	b
	4th	a	a	b
ENDOSPERM +	1st	714	693	8.57
CAVITY LIQUID	2nd	21 (0.03)	22 (0.03)	0.26 (0.03)
(dwt:0.157mg)	3rd	a	a	b
	4th	a	a	b
CAVITY LIQUID	1st	626	2054	7.31
(dwt:0.038mg)	2nd	12 (0.02)	17 (0.01)	b
	3rd	a	a	b
	4th	a	a	b

EXPLANATORY NOTES.

a = within 10 dpm and indistinguishable from background

b = within 0.5 nmoles and indistinguishable from zero.

was blended for 10 seconds (Kinematica* laboratory blender) and 3 extractions were performed (4 ml, 2.5 ml, 2.5 ml). The supernatants from each extract were pooled after centrifugation (3000 xg for 10 min) and made up to 10 mls.

2.11 SEPARATION OF NEUTRAL AND CHARGED FRACTIONS IN AQUEOUS AND ETHANOLIC EXTRACTS

Ion exchange chromatography is a simple procedure, used frequently to fractionate plants extracts. Cationic exchange resins remove cationic and zwitterionic species (including amino acids) allowing neutral and anionic species (including simple sugars and organic acids) to pass through (Barlow et al., 1983; Redgewell, 1980).

The purpose of the study reported here was (1) to devise the chromatographic techniques to separate amino acids from simple sugars in aqueous extracts and (2) to determine separation characteristics when an ethanolic (90%) extract was placed directly onto the column, instead of taking the usual first step of solvent evaporation and redissolving the solutes in water.

The cation-exchange resin used in all experiments was Dowex*-50W, hydrogen form, 8% cross-linked, 100-200 mesh, capacity 1.7 meq. per ml of bed volume. Assuming that a single wheat endosperm contains 1.4 umole of compounds that would bind to this resin (A.C. Jennings pers. comm.), the number of exchangeable sites in 1 ml of resin is in excess of 10^3 times the maximum number of sites required. This volume (1 ml) was used in all separation procedures.

Washed resin was layered into a modified pasteur pipette and preconditioned with successive washes (each 5 x 1 ml) of NaOH (1 N), HCl (1 N) and water (distilled and deionized). Wheat endosperm (cv. Gabo, 20

days p.a.) was blended and extracted in 90% hot ethanol (section 2.10). After evaporating the extract to dryness (55°C and 80 kPa in Buchi* rotavapor-R) it was redissolved in an aqueous solution of ^{3}H-glutamine and ^{14}C-sucrose.

An aliquot of the aqueous extract (0.2 ml; equivalent to 1 endosperm) was placed onto the column and eluted sequentially with water (8 x 0.5 ml) and ammonia solution (12 x 0.5 ml, 1 M w.r.t. NH_3). Essentially all of the ^{14}C was eluted with the water; however, 16% of the ^{3}H was eluted also in this fraction (Table 2-2, Test 1).

This test was repeated with several modifications. These were aimed at increasing the efficiency of the chromatographic separation: (1) the water used to precondition the column and elute the neutral fraction was adjusted with HCl to pH 2.8 - 3.0, (2) the aqueous extract was adjusted also to this pH value and (3) the ammonia solution used to elute the charged fraction was increased to 2 M (w.r.t. NH_3). However, there was no noticeable change in column performance (Table 2-2, Test 2).

Glutamine is unstable and decomposes non-enzymically to pyrrolidonecarboxylic acid and in this form would chromatograph into the neutral fraction (Greenstein and Winitz, 1961; Gortner, 1938). This is taken as the reason for some of the ^{3}H appearing in the neutral fraction. Evidence, showing that the performance of the column was satisfactory follows:

1. The capacity and efficiency of the column was tested by examining the binding of cations (NH_4^+) that were used to elute other charged solutes from the resin. The void volume of these columns was less than 1 ml; total bed volume was only 1 ml and appreciable amounts (20% - 65%) of the ^{14}C added to the column appeared in the first 1 ml of solution collected from the column (Tables 2-2 and 2-3). However, more than 2.5 mls of 1 M ammonia solution or 2.0 mls of 2 M ammonia solution were added to the column before

Table 2-2. Proportional distribution of radioactive label into sequential 0.5 ml eluate fractions during ion-exchange chromatography. Dpm's added; ^{3}H: 26,448 ± 32, ^{14}C: 24,397 ± 147 (Mean ± SE of 3 replicates).

FRACTION NUMBER	TEST 1				TEST 2	
	^{3}H		^{14}C		^{3}H	^{14}C
	Rep I	Rep II	Rep I	Rep II		
Eluting With Water						
1	.000	.000	.000	.000	.000	.000
2	.021	.013	.460	.192	.002	.213
3	.085	.079	.494	.626	.037	.625
4	.034	.051	.051	.136	.063	.121
5	.007	.015	.005	.025	.026	.017
6	.002	.004	.001	.008	.007	.008
7	.001	.002	.000	.002	.002	.002
8	.001	.001	.000	.000	.001	.000
Recovery	.151	.165	1.011	.989	.138	.986
Eluting With NH_4OH						
1	.001	.001	.000	.000	.001	.000
2	.001	.001	.000	.000	.001	.000
3	.001	.001	.000	.000	.002	.000
4	.001	.001	.000	.000	.003	.000
5	.022	.002	.000	.000	.015	.000
6	.403	.041	.003	.000	.475	.006
7	.283	.431	.002	.004	.290	.002
8	.097	.235	.000	.002	.043	.000
9	.037	.097	.001	.001	.013	.000
10	.003	.014	.000	.000	.007	.000
11	.000	.002	.000	.000	.005	.000
12	.000	.001	.000	.000	.004	.000
Recovery	.849	.827	.006	.007	.859	.008
TOTAL RECOVERY	1.000	.992	1.017	.996	.997	.994

Table 2-3. Proportional distribution of radioactive label into sequential 1.0 ml eluate fractions during ion-exchange chromatography. Label applied either in 90% ethanol or in water. All values are mean ± SD of 4 replicates. Dpm's added; ^3H : 24,868 ± 330, ^{14}C : 49,855 ± 780 (x ± SD of 8 replicates).

FRACTION NUMBER	ETHANOL EXTRACT		AQUEOUS EXTRACT	
	^3H	^{14}C	^3H	^{14}C
Eluting With Water				
1	.124 ± .002	.673 ± .028	.075 ± .011	.370 ± .023
2	.151 ± .004	.307 ± .033	.179 ± .008	.579 ± .026
3	.011 ± .002	.010 ± .001	.033 ± .003	.042 ± .004
4	.001 ± .000	.001 ± .000	.003 ± .001	.004 ± .002
5	.000 ± .000	.000 ± .000	.001 ± .001	.001 ± .001
Recovery	.287 ± .005	.990 ± .009	.291 ± .008	.996 ± .022
Eluting With NH_4OH				
1	.002 ± .000	.000 ± .000	.002 ± .001	.000 ± .001
2	.004 ± .000	.000 ± .001	.004 ± .001	.000 ± .001
3	.590 ± .029	.005 ± .001	.599 ± .071	.004 ± .001
4	.113 ± .028	.001 ± .000	.102 ± .064	.001 ± .001
5	.001 ± .000	.000 ± .000	.008 ± .014	.000 ± .001
Recovery	.710 ± .007	.006 ± .002	.715 ± .009	.006 ± .002
TOTAL RECOVERY	.997 ± .012	.996 ± .011	1.006 ± .017	1.002 ± .024

^{3}H appeared in the eluate (Tables 2-2 and 2-3) and before the eluate was basic (not shown).

2. ^{3}H from glutamine was again observed in the neutral fraction when small amounts of charged material (<1% the stated amount) was placed onto the column (Table 2-3, for explanation of table 2-3 see below).

For some studies (section 3.4, 3.5 and 3.6), it was an advantage to fractionate the solutes by placing an aliquot (0.2 ml) of the ethanolic extract directly onto the column. A solution of either 90% ethanol or water which contained ^{3}H-glutamine (40 uM, which is twice the expected amino acid concentration) and ^{14}C-sucrose was placed onto columns and eluted as before. There was no difference in the elution patterns (Table 2-3) demonstrating effective separation with an ethanolic extract.

Note: Different batches of ^{3}H-glutamine were used in the experiments reported here, which accounts for the varying proportion of ^{3}H appearing in the neutral fraction (Table 2-3 cf. Table 2-2).

2.12 MEASURING RADIOACTIVITY

Radioactive solutes were used as tracers in experiments reported in sections 2.10, 2.11, 3.2, 3.4, 3.5 and 3.6. ^{14}C and ^{3}H were assayed by liquid scintillation spectrometry using a Beckman* LS counter, model 7500. Counts per minute were converted to disintegrations per minute by appropriate quench correction programmes calibrated for each isotope and for the counting conditions used.

2.12.1 Ethanolic Extracts

An aliquot (0.2 ml) of an ethanolic extract (80%) was mixed in a plastic vial insert (Medical Plastics* BGC5C) with 1.8 ml of scintillant (ACS* Amersham*: Toluene, 2:1 v/v) and counted immediately, The window settings

used and the coefficients for the quench correction programme are shown in Table 2-4. This protocol provided very good separation between the ^{14}C and the ^{3}H channels (Table 2-5).

2.12.2 Aqueous Extracts

Aqueous solutions were evaporated to dryness (95°C), redissolved in 80% ethanol and radioactivity was measured as described above (section 2.12.1).

2.12.3 Ethanol Insoluble Material or Whole Tissue

Plant material (< 70 mg dwt) was dried (95°C for 24 hours) and autoclaved in 0.1 ml of water for 160 minutes at 121°C, primarily to gelatinize starch. This procedure to gelatinize starch was formulated previously by Dr. T. ap Rees (C.F. Jenner - pers. comm.). The tissue was chemically digested (1.35 mls of NCS* tissue solubilizer, Amersham*, at 50°C for at least 24 hours, shaking occasionally) and mixed with scintillant (15 mls of Ready-Solv N/A*, Beckman*). Normally, glacial acetic acid (40 ul) was added to reduce chemiluminescence; for exception see section 2.12.4. The scintillation cocktail was dark adapted (21°C) for 48 hours (also to reduce chemiluminescence) before the radioactivity was measured. The window settings and the coefficients for the quench correction programme used here are shown in table 2-4.

2.12.4 Photosynthetic Tissues of the Spikelet

When the standard procedure (section 2.12.3) was used to measure radioactivity in the photosynthetic tissues of the spikelet, i.e. the glumes, palea, lemma and rachilla (section 3.6), the final scintillation cocktail had a yellow appearance and substantial quenching occurred which could not be compensated for adequately by the quench-correction procedure (section 2.12.3, Table 2-4). The photosynthetic tissue of the grain itself,

Table 2-4. The window settings and the coefficients for the quench correction programme that were used in measuring radioactivity in ethanolic extracts (section 2.12.1) and ethanol-insoluble tissue or whole material (section 2.12.3). These protocols were devised experimentally by previous workers in Dr. Jenner's laboratory.

A. Ethanolic extracts

Window settings; Channel 1 (^{3}H), Channel 2 (^{14}C)

Lower limit; 0, 397

Upper limit; 317, 665

Coefficients for quench correction (A,B,C,D)

	A	B	C	D
ISO1-CH1:	55.56492,	-0.38922367,	-0.00004750,	0.00000314
ISO1-CH2:	-0.03629837,	0.01630766,	0.00002105,	0.00000004
ISO2-CH1:	4.077356,	0.05327141,	-0.00072997,	0.00000186
ISO2-CH2:	64.86495,	-0.07931360,	0.00111227,	-0.00000458

B. Ethanol-insoluble tissue or whole tissue

Window settings; Channel 1 (^{3}H), Channel 2 (^{14}C)

Lower limit; 0, 397

Upper limit; 317, 655

Coefficients for quench correction (A,B,C,D)

	A	B	C	D
ISO1-CH1:	48.75212,	-0.19234839,	-0.00186492,	0.00000822
ISO1-CH2:	1.177502,	-0.00788847,	0.00019983,	-0.00000037
ISO2-CH1:	6.562529,	-0.02378843,	-0.00012308,	0.00000042
ISO2-CH2:	55.76717,	-0.02056606,	0.00016286,	-0.00000063

Table 2-5. Separation of ^{3}H and ^{14}C channels in ethanolic extracts using the procedure detailed in section 2.12.1 (2 sigma % value of 2.00, 2 replicates shown).

	Channel 1		Channel 2	
	Measured cpm	Calculated dpm	Measured cpm	Calculated dpm
Aliquot of ^{3}H	12155.3	38392.7	513.6	153.9
	12106.6	37394.2	472.9	129.6
Aliquot of ^{14}C	1014.7	-317.7	14043.3	22249.9
	999.9	-350.2	14017.9	22208.2
Aliquot of ^{3}H and ^{14}C together	12744.3	37713.3	14165.8	21760.6
	12674.7	37007.2	14315.2	22023.6

produced negligible discoloration. The aim of the experiments reported here was to devise a procedure to decolorize the NCS-digests of these tissues and so to allow accurate measurement of radioactivity.

Theory: The primary effect of any quenching agent is to decrease the intensity of light emitted from the scintillant, i.e. the energy (pulse-height) of photons is reduced. Consequently, there is a reduced probability that photons generated as a result of a nuclear disintegration will be detected by the photomultiplier tube.

One, and probably the most useful, way to monitor quenching is the 'external standard' technique. This uses a high energy gamma source, such as ^{137}Cs, placed close to the sample vial to generate photons analagously to the internal radioactivity of the sample. The distribution of the resulting pulse-heights is called the 'Compton distribution' and the level of quenching can be expressed as a shift in the 'Compton edge' (Fig. 2-4). The H number is a measure of this shift, based on an arbitary scale of 0 to 1000.

Pigmented solutes absorb energy at their unsaturated sites, so one strategy to overcome colour quenching is to oxidize these sites. However, unsaturated sites are required within the scintillation fluorochromes to convert the energy of a disintegrating nucleus to measurable photons and these too can be oxidized readily. Hence the use of an oxidizing agent within a scintillation cocktail must be precise. In other applications, benzoyl peroxide has been used successfully to alleviate problems of colour quenching (information from Amersham) so this agent was tried again in the study reported here.

Experimental: The palea and lemma from florets of wheat, Triticum aestivum L. cv. Sonora, were digested as described in section 2.12.3 (1.35 mls of NCS* tissue solubilizer per floret). A 10 ul aliquot of aqueous ^{14}C-sucrose and ^{3}H-glutamine (or water) and varying amounts of benzoyl peroxide

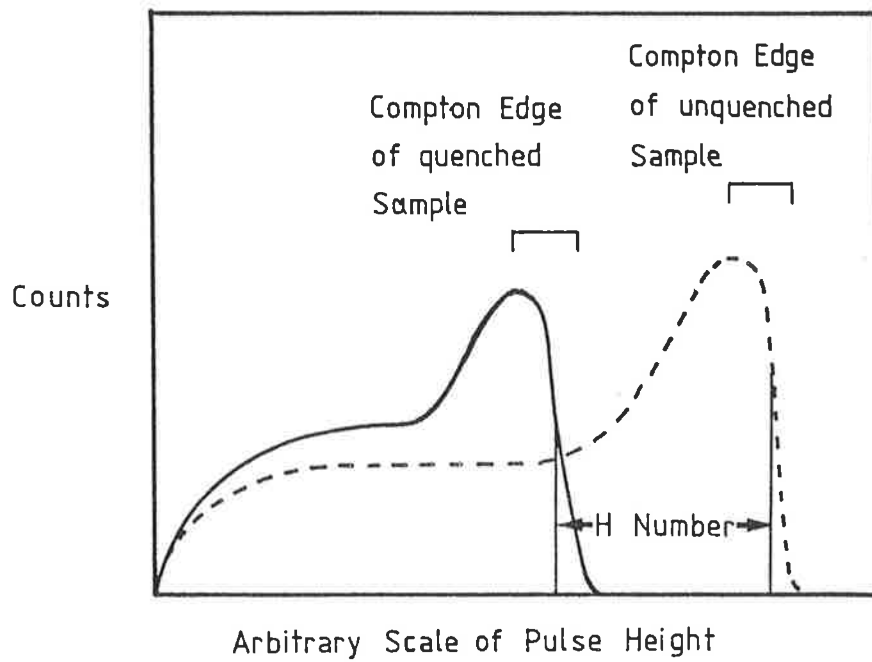


Figure 2-4. Typical distribution of pulse-heights in quenched and unquenched scintillation solutions. The H number is a measure of the shift in the inflexion point of the 'Compton edge'.

solution (see below) were added to aliquots (1.35 mls) of the tissue digest. This mix was allowed to stand for 4 hours before the scintillant was added (section 2.12.3). Glacial acetic acid was not used in conjunction with benzoyl peroxide (instruction from Amersham).

The benzoyl peroxide solution used in this experiment comprised 1 g dissolved at 60°C in 5 ml of toluene. Commercial benzoyl peroxide was supplied with 25% added water, hence two phases developed during the mix with toluene. The upper phase was filtered through phase-separation paper (Whatman 1PS) before use.

The amount of ^{14}C and ^{3}H added to a sample was determined by measuring radioactivity in similar aliquots using both the procedure for aqueous extracts (section 2.12.2) and the procedure for whole tissue (section 2.12.3). Both techniques gave similar results (not shown); the amount of radioactivity added, referred to as 'added dpm' was taken to be 320,500 ± 20,700 for ^{14}C and 429, 300 ± 12,000 for ^{3}H ($\bar{x}$ ± S.E. of 4 reps using procedure for whole tissue, section 2.12.3).

Relative absorbance of the scintillation cocktail (measured on a Unicam* SP1800 duel-beam spectrophotometer) is the absorbance of the solution containing the NCS-digest of photosynthetic tissue (see above) relative to a solution containing a 'blank' digest.

Results:

A. Quenching Effect of the Photosynthetic Tissue

Scintillation cocktails containing the NCS-digest of the palea and lemma absorbed light between 280 nm and 500 nm (Fig. 2-5). Absorbance at 420 nm was directly related to the amount of NCS-digest in the cocktail (Fig. 2-6) and to the H number determined by the external standard technique (Fig. 2-7). The presence of the photosynthetic tissue substantially decreased the calculated counting efficiency (Fig. 2-8). The effect of the NCS-digest of photosynthetic tissue on the measured dpm values was complicated however,

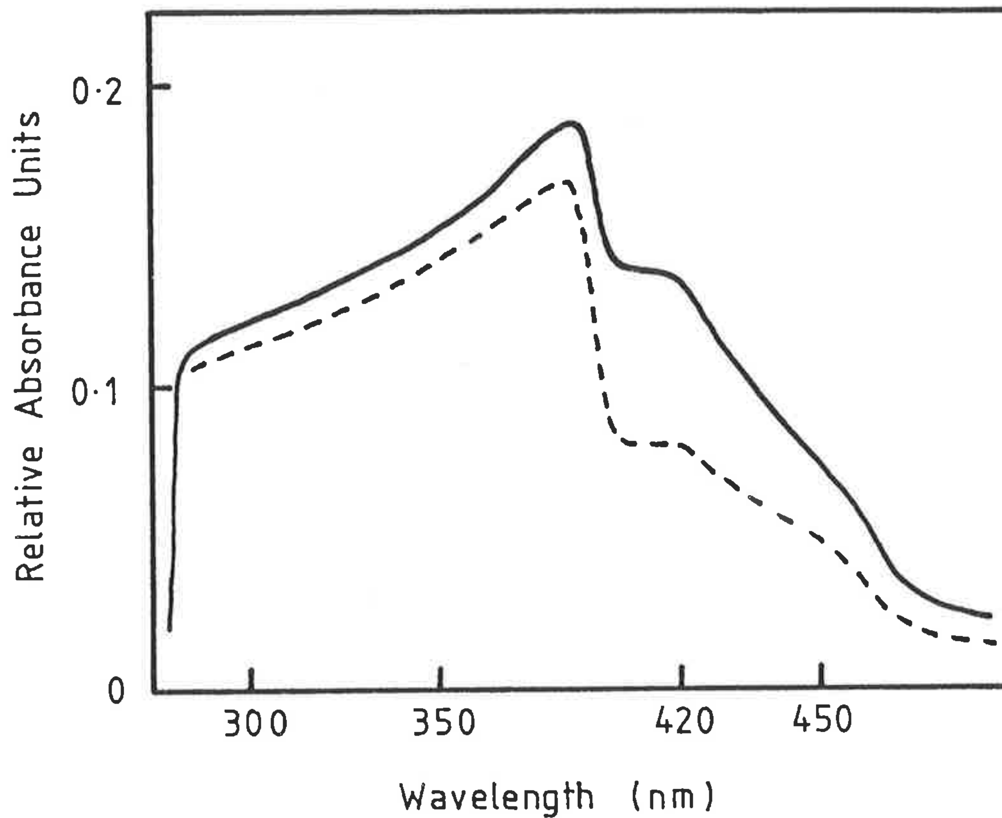


Figure 2-5. Ultraviolet/visible absorption spectrum of scintillation cocktails containing NCS-digests of photosynthetic tissue, relative to a cocktail containing a 'blank' digest. The broken line shows the spectrum when the digest contained 0.6 the amount of photosynthetic tissue as was contained in the digest denoted by the solid line.

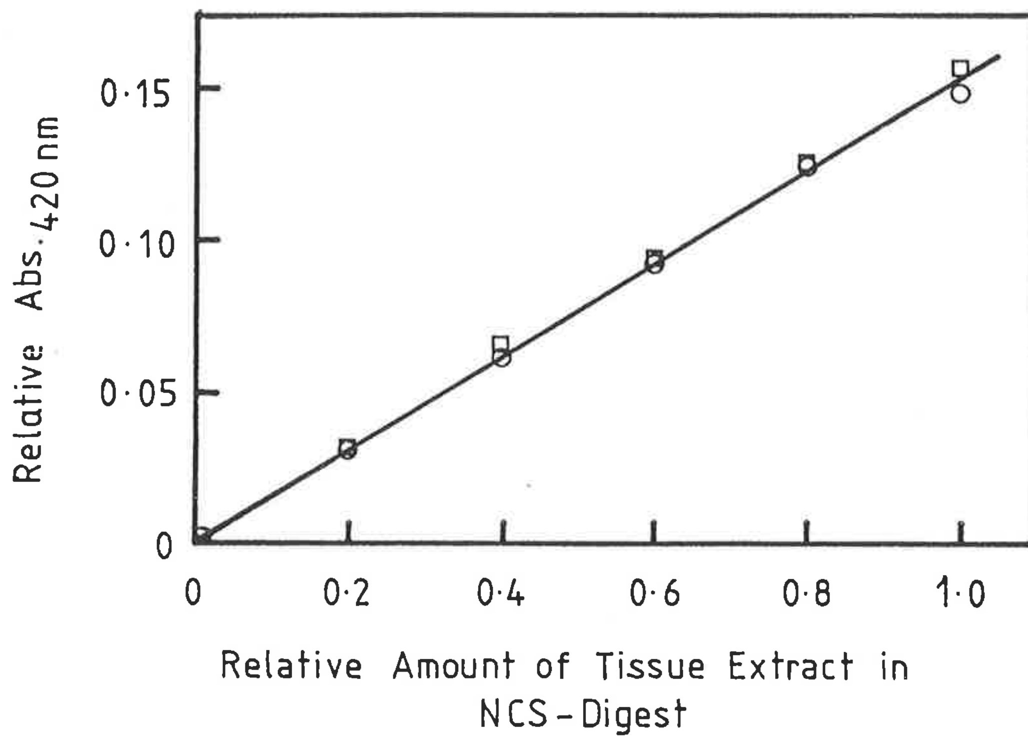


Figure 2-6. Relative absorbance at 420nm of the scintillation cocktail as a function of the relative amount of photosynthetic tissue in the NCS-digest for samples containing added radioactivity (□) and samples with no added radioactivity (O).

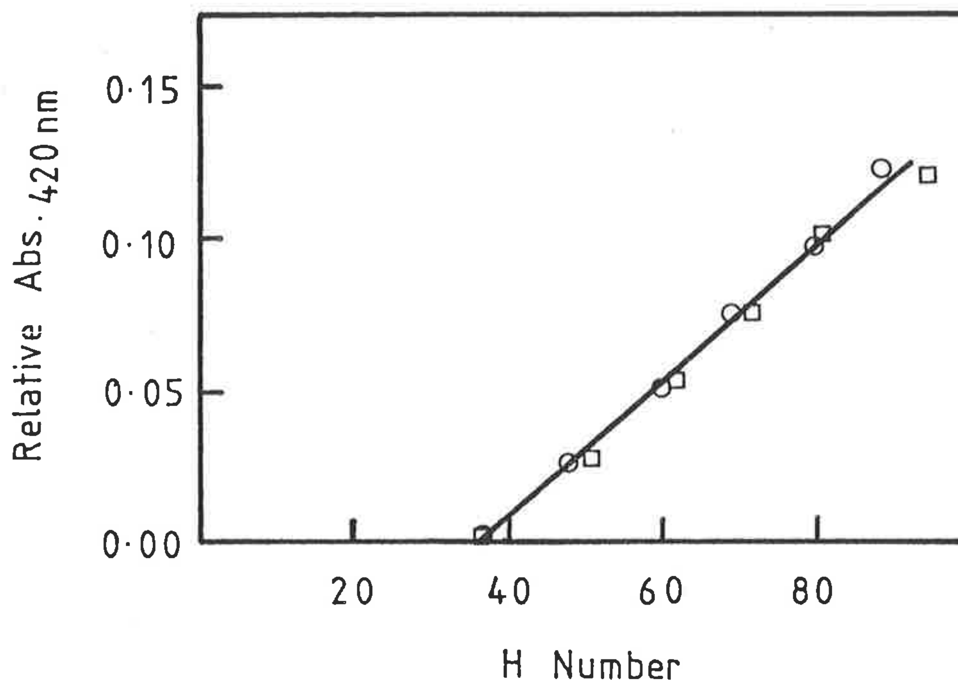


Figure 2-7. Relationship between relative absorbance at 420nm and H number of scintillation cocktails produced from NCS-digests containing varying amounts of photosynthetic tissue; (□), samples containing added radioactivity; (○), samples with no added radioactivity.

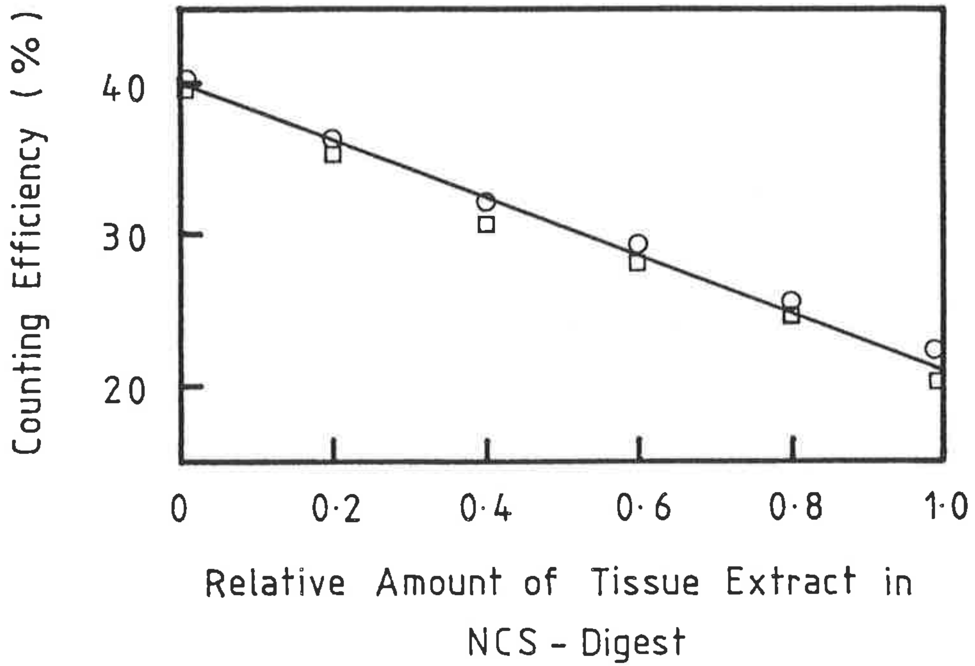


Figure 2-8. Counting efficiency (%) for scintillation cocktails as a function of the relative amount of photosynthetic tissue in the NCS-digest for samples containing added radioactivity (□) and samples with no added radioactivity (o).

and the quench correction programme of the scintillation counter did not compensate correctly. With ^{3}H there was a 15% decrease in measured dpm and a 22-fold increase in background (Fig. 2-9a) while with ^{14}C the measured dpm values were unaffected but the background values substantially decreased (Fig. 2-9b).

B. Effect of Benzoyl Peroxide Added to the NCS-Digests of Photosynthetic Tissue

Additions of benzoyl peroxide to the NCS-digest of photosynthetic tissue substantially affected visual and counting characteristics of the final scintillation cocktail. This oxidizing agent removed pigmentation (Fig. 2-10a) which in turn decreased H number (Fig. 2-10b) and increased counting efficiency (Figs. 2-10c and 2-10d). Excessive additions of benzoyl peroxide reversed the trend, however, presumably due to degradative effects on the fluorochromes of the scintillant.

A volume of 0.8 ml of benzoyl peroxide solution added to the NCS-digest resulted in the measured dpm values being within 10% of the actual values for all conditions tested: ^{3}H and ^{14}H in NCS-digests of photosynthetic tissue (Figs. 2-11a and 2-11b) and in NCS-digests without photosynthetic tissue (Figs. 2-11c and 2-11d). The H number of the scintillation cocktail of the NCS-digest without photosynthetic tissue containing 0.8 ml of benzoyl peroxide solution was 64 $\pm$ 2.

Conclusion and Summary of Procedure:

Benzoyl peroxide effectively decolourized the NCS-digest of photosynthetic tissues, in so doing allowed satisfactory measurement of radioactivity (^{3}H and ^{14}C) using the standard procedure (section 2.12.3). Benzoyl peroxide solution (1 g in 5 mls toluene) was made fresh each day and filtered through phase separation paper before use. A volume of 0.8 ml of solution was used for each 1.35 ml lot of NCS-digest, the mixture stood for 4 hours

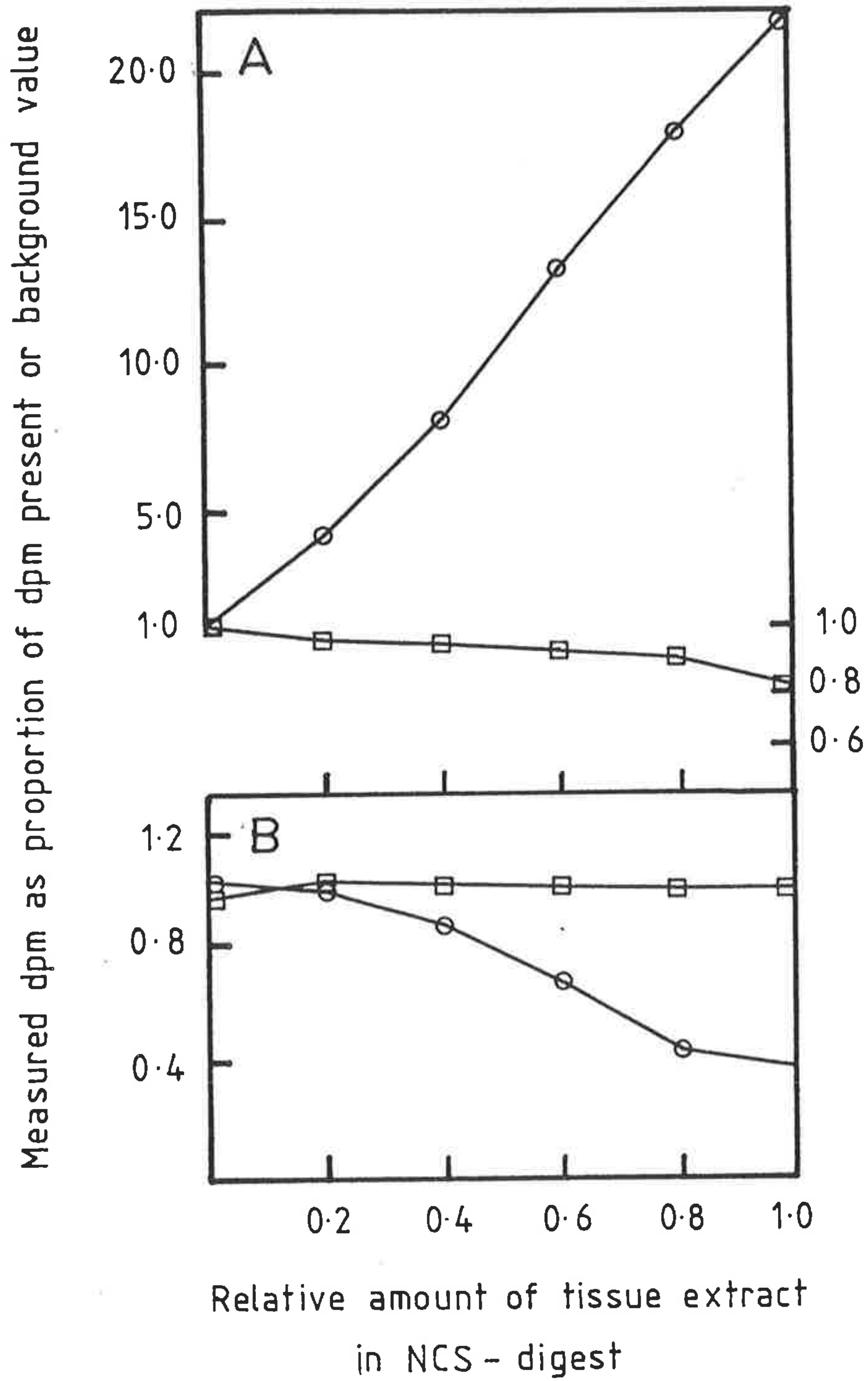


Figure 2-9. Effect of photosynthetic tissue in the NCS-digest on the measured dpm values for (A) ^{3}H and (B) ^{14}C; (□) samples containing added radioactivity; (O) samples with no added radioactivity.

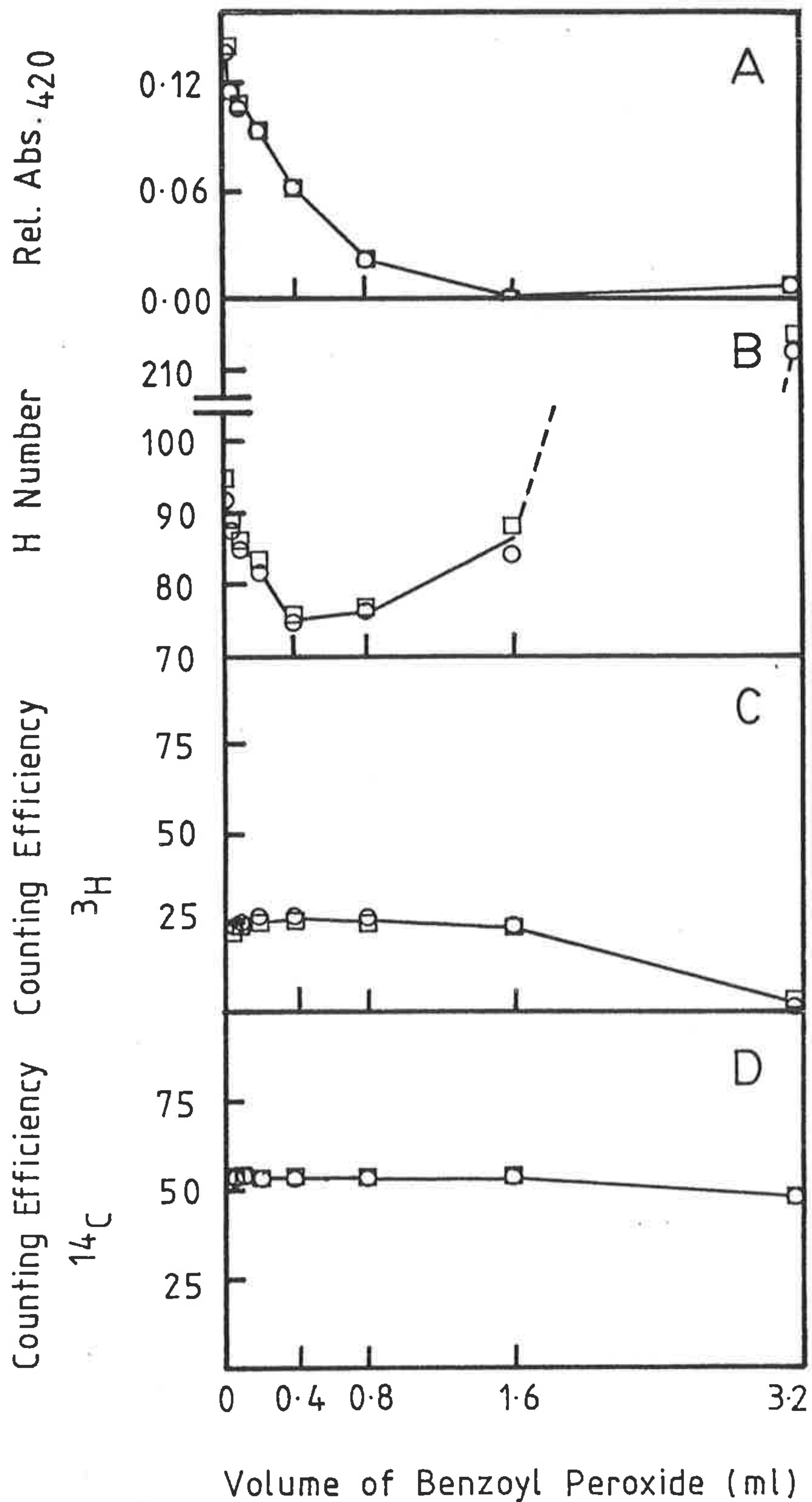


Figure 2-10. Effect of benzoyl peroxide added to the NCS-digest of photosynthetic tissue on (A) relative absorbance at 420nm, (B) H number, (C) counting efficiency of ^{3}H, and (D) counting efficiency of ^{14}C; (□) samples with added radioactivity, and (○) samples with no added radioactivity.

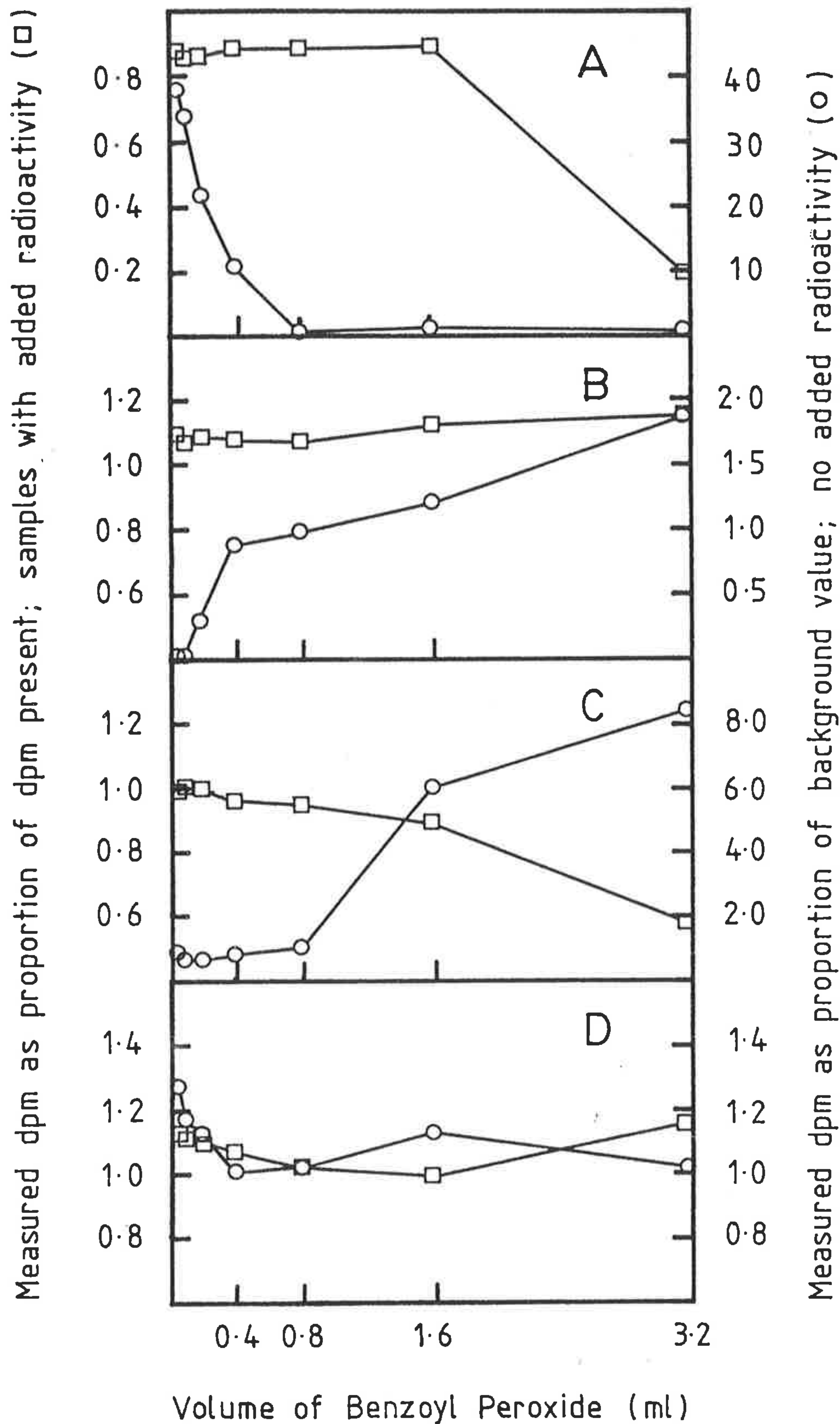


Figure 2-11. Effect of benzoyl peroxide on measurement of radioactivity; (A) ^{3}H in solutions containing NCS-digested photosynthetic tissue, (B) ^{14}C in solutions containing NCS-digested photosynthetic tissue, (C) ^{3}H in solutions containing 'blank' NCS-digest, and (D) ^{14}C in solutions containing 'blank' NCS-digest; for samples containing added radioactivity (□), and samples with no added radioactivity (○).

before the addition of Ready-Solv N/A. Glacial acetic acid was not added to the cocktail.

This technique may not be valid where the quantity of pigment is greater than in the tests reported here. (Ensure that $Rel.Abs._{420}$ of final scintillation cocktail is below 0.06 units.)

2.13 MEASURING PLANT COMPONENTS BY HPLC

High performance liquid chromatography (HPLC) was used to measure the principle soluble carbohydrates, the free amino acids and the component amino acids of protein in wheat endosperm and the fluid of the endosperm cavity (sections 3.2 and 3.3). The techniques needed to be sufficiently sensitive to analyse the solutes in small sections of tissue (150-250 ug, section 2.5.2) taken serially from the grain, in the experiments (section 3.3) to determine the gradient pattern of material along a proposed transport route. This section describes the development of techniques that were sufficiently sensitive for the required task.

The HPLC system comprised pumps (2 Waters* 510, 1 Milton-Roy* 396-31), autoinjector (Waters* WISP 710B), column heater blocks (2, controlled by Waters* Temperature Control Module), variable wavelength spectrophotometer (Waters* model 481), refractive index detector (Varian* RI-3), fraction collector (Pharmacia* Frac-100) and the Waters* 840 Control Station. High sensitivity pulse dampeners were used for some purposes.

2.13.1 Soluble Carbohydrates

Sugars are detectable by differential refractometry or by absorbance in the UV range (around 200 nm). However, both these techniques were of insufficient sensitivity, so a procedure based on derivatization was required.

Post-column derivatization of the sugars was favoured because (1) pre-

column derivatization is time-consuming and multiple derivatives of glucose and galactose are formed (Bjorkqvist, 1981), and (2) separation of un-derivatized sugars allows the option of collecting them for further analysis (see section 3.2).

Sulphuric acid is used frequently in post-column derivatizing systems to form furfural derivatives which in turn react with compounds such as phenols, aromatic amines or heterocyclic hydrocarbons to form coloured products. While these techniques detect a wide range of carbohydrates they have a number of disadvantages; the reagents are corrosive and relatively unstable, insoluble products may form and the sensitivity is quite low (Churms, 1982). Recently a system was proposed that uses a non-corrosive cuprammonium ion, likewise, to detect a wide range of carbohydrates (Grimble et al., 1983). However the reaction system is highly complex which, at present, makes it difficult to use and unreliable (Vratney et al., 1985; B. Walker, pers. comm.).

Post-column techniques that measure reducing sugar only, on the other hand, are simple, use reagents that are less-corrosive or non-corrosive, and are highly sensitive. Of these, the technique based on tetrazolium blue (e.g. Mopper and Degens, 1972) has the drawback that the resulting dye is poorly soluble in aqueous solutions, so a mobile phase containing ethanol or some other organic solvent is required. A technique developed by Mopper and Gindler (1973) using the copper bicinchoninate reagent is also available (e.g. Sinner and Puls, 1978). It is simple, compatible with most buffer systems and the reagents are stable. This technique, which was the one chosen for the current study, is based on the reduction of Cu(II) by reducing sugars and the subsequent formation of a deep lavender complex between Cu(I) and 2,2'-bicinchoninate.

Recently, another technique for analysing sugars has been developed and this is based on the reduction of p-hydroxybenzoic acid hydrazide

(Woollard, 1983) or the structurally related p-amino acid hydrazide (Vratney et al., 1985). While showing promise, this technique has yet to be used routinely, and is, at present, not as sensitive as the copper bicinchoninate system.

The copper bicinchoninate system was devised originally to detect sugars after they were eluted from a column with 89% ethanol (Mopper and Gindler, 1973). It was then developed further to detect sugars eluted with borate buffer (0.5 M, pH 8.8; Mopper, 1978; Sinner and Puls, 1978). Now there are columns that allow the sugars to be eluted with water only (see below in this section and section 3.2). Accordingly, one aim of this section was to investigate modifications of the copper bicinchinonate system so to maximize sensitivity in this new application.

Many oligosaccharides (e.g. sucrose, raffinose, stachyose, fructans) are hydrolysed readily to component reducing sugars when they pass through a strongly acidic cationic exchanger. An experimental column for this purpose was made and supplied by B. Walker of Millipore-Waters. It was placed between the analytical column and the copper bicinchoninate system, and its performance in measuring low levels of non-reducing oligosaccharides is described also in this section.

Experimental:

Sugars were separated on a Waters* SugarPak* column using water only as the mobile phase (0.5 ml/min, 75°C; see section 3.2 for more details). The hydrolytic column (12 cm x 4.6 mm i.d.) was packed with Dowex* 50 W, 16% cross-linked 200-400 mesh, which was regenerated periodically with HNO_3 (1 N, 2 mls). This resin material was chosen because it is extremely rigid which in turn gave long column life, it is cheap and readily available. The copper bicinchoninate reagent was made as detailed by Churms (1981) and absorbance was read at 562 nm. Boiling and outgassing in the hydrolytic column and the reaction coil was prevented by inserting a restrictor and a

cooling coil (8 psi) before the spectrophotometer and a back-pressure valve (50 psi) after it.

Results:

1. Optimizing the Copper Bicinchoninate System for Reducing Sugar Analysis

A. Flow Rate of the Derivatizing Solution

The volume-ratio of copper bicinchoninate reagent to column eluent used previously varies, but generally it is between 0.7:1 and 4.0:1 (e.g. Barr and Nordin, 1980; Sinner and Puls, 1978). However, when sugars were eluted from the column with water it was deemed advantageous to reduce this ratio substantially. Based on the findings reported in Fig. 2-12, the volume-ratio for all further work was 0.3:1.

B. Interaction of Temperature and Reaction Time on the Formation of Colour.

The effect of temperature and reaction time on the colour yield for both glucose and fructose is shown in Fig. 2-13. The maximum colour yield i.e. the optimum balance between the formation of colour and its subsequent decay, occurred at 110°C with a reaction time of 1.8 minutes. These conditions, which were adopted for all analytical studies (below in this section, and sections 3.2 and 3.3), also produced a high signal to noise ratio (Fig. 2-14) and low band spreading (Fig. 2-15).

2. Linearity and Range

Using the system described above, colour yield was linear (<5% deviation) for glucose and sucrose between 10 ng and 5 ug and for fructose between 5 ng and 5 ug. The lowest limit of detection (defined as the amount of substance injected onto the column which produces a peak height twice the noise level, see Hupe et al., 1985) was 1 ng for glucose and sucrose and 0.5 ng for fructose. The standard deviations for repeated injections (n=6) of 10 ng of sucrose, 10 ng of glucose and 5 ng of fructose were less than 9% for each sugar.

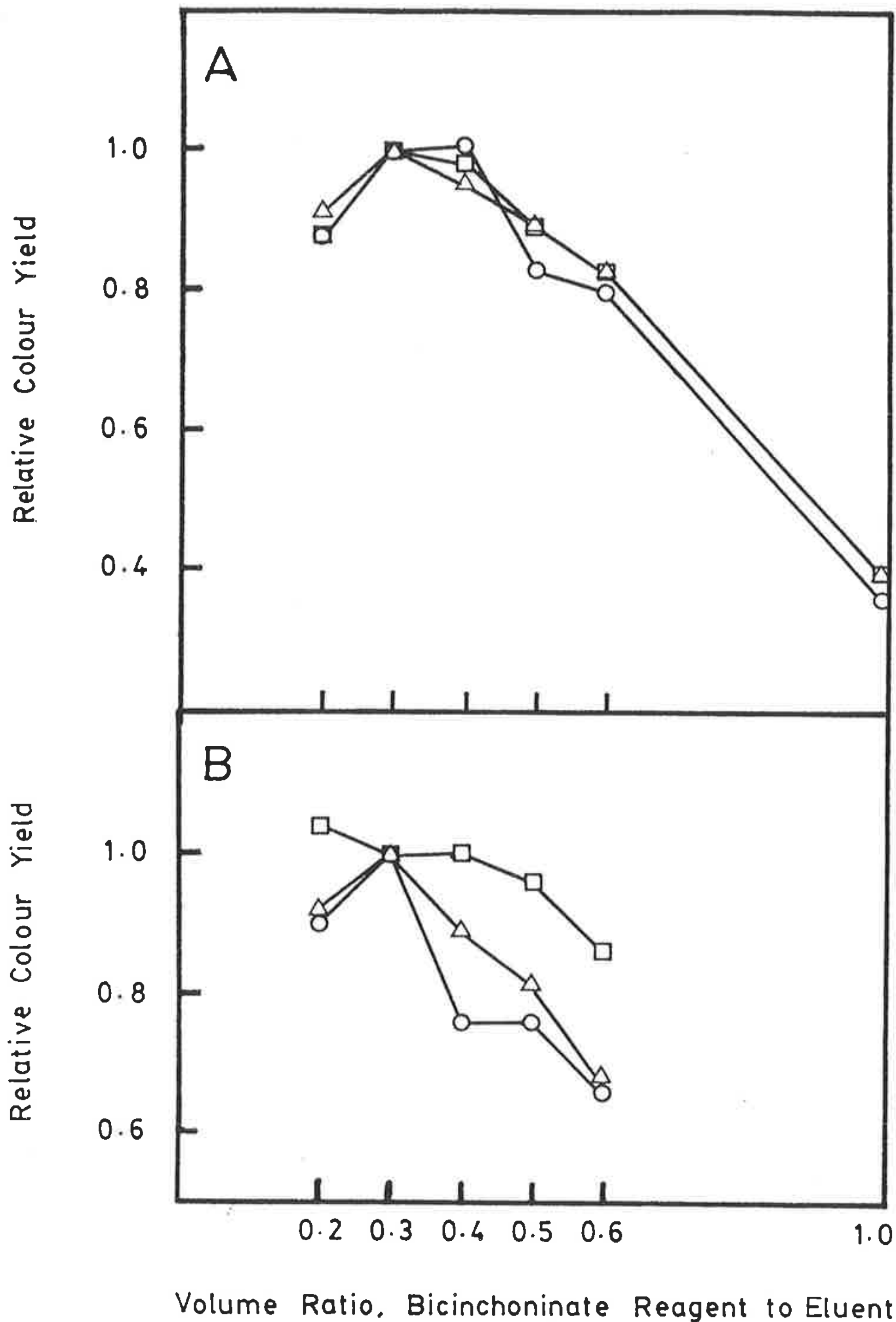


Figure 2-12. Colour yield as a function of the volume ratio of the copper bicinchoninate reagent to eluent, expressed as a proportion of colour yield at 0.3 for sucrose (△), glucose (○), and fructose (□) at 500ng on column (A) and 50ng on column (B). The hydrolytic column was used to analyse sucrose (see below). Flow rate through the analytical column was 0.5 $ml.min^{-1}$ and the post-column reaction coil consisted of a 6.1 m length of 0.5 mm id stainless steel tubing at 90°C.

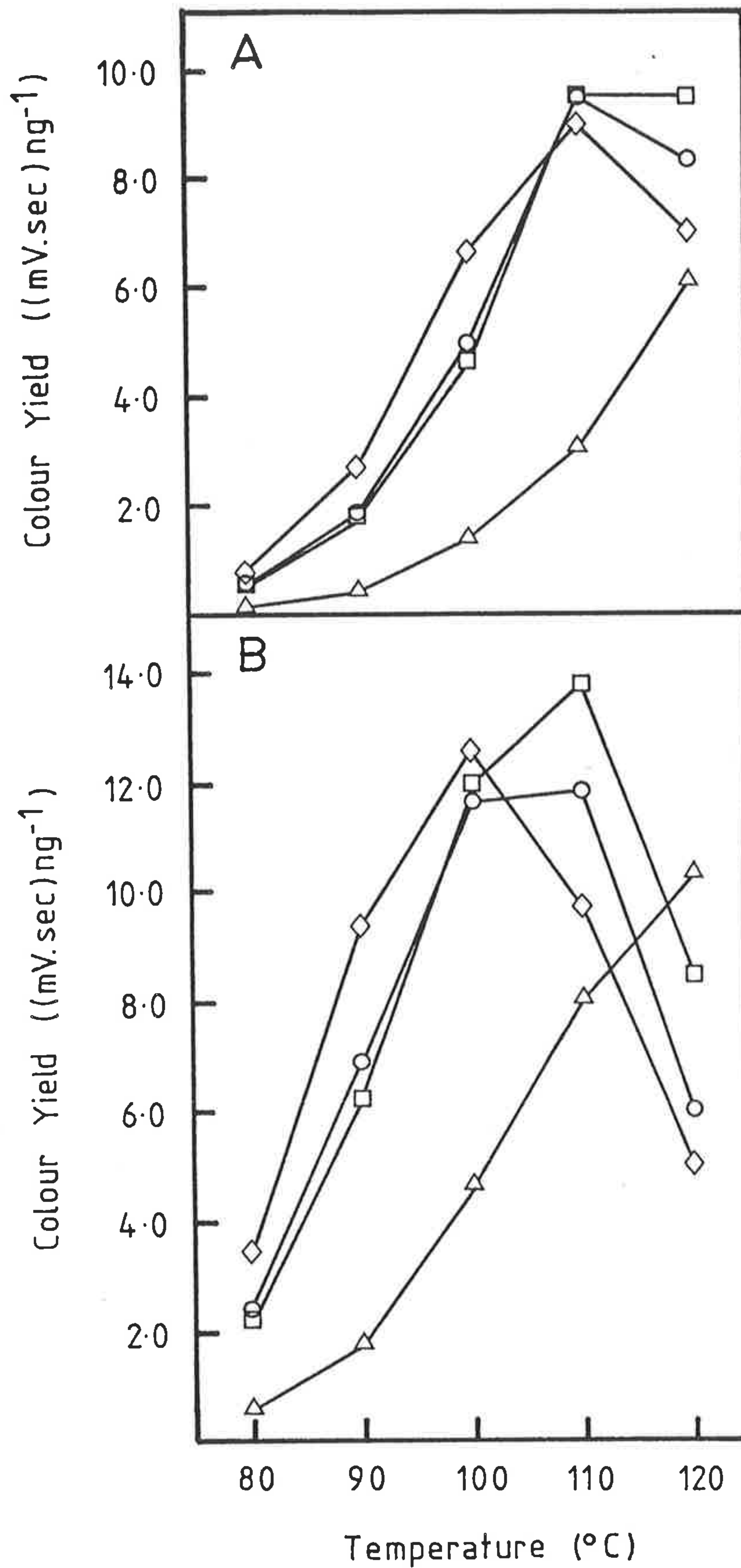


Figure 2-13. Colour yield as a function of temperature and reaction time; (◇), 3.6 min; (○), 2.7 min; (□), 1.8 min; (△), 0.9 min, for glucose (A) and fructose (B).

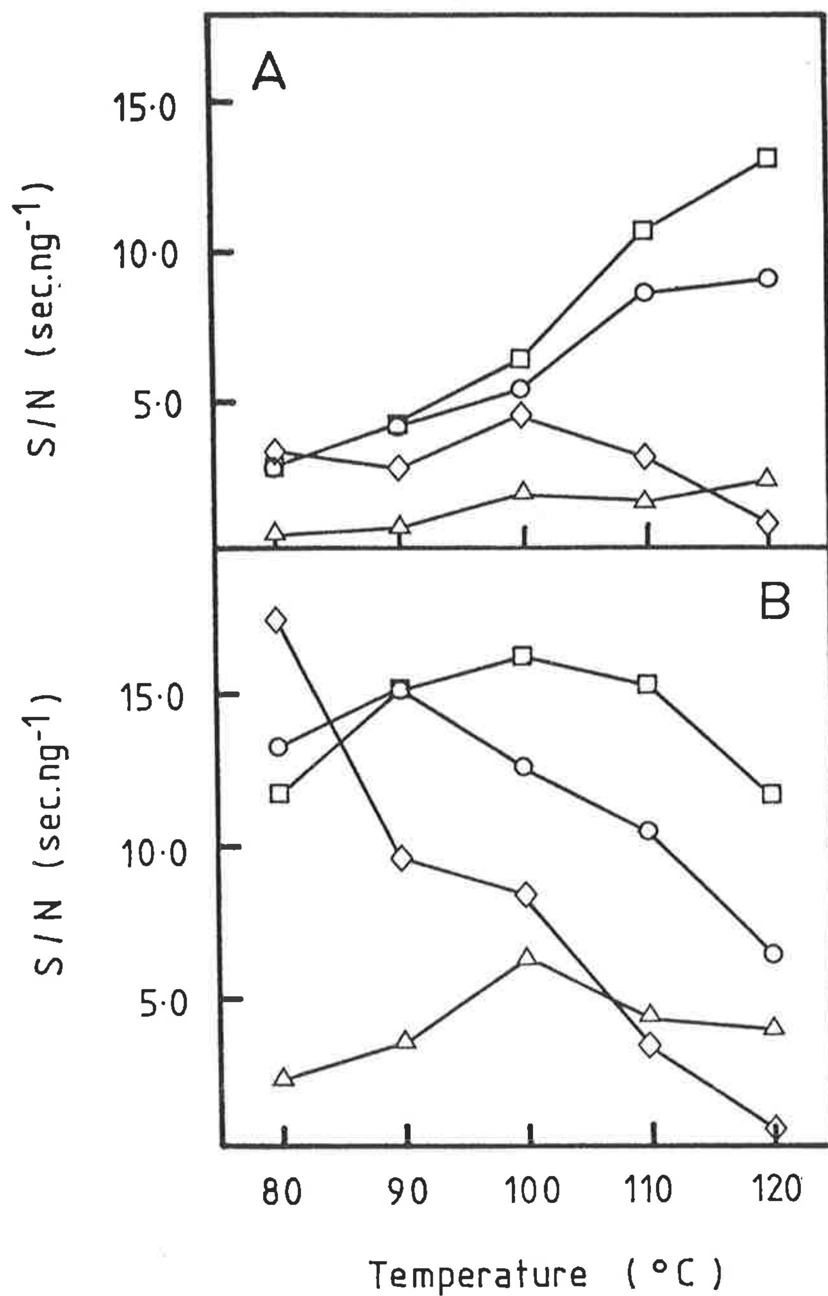


Figure 2-14. Ratio of signal (response factor; (uV.sec)ng^{-1}) to noise level (uV) as a function of temperature and reaction time; (◇), 3.6 min; (○), 2.7 min; (□), 1.8 min; (△), 0.9 min, for glucose (A) and fructose (B).

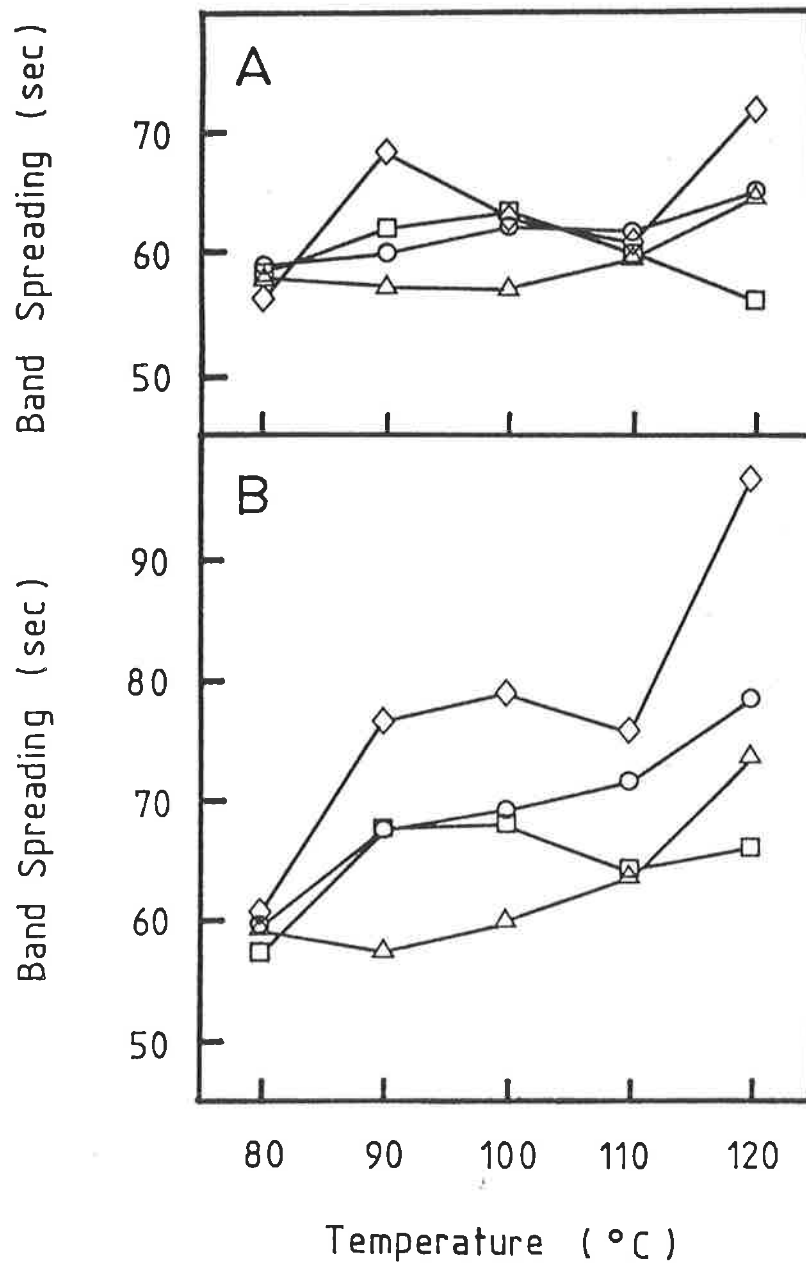


Figure 2-15. Band spreading (area.height^{-1}, i.e. (uV.sec)uV^{-1}) as a function of temperature and reaction time; (◇), 3.6 min; (○), 2.7 min; (□), 1.8 min; (△), 0.9 min, for glucose (A) and fructose (B).

3. Colour Yield of a Number of Sugars

Colour yield of a number of sugars, with and without the hydrolytic column, together with the minimum amount of these sugars (on column) that reasonably can be analysed (s.d. < 10%) is shown in table 2-6.

4. Performance of the Hydrolytic Column

The degree of hydrolysis of various oligosaccharides was measured first by placing the hydrolytic column (see above) before the analytical column and using refractive index for detection (Table 2-7). In this way, the appearance of the products of hydrolysis was measured concurrent with the decrease in the original compound. At 110°C sucrose was hydrolysed totally producing equal amounts of glucose and fructose. Hydrolysis of stachyose produced fructose and a trisaccaride, presumably manninotriose, while hydrolysis of raffinose produced fructose and a disaccharide, presumably melibiose.

In a second test, the hydrolytic column was placed between the analytical column and the post-column reaction system (copper bicinchoninate, see above). Equal amounts of sucrose, glucose and fructose were injected; the degree of sucrose hydrolysis being determined by colour yield at the sucrose retention time relative to the mean colour yield of the other two sugars. The degree of hydrolysis reached 100% at about 110°C (Fig. 2-16), total hydrolysis being achieved on quantities ranging from 20 ng to 5 ug on column (not shown).

SUMMARY

Sugars were separated on either the Waters* SugarPak* column (75°C) or the Waters* DextroPak* column (ambient temperature) using water (section 2.1.4) as the mobile phase (0.5 ml/min). A hydrolytic column placed after the analytical column allowed analysis of reducing sugars as well as non-reducing oligosaccharides. The copper biinchoninate reagent (Churms, 1981)

Table 2-6. Colour yield of a number of sugars relative to the colour yield of glucose (500 ng of each sugar) and the minimum amount of each sugar that upon repeated injections (n = 6) gave a s.d. value less than 10%.

Sugar	Relative Colour Yield		Minimum analyzable amount (ng)
	Without hydrolytic column	With hydrolytic column	
Glucose	1.00	1.00	10
Fructose	1.90	1.90	5
Sucrose	0.00	0.94 [a]	10 [b]
Stachyose	0.00	0.11 [a]	90 [b]
Raffinose	0.00	0.43 [a]	25 [b]
Maltose	0.45	0.47	20
Maltotriose	0.30	Not determined	Not determined

EXPLANATORY NOTES.

a = Irreversible decrease in hydrolytic activity of the column decreased colour yield in this experiment to between 60% and 70% the value obtained earlier in the experimental programme (e.g. Table 2-7, Fig. 2-16). This table should be used primarily to compare the yield of different sugars.

b = With a new hydrolytic column this value is likely to be 30% to 40% lower.

Table 2-7. Hydrolysis of a number of oligosaccharides (20 ug on column) during passage through the hydrolytic column at 110°C.

Oligosaccharide	% Hydrolysis
Sucrose	100
Maltose	not detectable
Raffinose	58
Stachyose	18

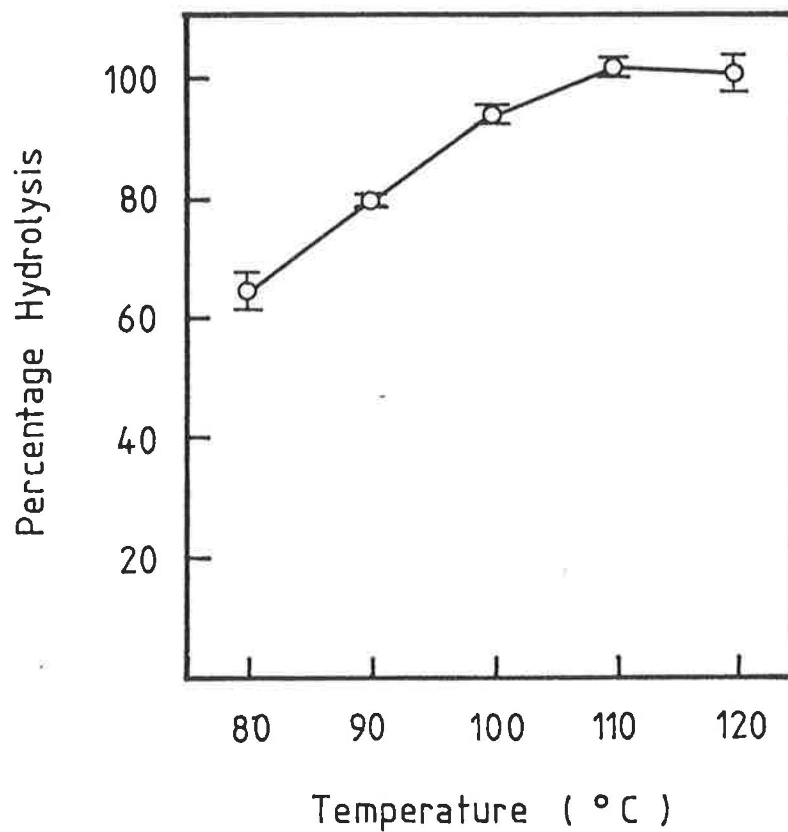


Figure 2-16. Hydrolysis of sucrose (100 ng on column) as a function of temperature; mean ± 1 s.d. of 3 replicates shown.

was delivered at 0.15 ml/min, the reaction proceeding for 1.8 minutes at 110°C. Colour yield was measured at 562 nm.

2.13.2 Free Amino Acids

After extraction (section 2.10) amino acids were derivatized by the Edman reagent, phenylisothiocyanate (PITC), and separated by reverse-phase chromatography, essentially as described in detail by Bidlingmeyer et al. (1984) and in the technical information supplied by Millipore-Waters* (Pico-tag* Work Station, Operator's Manual, Manual number 86746, May 1984; Pico-tag* Amino Acid Analysis System, Operator's Manual, June 1984). The solvents were as described by Bidlingmeyer et al. (1984). Minor modifications to the separation procedure resulted from a study that examined qualitatively the characteristics of separation (chromtograms not shown).

1. The solvent gradient between zero time and 10 minutes was changed to a linear curve (Waters* curve 6) instead a convex curve (Waters* curve 5) to aid separation of arginine, threonine, alanine and proline.

2. Column temperature was 35°C instead of 38° to aid separation of proline and ammonia, and cystine and isoleucine.

2.13.3 Component Amino Acids of Protein

The component amino acids of protein in wheat endosperm were analysed by a smaller-scale version of the procedure described in detail by Frost (198?) and in the technical information supplied by Millipore-Waters* (Pico-tag* Work Station, Operators Manual, Manual number 86746, May 1984). One modification was made: phenol (0.5 mg/ml) was included in the 6 N HCl to remove free chloride which in turn decreased breakdown of some amino acids during hydrolysis in particular methionine and cystine. The separation procedure was as described above (section 2.13.2).

2.14 ISOLATING STARCH

Starch was isolated mainly to determine the radioactivity therein (sections 3.4 and 3.6). It was quantified only in experiments testing the effectiveness of the isolation proceedure.

In the experiments of sections 3.4 and 3.6, starch was isolated by chromatographic purification (ionic exchange, section 2.11) of the soluble product of enzymic digestion (α-amylase and amyloglucosidase, as detailed by Bhuller, 1984). The effectiveness of this technique was assessed by comparing the results after digesting a known amount of purified starch and samples of wheat endosperm and measuring the resulting glucose enzymically (Jones et al., 1977), with analyses using the perchloric acid technique of extraction (Pucher et al., 1948, as adapted for the wheat grain, Jenner, pers. comm.) and analysis of the extracted material by the anthrone technique (e.g. Ough, 1964).

In the analysis of a known amount of purified starch the enzymic and perchloric acid techniques gave recovery values of 89% and 105% respectively. For wheat endosperm starch, the enzymic analysis gave a result 86% that of the perchloric acid technique (results not reported in detail).

2.15 ISOLATING PROTEIN

Protein was isolated to determine radioactivity therein (sections 3.4 and 3.6). After the wheat tissue was blended (Kinematica* laboratory blender for 20 seconds), protein was isolated by trichloroacetic acid (10%) precipitation of the NaOH (0.1N) soluble extract (Cruz et al., 1970, as modified by Singh et al., 1978).

2.16 HYDROLYSING OLIGOSACCHARIDES FOR COMPONENT ANALYSIS

Oligosaccharides from wheat tissue were hydrolysed to enable analysis of component sugars in the study on sugar identification (section 3.2). The most common technique for the hydrolysis step is to use 0.7N HCl at 100°C for 2.5 hours (e.g. Adams, 1964; Hassid, 1964; Pucher et al., 1948). However, in a preliminary test two problems were encountered while using this technique.

1. Acidity had to be removed after hydrolysis to allow the analysis of sugars by HPLC techniques (section 2.13.1). However, during evaporation (Savant* Speed Vac Concentrator*) an azeotropic solution forms (6N HCl) which even over a short time period destroyed almost totally the sugars for analysis. The addition of ammonium hydroxide to the solution to excess (pH 10) before drying allowed analysis of glucose, galactose and fructose with no detectable loss (results not shown in detail).

2. The hydrolysis step, itself, caused a substantial loss of fructose (20-30% recovery) although no loss was detected for glucose and galactose. However, 0.4N HCl for 2.25 hours resulted in 98% hydrolysis of the α-D-(1-4) linkage of maltose and an 84% recovery of fructose (Fig. 2-17). In a further study (results not plotted) these conditions hydrolyse to the same extent (98-99%) the α-D-(1-6) linkage and the α-D-(1-2) linkage of raffinose, contrary to claims (Adams, 1964) that the α-D-(1-6) linkage is more acid resistant. The conditions of hydrolysis described here (see also Fig. 2-17) were used subsequently.

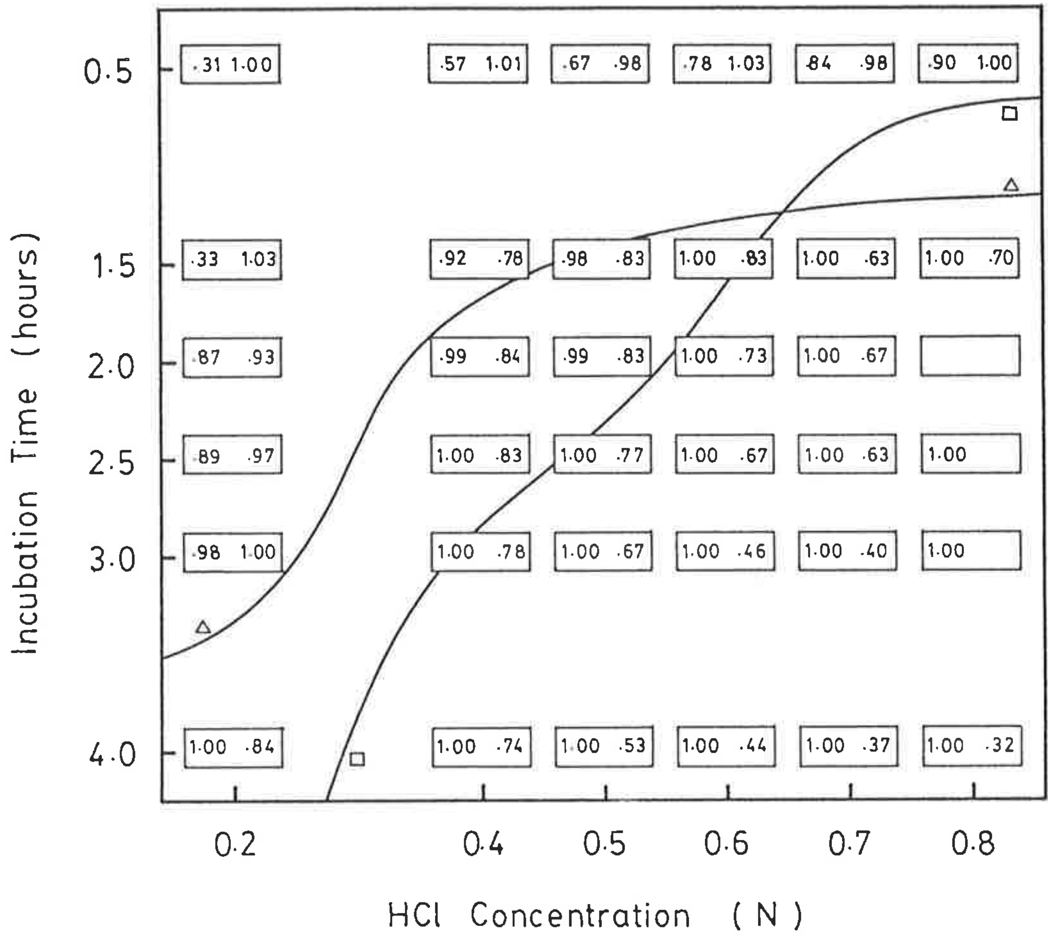


Figure 2-17. The proportion of maltose hydrolysed (left hand value) and the proportion of fructose recovered (right hand value) when 20 ug of each sugar was hydrolysed under a range of conditions. Twenty-five ul of HCl solution was used throughout and hydrolysis was performed in sealed small (6 mm x 50 mm) borosilicate glass culture tubes (Kimble*) at $98^{\circ}C$. The lines drawn estimate 98% hydrolysis of maltose△ and 80% recovery of fructose□.

SECTION 3

RESULTS

3.1 PHYSICAL CHARACTERISTICS OF THE ENDOSPERM CAVITY IN SIX VARIETIES OF WHEAT AND THE EFFECT OF REDUCING THE NUMBER OF GRAINS PER EAR

INTRODUCTION

The relationship between substrate supply and deposition of dry matter in the wheat grain is complex (see Jenner, 1985). Variations in substrate supply in the first 2 weeks after anthesis influence the rate of cell division, and in so doing influence the capacity (potential) of the grain to accommodate dry matter.

In the experiments of Radley (1978) mitotic activity in the endosperm of c grains was increased by removing all a and b grains 7 days after anthesis, a treatment presumed to increase substrate supply to the remaining grains. However, this increase in cell number was accompanied by changes in grain morphology. Radley (1978) described the development of a disproportionately large endosperm cavity. The grains themselves were larger than normal, both in fresh weight and dry weight. As the volume of the grains decreased during ripening, the enlarged endosperm cavity disappeared. It is not known whether the larger endosperm cavity during development was a contributing factor to the higher growth rate or merely a consequence of it.

It seemed that partially degraining spikelets or trimming the ear may be a useful technique to use to create grains that would be suitable for the experiments proposed in the current study programme (section 1.10). The preferred grain was a large one with a large evenly rounded and accessible endosperm cavity. Such techniques have been used previously to study the physiological effects of variation in substrate supply (e.g. Jenner, 1980) or to measure the grain's capacity for growth (e.g. Bremner and Rawson, 1978; Fischer and Laing, 1976; Radley and Thorne, 1981). The purpose of the study reported here (section 3.1) was to examine in detail the morphological

changes that occur in the endosperm cavity in grains enlarged as a result of reducing the number of grains per ear, so to test the suitability of this technique to produce grains that were large enough and well-adapted for the proposed experimentation.

MATERIALS AND METHODS

Wheat Plants

Six cultivars were used in this study

1. _Triticum aestivum_ L. cv. Gabo. Australian Wheat Collection access no (AUS no) 246. The result of a cross Gular/Gaza (a durum wheat) // Gular (Wrigley and Shepherd, 1977). It has short straw, is fairly strong and erect. The ears are compact and large with 17-18 spikelets, each spikelet has 3-4 grains. A few of the top florets are awned. The grains are moderate to large in size. During the late 1950's it was Australia's most widely grown variety.

2. _Triticum aestivum_ L. cv. Nainari 60. AUS no 680, Supremo/Mentana//Gabo/3/Thatcher/Queretaro/Kenya/Mentana/5/Gabo, origin Mexico. Similar form, grain size and ear characteristics to Gabo except that the lemmas have long awns.

3. _Triticum aestivum_ L. cv. Sonora, AUS no 15604, Yaktana 54//Norin 10/Brevor/3/2* Lerma Rojo 54, origin Mexico. A short compact variety producing many quite small grains.

4. _Triticum aestivum_ L. cv. Hornbill, AUS no 332, Florence/Huguenot (a durum wheat) origin Australia. A tall upright variety with 12-13 spikelets per ear, usually 2 large grains per spikelet, awnless. The spike is open, the large distance between spikelets makes it amenable to manipulative

techniques.

5. Triticum turgidum var. durum cv. Fransawi, AUS no 15482. This is a cultivar of tetraploid wheat grown by Arab farmers in the area near Hebrew in the Judean mountains. It is a tall upright variety, tillers quite profusely and produces large grains. There are usually 18-20 awned spikelets per head, but often the lowest 2-3 are infertile. Unless the ear is trimmed or degrained there are usually only 2 grains per spikelet.

6. Triticum polonicum var. martinari, AUS no 3824. The pedigree and origin are unknown. It is a tall upright variety with many tillers per plant. It has long glumes enclosing the spikelet and the lemmas have long awns. There are usually 15-16 fertile spikelets per head and 3 infertile ones at the base. Mostly, there are 3, but often 4, grains per spikelet. The grains are large, long and slender.

Plant Culture

Plants were grown in controlled environment conditions as described in section 2.2.

Manipulative Treatments

Ears were trimmed and spikelets were degrained 2 days after anthesis as described in sections 2.3.1 and 2.3.2.

Measuring the Volume of the Endosperm Cavity

The volume of the cavity was measured two ways; by planimetry (section 2.8.1) and by expressing and weighing the cavity sap (section 2.8.2).

Measuring the Area of the Endosperm Cavity - Endosperm Interface

The area of the endosperm cavity - endosperm interface was measured by planimetry (section 2.8.3).

RESULTS

1. Effect of Trimming and Degraining on the Fresh Weight and Shape of the Grain

All treatments that reduced the number of grains in the wheat ear 2 days after anthesis caused an increase in the fresh weight of the remaining grains (Table 3.1-1). This occurred with both b and c grains and with most cultivars studied. The b grain of cv. Hornbill was the exception; a reduction in the number of grains per ear had very little effect on the fresh weight of the remaining grains.

In intact ears, the b grains at 20 days were usually larger than the c. However, the proportional increase in fresh weight in response to a reduction in grain number was greater in the c grain, so the b and c grains were of similar weight at 20 days in the treated ears.

Trimming the ear increased the size of grains between 15% and 35%. Degraining generally caused a larger increase. A combination of these treatments caused the size to increase still further; albeit by only a small amount.

Enlarged grains had an increase in both length and cross-sectional area (not shown). The increase in length was small, however, and this contributed only marginally to the increase in fresh weight. The increase in cross-sectional area was isodiametric.

2. Volume of the Endosperm Cavity as Related to the Induced Changes in Grain Size.

There was always a positive relationship between the volume of the endosperm cavity and the fresh weight of the grain (at 20 days) irrespective of the method used to produce the enlarged grains (Table 3.1-1). There was good agreement in the volume of the cavity as measured by two independent techniquees (denoted Exp. 1 and Exp. 2 in table 3.1-1). A

Table 3.1-1 Fresh weight of the grain and characteristics of the endosperm cavity at 20 days after anthesis in the b and c grains of a number of cultivars as influenced by trimming the ear and degraining spikelets 18 days earlier. Missing values are due to insufficient number of these grains.

1. THE b GRAIN

Cvs. and T'ments	EXPERIMENT 1. Cavity Volume and Area of Cavity-Endosp'm Interface Measured by Planimetry				EXPERIMENT 2. Cavity Fluid Expressed and Weighed		
	n	Fresh Wt. (mg)	Cavity Volume (ul)	Cavity-Endo. Interface (mm^2)	n	Fresh Wt. (mg)	Volume of Cavity Fluid (ul)
		$\bar{x}$,SE, *	$\bar{x}$,SE , *	$\bar{x}$,SE , *		$\bar{x}$,SE, *	$\bar{x}$,SE , *
Gabo							
Control	4	74,6	1.2,0.4	14.1,1.4	8	72,4	0.6,0.1
Trimmed	4	97,3,1.3	5.8,0.8, 4.7	25.1,1.5,1.8	8	84,4,1.2	3.8,1.0, 6.5
Degra'd	4	95,5,1.3	5.3,1.5, 4.3	23.2,3.5,1.7	8	99,4,1.4	9.5,2.4,16.3
Tri&Deg	4	107,3,1.5	11.3,2.5, 9.2	38.7,6.8,2.8	7	101,3,1.4	9.1,1.4,15.6
Nainari							
Control	4	61,2	1.1,0.2	14.8,0.3	8	62,1	0.3,0.1
Trimmed	4	77,2,1.3	3.8,0.2, 3.5	21.6,0.6,1.5	8	66,3,1.1	1.3,0.2, 3.9
Degra'd	4	95,3,1.6	10.4,1.3, 9.6	37.1,3.8,2.5	8	94,3,1.5	7.7,0.8,23.4
Tri&Deg	4	95,5,1.6	10.6,3.0, 9.8	36.9,7.0,2.5	8	94,5,1.5	11.8,2.2,35.7
Sonora							
Control	4	55,1	0.3,0.1	9.6,1.0	8	52,2	0.2,0.1
Trimmed	4	62,3,1.1	1.1,0.1, 3.6	11.4,0.3,1.2	8	61,2,1.2	0.5,0.1, 2.4
Degra'd	4	78,5,1.4	2.6,0.4, 8.4	15.1,1.9,1.6	8	73,3,1.4	1.5,0.2, 7.3
Tri&Deg	4	78,1,1.4	2.9,0.2, 9.5	16.7,0.7,1.8	8	70,4,1.4	1.6,0.2, 8.0
Hornbill							
Control	4	96,1	4.7,0.1	25.4,1.5	7	85,4	4.0,0.7
Trimmed	4	81,3,0.8	3.4,0.1, 0.7	20.8,1.1,0.8	6	87,2,1.0	5.1,1.0, 1.3
Degra'd	4	94,1,1.0	10.0,0.9, 2.1	40.0,2.9,1.6	7	86,5,1.0	7.2,1.1, 1.8
Tri&Deg	4	92,3,1.0	9.5,0.9, 2.0	36.7,1.1,1.5	5	98,2,1.2	10.4,0.9, 2.6
Fransawi							
Control	4	81,6	2.3,0.2	17.2,0.7	7	83,3	1.0,0.1
Trimmed	4	108,4,1.3	6.1,0.8, 2.7	28.5,3.8,1.7	6	104,3,1.3	2.1,0.2, 2.2
Degra'd	4	103,7,1.3	5.2,0.8, 2.3	24.5,0.5,1.4	8	104,7,1.3	2.8,0.7, 2.9
Tri&Deg	4	104,4,1.3	5.3,0.4, 2.3	24.7,0.5,1.4	8	104,4,1.3	3.9,0.5, 4.0
T.Pol.3824							
Control	4	99,3	4.0,0.4	21.8,1.4	7	102,2	2.2,0.2
Trimmed	4	121,3,1.2	8.8,0.8, 2.2	32.3,1.6,1.5	7	116,6,1.1	7.4,1.4, 3.5
Degra'd	4	130,1,1.3	10.1,0.2, 2.6	34.6,0.5,1.6	8	121,8,1.2	9.3,1.7, 4.3
Tri&Deg	3	140,1,1.4	9.8,0.4, 2.5	34.4,0.6,1.6	5	130,6,1.3	7.9,1.8, 3.7

* = Proportion of Control Values

Continued next page

Table 3.1-1 Continued from previous page.

2. THE c GRAIN

Cvs. and T'ments	EXPERIMENT 1. Cavity Volume and Area of Cavity-Endosp'm Interface Measured by Planimetry				EXPERIMENT 2. Cavity Fluid Expressed and Weighed		
	n	Fresh Wt. (mg)	Cavity Volume (ul)	Cavity-Endo. Interface (mm^2)	n	Fresh Wt. (mg)	Volume of Cavity Fluid (ul)
		$\bar{x}$,SE, *	$\bar{x}$,SE , *	$\bar{x}$,SE , *		$\bar{x}$,SE, *	$\bar{x}$,SE , *
Gabo							
Control	4	54,2	0.7,0.1	10.1,0.6	7	51,3	0.4,0.2
Trimmed	4	77,1,1.4	4.1,0.4, 5.8	21.3,1.5,2.1	5	72,5,1.4	2.9,0.7, 8.1
Degra'd	4	99,7,1.8	5.7,2.1, 8.0	25.1,3.4,2.5	8	103,5,2.0	12.6,2.0,35.5
Tri&Deg	4	101,7,1.9	9.0,2.0,12.7	33.7,6.4,3.3	6	97,5,1.9	9.3,1.7,26.1
Nainari							
Control	4	54,3	1.1,0.2	13.2,0.7	8	51,3	0.3,0.1
Trimmed	4	72,3,1.3	3.2,0.2, 3.0	21.1,1.3,1.6	8	64,3,1.3	1.5,0.4, 5.6
Degra'd	4	96,7,1.8	11.6,2.4,10.9	38.8,4.7,3.0	8	95,4,1.9	7.3,0.8,27.1
Tri&Deg	4	105,2,2.0	14.3,2.8,13.3	43.7,4.8,3.3	7	96,2,1.9	10.2,1.5,37.6
Sonora							
Control	4	40,2,	0.1,0.1	7.1,1.2	8	37,2	0.2,0.1
Trimmed	4	49,2,1.2	0.7,0.1, 4.9	9.6,0.2,1.4	8	48,2,1.3	0.3,0.1, 1.8
Degra'd	4	65,5,1.6	2.2,0.5,16.0	13.8,2.0,1.9	8	67,2,1.8	1.6,0.1, 8.6
Tri&Deg	4	69,3,1.7	2.1,0.2,15.8	14.8,0.6,2.1	7	66,4,1.8	2.0,0.4,10.7
Hornbill							
Control	1	72	4.7	21.9	0		
Trimmed	4	70,3,1.0	3.4,0.1, 0.7	20.9,1.6,1.0	0		
Degra'd	4	97,4,1.3	10.0,0.9, 2.1	46.3,5.7,2.1	8	91,4	8.7,1.9
Tri&Deg	4	104,1,1.5	9.5,0.9, 2.0	44.7,3.2,2.0	7	97,3	12.7,1.0
Fransawi							
Control	0				2	52,1	0.5,0.1
Trimmed	3	87,8	5.2,0.5	23.6,2.5	4	88,5,1.7	2.1,0.2, 4.6
Degra'd	4	91,7	5.7,0.6	23.3,1.5	6	92,5,1.8	2.3,0.4, 5.0
Tri&Deg	4	89,4	5.2,0.4	21.5,0.5	5	91,3,1.8	3.2,0.6, 7.0
T.Pol.3824							
Control	4	86,6	3.2,0.3	18.7,1.3	5	75,6	1.6,0.2
Trimmed	4	99,3,1.2	6.2,0.7, 2.0	26.0,1.3,1.4	3	100,3,1.3	5.5,0.7, 3.5
Degra'd	2	137,3,1.6	16.0,0.6, 5.0	39.3,1.8,2.1	4	130,6,1.7	14.7,1.7, 9.3
Tri&Deg	4	131,3,1.5	15.1,1.2, 4.8	38.8,1.5,4.8	5	128,7,1.7	12.5,2.8, 7.9

* = Proportion of Control Values

positive relationship between cavity volume and fresh weight of the grain was also the general trend between individual grains of any one treatment (Fig. 3.1-1). These relationships between cavity volume and grain size were similar for both the b and the c grains.

The nature of the positive relationship varied. It appeared linear in some cultivars (Sonora, Fransawi, T. Pol. 3824) while in others (Gabo, Nainari) the larger grains had a disproportionately large endosperm cavity. Figure 3.1-1 shows examples of this difference.

In cv. Hornbill there seemed to be an upper limit to the fresh weight at 20 days independent of treatment. The volume of the endosperm cavity varied substantially in grains of similar weight. Grains from degrained florets had a larger endosperm cavity than did grains from untreated or trimmed ears (Fig. 3.1-1).

3. Nature of the Endosperm Cavity - Endosperm Interface and the Shape of the Endosperm Cavity as Related to Grain Size.

The modified aleurone, reported usually as an intact interface between the endosperm cavity and the endosperm (e.g. Sakri and Shannon, 1975), was observed frequently to be ruptured. The rupturing appeared as one or a number of tears which extended between the cells of the endosperm (Fig. 3.1-2). In a few cases the rupturing was massive (Fig. 3.1-3).

The rupturing of the modified aleurone layer was rare in grains grown in intact ears. In some cultivars (Gabo, Nainari, T. Pol. 3824) it occurred in most of the enlarged grains while in others (Hornbill, Sonora) it occurred predominantly only when the spikelets were degrained, either as the sole treatment or in combination with trimming. In cv. Fransawi it was rare.

The area of the endosperm cavity - endosperm interface is related positively to grain size (Table 3.1-1) but in the absence of ruptures the

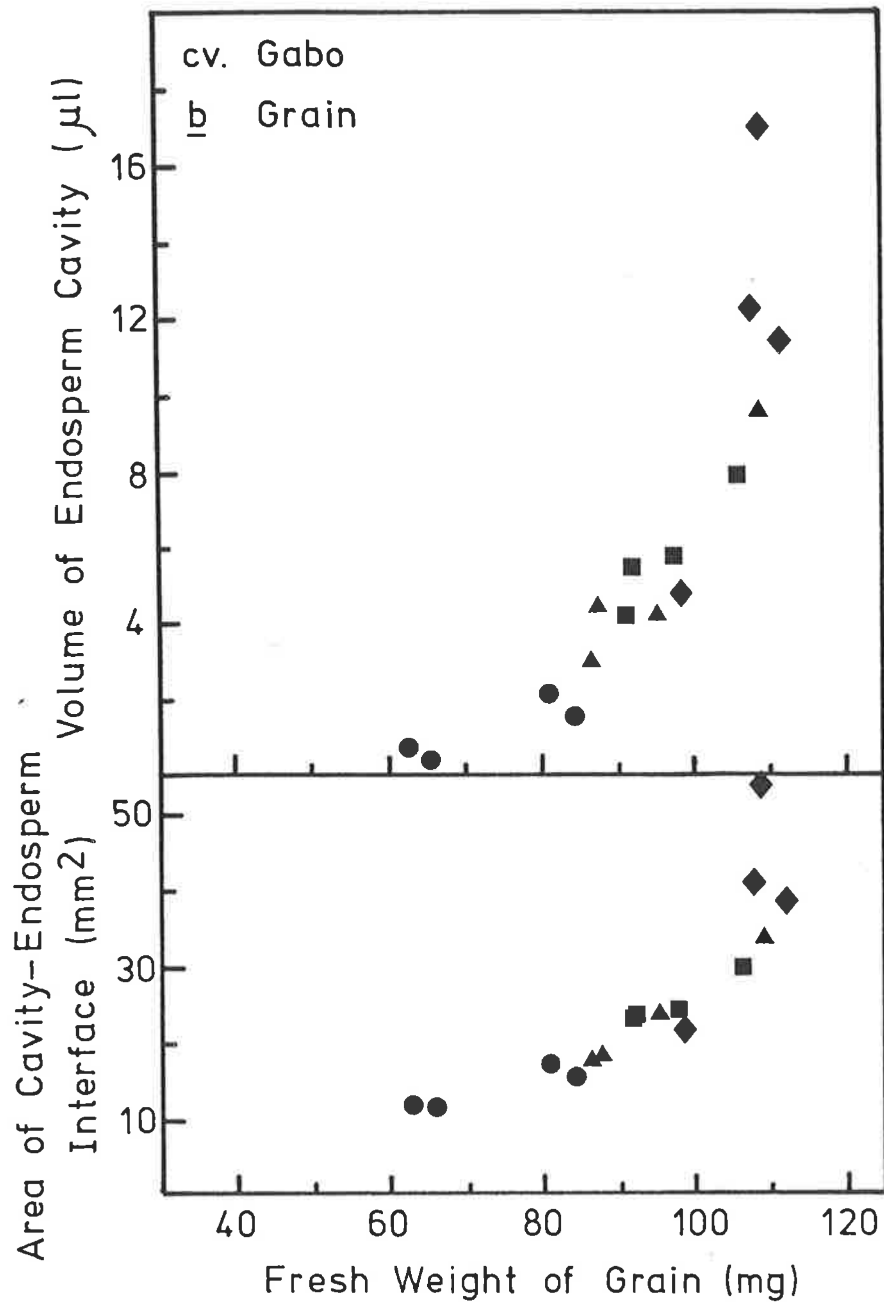


Figure 3.1-1A. Volume of the endosperm cavity and area of the cavity-endosperm interface at 20 days after anthesis for b grains of the cultivar Gabo as related to the fresh weight of the grain and to the technique used to reduce the number of grains 18 days earlier; grains in intact ears (control, ●), trimmed ears (■), degrained spikelets (▲), and trimmed ears together with degrained spikelets (◆).

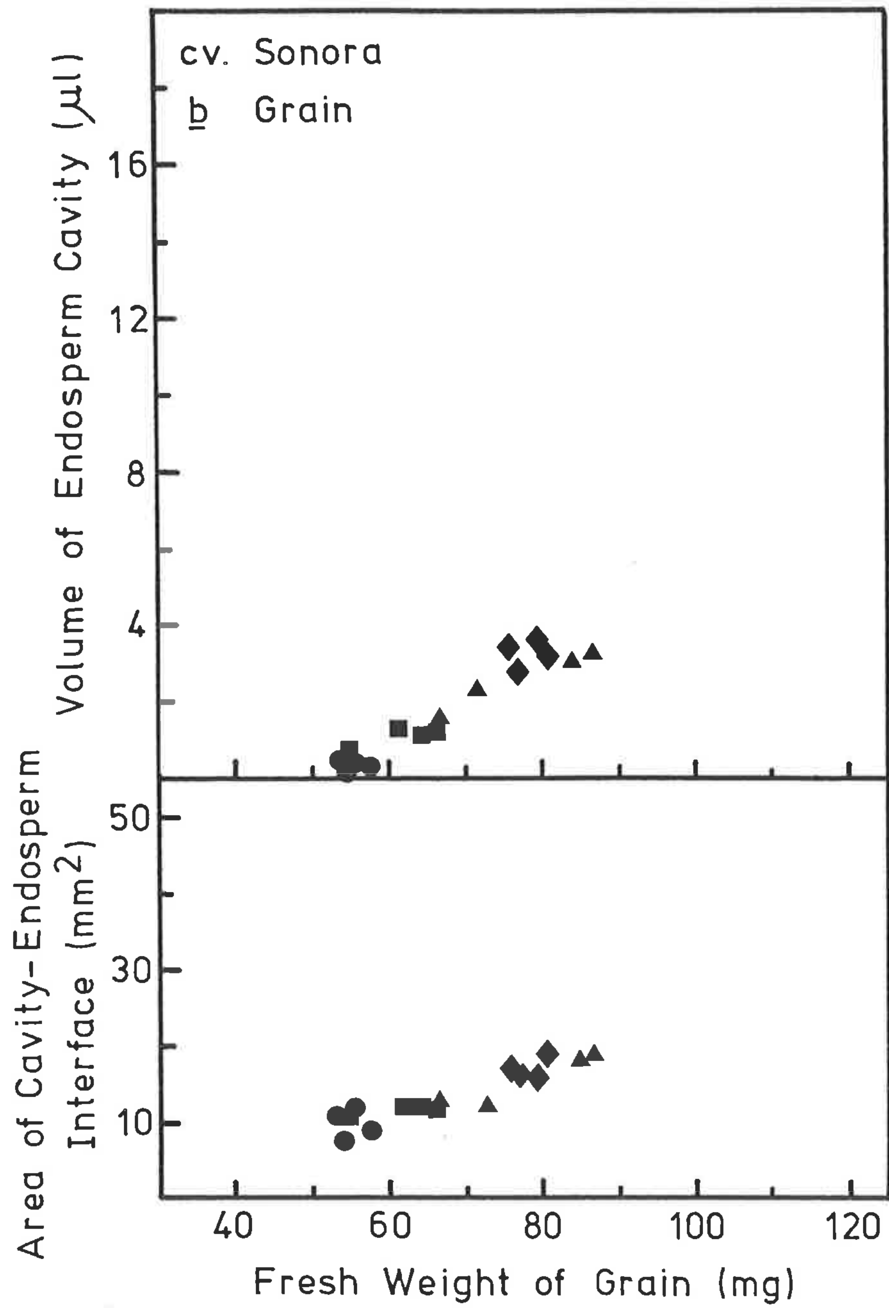


Figure 3.1-1B. As Fig. 3.1-1A, b grains of the cultivar Sonora.

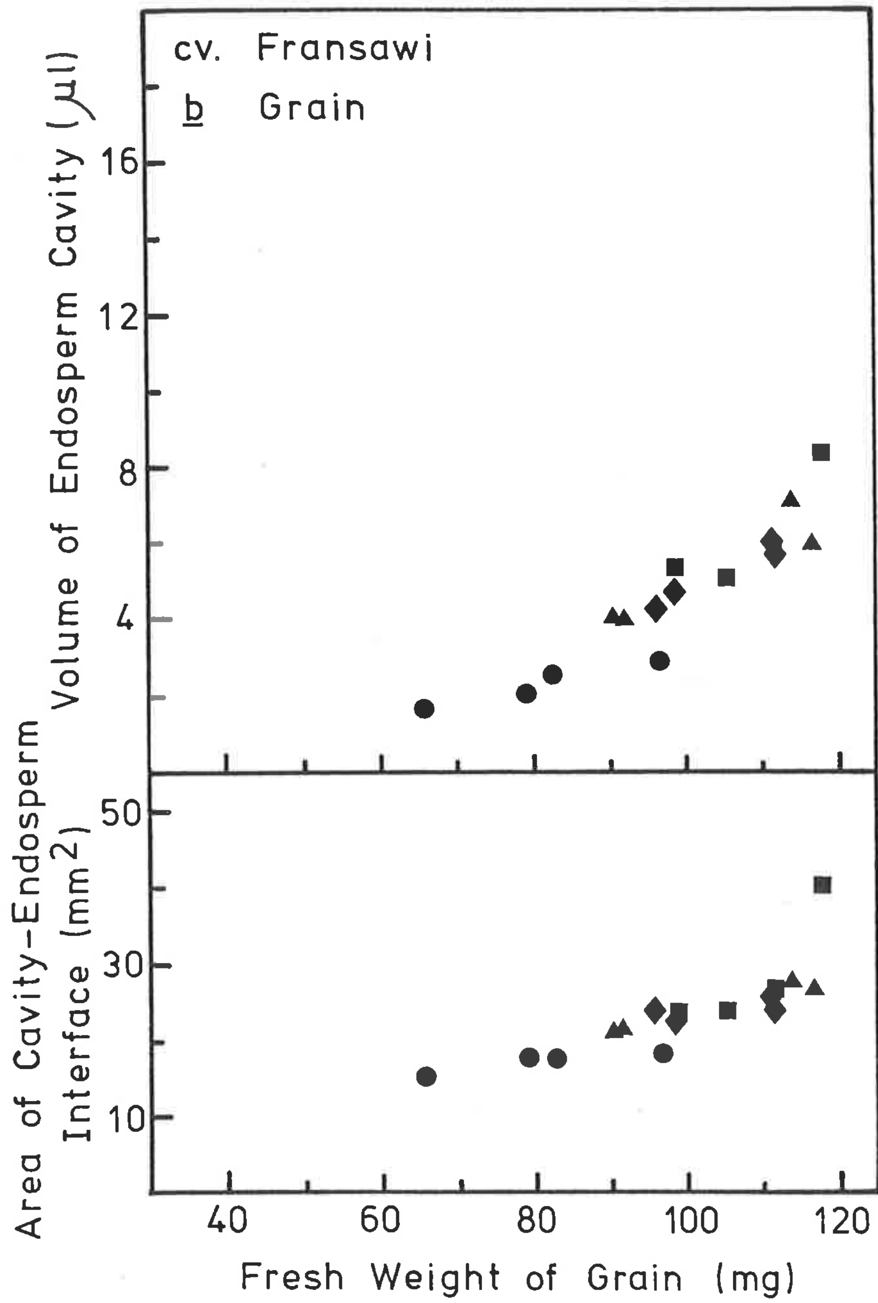


Figure 3.1-1C. As Fig. 3.1-1A, b grains of the cultivar Fransawi.

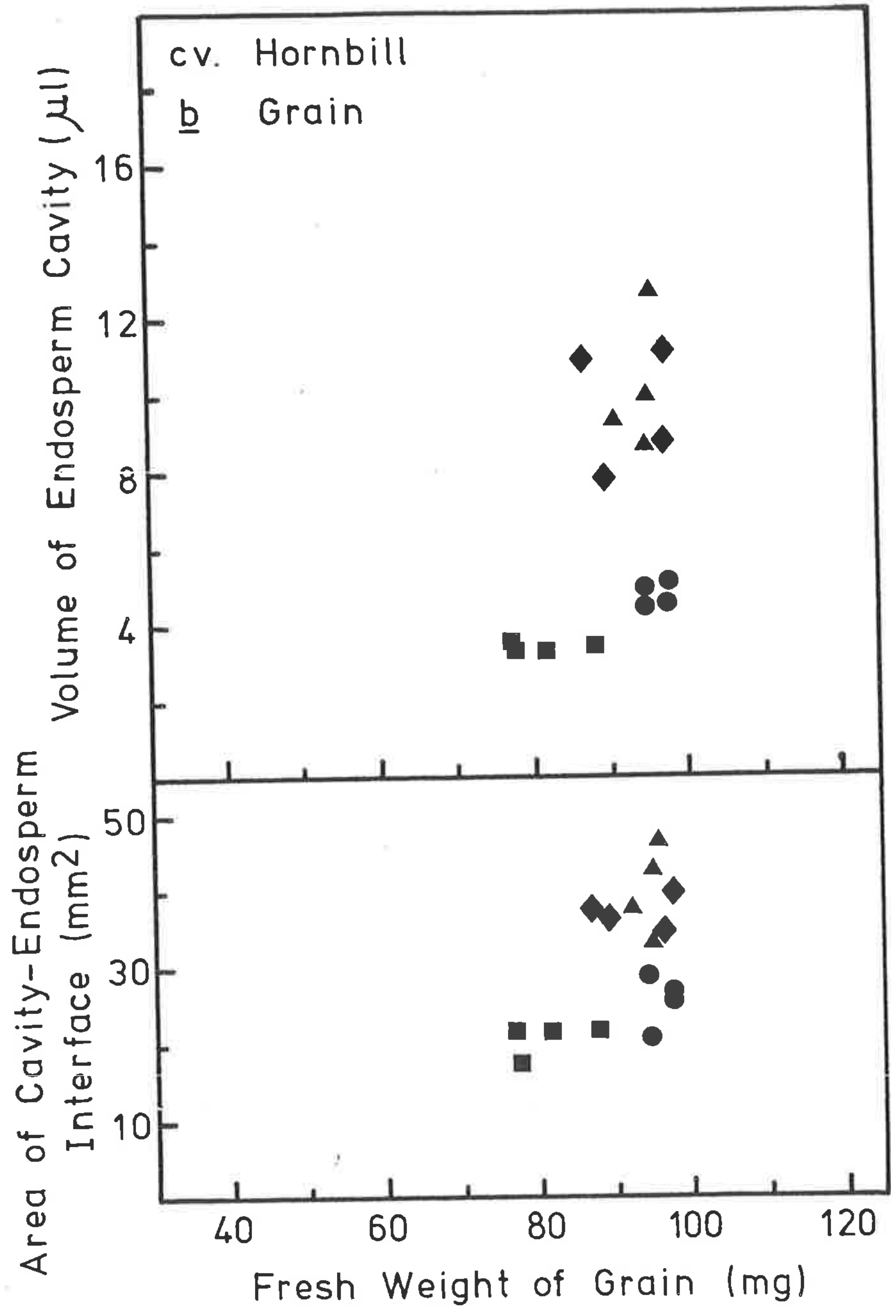


Figure 3.1-1D. As Fig. 3.1-1A, b grains of the cultivar Hornbill.

NB. Anatomical features of the grain seen in figures 3.1-2 to 3.1-10 are shown diagramatically and labelled in figure 1-6. Magnification scale for figures 3.1-2 to 3.1-10 is x40, except figure 3.1-7 which is x30.

Figure 3.1-2. Cross-sectional photograph of a wheat grain (cv. Gabo, c grain from a trimmed ear, fwt 90.1 mg) showing ruptures of the modified aleurone layer.

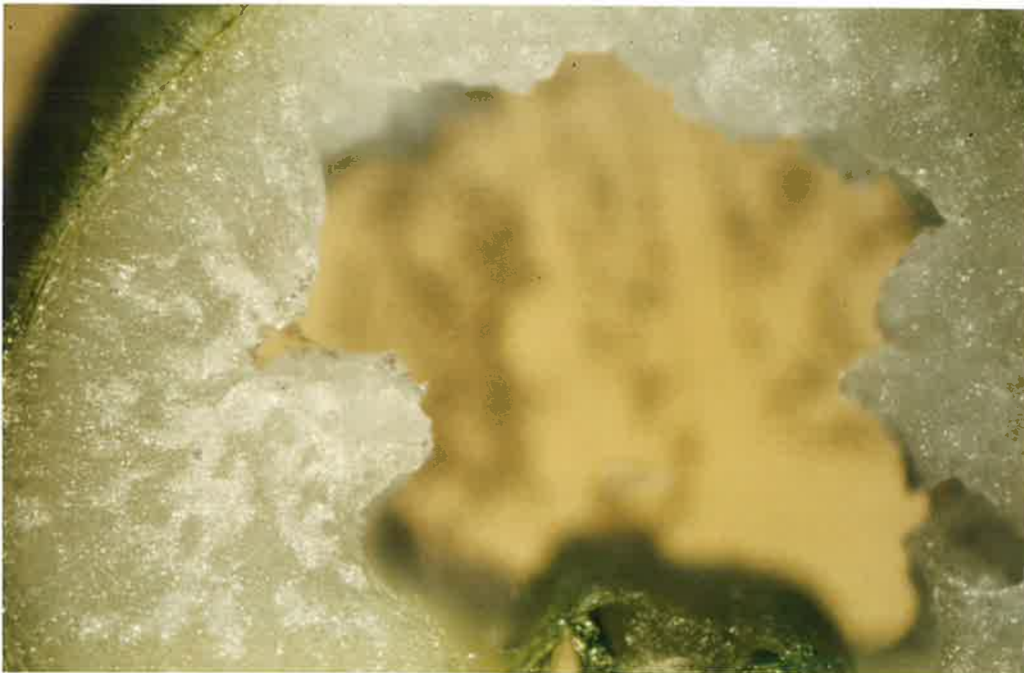


Figure 3.1-3. Massive ruptures of the modified aleurone layer and the endosperm in a wheat grain (cv. Nainari, b grain from a degrained spikelet, fwt 100.8 mg).

increase in area was small (cvs. Sonora and Fransawi cf. cv. Gabo, Fig. 3.1-1).

Other features contribute to a large cavity. The shape of the cavity may be flat (Fig. 3.1-4) or cylindrical (Fig. 3.1-5). In addition the pericarp tissue in the base of the crease may be positioned nearer to the circumference of the grain than to its axis (Fig. 3.1-6, see also Fig. 3.1-10, cf. Figs. 3.1-2, 3.1-4 and 3.1-5).

4. Evidence for an Embryo Cavity

A cavity was observed in developing grains between the endosperm and the embryo (Fig. 3.1-7). This embryo cavity was larger in cross-section than the endosperm cavity near the proximal end of the grain (Fig. 3.1-8), and like the endosperm cavity was filled with fluid (see especially 3.1-7). The two cavities are connected directly (Figs. 3.1-7 and 3.1-8) and linked by apoplastic channels between the endosperm cells (Fig. 3.1-9).

DISCUSSION

The phenomenon of an increase in the size of the endosperm cavity previously observed by Radley (1978) as a response in the c grain after all other grains in the ear were removed, appears to be a general one. The current study has shown a positive relationship between the volume of the endosperm cavity and grain fresh weight within any one cultivar irrespective of the technique used to alter the rate of grain growth. This was observed in a range of cultivars selected for their diverse background and varying grain size, and in both the b and the c grains.

It is reasonable to assume that the measurements of cavity volume made during this study are a valid representation of the *in vivo* state on two accounts. First, the external dimensions of grains were measured before and after slicing using fine callipers. There was no change in these

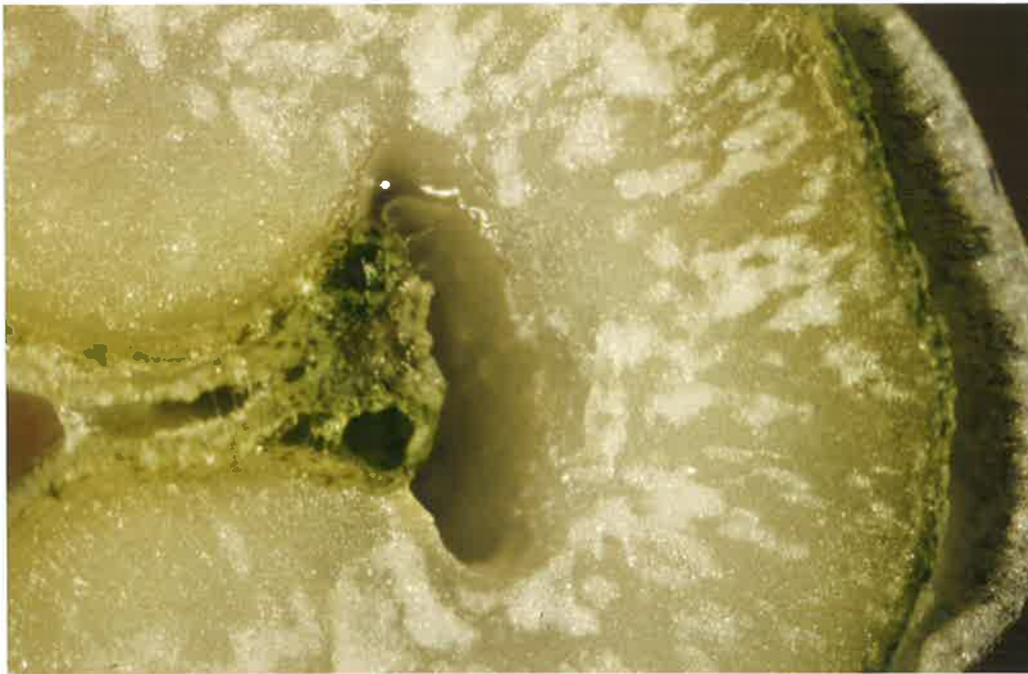


Figure 3.1-4. The endosperm cavity flat in cross-section (cv. Nainari, b grain from a trimmed ear, fwt 78.8 mg).

Figure 3.1-5. The endosperm cavity rounded in cross-section (cv. T. Pol. 3824, b grain from a trimmed ear, fwt 114.8 mg).

Figure 3.1-6. Cross-sectional photograph of a wheat grain (cv. T. Pol. 3824, c grain from a degrained spikelet on a trimmed ear, fwt 125.0 mg) showing the pericarp tissue of the crease located near the circumference of the grain.

Figure 3.1-7. Longitudinal section of a wheat grain (cv. Fransawi, b grain from a degrained spikelet, fwt 125.0 mg) showing the endosperm cavity, the embryo cavity, and the connection between them.

Figure 3.1-8. Embryo cavity near the proximal end of the grain (cv. Gabo, c grain from a degrained spikelet, fwt 94.6 mg).

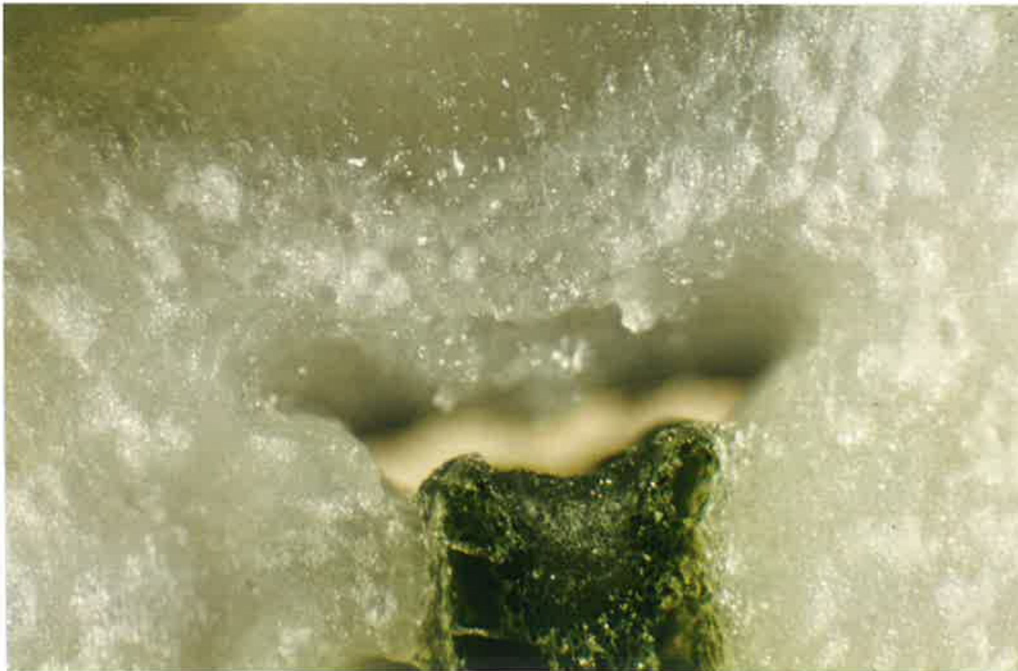


Figure 3.1-9. Apoplastic channels between the endosperm cells connecting the endosperm and embryo cavities near the distal end of the embryo (cv. Gabo, b grain from a degrained spikelet, fwt 87.7 mg).

external dimensions (see Materials and Methods: i.e. section 2.8.1). One-mm-thick sections of the wheat grain, 20 days after anthesis, have sufficient resilience to make unlikely any error caused by deformation. Secondly there is good agreement in the volume of the endosperm cavity as measured by two independent techniques (Table 3.1-1).

This good agreement between the volume of fluid expressed from the endosperm cavity and the cavity's physical dimensions means also that the cavity is filled essentially with fluid, not air. In addition no evidence was found for the presence of air, either during microscopic examination of the cavity or during the expression of fluid from the cavity (no results shown). Hence, regardless of the size of the cavity it seems that there would be a diffusive pathway of low resistance for solute movement from the tissues of the crease to the endosperm.

Three different anatomical characteristics were observed that contribute to enlargement of the endosperm cavity.

1. Shape. The cavity may be flat or elliptical in cross-section, or it may be more rounded (Figs. 3.1-4 and 3.1-5).

2. Position of pericarp. In larger grains the pericarp tissue in the region of the crease may become taut and this has the effect of drawing the tissue at the base of the crease away from the endosperm (Fig. 3.1-6 and 3.1-10).

3. Rupture of aleurone layer. The modified aleurone layer may become ruptured (Figs. 3.1-2 and 3.1-3).

From observations made during the current study it is not possible to describe the forces that act to cause these transformations, but clearly neither the pericarp tissue within the crease nor the modified aleurone layer expand sufficiently to prevent tension at these sites.

The function of the modified aleurone layer during grain development

is not understood but some anatomical features suggest that it has a role in solute transfer. First the thick walls of the modified aleurone cells fluoresce in a way characteristic of callose, suggesting (Cochrane and Duffus, 1980) that there may be high resistance to apoplastic movement of solutes through this layer. The cells contain membranous loops and while these may not be identical anatomically to the wall ingrowths of transfer cells (Cochrane and Duffus, 1980; Harrison et al., 1978; Zee and O'Brien, 1971; but see also Ayre and Angold, 1979), it seems not unreasonable to propose a similar function. Plasmodesmatal connections are abundant between cells of the modified aleurone layer and cells of the endosperm which may allow a high rate of symplastic transfer (Cochrane and Duffus, 1980; Duffus and Cochrane, 1982; MacMasters et al., 1971; Morrison et al., 1975, 1978). The development of ruptures in the modified aleurone layer, however, brings the cells of the inner endosperm into direct apoplastic contact with the fluid of the endosperm cavity. Accordingly, this would circumvent either in part or totally any regulatory role of the modified aleurone layer on the movement of solutes to the endosperm symplast. The effect on the developmental ontogeny of the grain could be substantial.

Treatments that reduce the number of grains competing for solutes have been used frequently to study whole plant interactions; for instance to estimate the extent of source limitation to grain weight (e.g. Fischer and HilleRisLambers, 1978; Pinthus and Millit, 1978; Radley and Thorne, 1981, Thorne, 1981). In these studies the underlying assumption, mostly a tacit one, is that the remaining grains although larger are not abnormal. In other words, it is assumed that the mechanism of growth within the grain, itself, is not altered by these treatments and that any induced change to grain growth is an expression essentially of a change in the levels of substrate delivered to the grain.

It is surprising therefore, that the response of grains to these treatments is not predictable, For example, the concentration of sucrose in

the endosperm may increase (Radley, 1978), decrease (Jenner, 1979, 1980; data for whole grain given by Konovalov, 1966), or remain unchanged (Radley and Thorne, 1981), while the rate of dry matter deposition may follow the sucrose concentration (Radley, 1978), be inversely related (Konovalov, 1966) or appear unrelated (Jenner, 1979, 1980; Radley and Thorne, 1981).

The current study has shown that degraining or trimming the ear may influence both the size of the endosperm cavity and the integrity of the modified aleurone layer, and importantly there are large differences in the responses between cultivars. It seems not unreasonable to consider whether or not these varying morphological responses and the varying growth responses are related. In any case, these observed changes in grain morphology strongly challenge the validity of experiments that use the growth of grains in trimmed or degrained ears to describe the physiology of systems external (even partly so) to the grain.

The b grain of the cv. Hornbill either did not increase in fresh weight, or increased very little in response to a reduction in the number of grains per ear, even though the volume of the endosperm cavity increased (Table 3.1-1; Fig. 3.1-1). On the other hand the c grain responded positively (Table 3.1-1). The bracts and glumes of this cultivar are extremely strong and rigid and the grains were frequently quite distorted in shape (not shown). Possibly in this cultivar physical constraints within the spikelet imposed by the floral organs may have restricted the expansion of the grain, especially in the grains within the lower regions of the spikelet.

Changes to the nutrient supply of an ear affect to a greater extent the growth of grains in the distal positions (Bremner and Rawson, 1978; Simmons et al., 1982; Singh and Jenner, 1983; Wardlaw et al., 1980). This had lead to the view that grain growth is influenced by gradients of nutrient supply within the rachis and rachilla (Thornley et al., 1981). The

results of the current study are another example of the equilizing of grain size within a spikelet in response to increased substrate supply (Table 3.1-1).

A cavity was observed between the endosperm and the embryo which is continuous with the endosperm cavity (Fig. 3.1-7, 3.1-8 and 3.1-9). Presumably solutes in the fluid of this cavity are a source of nourishment for the developing embryo.

The main purpose of the experiments reported in this section (3.1) was to provide information upon which a cultivar and growing technique could be chosen to provide grains suitable for the physiological studies on substate transport within the grain (sections 3.2, 3.2, 3.4, 3.5 and 3.6). The preferred type of grain was large with a large evenly rounded and accessible endosperm cavity, and in view of the results presented above, the modified aleurone layer was required also to be intact. Accordingly, the b grain of cv. Fransawi grown on trimmed ears was chosen for subsequent work. A cross-sectional photograph of such a grain is shown in figure 3.1-10.

The principal question arising from this study is whether or not the morphological changes that occur in the endosperm cavity and the modified aleurone layer, in themselves, influence the physiological development of the grain. The first step to answer this question may be to monitor carefully the morphological development, the substrate supply and the deposition of dry matter within the grain, and the endosperm in particular, from anthesis to maturity on cultivars that differ in their response to degraining or trimming (e.g. Gabo and Fransawi). This question could not be pursued further at this time.

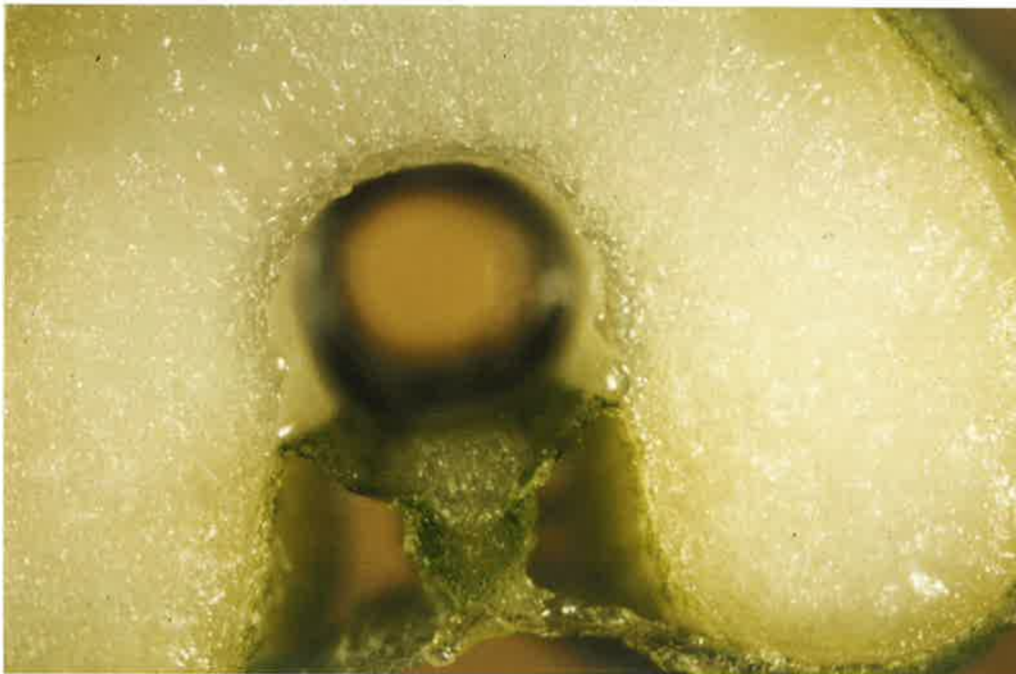


Figure 3.1-10. Cross-sectional photograph of the type of grain used for the physiological studies on substrate transport (cv. Fransawi, b grain from a trimmed ear, fwt 95.0 mg).

3.2 SOLUTES OF THE DEVELOPING WHEAT GRAIN: USING HPLC TO MEASURE THE PRINCIPAL FREE AMINO ACIDS AND SOLUBLE CARBOHYDRATES IN THE ENDOSPERM AND ENDOSPERM CAVITY FLUID.

INTRODUCTION

One has to go back 20-25 years to find research studies that attempted to analyse in a comprehensive way the solutes of the developing wheat grain (e.g. Jennings and Morton, 1963a; Menger, 1960). The analytical techniques available then made such a task difficult and time consuming. Precision and sensivity were low, and usually only one tissue type was studied.

The endosperm cavity has been viewed with interest for a long time (e.g. Bradbury et al., 1956a; Brenchley, 1909), being a part of the fluid connection between the maternal and filial tissues (e.g. Ho and Gifford, 1984; Sakri and Shannon, 1975). While this fluid can be extracted easily from the grain (e.g. Jenner, 1974) there are no studies that attempt to analyse in a comprehensive way the solutes therein.

With the advent of improved analytical techniques, based principally on gas liquid chromatography and high performance liquid chromatography (see e.g. Advances in Chromatography, Vols. 1-25, Marcel Dekker Inc.), the time is ripe to examine again, or in some cases for the first time, the solute composition of tissues within the developing wheat grain. The study reported in this section (3.2) is a start.

The aims of the study were as follows.

1. To identify and measure the free amino acids and soluble carbohydrates in the endosperm and in the fluid of the endosperm cavity. This study had to be placed within the constraints imposed by the availability of time and finance, and access to a modern analytical system.

2. To develop and test analytical techniques; in so doing to provide the foundation for a study on the gradients of solutes throughout the endosperm, a study (section 3.3) in which the same techniques are to be used but in which the material available for analysis will be very small.

3. To identify the form in which carbohydrate enters the grain and the form in which it is transported through the endosperm cavity and endosperm.

MATERIALS AND METHODS

Plant Material

Wheat plants, cv. Fransawi (see section 3.1 for a description of this cultivar) were grown in controlled environment conditions as described in sections 2.1.1, 2.1.2, 2.2.1, 2.2.2, and 2.2.3. The grains used in this study were from ears trimmed to 6 spikelets 2 days after anthesis (sections 2.3.1 and 3.1). The b grain from the 4 central spikelets were used for experimentation at 20 days after anthesis. Only ears in which each of these 4 central spikelets contained also at least the a and c grains were used.

Harvesting the Grains and Solute Extraction

Grains were harvested after 7 hours of the day cycle. Two batches each of two tissue-types and appropriate controls were taken. The overriding aim during tissue preparation was to minimize the chance of chemical or enzymic transformation of solutes.

1. Endosperm. All other tissue was peeled away from around the endosperm as quickly as possible and the endosperm cavity was flushed with water (section 2.5.1). As soon as each endosperm was prepared (always within 1 minute of harvest) it was plunged into hot (75°C) 90% ethanol.

Each batch comprised 6 endosperms and the tissue was extracted

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immediately (section 2.10).

Fresh weight and dry weight of the endosperm tissue was not measured directly. Rather estimates were made from the fresh weight of the whole grains before dissection.

The total fresh weight of the grains was 725 mg in Batch A and 752 mg in Batch B. Assuming that the percentage dry weight of the endosperm was the same as that measured for the whole grain (i.e. 38%, see section 3.3) and that the endosperm comprised 63% the weight of the whole grain (Jennings and Morton, 1963a), the fresh weight and dry weight of the endosperm tissue in Batch A were 457 mg and 174 mg respectively, while in Batch B they were 474 mg and 180 mg.

2. Endosperm Cavity Fluid. Fluid from the endosperm cavity of 10 grains for each batch was expressed and collected as described in section 2.9. The weight of fluid extracted and its volume was 19.67 mg and 18.54 ul for Batch A and 20.13 mg and 19.01 ul for Batch B. This fluid was 'extracted' immediately as described in section 2.10.

Measurement of Free Amino Acids

Amino acids in the ethanolic extract were measured by HPLC techniques (reverse-phase) after the formation of the phenylthiocarbamyl derivatives as described in section 2.13.2. Hereafter, this method of analysis is referred to as 'Method 1'.

Duplicate samples of the ethanolic extracts were analysed using other HPLC systems and procedures. I am grateful to A. Jennings (Agricultural Biochemistry Dept., Waite Agric. Res. Inst.) and to D. Boehm (Animal Sciences Dept., Waite Agric. Res. Inst.) for these analyses. A brief description of these alternative procedures follows.

Method 2. Ion-exchange of the underivatized amino acids on a Varian* 5000

LC system using a lithium citrate/lithium hydroxide solvent gradient and a Waters* 'Amino Acid Analysis' column in the lithium form. Detection was by fluorometry after post-column derivatization with ortho-phthalaldehyde.

Method 3. Ion-exchange of the underivatized amino acids on a Varian* 5000 LC system, using a lithium-citrate-chloride solvent gradient and a Varian* Micropak* column in lithium form. Detection was by fluorometry after a 2-stage post-column derivatizing system involving hypochlorite and ortho-phthalaldehyde.

The run time of a single analysis using Methods 2 and 3 is 4 hours. Because of this, only Batch A of the extracts (and appropriate blanks and standards) was analysed using Methods 2 and 3 and then only one replicate of each. While this may be less than ideal, it still provided sufficient information for the comparative purposes of this study (see Table 3.2-1).

Measurement of Soluble Carbohydrates

Two HPLC columns were used to separate carbohydrates; the Waters* SugarPak* and the Waters* DextroPak*. Hereafter, these columns will be referred to only as the SugarPak and the DextroPak. Both these columns were coupled in turn to 3 detection methods; differential refractometry, the copper bicinchoninate post-column derivatizing system and the copper bicinchoninate post-column derivatizing system following the hydrolytic column (see section 2.13.1 for detailed descriptions). The following commercially prepared sugars were used as standards; inositol, galactose, sorbitol, glucose, fructose, ribose, melibiose, maltose, sucrose, maltotriose, stachyose, raffinose, maltotetrose.

Component Analysis of Solute Peaks

Fractions were collected (Pharmacia* Frac 100) mostly at 15 seconds intervals from the outlet of the refractive index detector, after

separation on the DextroPak column. Appropriate fractions (described below in the Results section) were pooled and dried under vacuum (Savant* Speed Vac Concentrator* 100H). Solutes in these fractions were hydrolysed in 0.4N HCl at 98°C for 2.25 hours within small sealed glass culture tubes (section 2.17). Ammonia solution was then added until the pH of the solution was 10, before again the solutes were dried. The solutes were redissolved in Pierce* pHix* buffer preservative solution (60 $ul.l^{-1}$ pentachlorophenol solution; 5 $mg.ml^{-1}$ in 95% ethanol), separated on the SugarPak column, and detected by differential refractometry and by the copper bicinchoninate post-column derivatizing system (section 2.13.1).

RESULTS

1. Analysis of Amino Acids Using Precolumn Derivatization

A chromatogram of the Pierce* amino acid standard 'H' derivatized before injection by phenylisothiocyanate (PITC) and separated by reverse-phase chromatography (Method 1; see section 2.13.2) is shown in figure 3.2-1. Each amino acid in this mixture, together with the amides glutamine and aspargine were analysed individually to confirm peak identification (chromatograms not shown).

The method for analysing amino acids extracted from tissues of the wheat grain (section 2.13.2) was essentially the same as the method for analysing the amino acid standards; amino acids in the ethanolic extract were derivatized directly without purification. Such a technique had two advantages. (1) The in vivo state of the amino acids was preserved as much as possible and (2) the risk of solute loss during purification was avoided, this being of concern especially when the amount of material for analysis was very small (see section 3.3).

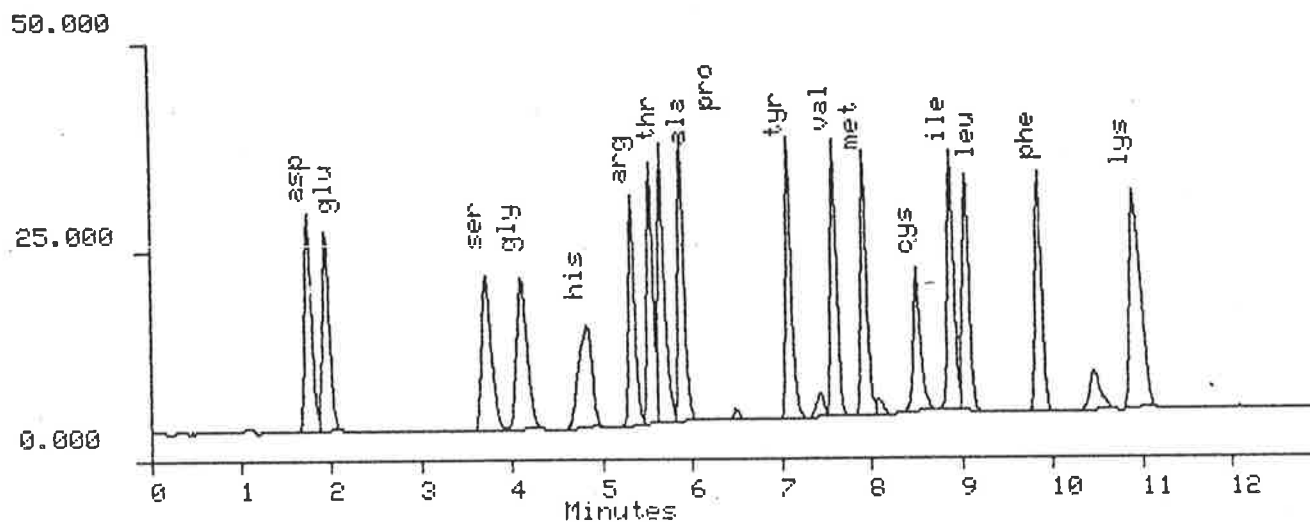

Figure 3.2-1. Chromatogram of the Pierce Amino Acid Standard Mixture 'H' after analysis by the PITC method described in section 2.13.2. This chromatogram was of 250 pmole of each amino acid on column, except cystine which was 125 pmole.

On the other hand, other solutes in the crude extract such as salts and carbohydrates may generate interfering peaks on the chromatogram. Indeed, the derivatizing agent (PITC) reacts with the free hydroxyl groups of carbohydrates and sugar alcohols to form complexes that can be separated and analysed by reverse-phase chromatography (Bjorkqvist, 1981). However, the tests described below showed that other solutes in the ethanolic extracts did not, in themselves, interfere with the amino acid analysis.

Salts in the derivatized extract eluted in the void volume (B. Walker, pers. comm., additional results not shown).

A range of sugars (glucose, fructose, sucrose and raffinose) and solutes of the soluble carbohydrate fraction from wheat endosperm (see sections 2.11, 3.4, 3.5 and 3.6, also later in this section), each in amounts 20 times the expected maximum level of total carbohydrate in the extract were derivatized separately or with the amino acid standard mixture (Pierce* 'H'). No peaks were produced as a result of these other solutes in the region of the chromatogram occupied by the amino acids and no other interference with the amino acid analysis was detected (chromatograms not shown).

Notwithstanding the above result, there were a number of peaks on the amino acid section of the chromatogram during analysis of both standard mixtures and the extracts from the grain which are not amino acids. These are listed below.

1. A peak between the proline and γ-aminobutyric acid peaks (approx. 6.15 mins., not obvious in Fig. 3.2-1). This peak is phenylthiourea (Cohen et al., 1986), a breakdown product of the derivatizing reagent. It co-chromatographs with ammonia (Manual for the Waters* PicoTag* column).

2. In some samples a peak appeared in the position of γ-aminobutyric acid (approx. 6.50 mins.). The compound and the reason for its intermittant appearance was not known. However, the presence of this peak meant that γ-

aminobutyric acid could not be used as an internal standard with this procedure.

3. A peak between the tyrosine and valine peaks (approx. 7.45 mins.). This peak was usually small. Its size was associated with the purity of triethylamine used in the derivatization procedure.

4. A peak between methionine and cystine (approx. 8.15 mins.). Its origin was unknown, it was always small, but care had to be taken to distinguish it from known adjacent peaks.

5. A peak just before, but distinguishable from lysine (approx. 10.60 mins.). This peak was diphenylthiourea, a breakdown product of PITC (B. Walker, pers. comm.). The final drying down step of the derivatizing procedure was found to be critical. Drying was continued long enough to remove essentially all the unreacted PITC; excessive drying caused, among other things, this diphenylthiourea peak to become quite large. It could have been a breakdown product of the phenylthiocarbamyl-water complex as there was no noticable relationship between the build-up of this peak and the decay of any amino acid peak.

2. Identification of the Free Amino Acids in Wheat Extracts by Comparative Analysis.

The composition of free amino acids in the endosperm and in the fluid of the endosperm cavity as analysed by 3 independent techniques is shown in Table 3.2-1.

The descriptive term 'not detectable' used in table 3.2-1 refers either to the inability of the technique to detect as is the case of proline, hydroxyproline, and cysteine with Method 2, or to the inability to quantify an observed peak at this time because no appropriate amino acid standard was analysed concurrently. This is probably the case for γ-

Table 3.2-1. Composition of free amino acids in the endosperm and in the fluid of the endosperm cavity as analyzed by 3 independent techniques.

TISSUE:	ENDOSPERM				FLUID OF ENDOSPERM CAVITY			
UNITS:	nmoles.mgfwt^{-1}				nmoles.ul^{-1}			
BATCH:	A			B	A			B
METHOD OF ANALYSIS:	1	2	3	1	1	2	3	1
AMINO ACID								
alanine	5.14	5.63	4.60	4.23	13.75	14.30	13.10	12.07
asparagine	3.73	5.45	7.58	2.93	2.94	6.03	5.81	2.85
glutamate	3.89	1.09	2.77	2.90	1.01	DND	.46	.86
serine	3.66	1.35	1.29	2.54	5.70	6.27	4.56	5.80
glycine	2.74	1.49	1.61	2.19	4.67	2.26	2.00	5.20
glutamine	1.90	1.18	2.47	1.49	9.61	*	18.38	10.35
proline	1.76	ND	DND	1.38	4.12	ND	DND	4.35
aspartate	1.41	.74	.71	.84	.88	.57	.52	.52
valine	.76	.25	.41	.65	DND	.95	.99	1.02
histidine	.63	.07	.11	.62	.96	.79	.38	1.30
threonine	.56	.53	.56	.49	1.34	2.45	1.53	1.37
tyrosine	.57	.12	.07	.43	.07	.14	DND	.78
arginine	.46	.14	.27	.36	.86	.22	.39	.68
methionine	.22	.37	.14	.22	DND	.17	.50	DND
phenylalanine	.21	DND	.15	.10	DND	DND	.07	DND
leucine	.19	DND	.16	.11	DND	.09	.21	DND
isoleucine	.17	.95	.16	.08	DND	3.29	.34	DND
lysine	.13	.05	.25	.10	.39	.38	1.17	.68
cystine	.11	1.06	.02	.11	DND	1.47	1.79	DND
γ-aminobutyric acid	ND	.31	.23	ND	ND	.50	.27	ND
tryptophan	ND	.16	DND	ND	ND	.12	DND	ND
ornithine	ND	DND	.29	ND	ND	.53	.83	ND
ethanolamine	ND	ND	.11	ND	ND	ND	.62	ND
cystathionine	ND	ND	.12	ND	ND	ND	.09	ND
P-ethanolamine	ND	ND	.06	ND	ND	ND	.18	ND

EXPLANATORY NOTES

The numbers denoting Method of Analysis correspond to the description of methods in the text.

ND = Not Detectable.

DND = Did Not Detect, although detectable.

* = Value lost during integration.

Conversion factor, nmoles.mgfwt^{-1} to nmoles.mgdwt^{-1} is 2.63; see Fig.3.3-1

All values for the PITC analysis (Method 1) are the mean of 2 analyses; rarely was there greater than 5% between measurements.The values for Methods 2 & 3 are single analysis values only.

aminobutyric acid and ornithine with Method 1. Nevertheless, with Method 1, the peaks that could be identified positively accounted for 97-98% of the total peak area of the chromatogram (not shown), consistent with the amounts of γ-aminobutyric acid, ornithine, and other minor amino acids detected by other methods.

The low level or absence of proline in the analysis of Method 3 seems an unlikely result in view of (1) its detection previously (Jennings and Morton, 1963a) in a proportion similar to that observed in the current analysis using Method 1, and (2) the high levels of proline in the protein of wheat endosperm (e.g. review by Kasarda et al., 1971).

Alanine and glutamine are the most abundant amino acids in the fluid of the endosperm cavity. Serine, glycine, proline, and asparagine are present also in high amounts. The most abundant in the endosperm are alanine, asparagine, glutamate, serine, glycine, glutamine, and proline.

Also detected in the endosperm using Method 3 (not shown in Table 3.2-1) were low levels (0.02-0.07 $nmole.mgfwt^{-1}$) of taurine, α-aminoadipic acid, β-alanine, carnosine, α-amino-n-butyric acid, phosphoserine, and β-aminoisobutyric acid. Of these only β-alanine and α-amino-n-butyric acid were detected in the fluid of the endosperm cavity (0.07 and 0.06 $umole.ul^{-1}$ respectively).

Following is a list of amino acids that were detectable but not detected by Method 3: methionine sulphone, hydroxyproline, sarcosine, citrulline, hydroxylysine, 1-methylhistidine, anserine. The limits of detection were 0.02 $nmoles.mgfwt^{-1}$ in the endosperm and 0.05 $nmoles.ul^{-1}$ in the fluid of the endosperm of the endosperm cavity.

3. Identification of the Principal Soluble Carbohydrates in the Wheat Extracts

Part A. Using the SugarPak Column

One of the merits of the new generation of columns using a polystyrene cation-exchange resin in the calcium form (of which the SugarPak column is one) is that most of the common monosaccharides separate discretely using a very simple isocratic mobile phase that is compatable with detection methods of high sensitivity (section 2.13.1). The disaccharides sucrose and maltose, although eluting close to one another ($\triangle$RT = 0.1 min.) separate well from other major sugars.

These columns are not particularly useful for separating oligosaccharides with degrees of polymerization of 3 or more as these solutes separate early with poor resolution.

Amino acids, salts and organic acids eluted close to the void volume (results not shown) and did not interfere with the analysis of neutral carbohydrates. No erroneous peaks interfered with the use of this column with any of the detection methods used (section 2.13.1).

Chromatograms of the wheat endosperm extract using 3 methods of detection are shown in figure 3.2-2. Corresponding chromatograms of the endosperm cavity fluid were similar (not shown).

Detection by differential refractometry showed appreciable levels of oligosaccharides with degrees of polymerization of 3 or more, appreciable levels of maltose and/or sucrose but low levels of reducing monosaccharides.

Detection using the copper bicinchoninate system showed very little reducing activity associated with the oligosaccharides (DP > 2) but it did reveal activity consistent with maltose.

The hydrolytic column, inserted after the analytical column allowed detection of sucrose. Quantification of the mono- and disaccharide peaks is shown in table 3.2-2.

Ribose is detectable with the copper bicinchoninate system and has a retention time in this system of 19.90 minutes. However, no ribose peak was detected in the wheat extracts (detection limits 0.005 ug.mgfwt^{-1} in the

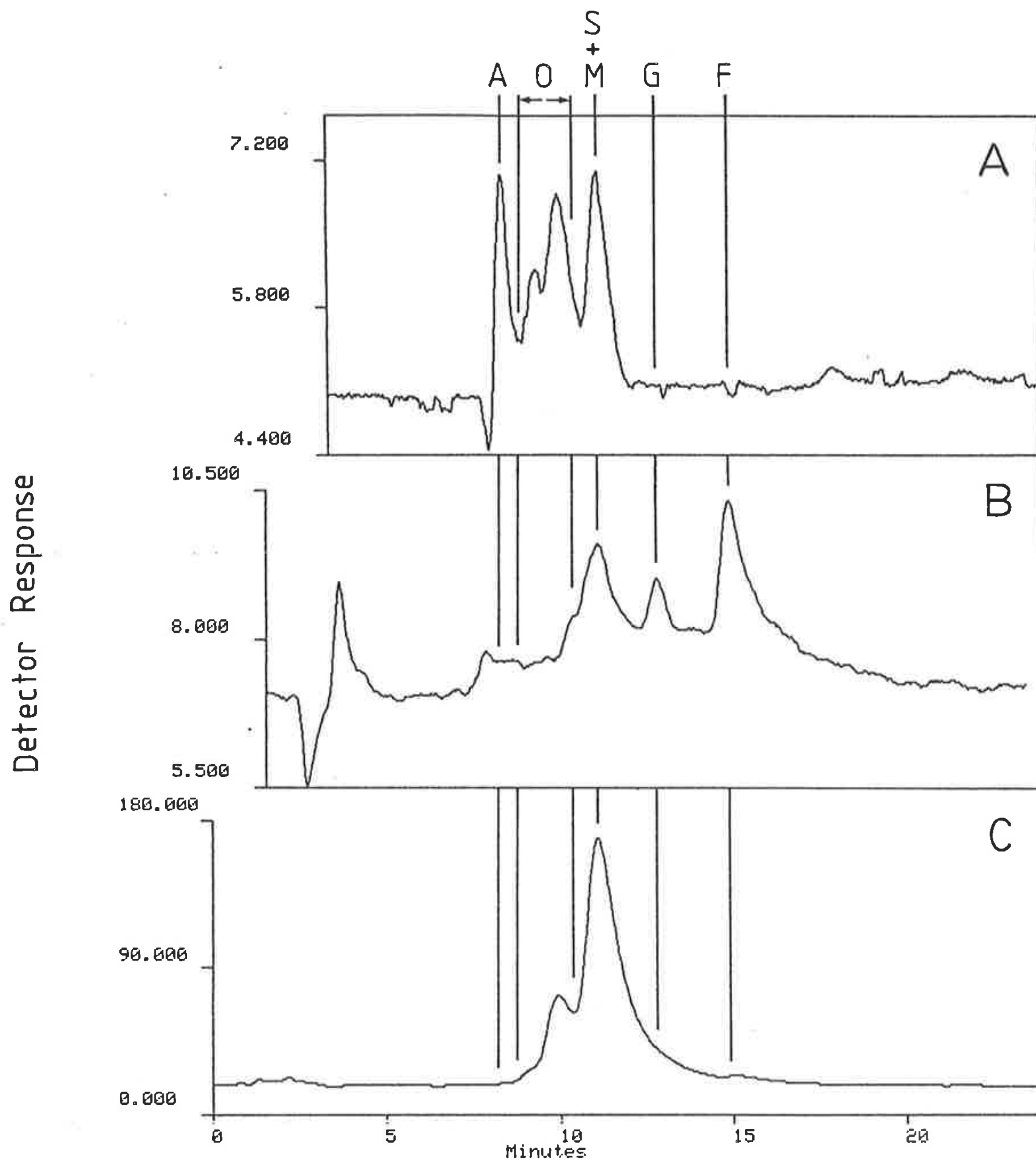

Figure 3.2-2. Analysis of the ethanolic extract of wheat endosperm using the SugarPak column and 3 methods of detection; differential refractometry (A), the copper bicinchoninate system (B), and the copper bicinchoninate system together with the hydrolytic column (C).

A = Amino acids, salts.
O = Oligosaccharides with DP > 2.
S + M = Sucrose and Maltose.
G = Glucose.
F = Fructose.

Table 3.2-2. Soluble sugars in the endosperm and in the fluid of the endosperm cavity (2 preparations of each; represented as A & B) as separated using the SugarPak column and detected with 3 methods (see Fig. 3.2-2).

TISSUE:	ENDOSPERM		ENDOSPERM CAVITY	
UNITS:	(ug.mgfwt^{-1})		(ug.ul^{-1})	
BATCH:	A	B	A	B
1. Detection by Differential Refractometry				
Sucrose + Maltose	3.14	3.04	14.86	14.60
Fructose	0.11	0.12	CD	CD
2. Detection Using Copper Bicinchoninate System				
Maltose	0.61	0.74	0.25	0.30
Glucose	0.08	0.09	0.08	0.10
Fructose	0.10	0.11	0.11	0.10
3. Detection Using Hydrolytic Column plus Copper Bicinchoninate System				
Sucrose*	3.13	3.15	15.77	14.01

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CD = Could not detect due to large sucrose peak nearby.

* = Allowance made for contribution at this retention time by maltose

endosperm and 0.005 ug.ul^{1-} in the fluid of the endosperm cavity).

Inositol elutes close to fructose ($\triangle$RT = 0.1 min.), but unlike fructose it does not react with the copper bicinchoninate detection system. For the extracts from endosperm the relative response factor for the peak eluting at the inositol/fructose position (i.e. response to the copper bicinchoninate system relative to the differential refractometry response, see note in table 3.2-3) was 1657 for Batch A and 1631 for Batch B. The relative response factor for authentic fructose was 1626 (n = 2). Thus, the peak at the inositol/fructose position was accounted for fully by fructose. (For the endosperm cavity fluid there was insufficient response to detection by differential refractometry at the inositol/fructose position to determine the relative response factor in this case.)

Sorbitol elutes discretely at 16.40 minutes with differential refractometry, but it was not detected (detection limits 0.1 ug.mgfwt^{-1} in the endosperm and 0.1 ug.ul^{-1} in the fluid of the endosperm cavity). It is not detectable by the other two methods.

Galactose elutes between glucose and fructose and unless it is present in amounts at least 10-20% that of glucose and fructose it is not identifiable positively using the copper bicinchoninate system. No peak corresponding to galactose was detected, so if present, its quantity does not exceed 0.01 ug.mgfwt^{-1} in the endosperm and 0.01 ug.ul^{-1} in the cavity fluid.

There was always a small peak at 8.50 minutes on the chromatogram of wheat extracts when the copper bicinchoninate system was used. This is the retention time of maltotriose. If this peak does represent maltotriose, the amount of maltotriose in the endosperm was 0.004 ug.mgfwt^{-1} while in the fluid of the endosperm cavity it was 0.13 ug.ul^{-1}. Maltotetrose which also has reducing activity and elutes at 7.97 minutes with the copper bicinchoninate system was not detected.

Part B. Using the DextroPak Column

The DextroPak column is a radial-pack cartridge containing octadecylsilane bonded to silica. It is particularly suited to the separation of carbohydrate oligomers, with sugars of lower molecular weights generally eluting first. Other compounds, including amino acids, are also separated to some extent, however.

The amino acid fraction from wheat endosperm, recovered from cationic exchange resin (section 2.11) was injected onto the DextroPak column. Also injected was a sample sample of the Pierce* 'H' standard amino acid mixture. Amino acids eluted in two distinct peaks (detected by differential refractometry, chromatograms not shown). The first was close to the void volume (5.4 mins.) and the second was in the hexose position (7.3 mins.). In the extract from wheat endosperm these two peaks were of equal size, while for the Pierce* 'H' mixture the first peak was 4 times the size of the second.

There was no positive detection of these amino acid peaks using the copper bicinchoninate detection systems.

Chromatograms of the extract from wheat endosperm using the DextroPak column and 3 methods of detection are shown in figure 3.2-3. The standard sugars inositol, galactose, glucose, sorbitol, fructose, melibiose and ribose all eluted around the region of peak number 3 (see Fig. 3.2-3; as also did some amino acids - see above). Two erroneous peaks appeared when using the DextroPak column coupled to detection by differential refractometry. These were at 15.6 minutes (peak number 9) and at 37 minutes (peak number 12).

Peaks 1 and 2 (salts, amino acids, organic acids, sugar phosphates), peak 3 (hexoses, amino acids, melibiose) and peaks 9 and 12 (artifacts) are not considered further.

Neither stachyose (peak position 7) nor maltotetrose (anomers between

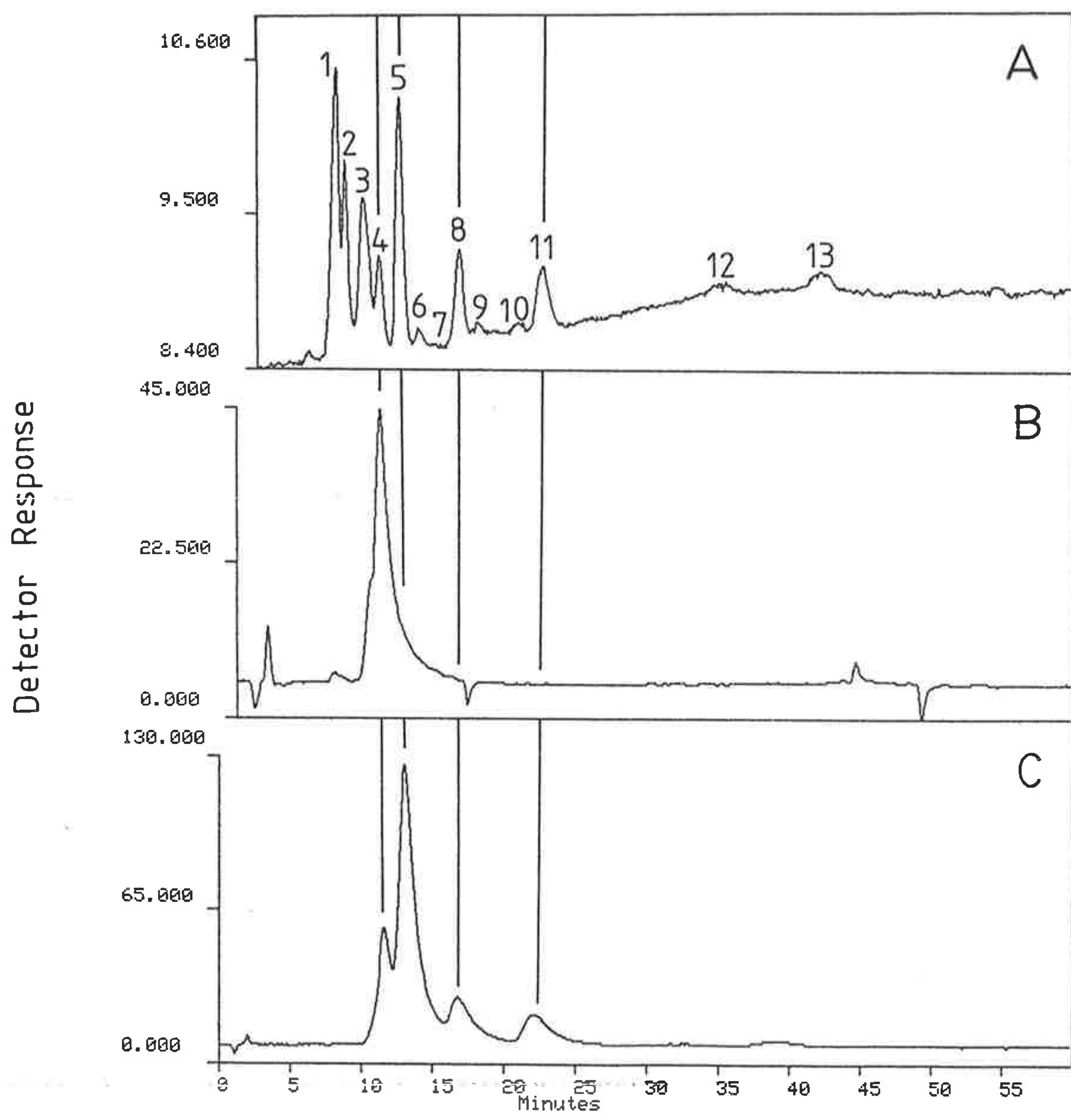

Figure 3.2-3. Analysis of the ethanolic extract of wheat endosperm using the DextroPak column and 3 methods of detection; differential refractometry (A), the copper bicinchoninate system (B), and the copper bicinchoninate system together with the hydrolytic column (C). See text for details of peak numeration.

detection for these sugars was 0.05 and 0.01 ug.mgfwt^{-1} respectively in the endosperm and 0.05 and 0.01 ug.ul^{-1} respectively in the fluid of the endosperm cavity. Maltotriose elutes close to sucrose ($\triangle$RT = 0.1 min.) and small amounts, as proposed from the study using the SugarPak column, could have escaped detection.

Peaks 4, 5 and 8 (Fig. 3.2-3) elute with the retention time of maltose, sucrose and raffinose respectively (Table 3.2-3). While on the basis of detection response peaks 4 and 5 correspond to maltose and sucrose, peak 8 does not seem to be raffinose as its response factor is much higher than authentic raffinose.

The peak fractions (Fig. 3.2-3) eluting from the DextroPak column were collected and hydrolysed (section 2.17). The component sugars were separated by the SugarPak column and detected by both differential refractometry and the copper bicinchoninate system. The results for the peaks of interest are listed below:

Peak 4. Composed exclusively of glucose, consistent with maltose.

Peak 5. Composed of glucose and fructose in the ratio 1.0:1.0, consistent with sucrose.

Peak 8. This peak contained glucose and fructose only - no galactose was detected. Therefore this peak did not satisfy the test for raffinose. In the extracts from both the endosperm and the endosperm cavity the ratio of glucose to fructose was 1.0:1.7. Possibly there was more than one oligosaccharide in this peak.

Peak 11. Contained glucose and fructose only, in the ratio of 1:2. (Actual values were 1.0:1.9 in the endosperm extract and 1.0:1.8 in the endosperm cavity extract.)

Peak 13. Contained glucose and fructose only, probably in the ratio 1:3.

Table 3.2-3. Retention times and relative response factors for some standard sugars and for some of the soluble carbohydrates extracted from wheat tissue when separated using the DextroPak column. The extracted carbohydrates are identified by peak number as described in figure 3.2-3. Values for the standard sugars are the mean of 2 replicates. Values for the extracted carbohydrates are the mean of 2 replicates each of the endosperm tissue and of the fluid of the endosperm cavity, there being no difference between the two sets of results.

	STANDARDS			EXTRACT				
	Maltose	Sucrose	Raffinose	Peak 4	Peak 5	Peak 8	Peak 11	Peak 13
1.Det'n by Differential Refractometry								
Retention Time (min)	8.70	10.13	14.40	8.65	10.10	14.52	20.23	42.04
2.Det'n Using Copper Bicinch'ate System								
Retention Time (min)	10.23			10.10				41.47
Rel. Response Factor	464	NIL	NIL	547	ND	ND	ND	13
3.Det'n Using Hydrolytic Column Plus Copper Bicinch'ate System								
Retention Time (min)	11.77	13.20	16.70	11.67	13.13	16.77	22.09	38.98
Rel. Response Factor	507	1205	190	569	1357	777	492	149

EXPLANATORY NOTES.

Relative Response Factor = The ratio of the response factor value for detection using the described method to the response factor value for differential refractometry. In effect, it is a measure of the colour yield per unit of refractive index.

ND = Not Detected.

(Actual values 1.0:3.2 in the endosperm extract and 1.0:2.8 in the endosperm cavity extract.)

On the basis that peak 4 was maltose and that peak 5 was sucrose, the calculated amount of these carbohydrates in the endosperm and the fluid of the endosperm cavity (Table 3.2-4) corresponds well with the amount of these carbohydrates as analysed using the SugarPak column (Table 3.2-2).

The standard deviation of the response factors for differential refractometry of all standard sugars tested (n = 13) was 8.7% of the mean. The amount of sugar in the unknown peaks numbered 6, 8, 10, 11 and 13 in the endosperm and the fluid of the endosperm cavity, calculated using this mean value for response factors is shown also in table 3.2-4.

4. The Form in Which Carbohydrate is Transported Within the Wheat Grain and its Conversion <u>en Route</u>

The purpose of the experiment reported here was to identify the form in which carbohydrate is transported within the wheat grain, in so doing to identify the principal sugar(s) providing substrate for carbohydrate metabolism within the grain.

In addition, this experiment provided the opportunity to test again, but in another way, the performance of the cation-exchange chromatography used in sections 3.4, 3.5 and 3.6 to separate the ethanolic extracts of wheat tissue into the carbohydrate and amino acid fractions (see section 2.11). The results of this test also are described here.

A wheat grain, taken from the detached spikelet system, previously cultured for 90 minutes on ^{14}C-sucrose and glutamine (sections 2.4.2 and 3.6), was sectioned (section 2.5.2), and the ethanolic extracts of these sections (section 2.10) were pooled as shown in table 3.2-5. Aliquots of the ethanolic extracts from wheat tissue, used in the experiments described above (results sections 1, 2, and 3) were added to these radioactive

Table 3.2-4. Soluble sugars in the endosperm and in the fluid of the endosperm cavity (2 preparations of each; A & B) as separated using the DextroPak column and as detected using 3 detection methods (see Fig. 3.2-3).

	TISSUE:	ENDOSPERM		ENDO'M CAVITY	
	UNITS:	($ug.mgfwt^{-1}$)		($ug.ul^{-1}$)	
	BATCH:	A	B	A	B
1. Detection by Differential Refractometry					
	Maltose	.85	.75	.52	*
	Sucrose	2.08	2.13	12.86	11.38
	Peak 6	.31	.11	.61	*
	Peak 8	1.29	.94	2.64	2.16
	Peak 10	.18	.10	DND	*
	Peak 11	1.07	.95	9.54	8.39
	Peak 13	.64	.40	4.16	4.02
2. Detection Using Copper Bicinchoninate System					
	Maltose	.98	1.14	.77	.57
3. Detection Using Hydrolytic Column Plus Copper Bicinchoninate System					
	Maltose	.85	.98	.25	.27
	Sucrose	2.76	2.80	13.00	11.48

EXPLANATORY NOTES.

DND = Did Not Detect, although detectable.

* = Not detected, probably due to a fault with the RI detector during the analysis of endosperm cavity extract batch B, which decreased sensitivity although accuracy was largely unimpared. The fault could not be fixed in time to repeat the analysis.

Table 3.2-5. Protocol for the experiment designed to establish the form of carbohydrate transport and its conversion to other solutes within the wheat grain

Section No. (*)	Description of Section (*)	Dwt. (ug)	Pooled Fractions	Extract Used as Carrier	Type of Analysis
3	Endosperm segment 1	123	Endosperm near periphery (see Fig. 3.3-1D)	Endosperm	Form of radioact'ty
4	Endosperm segment 2	146			
5	Endosperm segment 3	144			
6	Endosperm segment 4	172	Endosperm near cavity (see Fig. 3.3-1C)	Endosperm	Form of radioact'ty
7	Endosperm segment 5	134			
8	Mainly endo. plus cavity	83		Nil	Measurement of sugars
10	Cavity fluid only	24	(see Fig. 3.3-1B)	Endosperm cavity	Form of radioact'ty
12	Vascular Bundle	161	(see Fig. 3.3-1A)	Endosperm cavity	Form of radioact'ty

EXPLANATORY NOTE.

* = See sections 3.4, 3.5 and 3.6 for descriptions of the tissue sections and the numbering system used to denote the sequence with which they were taken from the grain.

extracts as also described in table 3.2-5. The function of these added solutes was to serve as a 'carrier' through the HPLC system and as a 'marker' for peak detection by differential refractometry.

Solutes in the radioactive extracts were separated using the DextroPak column and collected in fractions from the outlet of the refractive index detector. Radioactivity in each fraction was measured by liquid scintillation spectrometry (section 2.12.2).

In addition, aliquots of the pooled radioactive extracts were taken for measurement of total radioactivity (section 2.12.2) and of the radioactivity in the carbohydrate and amino acid fractions after cation-exchange chromatography (sections 2.11 and 2.12.2).

The extract taken from the tissue in section number 8 (see Table 3.2-5) was analysed using the SugarPak column , the hydrolytic column and the copper bicinchoninate system. The ratio of sucrose peak area to reducing-sugar peak area in the extract was 1.00:0.08. This ratio value compares favourably with 1.00:0.09 (Batch A) and 1.00:0.10 (Batch B) in the extracts of endosperm that were prepared specifically in a way to minimize the chance of chemical and enzymic transformation of the solute (see Materials and Methods, this section; values calculated from values presented in results section 3A above). Accordingly, it seems unlikely that any radioactivity appearing in the monosaccharide position of the Dextropak chromatogram would have derived from hydrolysis of oligosaccharides (including sucrose) during the preparation procedure.

Nevertheless, since some amino acids also elute at the monosaccharide position (see above) and ^{14}C from sucrose becomes incorporated into amino acids (see sections 3.5 and 3.6), the measurement of radioactivity at this position is likely still to be greater than the activity in hexose form <u>in vivo</u>.

The solute distribution of radioactivity in the extracts together with

the chromatograms of differential refractometry obtained at the same time is shown in figures 3.2-4A, 3.2-4B, 3.2-4C and 3.2-4D.

Radioactivity in the tissue surrounding and including the vascular bundle at the base of the crease was mainly in sucrose. It was present also in solutes that eluted in the first amino acid position and in the position of the remaining amino acids and monosaccharides.

Essentially all (99%) of the radioactivity in the carbohydrates of the endosperm cavity was in sucrose.

Throughout the endosperm the proportion of total activity contained in sucrose decreased as a function of distance from the cavity (73% to 60% in the two sampled regions).

In all extracts there was no radioactivity in solutes that eluted later than sucrose, i.e. in oligosaccharides of peaks number 6 to 13 (see Fig. 3.2-3). Hence, sucrose is the form in which carbohydrate enters the grain (i.e. appears in the main vascular bundle and in the endosperm cavity), and it remains mostly in this form during transport throughout the grain.

The total amount of radioactivity in the ethanolic extracts, and the radioactivity in each of the fractions separated by cation-exchange chromatography are shown in table 3.2-6. These results parallel closely those obtained after separation using the DextroPak column (summarized also in table 3.2-6). Most of the radioactivity in the extracts from the region of the vascular bundle, and from the endosperm cavity separated into the carbohydrate fraction, while the proportion of total radioactivity in the endosperm that separated into the carbohydrate fraction decreased as the distance from the cavity increased (78% to 69%).

Furthermore, the absolute amount of radioactivity in the carbohydrate fraction equalled the radioactivity in solutes identified as carbohydrates by the HPLC system in all the cases examined.

These results vindicate (see sections 2.11 and 3.5) the use of cation-

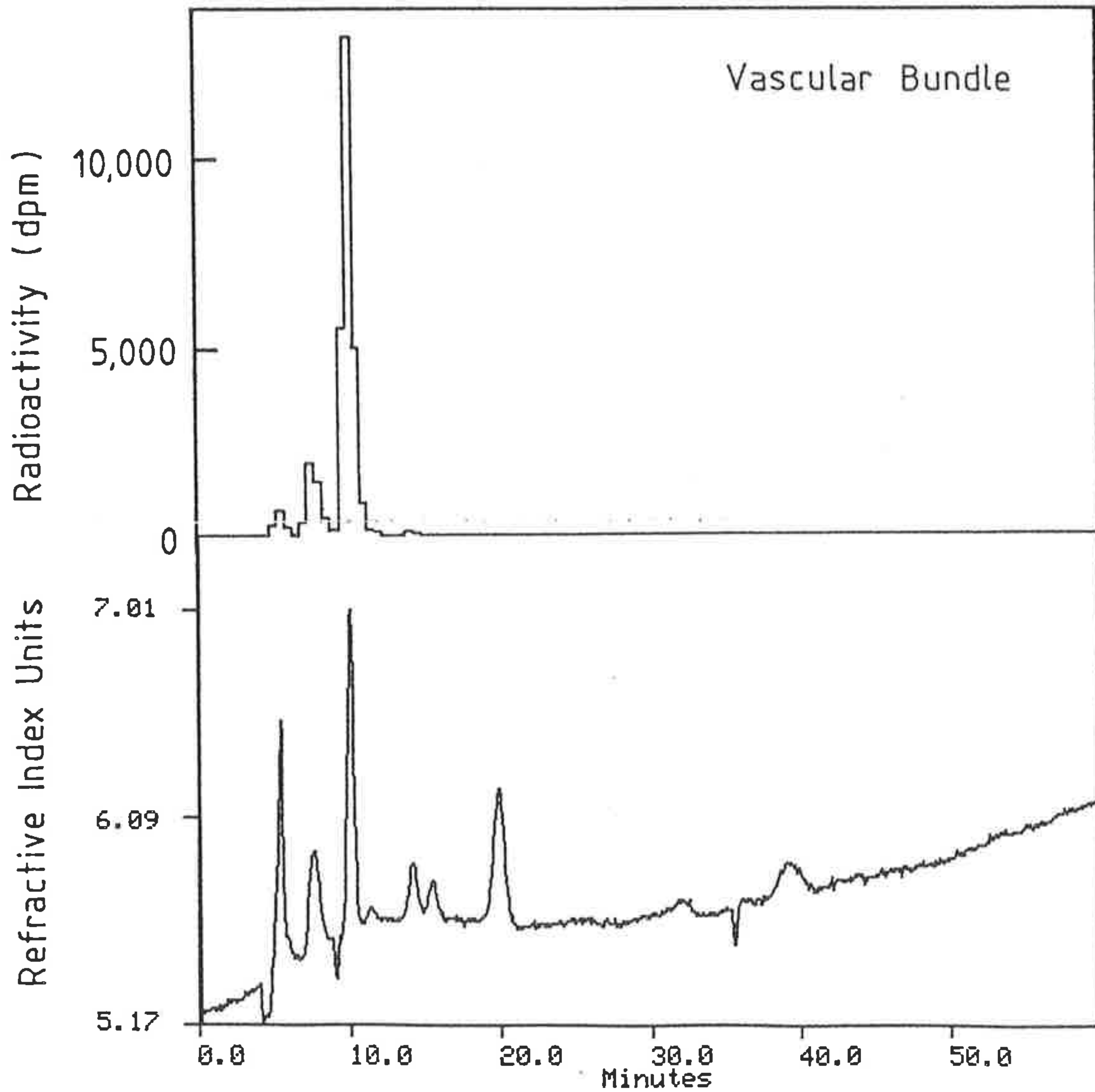

Figure 3.2-4A. Distribution of radioactivity after separation by HPLC (DextroPak column) of solutes extracted from pericarp tissue containing the main vascular bundle of the grain, and the plot of differential refractometry (of carrier solutes) obtained at the same time.

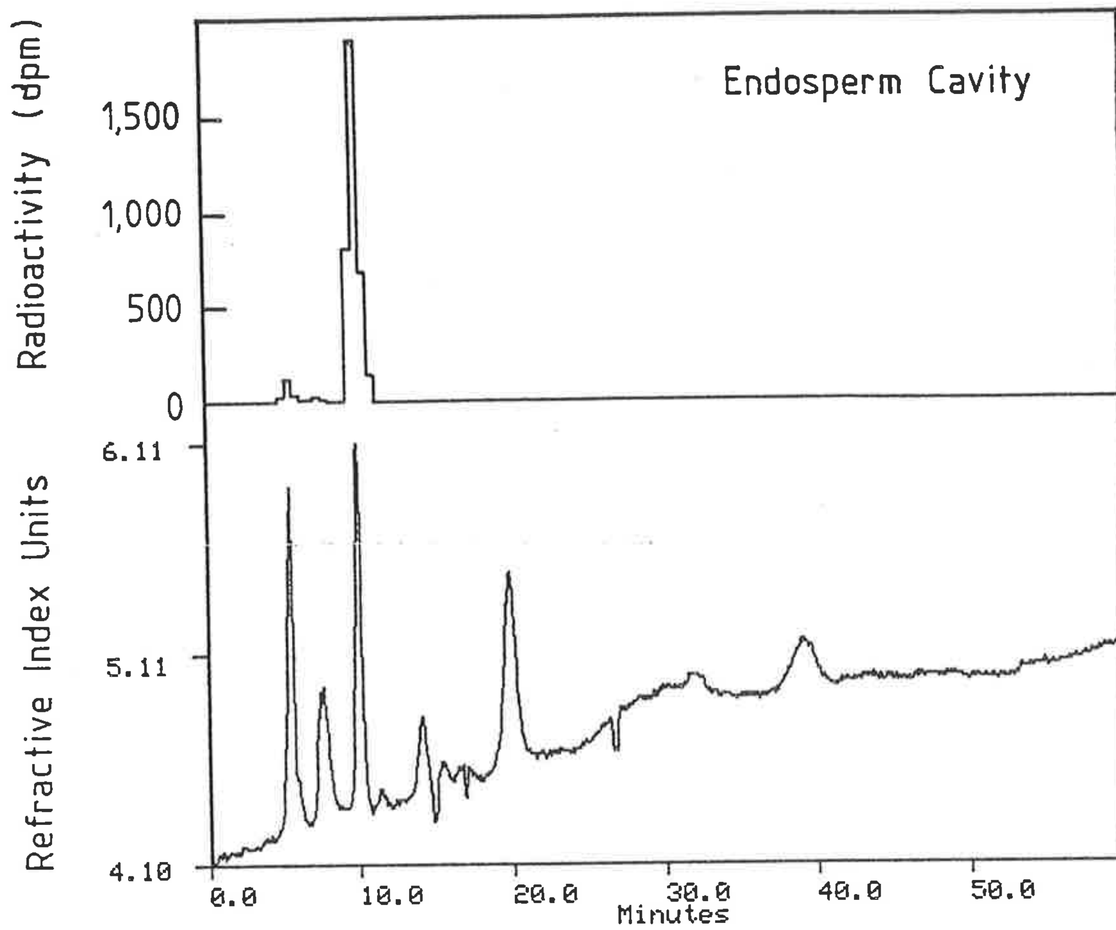

Figure 3.2-4B. As Fig. 3.2-4A, Solutes extracted from the endosperm cavity.

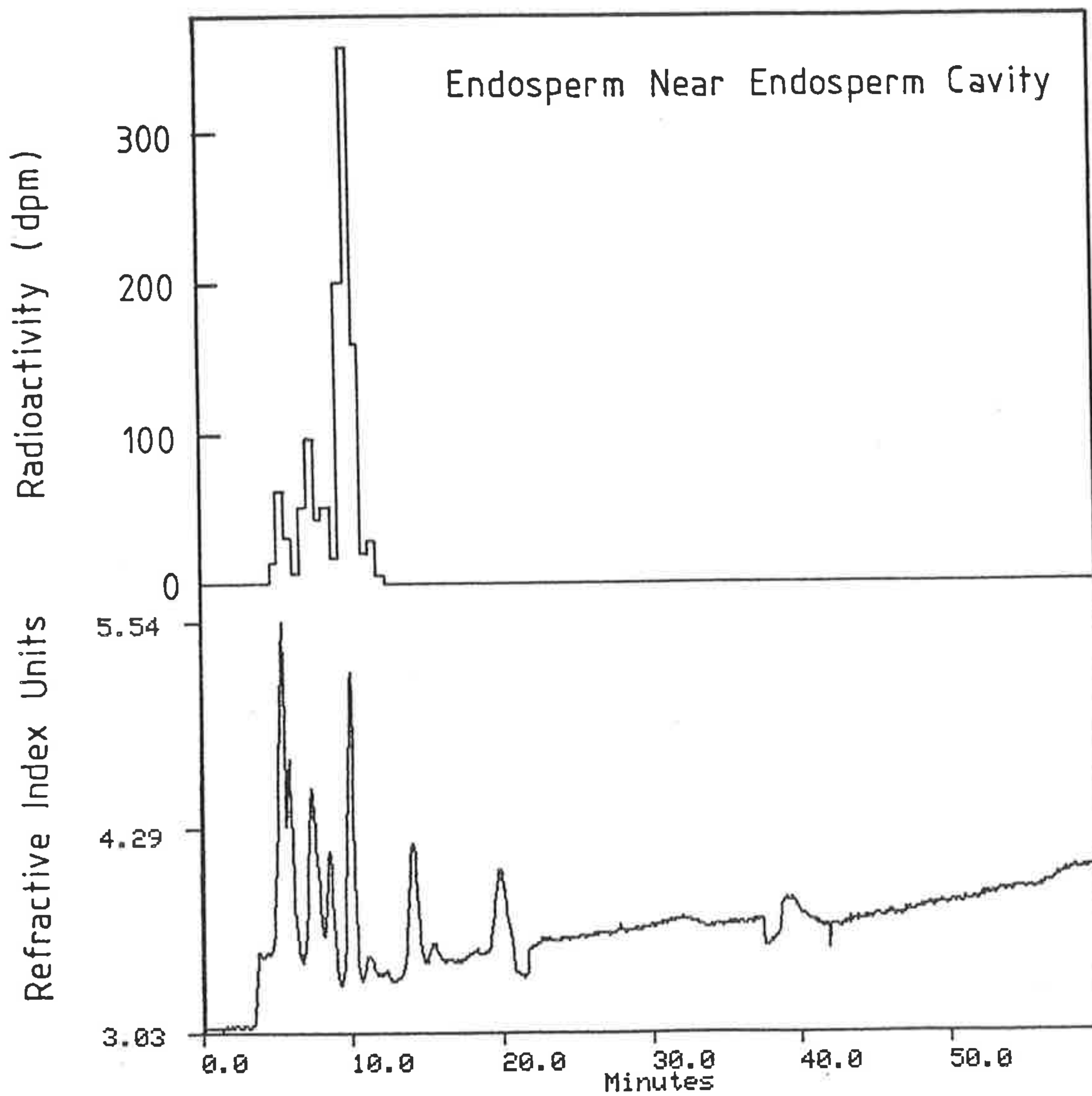

Figure 3.2-4C. As Fig. 3.2-4A, Solutes extracted from the endosperm in the region near the endosperm cavity.

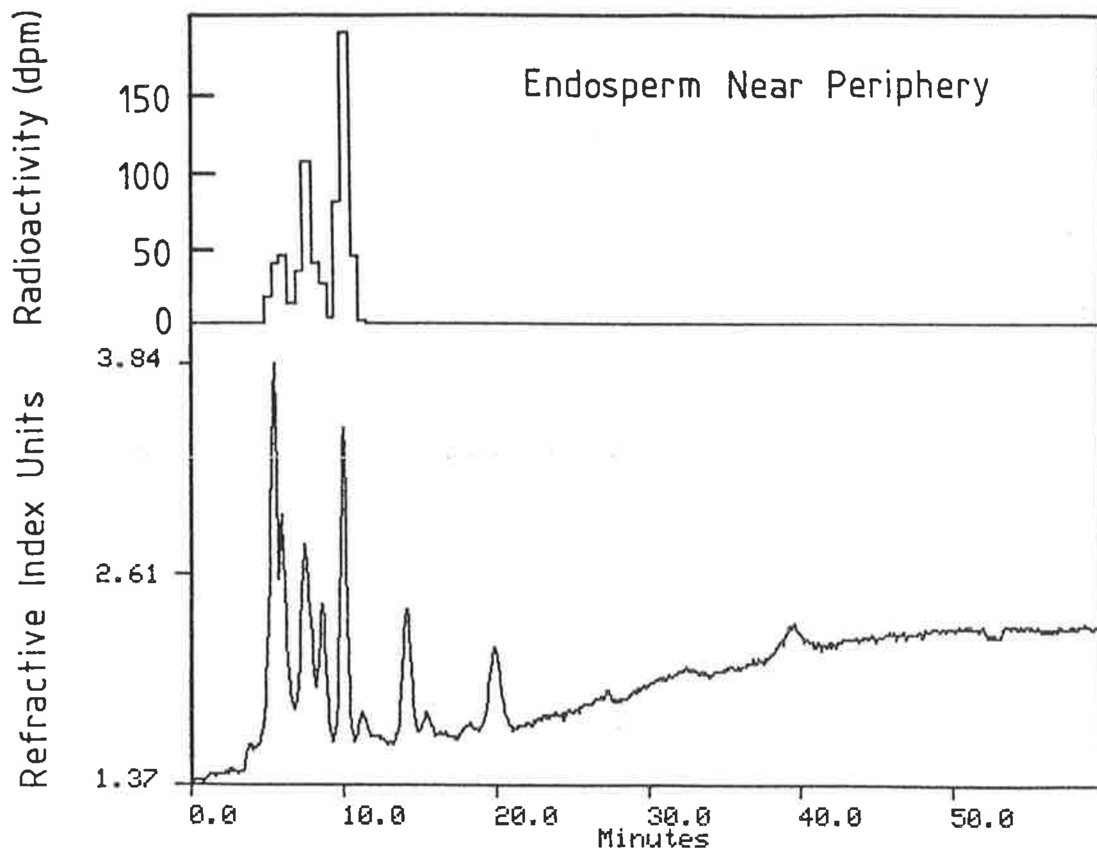

Figure 3.2-4D. As Fig. 3.2-4A, Solutes extracted from the endosperm in the region near the periphery of the grain on the dorsal surface.

Table 3.2-6. Form of radioactivity in the ethanolic extracts from the wheat grain (see Table 3.2-5). All values are dpm ^{14}C.

	Vascular Bundle (extract A)	Cavity Fluid (extract B)	Endosp'm Near Cavity (extract C)	Endosp'm Near Periph'y (extract D)
1. Total Amount	29,512	3,565	1,138	688
2. After Cation-Exchange Chromatography				
Neutral and Anionic Fraction (A)	30,112	3,214	926	490
Cationic Fraction (B)	800	215	265	212
3. After HPLC				
Total (C)	30,353	3,686	1,157	648
Soluble Carbohydrate (D)	29,163	3,510	1,045	531
4. Recoveries				
D/A	0.97	1.09	1.13	1.08
C/Total	1.03	1.03	1.02	0.94

exchange chromatography as the routine method for isolating radioactivity in the carbohydrate and amino acid fractions of the ethanolic extracts of wheat tissue (as employed in sections 3.4, 3.5 and 3.6).

5. Amount of Amino-Substrate and Carbohydrate-Substrate in the Endosperm and Endosperm Cavity Fluid

By assuming that all the free amino acids in the endosperm are available for protein deposition (see Discussion below), it can be shown from the values for the PITC analysis (i.e. Method 1 in Table 3.2-1) that the amount of amino substrate per endosperm was 237 ug. Likewise, by using a value of 6.1 ul as the volume of fluid in the endosperm cavity (Table 3.1-1) it can be shown that 34 ug of amino-substrate was contained in the cavity.

The rate of protein deposition in the endosperm was 166 $ug.day^{-1}$. This was calculated from the rate of dry matter deposition per grain of 2.3 $mg.day^{-1}$ (section 3.3), by assuming that 80% of the dry matter was deposited in the endosperm, and by assuming that the proportional deposition of protein in the endosperm was the same as the overall protein percentage at that time (see section 1.4.4.2), i.e. 9% (section 3.3).

From the above calculations there is sufficient amino-substrate in the endosperm alone to account for 1.4 days of protein accumulation. Including the endosperm cavity, this period extends to 1.6 days.

A similar calculation can be made for the amount of carbohydrate-substrate. By using the mean values from tables 3.2-2 and 3.2-4 (i.e. Endosperm, sucrose 2.7 $ug.mgfwt^{-1}$, total sugars 5.9 $ug.mgfwt^{-1}$; Endosperm cavity, sucrose 12.2 $ug.ul^{-1}$, total sugars 28.1 $ug.ul^{-1}$), and the assumptions described above, it can be shown that the endosperm contained 210 ug of sucrose and 458 ug of total soluble carbohydrate. Likewise, the endosperm cavity contained 74 ug of sucrose and 171 ug of total soluble carbohydrate.

Using 'total minus protein' as the estimate for the rate of deposition of starch in the endosperm, (i.e. 1.67 $mg.day^{-1}$) it can be shown that there is sufficient sucrose in the endosperm alone for 0.13 days (3.1 hours) of starch accumulation and sufficient total soluble carbohydrate for 0.27 days (6.6 hours). Taking the endosperm cavity into account, these time periods extend to 0.17 days and 0.37 days respectively.

Taking the case where all the soluble carbohydrate in the endosperm and endosperm cavity was an immediate source of substrate for starch deposition the turn-over of this pool would be 4.3 times more rapid than the turn-over of the pool of amino-substrate. However, when this calculation is made on the basis that sucrose only is the principal substrate for starch deposition (see above, results section 4), this turn-over ratio is 9.4.

DISCUSSION

Although the PITC method of amino acid analysis used in this study was designed principally for measuring amino acids in protein hydrolysates (e.g. Hienrikson and Meredith, 1984), its speed, reliability and good agreement with other methods of analysis (Table 3.2-1) makes it a useful technique for measuring also physiological amino acids in wheat tissue.

An improved procedure for the separation of the phenylcarbamyl derivatives has recently been developed (Cohen et al., 1986), allowing greater resolution of minor amino acids. It was unfortunate that this procedure was not available early enough for use in the current study.

Alanine and glutamine together comprise half the amino acids in the endosperm cavity. Serine, glycine and proline contribute another 10% each. Within the endosperm, however, the proportional content of these 5 amino acids is lower, comprising in total only 50%. The relative proportions of

glutamate and asparagine are considerably higher (Table 3.2-1).

It is not possible at present to design an experiment that will describe the form or forms in which nitrogen moves throughout the wheat grain, the pathways of transamination in the tissues of the grain, and the rates of turn-over of specific amino acids. This is because such an experiment relies on supplying to the grain a sharp front of radioactive amino acids in the same complement as the grain receives in vivo. Although the amino acid composition of the xylem stream to the ear has been established (Kirkman and Miflin, 1979), the composition in the phloem supplying the grain (see Zee and O'Brien, 1970a) has not.

However, for the purpose of obtaining an estimate of the amount of amino-substrate in the endosperm, itself, it seems reasonable to assume that most, if not all, of the amino acids in the endosperm would be available as substrate for protein deposition. This is because transaminases generally are abundant in nitrogen metabolising tissue (Hageman, 1979; Miflin and Lea, 1980; Miflin, 1980) and their action has been demonstrated, at least in the ear of wheat, by the ability of cultured ears to utilize asparagine, glutamine, a cocktail of amino acids or ammonium nitrate as the nitrogen source apparently with equal proficiency (Donovan and Lee, 1978).

With regard to soluble carbohydrates, there were no major discrepancies in the levels of sugars as measured by more than one technique (Tables 3.2-2 and 3.2-4). However, there are major differences between the levels of sugars found in this study and other reports in the literature. In view of the paucity of information on the levels of soluble carbohydrate in tissues of the wheat grain, spanning varieties, growing conditions and analytical techniques it is not possible to state with any assurance whether individual results are normal or the exception; likewise whether any particular reported value is likely to be subject to analytical error.

The fluid of the endosperm cavity contained low levels of hexose, only 1.6% (w/w) the amount of sucrose (cf. 2.4%, Jenner, 1974; 18% Ho and Gifford, 1984). Sucrose, itself, accounted for 43% of total soluble carbohydrate (cf. 10%, Ho and Gifford, 1984). The concentration of reducing sugar in the endosperm, relative to sucrose is higher (7%) than it is in the cavity (cf. 18%, Jenner, 1974; 168%, Ho and Gifford, 1984).

In both the endosperm and the endosperm cavity there were substantial amounts of a glucose disaccharide. In the endosperm it was 26% (w/w) the amount of sucrose, while in the endosperm cavity it was 3% (w/w) the amount of sucrose (Tables 3.2-2 and 3.2-4). Every test employed; retention time under two chromatography systems (Figs. 3.2-2, 3.2-3 and Table 3.2-3), component analysis (Results section 3B), and the reactivity to the copper bicinchoninate system with and without the hydrolytic column (Results section 3A and Table 3.2-3) produced results consistent with maltose.

Although maltose in the wheat grain has been reported before (Abou-Guendia and D'Appolonia, 1972a; see also D'Appolonia et al., 1971), the possible physiological role of this compound is still intriguing. Is it a breakdown product of starch, and if so what would be its fate? Is it involved in starch synthesis contrary to the most plausible biochemical route (see section 1.5)? Or has it a role in grain development not related directly to starch metobolism?

Ribose has been reported before (Abou-Guendia and D'Appolonia, 1973b) as has raffinose (Abou-Guendia, 1973a; D'Appolonia et al., 1971). Inositol is involved in phytic acid metabolism (Calhoun et al., 1958). In the current study however, none of these sugars were detected.

The structure of the oligosaccharides with degrees of polymerization >2 still needs to be described. The endosperm tissue and the cavity fluid contained a similar complement of oligosaccharides (Figs. 3.2-3, 3.2-4 and Results section 3B). Their relative concentrations in the endosperm and

the cavity fluid allows the suggestion that in the endosperm they may have been confined to apoplastic fluid continuous with the fluid of the cavity (especially peaks number 11 and 13, see Table 3.2-4).

Fructans, the name given to oligosaccharides with a predominance of fructose, are present in the internodes of wheat (Blacklow et al., 1984; Thome and Kuhbauch, 1985) in a series, glucose(fructose)$_2$, glucose(fructose)$_3$, etc., (Thome and Kuhbauch, 1985). From the component analysis of the oligosaccharides from the wheat endosperm and the cavity fluid (Results section 3B) it is likely that a similar series exists here. The form of these fructans may be complex. For instance, compounds with the formula glucose(fructose)$_2$ are known to exist in 4 structural forms (Stanek et al., 1965).

The relative amounts of carbohydrate-substrate and amino-substrate in the endosperm is now discussed.

The oligosaccharides with degrees of polymerization >2 contained little or no radioactivity after detached spikelets were cultured for 90 minutes on ^{14}C-sucrose. Likewise, Ho and Gifford (1984) found that even after 6 hours of feeding $^{14}CO_2$ to the flag leaf these oligosaccharides contained negligible activity. These results, while consistent with the general but unsubstantiated view (Duffus and Cochrane, 1982) that sucrose is the main source of carbon for starch synthesis in the endosperm are not sufficient, in themselves, to exclude unequivocally the involvement of longer chain carbohydrates over a longer period of time.

Nevertheless, whether or not these longer chain carbohydrates can be utilized as substrate for starch synthesis is of little consequence in the overall conclusion that the rate of turn-over of carbohydrate substrate in the endosperm is greater than the rate of turn-over of amino-substrate, at least by a factor of 4.3 and possibly up to 9.4 (Results section 5). Accordingly, the speed of carbohydrate movement through the endosperm will be greater than that of amino acids. Hence if resistances to substrate

movement throughout the endosperm are of consequence, one may expect a more steep supply gradient of carbohydrates than of amino acids. This question is examined in the following section (3.3).

3.3 ASSOCIATION BETWEEN SUBSTRATE GRADIENTS WITHIN DEVELOPING WHEAT ENDOSPERM AND REGIONAL PATTERNS OF DRY MATTER DEPOSITION

INTRODUCTION

Protein percentage throughout the endosperm of mature (dried) wheat grains increases as a function of distance from the endosperm cavity (Cobb, 1902-1904; Hinton, 1947; Morris et al., 1945, 1946; Normand et al., 1965). Staining from protein-selective dyes is more intense in the outer regions suggesting (Pomeranz and Shellenberger, 1961) that this gradient is due, at least in part, to a gradation of protein. To the contrary, data on cell size and component analysis suggests (Evers, 1970) that a gradation of starch, alone, is the main contributing factor. This issue is clouded further by the effects of grain shrinkage during maturation, by up to 35% of the fresh volume (Sofield et al., 1977). Shrinkage would have to be uniform for the gradients of polymeric products measured on the dried grain to reflect regional patterns of deposition during development.

Using an alternative approach, Briarty, et al. (1979) observed that early in development the A-type granules in the outer zones grew faster than those nearer the centre, but later this trend was reversed. From this they proposed that the rates of starch deposition throughout the endosperm may be due to regional patterns of substrate supply. The argument of this proposal is not strong, however, since there was no information on (1) the frequency distribution of A-type granules, (2) the size and frequency of B-type granules, (3) levels of substrate throughout the endosperm and, indeed, (4) whether or not it is valid to use changes in granule diameter as a measure of the rate of starch deposition.

The aims of the experiments reported in this section (3.3) were to describe the regional patterns of starch and protein deposition throughout

(the dorsal region of) the endosperm of developing grains and to establish whether or not such patterns of deposition may be due to regional patterns of substrate supply.

MATERIALS AND METHODS

Plant Material

Wheat plants, cv. Fransawi (see section 3.1 for a description of this cultivar) were grown in controlled environment conditions (sections 2.1.1, 2.1.2, 2.2.1, 2.2.2 and 2.2.3). The grains used in this study were from ears trimmed to 6 spikelets, 2 days after anthesis (sections 2.3.1 and 3.1). The b grains from the 4 central spikelets were used for experimentation, but only where each of these spikelets contained also at least the a and c grains.

Dry weight growth and fresh weight growth of such grains are shown in figure 3.3-1. Grains 20±1 days after anthesis were used for the study, being at their maximum rate of dry weight growth (2.3 $mg.day^{-1}$) even though fresh weight growth was entering a period of rapid deceleration.

Harvesting and Sectioning the Grains

Grains were harvested after 7 hours of the day cycle and immersed immediately in liquid nitrogen. The grains remained under liquid nitrogen until they were carved and sectioned. The aim of the carving procedure was to produce sections of tissue in series along the radius from the endosperm cavity to the inner pericarp on the dorsal surface at the mid-length of the grain. First, while frozen, tissue of the grain was carved away so to leave a block (2mm x 1mm in cross-section) that extended from the dorsal surface to the crease region on the ventral side. This block was sectioned transversely (i.e. across a radial axis of the grain) into pieces 150 um

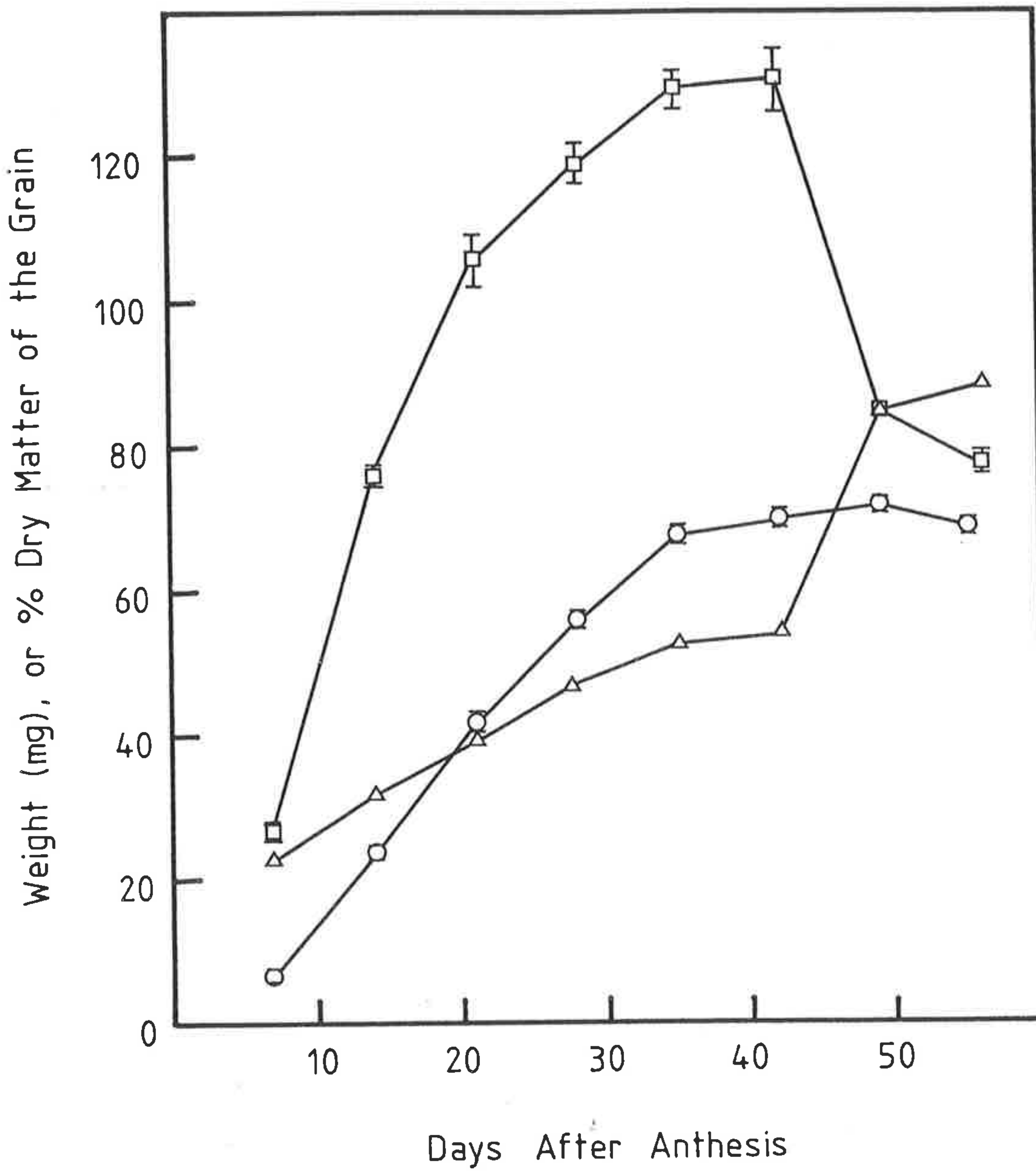

Figure 3.3-1. Dry weight (○), fresh weight (□), and percentage dry matter (△) of b grains of cv. Fransawi grown on trimmed ears. Mean ± S.E. is shown. The number of grains sampled at each sample date ranged from 12 to 21.

thick. Ice from the endosperm cavity and tissue from around and including the vascular bundle at the base of the crease were taken also. The pieces of tissue and cavity fluid remained frozen throughout until they were freeze-dried. This carving and sectioning procedure is described in more detail in section 2.5.2.

Relative Position of Each Slice of Endosperm Tissue

The frozen block of tissue carved from the grain was sectioned in an inward direction commencing at the inner pericarp (see above). The third piece of tissue sliced from this block was considered to be the first one that always comprised endosperm tissue alone. The endosperm cavity was encountered in slice number 8. The position of all slices comprising endosperm tissue only was expressed relative to a scale of 1. This was done to conform to nomenclature used elsewhere in this thesis, in sections (3.4, 3.5 and 3.6) where it was necessary to account for slight differences between-grain variation in diameter (see e.g. Table 3.4-1).

Estimating the Fresh Volume of the Sections of Tissue

Fresh volume of each section of endosperm was calculated using the measured value of dry weight (section 2.6.2) and the appropriate value of bulk density (dry weight per unit fresh volume) as determined throughout the dorsal region on a set of equivalent grains (Fig. 3.3-2, for description of methods see sections 2.6.2 and 2.7).

Analytical Techniques

Solutes in the small sections of endosperm were extracted using hot (75°C) 90% ethanol (section 2.10) and analysed by HPLC techniques. Amino acids were measured by reverse-phase separation of the phenylcarbamyl derivatives (sections 2.13.2 and 3.2). Two analytical methods were used for soluble

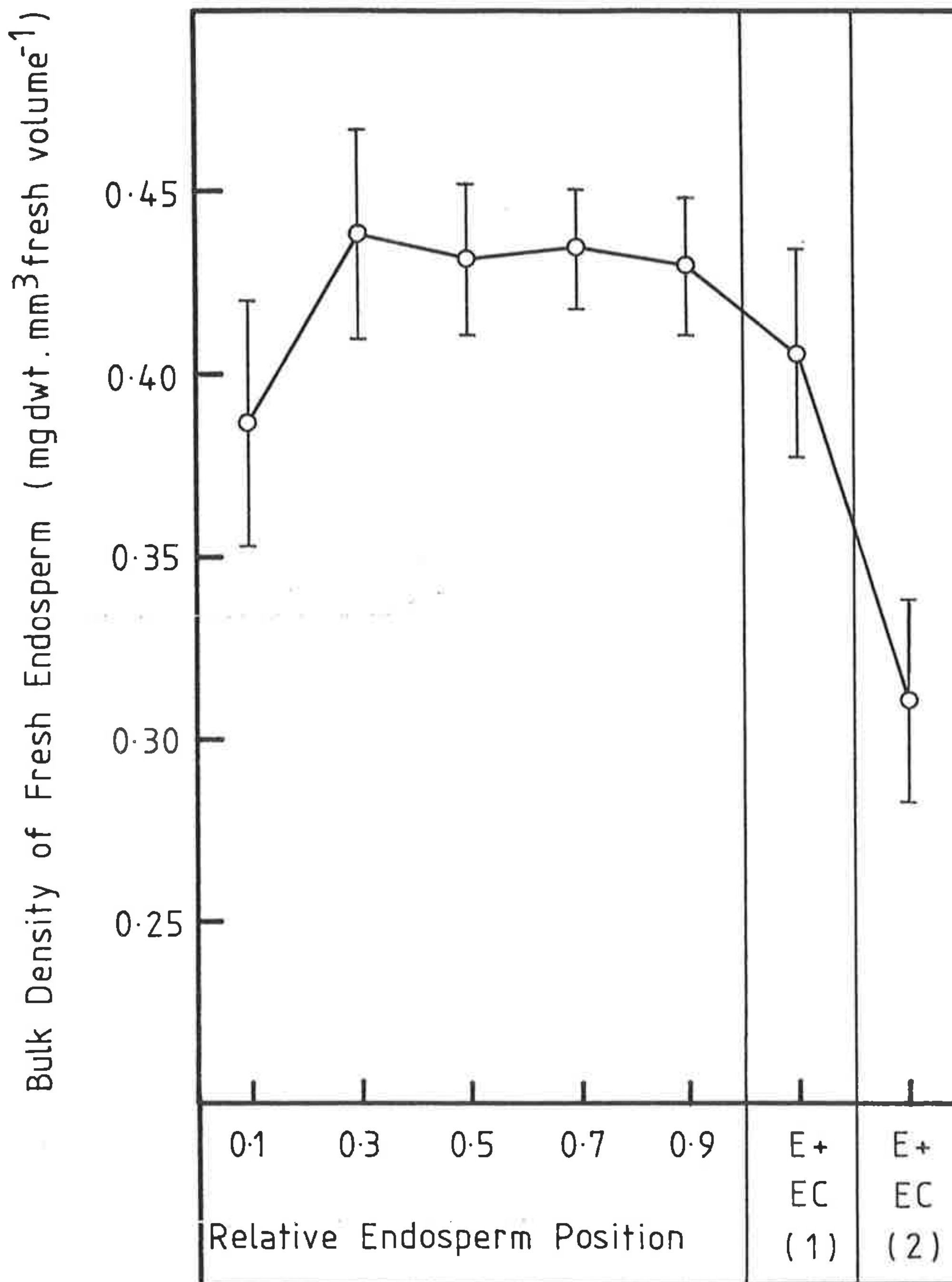

Figure 3.3-2. Profile of bulk density throughout the dorsal region of developing (fresh) endosperm (mean ± S.E. of 4 replicates).

Explanatory Notes.

E + EC (1) = Mainly endosperm plus a small amount of fluid from the endosperm cavity.

E + EC (2) = Mainly endosperm but containing a greater amount of fluid from the endosperm cavity than in the adjacent slice (see E + EC (1) above).

carbohydrates. The Waters* SugarPak* column with the copper bicinchoninate detection system (sections 2.13.1) was used to measure the levels of the glucose disaccharide (hereafter referred to as maltose, see section 3.2) and the reducing monosaccharides glucose and fructose. The Waters* DextroPak* column in series with the hydrolytic column and the copper bicinchoninate detection system (sections 2.13.1 and 3.2) was used to measure sucrose and the oligosaccharides with degrees of polymerization greater than 2. Raffinose, with an appropriate conversion factor (see Table 3.2-3) was used as the standard to quantify the unknown oligosaccharides. Total soluble carbohydrate is the sum of the major ones (see Fig. 3.3-5), these account for 96% the peak area attributable to soluble carbohydrates detected by differential refractometry (see section 3.2, this value calculated from the chromatogram shown in figure 3.2-3A).

Protein in the small sections of tissue was measured by analysing the component amino acids following acid hydrolysis of the ethanol-insoluble residue (section 2.13.3). Starch was estimated by the difference between the measured dry weight after ethanol extraction and the amount of protein.

RESULTS

1. Distribution of Starch and Protein

In the developing grains of this study, the distribution of starch along the radial profile of the dorsal region was even, apart from a slight decline in the peripheral zones (Fig. 3.3-3A). This decline was apparent also in the pattern of total dry matter deposited (Figs. 3.3-3B and 3.3-2). On the other hand, there was an increasing, near linear, relationship between the amount of protein per unit volume and distance from the endosperm cavity; the outer zones containing nearly twice the concentration of protein as the tissue adjacent to the cavity (Fig. 3.3-4A). Clearly the

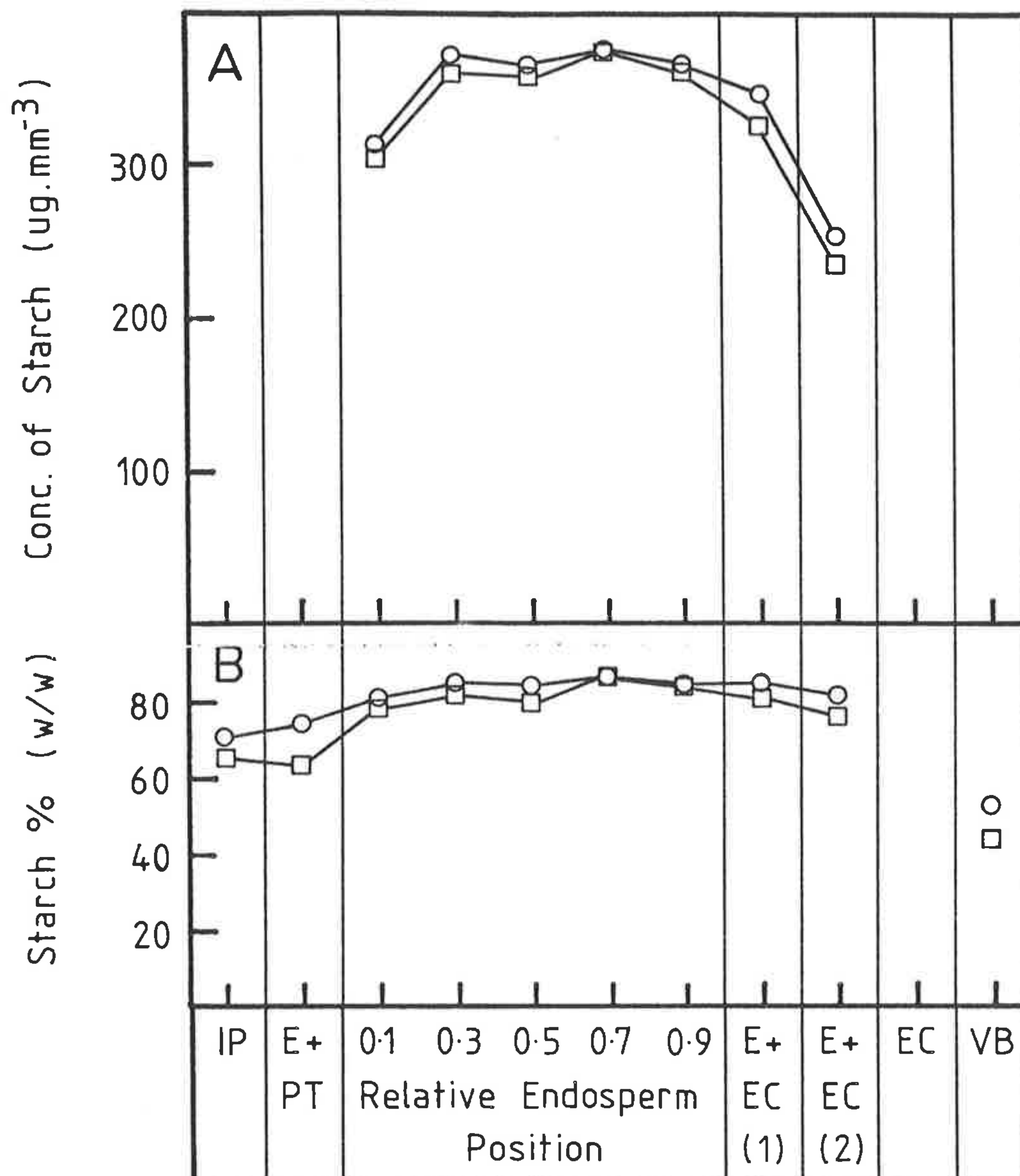

Figure 3.3-3. Concentration of starch (mass per unit fresh volume; A), and starch percentage (w/w; B) throughout the dorsal region of developing wheat grains. Results from two grains are shown.

Explanatory Notes.

IP = The section containing mostly inner pericarp tissue (dorsal surface).

E + PT = Mainly endosperm plus a small amount of peripheral tissue.

E + EC (1) = As Fig. 3.3-2.

E + EC (2) = As Fig. 3.3-2.

EC = Fluid from the endosperm cavity.

VB = Small piece of tissue taken from around and including the main vascular bundle of the grain.

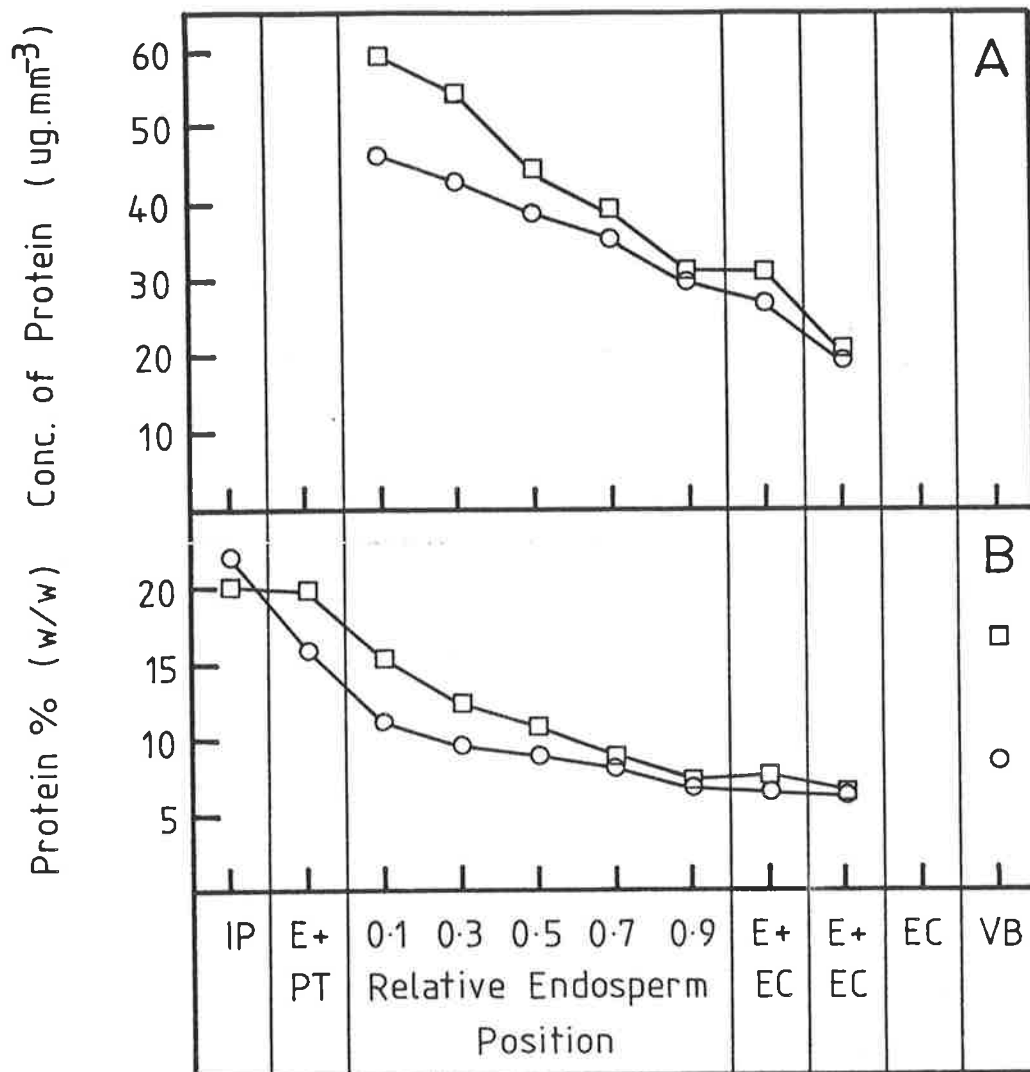

Figure 3.3-4. Concentration of protein (mass per unit fresh volume; A), and protein percentage (w/w; B) throughout the dorsal region of the same two grains of wheat as shown in figure 3.3-3. The notations IP, E + PT, E + EC (1), E + EC (2), EC, and VB are as in figure 3.3-3.

gradient of protein percentage throughout the endosperm of developing grains (Fig. 3.3-4B) is due primarily to a gradation of protein.

There was no major change in the component analysis of the protein throughout the endosperm, with glutamate (as measured, i.e. glutamine + glutamate) and proline most abundant (Table 3.3-1).

2. Distribution of Carbohydrate Substrate

Sucrose is considered to be the form in which carbon for starch metabolism is transported within the wheat grain (Duffus and Cochrane, 1982). Indeed, in short-term labeling experiments (up to 6 hours, section 3.2; Ho and Gifford, 1984) essentially all of the radioactivity in soluble carbohydrates extracted from the grain was contained in sucrose. There was a steep concentration gradient of sucrose throughout the dorsal region of the grain, decreasing as a function of distance from the cavity (Fig. 3.3-5A). This gradient was obvious both within the endosperm itself and in the slices of tissue that contained an increasing proportion of fluid from the endosperm cavity. This rise into the cavity shows that on a fresh volume basis, the concentration of sucrose in the cavity is greater than in the endosperm (as shown previously, Tables 3.2-2 and 3.2-4).

The distribution of other soluble carbohydrates was even throughout the endosperm (Figs. 3.3-5B to 3.3-5G). However, there was a rise in the concentration of the fructose-containing oligosaccharides (Figs. 3.3-5E, 3.3-5F and 3.3-5G) in the tissues containing increasing amounts of cavity fluid. These rises (also that of sucrose) and the lack of any corresponding rise in the concentration of glucose (Fig. 3.3-5B) and maltose (Fig. 3.3-5D) reflect the concentration of these solutes in the endosperm as a whole and in the fluid of the endosperm cavity as measured previously (Tables 3.2-2 and 3.2-4). The concentration of fructose appeared greater in the endosperm cavity than in the endosperm (Fig. 3.3-5C) contrary to the

Table 3.3-1. Component analysis, expressed as molar percentage, of protein throughout the dorsal region of the wheat grain. Values are the mean of 2 grains; the same grains as shown in Figs. 3.3-3 and 3.3-4.

	Inner Peric'p Section	Mainly Endo'm (1)	ENDOSPERM					Mainly Endo'm (+Cav. Fluid) (2)	Mainly Endo'm (+Cav. Fluid) (3)
			Relative Endosperm Position						
			1	3	5	7	9		
AMINO ACID									
Glutamate (+Glutamine)	27.4	33.8	33.6	31.3	33.2	32.2	34.0	28.9	27.5
Proline	9.0	11.1	12.4	12.7	12.0	11.2	11.7	10.5	9.7
Serine	6.1	6.3	6.7	7.0	6.7	6.7	6.8	7.1	6.6
Leucine	6.9	7.1	6.3	6.6	7.0	6.6	6.0	7.0	8.1
Glycine	7.3	6.0	6.2	6.5	6.0	5.5	5.3	6.2	6.5
Valine	5.1	4.7	4.5	4.7	4.5	4.6	4.5	4.8	4.6
Alanine	5.9	4.6	4.3	4.1	4.2	3.9	4.1	5.3	6.2
Tyrosine	3.6	3.5	3.7	3.8	3.7	3.7	4.0	3.6	3.7
Isoleucine	3.5	3.7	3.4	3.3	3.5	3.7	3.1	3.6	3.6
Aspartate (+Asparagine)	6.1	3.4	3.6	3.0	2.9	4.2	4.6	4.4	5.5
Phenylalanine	3.7	4.0	2.9	4.0	4.1	3.8	3.6	4.0	4.6
Arginine	4.1	3.2	3.3	3.2	3.3	3.3	3.4	3.9	3.7
Threonine	3.4	3.0	3.1	3.0	3.2	3.1	3.6	3.6	4.1
Lysine	2.7	2.0	1.7	1.9	2.1	2.1	2.2	3.0	3.6
Histidine	2.1	1.8	1.8	2.0	1.7	1.7	1.8	1.8	1.5
Methionine	1.6	0.9	1.4	1.7	0.8	0.6	0.9	0.7	0.6
Cystine	1.5	1.1	1.2	1.2	1.1	1.2	0.4	1.5	0.4

EXPLANATORY NOTES.

(1) = Mainly endosperm, but includes a small amount of peripheral tissue.

(2) = Mainly endosperm, but contains a small amount of cavity fluid.

(3) = Mainly endosperm, but contains a greater amount of cavity fluid than does the adjacent section of endosperm tissue (explanatory note 2 above).

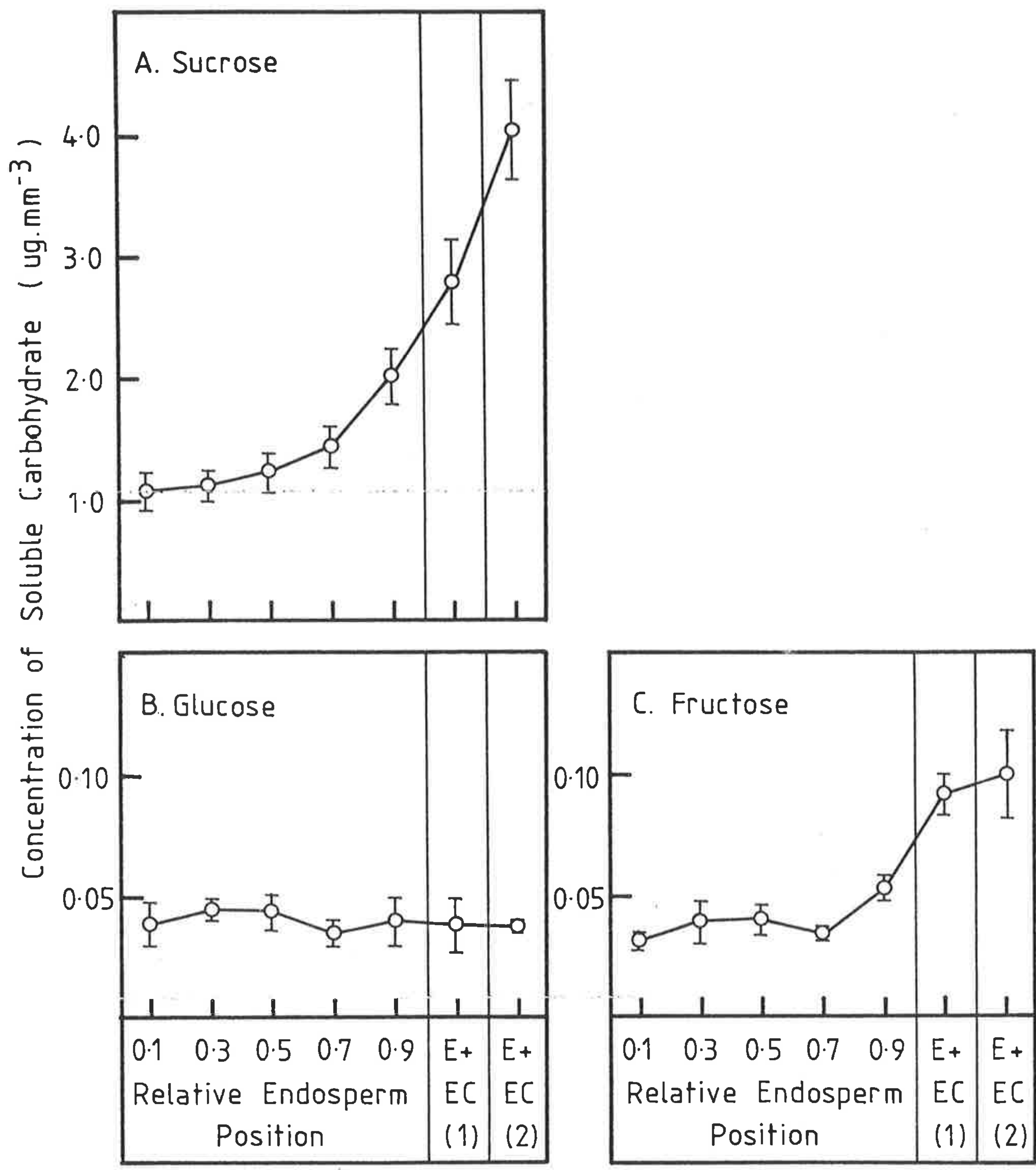

Figure 3.3-5, (Part A). Concentration of soluble carbohydrate (mass per unit fresh volume) throughout the dorsal region of developing wheat endosperm (mean ± S.E. of 5 replicates). The notations E + EC (1) and E + EC (2) are as in figure 3.3-2. See following page for part 2.

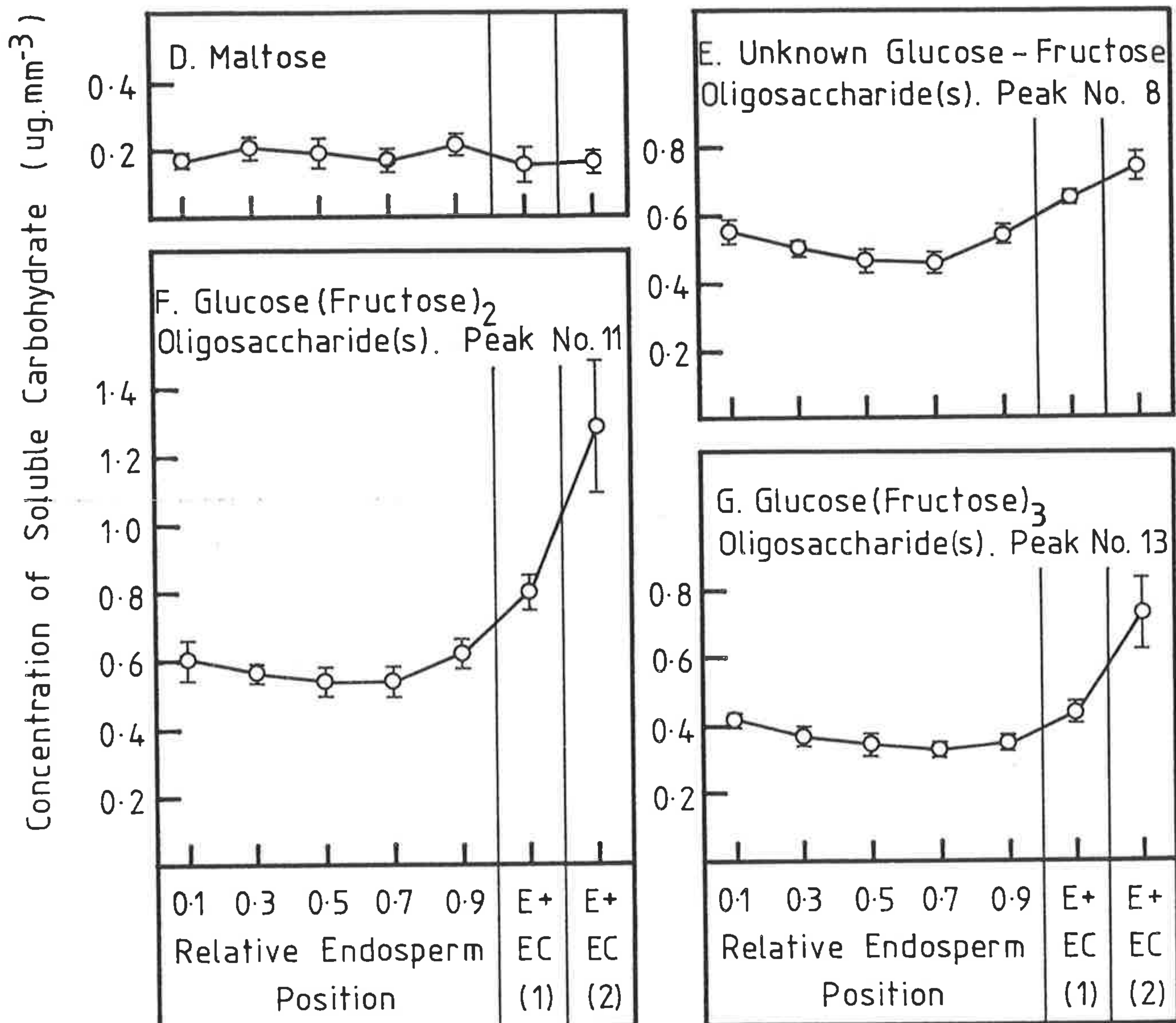

Figure 3.3-5, (Part 2). As figure 3.3-5 (Part 1), see preceding page. The peak numbers used here are the same as those used in section 3.2 to identify the carbohydrate peaks after separation of ethanolic extracts of wheat endosperm on the DextroPak* column.

earlier result where they appeared equal (Tables 3.2-2 and 3.2-4).

The standard error values shown in figures 3.3-5A to 3.3-5G may seem large, but this was always due to between-grain variation in the overall concentration of solute; the nature of the gradients was always the same (for example, Fig. 3.3-6).

Since the gradient of sucrose, especially within the endosperm itself, was of a different form to the gradients of other soluble carbohydrates, the proportional amount of sucrose in the pool of soluble carbohydrates varied also (Fig. 3.3-7), decreasing as a function of distance from the cavity.

The pattern of accumulated starch (Fig. 3.3-3A) did not follow directly the pattern of sucrose concentration (Fig. 3.3-5A). The ratio of sucrose to accumulated product (Fig. 3.3-8) decreased as a function of distance from the cavity, except for a slight rise in the outermost section where starch deposition was inordinately low (Fig. 3.3-3A). Thus in terms of substrate kinetics, the efficiency of conversion of substrate to product increases as a function of distance from the cavity except in the peripheral zone where a decrease is observed.

3. Distribution of Amino-Substrate

In both the vascular bundle section and the fluid of the endosperm cavity, glutamine, serine, glycine and alanine accounted for 80% (mole/mole) the complement of free amino acids (Table 3.3-2). Since these regions are a part of the main transporting pathway for amino acids (section 3.6) it is reasonable to expect that these amino acids are the main form in which amino-substrate is conveyed to the endosperm.

The composition of the pool of amino acids within the endosperm reflected largely the composition within the vascular bundle (Table 3.3-2). Exceptions being as follows. Glutamine and glutamate combined, formed a constant proportion of the total in all sections, but glutamate was more

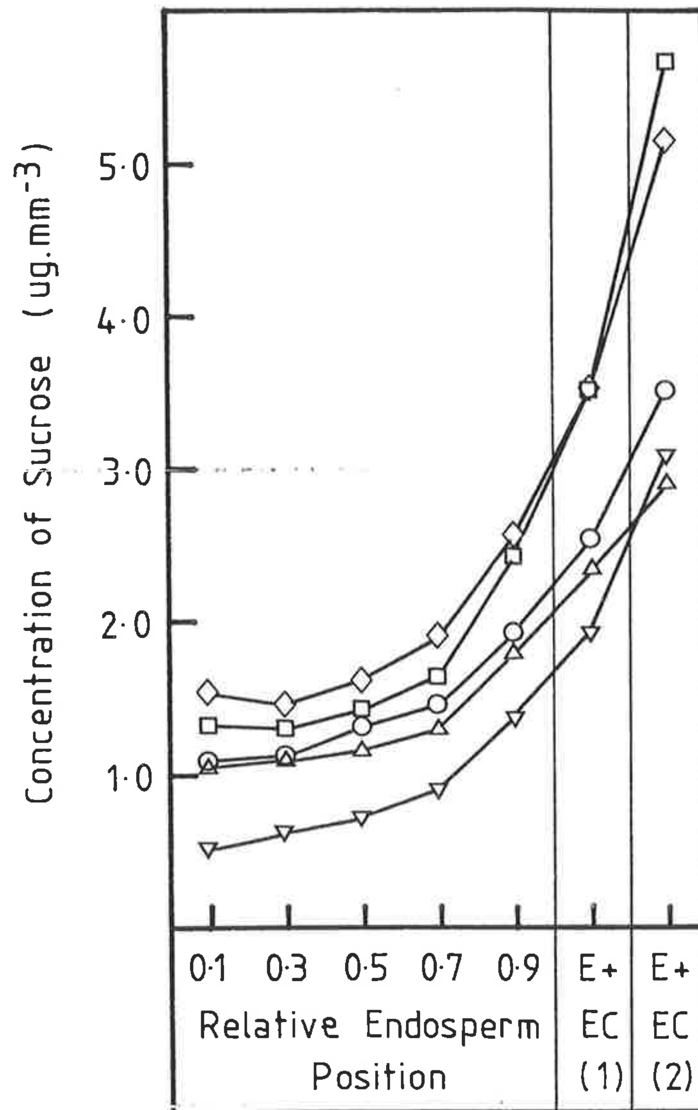

Figure 3.3-6. Individual plots of sucrose concentration throughout the dorsal region of 5 grains. The notations E + EC (1) and E + EC (2) are as in figure 3.3-2.

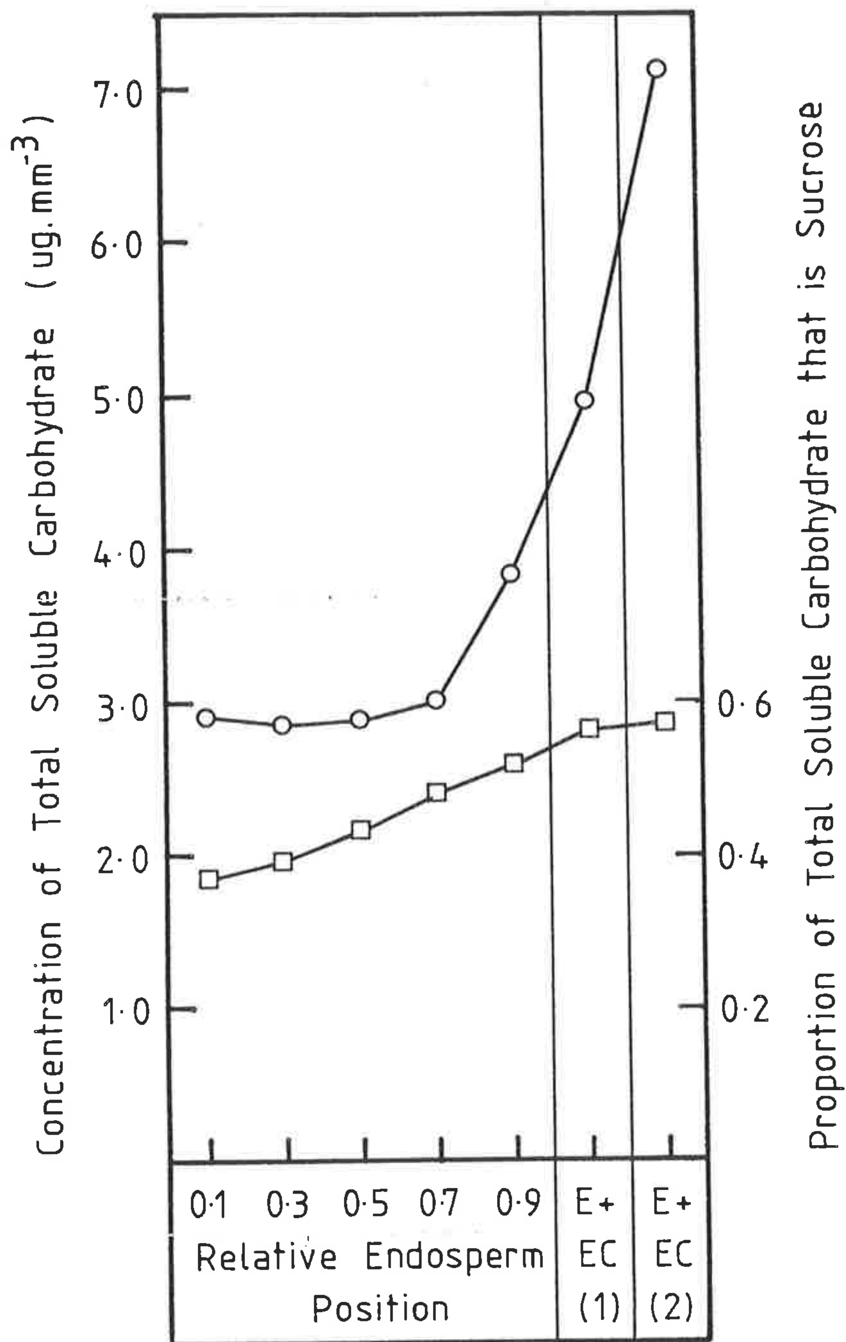

Figure 3.3-7. Concentration (mass per unit fresh volume) of total soluble carbohydrate throughout the dorsal region (o), and the proportion of this carbohydrate that is sucrose (□). The notations E + EC (1) and E + EC (2) are as in figure 3.3-2.

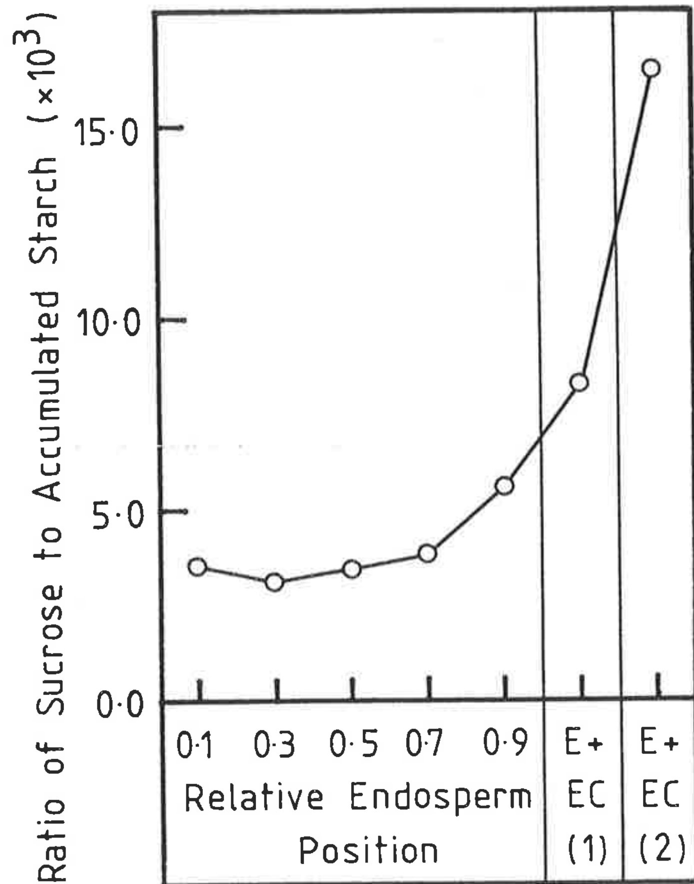

Figure 3.3-8. Ratio of sucrose to accumulated starch throughout the dorsal region of wheat endosperm. The notations E + EC (1) and E + EC (2) are as in figure 3.3-2.

Table 3.3-2. Composition of the pool of free amino acids, expressed as molar percentage throughout the dorsal region of the wheat grain. Values are the mean of 2 grains; the same grains as shown in figures 3.3-3, 3.3-4, 3.3-9A, 3.3-9B and table 3.3-1.

	Inner Peric'p Section	Mainly Endo'm (1)	ENDOSPERM Relative Endosperm Position 1	3	5	7	9	Mainly Endo'm (+Cav. Fluid) (2)	Mainly Endo'm (+Cav. Fluid) (3)	Endo'm Cavity Fluid	Vascular Bundle (4)
AMINO ACID											
Glutamine	16.4	8.0	7.0	7.5	8.6	8.5	9.1	9.9	14.0	22.5	32.6
Glutamate	19.5	22.7	21.9	22.5	20.1	20.7	20.1	20.1	15.7	1.5	3.6
Proline	2.0	1.0	1.0	1.2	1.3	1.0	0.9	0.8	0.6	0.5	0.4
Serine	8.8	9.6	9.0	8.3	7.4	8.5	8.0	7.4	8.0	8.5	5.8
Leucine	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Glycine	7.2	6.6	7.1	7.6	7.4	8.7	7.0	9.7	11.4	15.4	13.6
Valine	0.9	1.0	1.1	0.8	0.8	0.8	0.8	1.0	1.0	2.0	1.3
Alanine	20.3	28.2	30.6	30.5	33.6	32.5	36.1	32.6	32.4	32.2	27.3
Tyrosine	0.3	0.7	0.4	0.3	0.4	0.3	0.3	0.3	0.3	0.6	0.3
Isoleucine	0.2	0.3	0.3	0.3	0.3	0.3	0.3	0.5	0.5	0.5	0.4
Asparagine	6.6	2.1	4.6	4.7	3.4	3.3	2.8	2.7	3.2	3.0	3.5
Aspartate	7.6	8.1	6.2	5.4	3.6	2.8	1.7	1.3	0.9	0.9	0.7
Phenyl-alanine	0.2	0.3	0.5	0.3	0.8	1.1	1.0	1.0	0.9	1.5	0.7
Arginine	3.7	3.3	2.8	3.4	3.3	3.6	3.1	3.2	3.1	1.9	2.0
Threonine	3.7	3.0	3.2	3.3	3.1	3.2	4.1	4.4	3.7	3.2	3.8
Lysine	1.5	2.1	3.3	2.8	3.6	2.9	3.1	3.6	3.4	2.1	1.0
Histidine	0.6	0.6	0.5	0.5	0.5	0.5	0.5	0.6	0.7	1.0	0.9
Methionine	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Cystine	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	<0.1	<0.1

EXPLANATORY NOTES.

(1), (2) and (3) as for table 3.3-1.

(4) = A small sample of pericarp tissue taken from the base of the crease, surrounding and including the main vascular bundle.

abundant in the endosperm and glutamine was more abundant in the vascular bundle and cavity fluid. The proportion of glycine was lower in the endosperm than in the cavity and vascular bundle, and aspartate increased as a function of distance from the vascular bundle.

To some extent the component analysis of storage protein reflected the proportional content of the amino-acid species in the pool of free amino acids (Table 3.3-1 cf. Table 3.3-2). Contrary to this trend, however, alanine was less abundant in storage protein while some others (proline, leucine, valine, tyrosine, isoleucine, phenylalanine) were more so.

In section 3.2, it was argued that the many pathways of transamination in nitrogen metabolising tissue makes it reasonable to assume that the full complement of free amino acids in the endosperm is available as substrate for protein deposition. This seems equally valid when considering the amount of amino-substrate on a regional basis. Within the endosperm, itself, amino acids are distributed evenly, possibly with a slight increase in concentration towards the peripheral zones (Fig. 3.3-9A). The inclusion of fluid from the endosperm cavity increased the concentration of amino acids in tissue containing increasing amounts of the endosperm cavity contents.

The increase in the concentration of accumulated protein towards the peripheral zones (Fig. 3.3-4A) exceeds the increase in the concentration of amino-substrate (Fig. 3.3-9A; for ratio of the two see Fig. 3.3-10). Hence, as for carbohydrates, the outer regions of the endosperm are more efficient at the conversion of amino-substrate to insoluble product.

DISCUSSION

The milling industry has recognized for a long time that the composition of the endosperm is not uniform as flours from different mill streams have

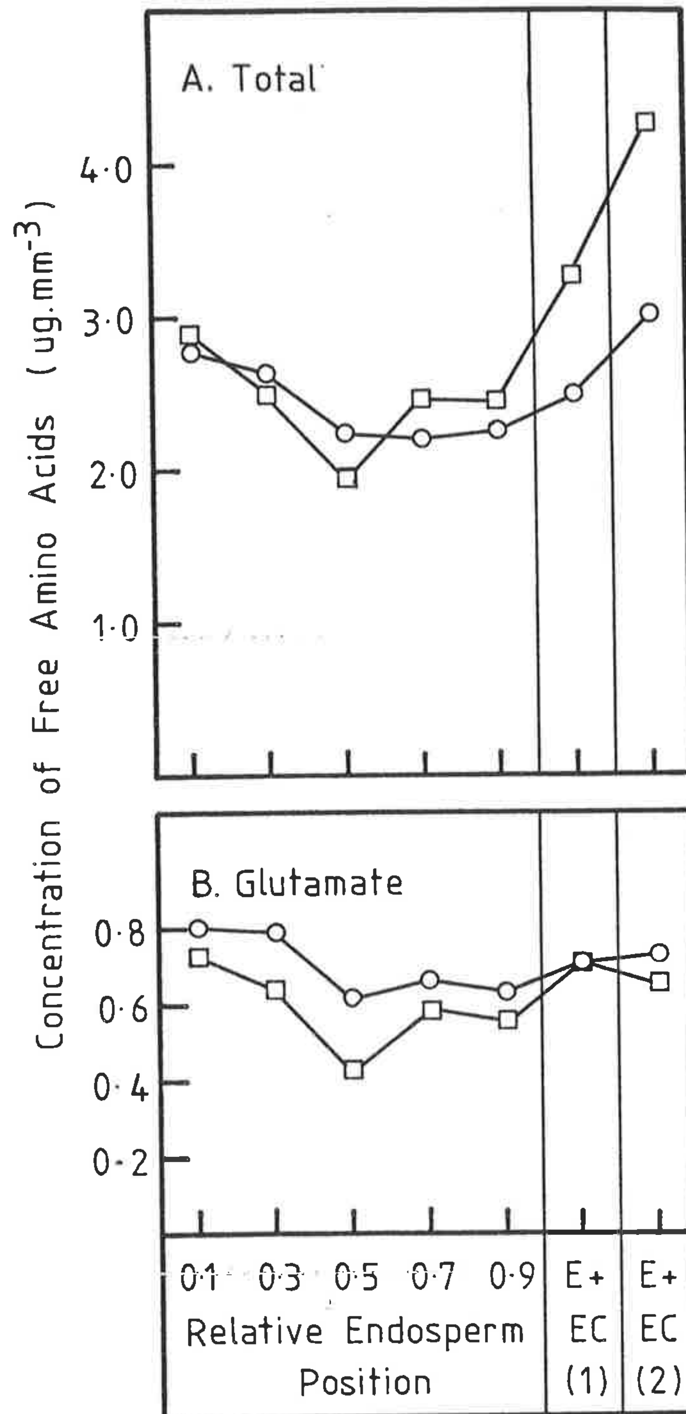

Figure 3.3-9. Concentration (mass per fresh volume) of total free amino acids (A) and of glutamate (B) throughout the dorsal region of developing wheat endosperm. Results from two grains are shown; the same grains as those shown in figures 3.3-3 and 3.3-4. The notations E + EC (1) and E + EC (2) are as in figure 3.3-2.

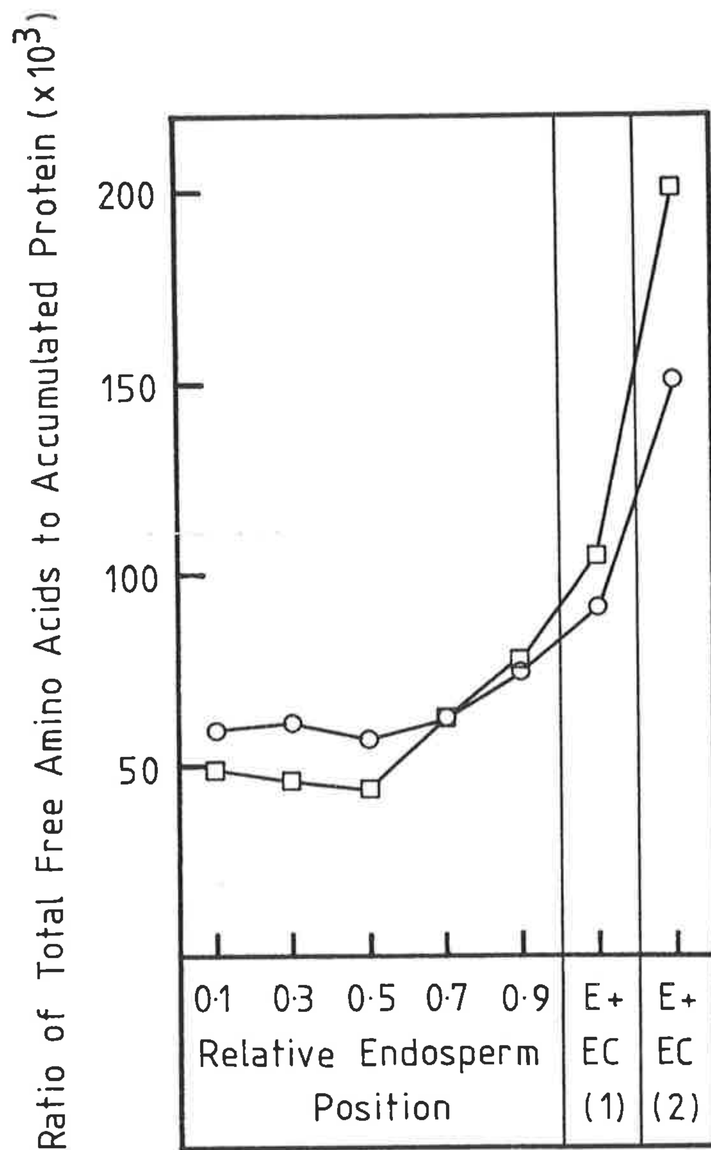

Figure 3.3-10. Ratio of total free amino acids to accumulated protein throughout the dorsal region of developing wheat endosperm. Results from two grains are shown; the same grains as those shown in figures 3.3-3, 3.3-4 and 3.3-9. The notations E + EC (1) and E + EC (2) are as in figure 3.3-2.

different rheological properties. The gradient of protein percentage increasing as a function of distance from the endosperm cavity frequently reported in mature (dry) grains (Cobb, 1902-1904; Hinton, 1947; Morris et al., 1945, 1946; Normand et al., 1965) is manifest midway through grain fill (Fig. 3.3-4B). At this time, the gradient is due almost entirely to a gradient of protein per volume (Fig. 3.3-4A), with starch essentially uniform throughout (Fig. 3.3-3A).

In comparing the regional patterns of dry matter deposition with the distribution of substrate throughout the endosperm (Figs. 3.3-8 and 3.3-10), the main assumption is that the regional patterns of accumulated dry matter measured on day 20 p.a. are a reasonable estimate of temporal deposition. In other words, it is assumed that the deposition profiles of starch and protein remain unchanged in the period leading up to day 20 p.a. This assumption seems reasonable since the overall rates of starch and protein deposition remain essentially unchanged during grain fill from about day 12 p.a. (Bilinski and McConnell, 1958; Donovan et al., 1977a, 1977b; Jennings and Morton, 1963a; McCalla, 1938), and the gradient of protein percentage at maturity is already established, at least qualitatively, by day 20 p.a. (see above). The overriding result is that in terms of substrate kinetics the efficiency of conversion of both sucrose to starch and amino acids to protein is lower in the zones of endosperm nearer the endosperm cavity than in more remote zones. Hence, in its simplest form, the proposal (Briarty et al., 1979) that the rates of dry matter deposition throughout the endosperm may be determined by gradients of substrate is not correct. One cannot exclude, however, a change in substrate gradients during ontogeny and a role of the early levels of substrate supply in the development of the systems involved in synthesizing dry matter. Later, during grain fill, the activity of these systems may be expressed independently of the substrate gradients that exist then.

The component amino acids of storage protein in the endosperm do not

reflect closely the composition of the pool of free amino acids in any of the sampled tissues (vascular bundle, endosperm cavity, endosperm; Table 3.3-1 cf. Table 3.3-2). Hence, as predicted (results section 3 above, and section 3.2), there must be substantial transaminase activity within the endosperm itself, as well as in the photosynthetic tissues of the ear (Donovan and Lee, 1978). For this transaminase activity within the endosperm , glutamine (which carries 2 amino groups), alanine and glycine are the main donors of nitrogen, while glutamate, proline, leucine, valine, tyrosine, isoleucine and phenylalanine are the main species synthesized.

The concentration of maltose in the endosperm is higher than in the endosperm cavity, by at least a factor of 2 (section 3.2). Maltose in the endosperm is not a breakdown product of any of the major soluble carbohydrates in the wheat grain since none contains a glucose dimer (section 3.2). Hence, maltose either accumulates from small amounts within the carbohydrate supply or is synthesized _de novo_ in the endosperm. Consider glutamate; its concentration in the endosperm also is higher than in the endosperm cavity, by at least a factor of 3 (Table 3.3-2, section 3.2). Like maltose, its presence in the endosperm is dependent on biochemical functions within the _cells_ of the endosperm (see Hageman, 1979; Miflin and Lea, 1980; Miflin, 1980). Therefore, the distribution profiles of maltose and glutamate may provide a guide as to the distribution of solutes that are compartmented within the endosperm cells.

Although the distribution profiles of maltose and glutamate are not identical (Figs. 3.3-5D and 3.3-9B) they are similar to the profile of total amino acids (Fig. 3.3-9A), but quite different to that of sucrose (Fig. 3.3-5A).

This raises again (see section 1.8) questions about the location of substrate within the endosperm, the direction of substrate supply and the mechanism of movement of substrate within the endosperm. For instance, is

the observed gradient of sucrose a result of a diffusional gradient within the apoplast from supply predominantly from the endosperm cavity? Is the more even distribution of amino acids due solely to the slower rate of turn-over within the endosperm (see section 3.2), or alternatively, are amino acids transfered more readily to the symplast where a supply gradient is dispersed?. As a third alternative, are amino acids supplied to the endosperm from the periphery of the grain in an inward radial direction (as is substrate to the endosperm of rice; Oparka and Gates, 1984)?

Before mechanisms and kinetics of substrate movement to and within the endosperm can be considered in any realistic way, the route(s) of substrate movement must be established unequivocally (unlike in the literature at present, see sections 1.8 and 1.9). The aim of the experiments described in the next sections (sections 3.4, 3.5 and 3.6) was to describe the route of substrate movement to and within the endosperm.

3.4 ROUTE OF ^{14}C-CARBOHYDRATE MOVEMENT WITHIN THE DEVELOPING WHEAT GRAIN

INTRODUCTION

The vascular system in a wheat floret comprises four tracheids that branch from a basal vascular bundle (Simmonds 1974b). One of these tracheids extends through the funiculus to the ovule and the other three extend through the ovary wall, one dorsal and one in each of the flanks. The lateral veins continue to the two stigmas of the flower while the dorsal one terminates blindly. Shortly after fertilization these three veins degenerate (Alexandrov, 1937; Alexandrov and Alexandrova, 1939a; Frazier and Appalanaidu, 1965; Simmonds, 1974b).

During the period 5-7 days after anthesis the tracheid that serves the ovule becomes extended the length of the grain in a process apparently unique to cereal caryopses (Alexandrov, 1937; Frazier and Appalanaidu, 1965). Concurrent with the development of the embryo sac, the ovule changes from atropic to anatropic; i.e. the axis of the ovule turns through 90° so that the micropyle is turned towards the funicle. The funicle, previously short and rounded in cross-section elongates greatly in the direction of the lengthening young grain. The funiculus and the chalazal areas remain in close contact with one another and fuse.

It is assumed that this fused tissue becomes the main conducting mass for longitudinal movement of solutes within the grain, and furthermore that solutes move from this tissue to the endosperm in a radial direction across the endosperm cavity. The main evidence for these assumptions is as follows.

1. It seems anatomically feasible (see above, also sections 1.4.3 and 1.4.4, and Sakri and Shannon, 1975)

2. The concentration of sucrose follows a decreasing gradient in the vascular bundle, the endosperm cavity and the endosperm (Jenner, 1974).

3. After culturing wheat ears on a solution containing ^{14}C-sucrose, the specific activity of sucrose in the endosperm follows the same pattern as the specific activity in the vascular bundle (Jenner, 1974).

4. Fluorescein, a symplastic tracer moves from the region of the crease into the endosperm cavity then to the endosperm (Cook and Oparka, 1983).

The studies mentioned above do not exclude the possibility that substrate is transported into the endosperm concurrently along other routes (see also section 1.8.1). Such alternative routes, circumferential or peripheral ones, may be (1) within or around the nucellar epidermis, (2) in the symplast of the aleurone layer or (3) from the pericarp tissue directly across the developing cuticular layers.

Different classes of substrate and their polymeric products differ in their patterns of distribution throughout developing endosperm (section 3.3). Soluble carbohydrates are distributed with a pronounced concentration gradient, decreasing as a function of distance from the endosperm cavity whereas amino acids are distributed more evenly. Correspondingly, the ratio of protein to starch is twice as high in the peripheral zones as in the zones closer to the endosperm cavity. The direction of substrate supply, i.e. from the endosperm cavity outwards, or from the peripheral zones inwards, is one of the factors that could contribute to this observed pattern of substrate concentration throughout the endosperm. Accordingly, studies were undertaken to describe more completely the pathways of substrate movement into the grain. The movement of labelled carbohydrate is described here and in section 3.6, while the movement of labelled amino acids is described in sections 3.5 and 3.6.

MATERIALS AND METHODS

Plant Material

Wheat plants, c.v. Fransawi (section 3.1) were grown under controlled environment conditions (sections 2.1.2, 2.2.1 and 2.2.2). Ears were trimmed to 6 spikelets 2 days after anthesis (sections 2.3.1 and 3.1). The b grains from the 4 central spikelets were used for experimentation 18±1 days later. Only ears in which each of these 4 central spikelets contained also at least the a and c grains were used for these experiments.

Treatments

Wheat plants were taken from the controlled environment facility to the laboratory (21°C) on the evening before the day of experimentation. In this way the plants did not experience any 'day' period of the diurnal cycle prior to experimentation. Spikelets and grains were prepared, always at the same time (mid-morning) using treatments designed to disrupt the putative transport pathways in the grain. The treatments used were coded as follows and reference is made to detailed descriptions made earlier.

1. Grains within intact spikelets (control grains); section 2.3.3.4

2. Grains prepared for experimentation; section 2.3.3.5

3. Detached grains; section 2.3.3.1

4. Grains with transport in the vascular bundle disrupted; section 2.3.3.2

5. Grains with transport in circumferential routes disrupted; section 2.3.3.3

Replicates of each treatment were allocated to different spikelet

positions on different ears so as to cancel any positional effects.

Feeding $^{14}CO_2$ to Illuminated Ears

Grains were supplied with radioactive substrate immediately after the surgical treatments (see above) were imposed. Ears were illuminated in a $^{14}CO_2$-rich atmosphere for a preset time (30, 60 or 90 minutes) as described in section 2.4.1.

Harvesting and Sectioning the Grains

Movement of radioactivity into and within the grains was stopped by removing the grains from the ear and immersing them immediately in liquid nitrogen. The grains remained under liquid nitrogen until they were carved and sectioned. The aim of this carving and sectioning procedure was to obtain small pieces of tissue in series along a proposed transport route. First while frozen, pieces of the grain were carved away so to leave a block (2 mm x 1 mm in cross-section) in the midpoint of the grain extending from the inner pericarp, usually on the dorsal side, to the crease region of the grain. This block was sectioned transversely (i.e. along a radial axis of the grain) into pieces 150 um thick. Ice from the endosperm cavity and tissue from around and including the vascular bundle at the base of the crease was taken also. The pieces of tissue and cavity fluid remained frozen throughout until they were freeze-dried. This carving and sectioning procedure is described in more detail in section 2.5.2.

Measuring Radioactivity in Fractions Extracted from the Small Pieces of Tissue Sectioned from the Grain

The small pieces of dried tissue were weighed (section 2.6.2) before the solutes were extracted in hot 90% ethanol (section 2.10) and fractionated by ion-exchange techniques into a cationic fraction and a neutral and anionic fraction (section 2.11). Radioactivity in these fractions and in

the insoluble residue was measured by liquid scintillation procedures (sections 2.12.2 and 2.12.3).

Form of the Radioactivity in the Neutral and Anionic Fraction

In a previous study (section 3.2), radioactivity in the neutral and anionic fraction after cationic exchange chromatography (section 2.11) was accounted for fully by radioactivity contained in solutes identified by HPLC as soluble carbohydrates (see also section 2.11). This was the case in extracts from the vascular bundle, the endosperm cavity, the endosperm near the endosperm cavity and the endosperm near the periphery of the grain. Thus, it was deemed reasonable, to use the cationic exchange procedure as the routine method for isolating from wheat grain tissue the radioactivity contained in soluble carbohydrates. Hereafter, the neutral and anionic fraction will be referred to as the soluble carbohydrate one.

The Relative Position of Each Section of Endosperm Tissue and the Normalizing of Radioactivity Values

The frozen block of tissue carved from the grain midway along its length was sectioned in an inward direction commencing at the inner pericarp (see above). The third piece of tissue sliced from this block was considered to be the first one that always comprised endosperm tissue alone. The endosperm cavity was encountered usually in slice number 8, but sometimes, due to variation in the dimensions of the grain, in slice 7 or 9 (for further details see section 2.5.2). The position of all slices comprising endosperm tissue only was expressed relative to a scale of 1 to account for this between-grain variation (for an example, see Table 3.4-1).

The total amount of radioactivity extracted from the grains varied also. In order to compare directly the profile of radioactivity along the sampled region in a number of grains, the specific activity value (dpm.

Table 3.4-1. An example of the procedures by which the position of slices comprising only endosperm tissue was expressed relative to 1, and the specific radioactivity of all slices was normalized to the specific activity in the slices of endosperm taken as a whole. This example is for the soluble carbohydrate fraction of a grain within an intact spikelet after the ear was illuminated for 90 minutes. The information here is reported in graphical form in Figs. 3.4-1C and 3.4-2B.

Slice Number	Type of Tissue	Relative Endosp'm Position	Dwt. (mg)	Dpm (^{14}C)	$Dpm.mg^{-1}$	Normal'd ^{14}C Value
1	Inner Pericarp (plus other peripheral tissue)		.166	486	2928	1.13
2	Mainly Endosperm (plus peripheral tissue)		.145	289	1993	0.77
3	Endosperm Section 1	0.1	.161	280	1739	0.67
4	Endosperm Section 2	0.3	.163	294	1804	0.69
5	Endosperm Section 3	0.5	.166	353	2127	0.82
6	Endosperm Section 4	0.7	.174	494	2839	1.09
7	Endosperm Section 5	0.9	.143	678	4741	1.82
	(Totals for Endosperm)		(.807)	(2099)	(2601)	
8	Mainly Endosperm (plus cavity fluid)		.132	1829	13,856	5.33

$mg dwt^{-1}$) in each slice was normalized relative to the specific activity value throughout the group of endosperm slices taken as a whole. An example of this procedure is shown also in table 3.4-1.

RESULTS

1. Pattern of Radioactivity as a Function of Time

After the ears were illuminated in the $^{14}CO_2$-rich atmosphere for 30 minutes, radioactivity was found in the tissue surrounding and including the main vascular bundle of the grain and in the fluid of the endosperm cavity (data not shown). Radioactivity was found also in the sections of endosperm tissue adjacent to the endosperm cavity and in the peripheral tissues on the dorsal side of the grain (Fig. 3.4-1A). After longer periods of time radioactivity was detected in all sections throughout the dorsal region (Figs. 3.4-1B and 3.4-1C). Within the endosperm itself, there was a gradient of radioactivity, decreasing away from the endosperm cavity. Over time this gradient became less pronounced.

At all times and in all pieces of tissue the majority of radioactivity was contained in the soluble carbohydrate fraction (Figs. 3.4-1A, 3.4-1B and 3.4-1C). Of the radioactivity in the insoluble fraction essentially all was contained in starch, only small amounts appeared in other polymeric products (details of this study are presented in section 3.6). Since the subject of the current study (section 3.4) is the movement of soluble carbohydrates, all results that follow will describe only the distribution of radioactivity in this fraction.

2. Effect of Preparing the Grain for Experimentation

It was necessary to manipulate the spikelets, florets and grains to provide access to the grain so that treatments designed to disrupt the putative transport pathways could be applied (see Materials and Methods, above). The

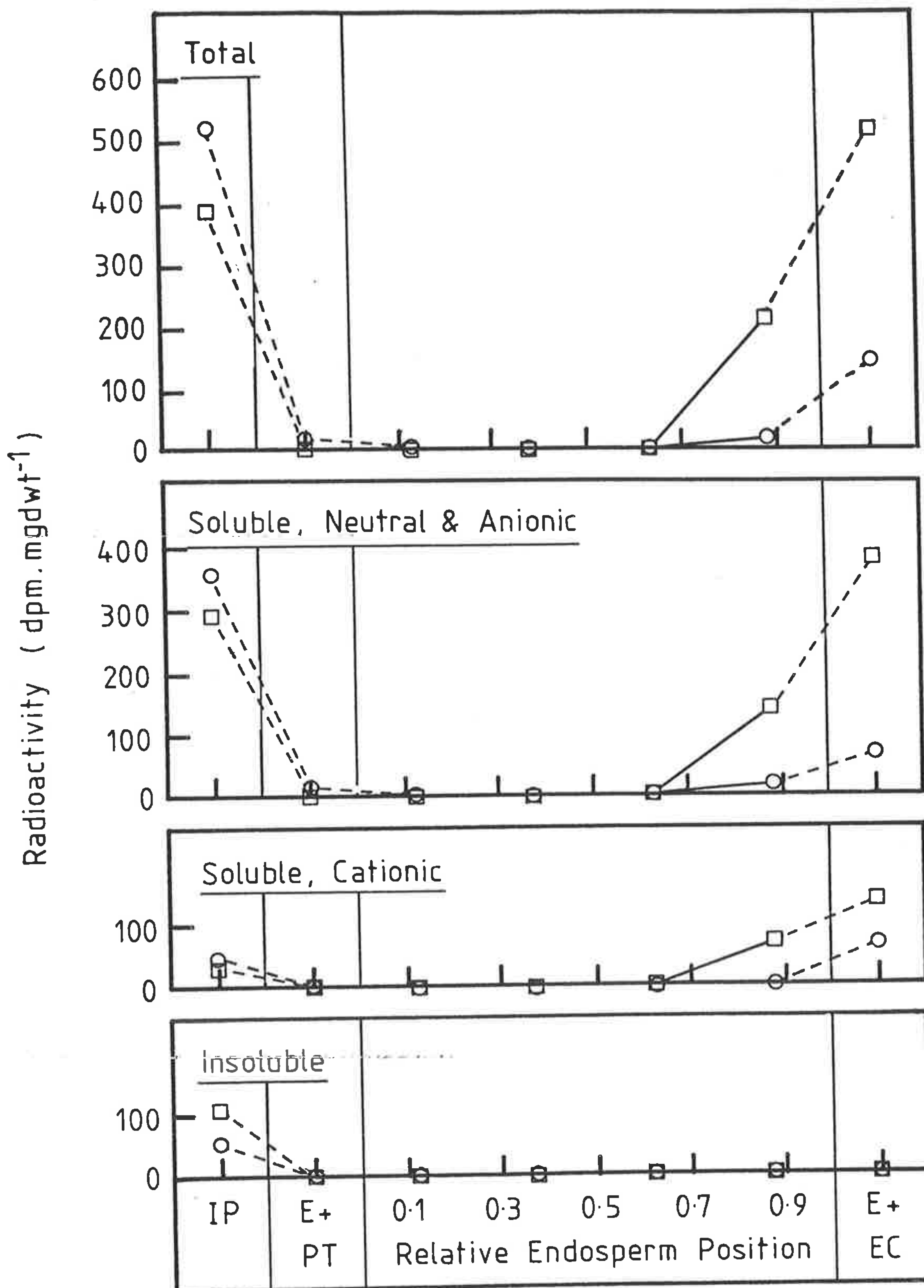

Figure 3.4-1A. Distribution of radioactivity across the sampled region (dorsal side) of grains after ears were exposed for 30 minutes to $^{14}CO_2$ in the light. Two grains are shown. Radioactivity in each of the three extracted fractions is plotted separately.

Explanatory Notes.

IP = The section containing mostly inner pericarp tissue (dorsal surface).

E + PT = Mainly endosperm plus a small amount of peripheral tissue.

E + EC = Mainly endosperm plus a small amount of fluid from the endosperm cavity.

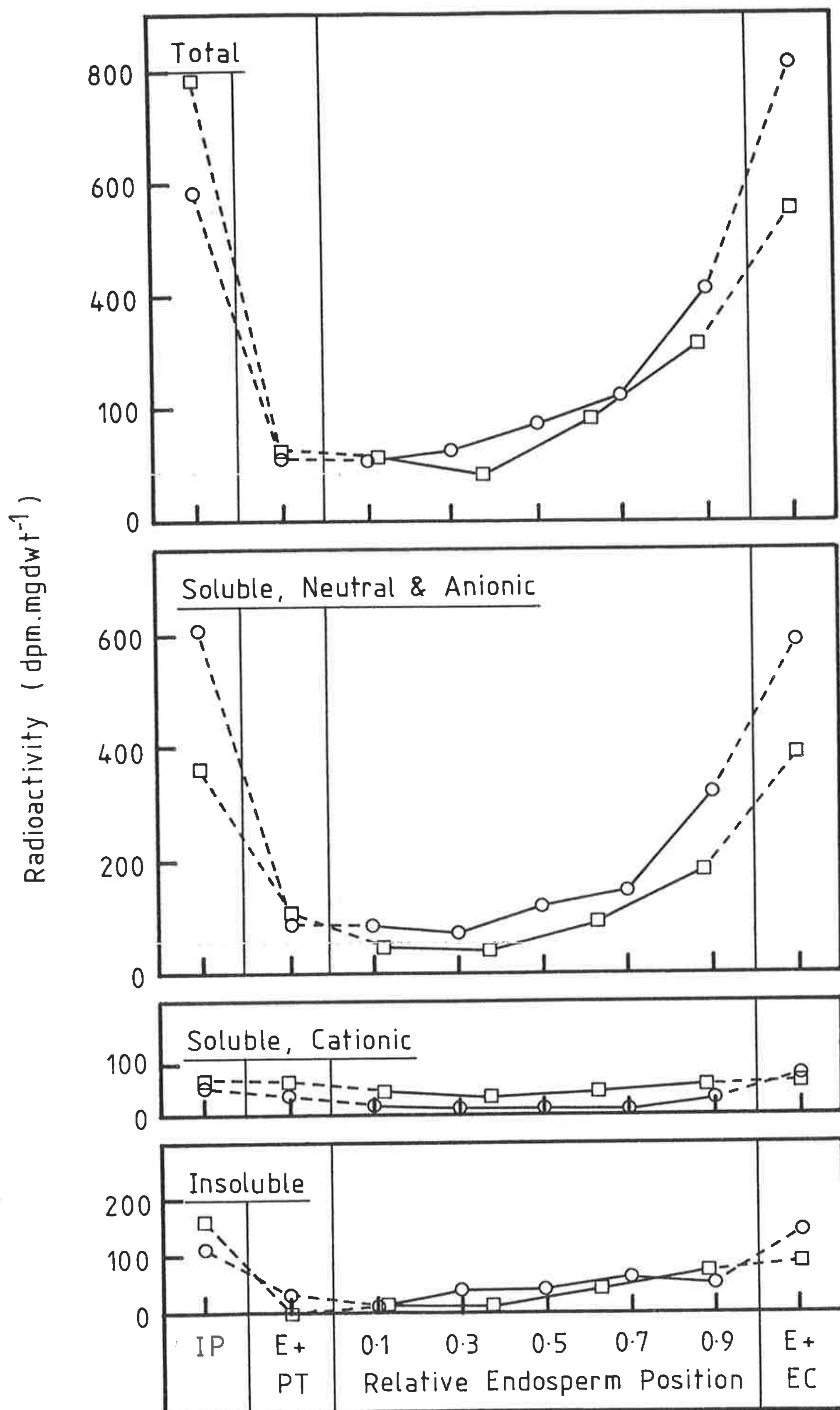

Figure 3.4-1B. As Fig. 3.4-1A, after ears were exposed for 60 minutes.

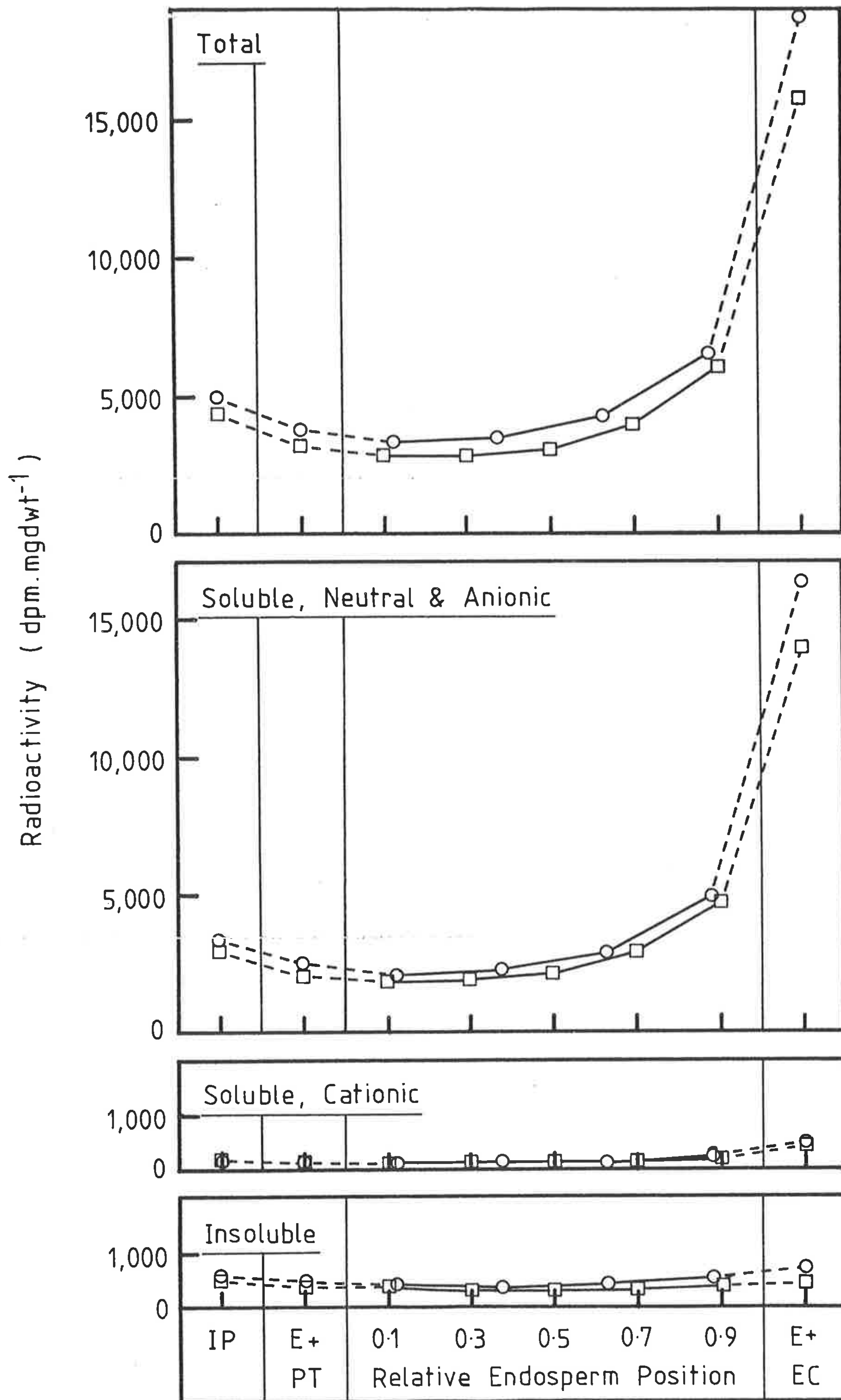

Figure 3.4-1C. As Fig. 3.4-1A, after ears were exposed for 90 minutes.

purpose of the experiment reported here was to test whether these manipulative treatments in themselves altered the magnitude or pattern of solute movement into the grain.

The manipulative treatments reduced by 50% the amount of radioactivity extracted from the grain after ears were illuminated for either 60 minutes or 90 minutes (Table 3.4-2). There was no effect, however, on the pattern by which radioactivity was distributed throughout the dorsal region of the grain (Figs. 3.4-2A and 3.4-2B).

3. Effect of Disrupting Transport in the Stalk

It is most unlikely that that radioactive solutes could move from the plant to the grain across the region of the broken stalk. Indeed, this was shown unequivocally to be the case in the experiments of section 3.6. Grains with transport in the stalk disrupted still contained radioactivity, albeit in amounts substantially less than in attached grains (Table 3.4-2), demonstrating photosynthetic activity in the grain (inner pericarp) itself.

The concentration of radioactivity in the inner pericarp of detached grains within intact spikelets was nearly twice the concentration in the pericarp of grains still attached to the plant (Table 3.4-2). This result is not considered further as it is not possible to distinguish between a possible stimulatory effect on the amount of ^{14}C fixed by the pericarp or a decrease in the amount exported from this tissue.

The distribution pattern of radioactivity within the sampled region of detached grains is now described.

After 60 minutes, radioactivity was found in the peripheral slice (inner pericarp tissue) and the tissue surrounding and including the main vascular bundle, but at this time not in the endosperm cavity or any of the endosperm slices. At 90 minutes, however, radioactivity was found also in the endosperm cavity and extending into the slices of endosperm adjacent to

Table 3.4-2. Radioactivity extracted from the grain and the inner pericarp segment on the dorsal side as influenced by manipulative treatment on the grain and duration for which the ear was illuminated in the $^{14}CO_2$ rich atmosphere.

Description of Grain Treatment	n	Dpm (^{14}C) in the grain (x, ± SE where n = 4)	Dpm (^{14}C) in detached grains proportional to dpm ^{14}C in attached grains	Radioactivity in inner pericarp section (dpm.mgdwt^{-1})
1. After 60 minutes				
Grain within intact spikelet	4	56,482±10,078		591±118
Grain detached within intact spikelet	2	20,189	0.36	1,005
Grain prepared for experimentation	4	29,441±9,184		498±122
Grain detached and prepared for experimentation	2	6,215	0.21	454
Grain with vascular bundle disrupted	2	15,420		610
2. After 90 minutes				
Grain within intact spikelet	2	589,321		3,549
Grain detached within intact spikelet	2	163,425	0.28	5,140
Grain prepared for experimentation	4	292,553±102,593		2,347±862
Grain detached and prepared for experimentation	2	48,524	0.17	1,907
Grain with vascular bundle disrupted	2	54,639		1,033

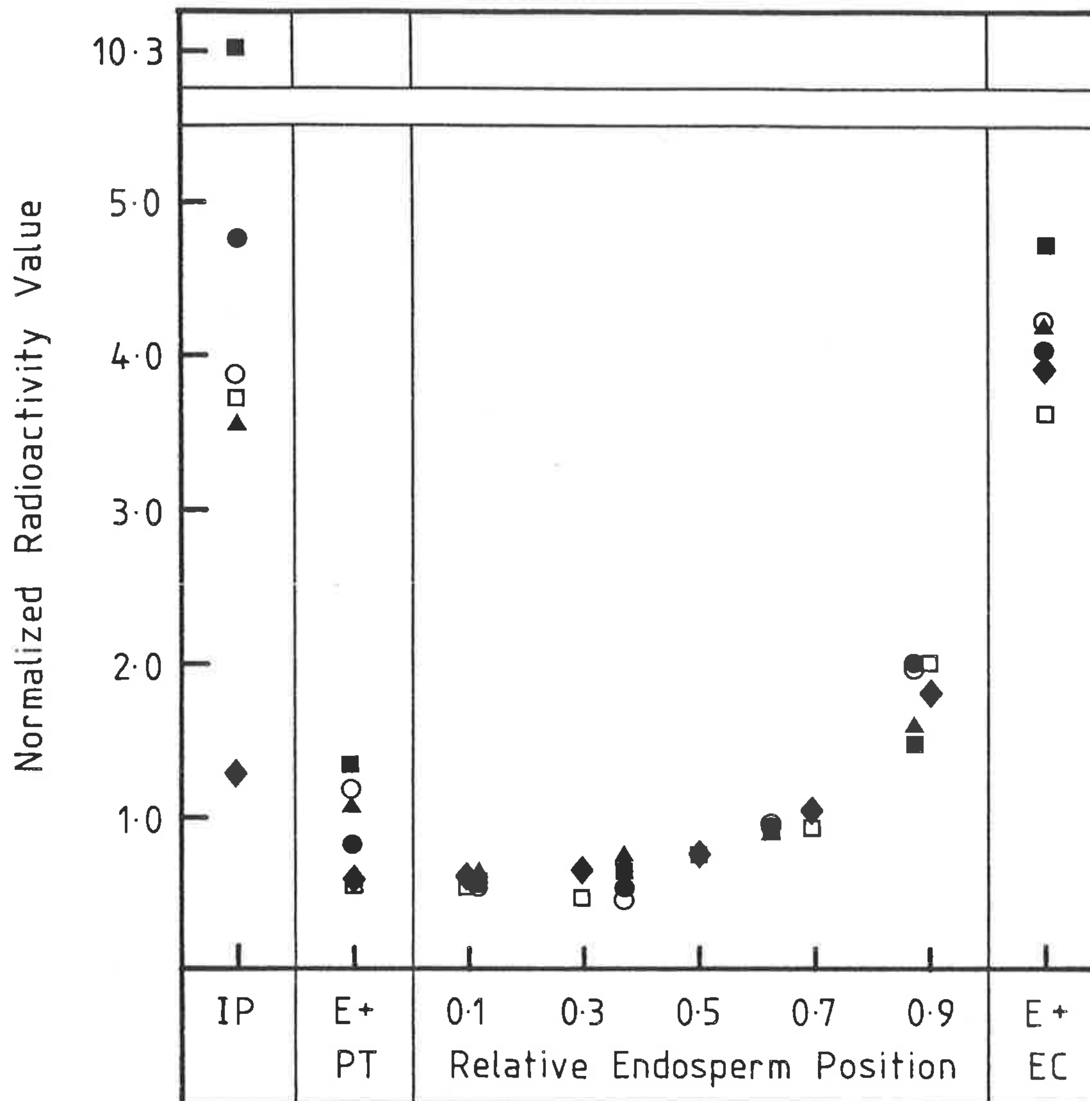

Figure 3.4-2A. Pattern of the normalized radioactivity value in the soluble carbohydrate fraction extracted from the sampled region (dorsal side) of wheat grains after ears were exposed for 60 minutes to $^{14}CO_2$ in the light. The open symbols are for grains within intact spikelets (n=2) and closed symbols are for grains prepared for experimentation (n=4). Different symbols denote separate grains. The symbols used here for the intact grains correspond to the same symbols and grains shown in Fig. 3.4-1B. The notations IP, E + PT and E + EC are as in Fig. 3.4-1A.

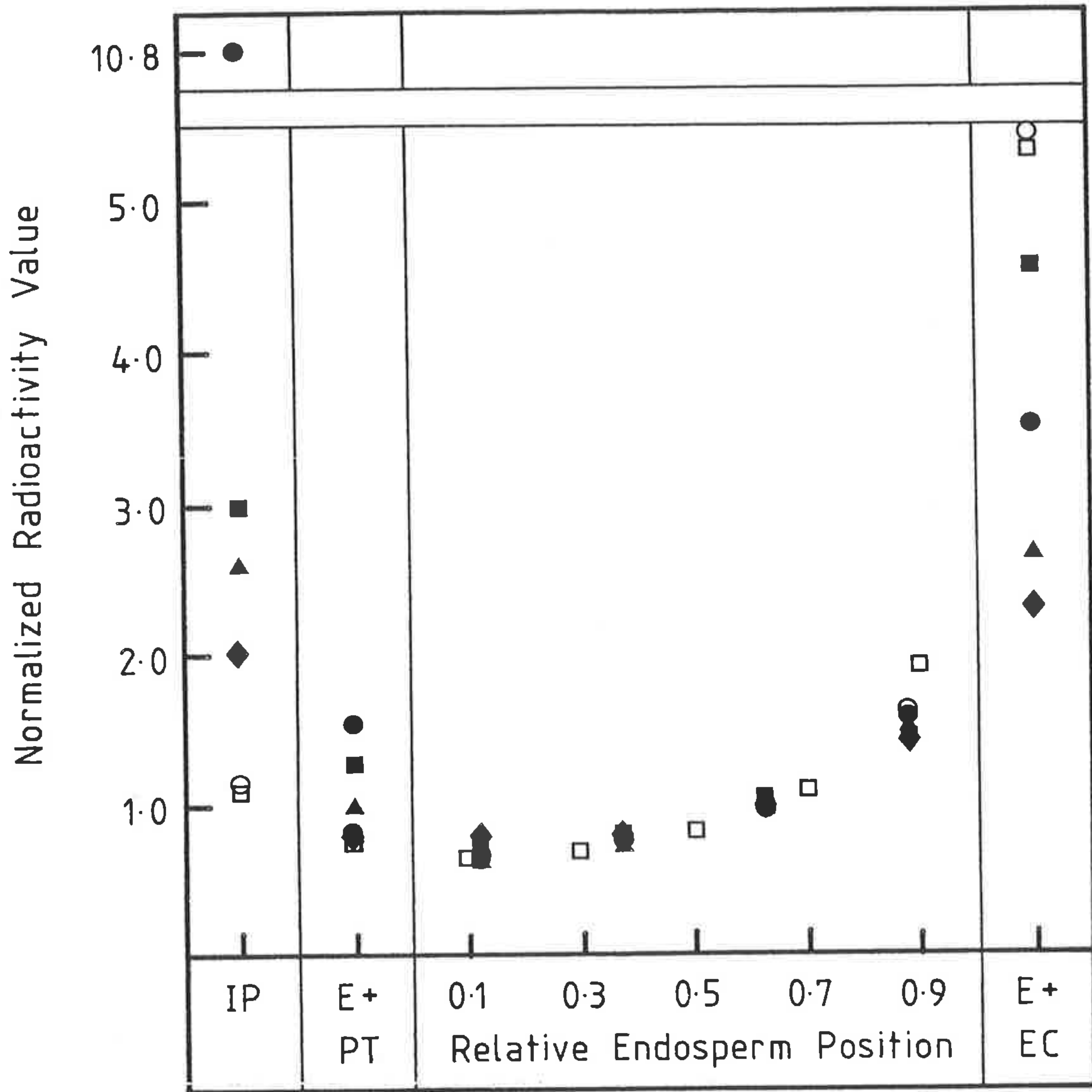

Figure 3.4-2B. As Fig. 3.4-2A, after ears were exposed for 90 minutes. The symbols used here for the intact grains correspond to the same symbols and grains shown in Fig. 3.4-1C.

the cavity (results not plotted in toto, but see Fig. 3.4-3 below).

In one grain that was detached and within an intact spikelet there was sufficient label to normalize the radioactivity values even though the amount of radioactivity in its endosperm was only 2% the radioactivity contained in the endosperm of a comparable attached grain (see Fig. 3.4-1C). The distribution profile of radioactivity in the endosperm of this grain (Fig. 3.4-3) was similar to that obtained when radioactive substrate was supplied external to the grain (Figs. 3.4-2A and 3.4-2B). In this grain there was a 50-fold difference in the concentration of radioactivity between the peripheral tissue and the adjacent (outermost) slice of endosperm (Fig. 3.4-3). In detached grains with less radioactivity in the endosperm, this value was even higher. This compares with a 4-fold gradient in attached grains (Fig. 3.4-2B).

The sequence of tissues into which radioactivity appeared, the profile of radioactivity throughout the endosperm itself and the apparent lack of any relationship between the steepness of the ^{14}C gradient between the peripheral tissue and the dorsal-most region of endosperm all lead to the conclusion that radioactive carbohydrates do not move from the inner pericarp to the endosperm directly in an inward radial direction. Instead solutes from the inner pericarp move to the endosperm principally via the endosperm cavity.

4. Effect of Disrupting Transport in the Vascular Bundle

Disrupting transport in the vascular bundle reduced substantially the amount of radioactivity that moved into the grain (Table 3.4-2). This treatment had less effect on the concentration of radioactivity in the peripheral tissues on the dorsal surface however (Table 3.4-2). No radioactivity could be detected in any section taken from the endosperm cavity and endosperm distal to the blockage after both 60 and 90 minutes (results not plotted). Therefore, the vascular bundle within the pericarp

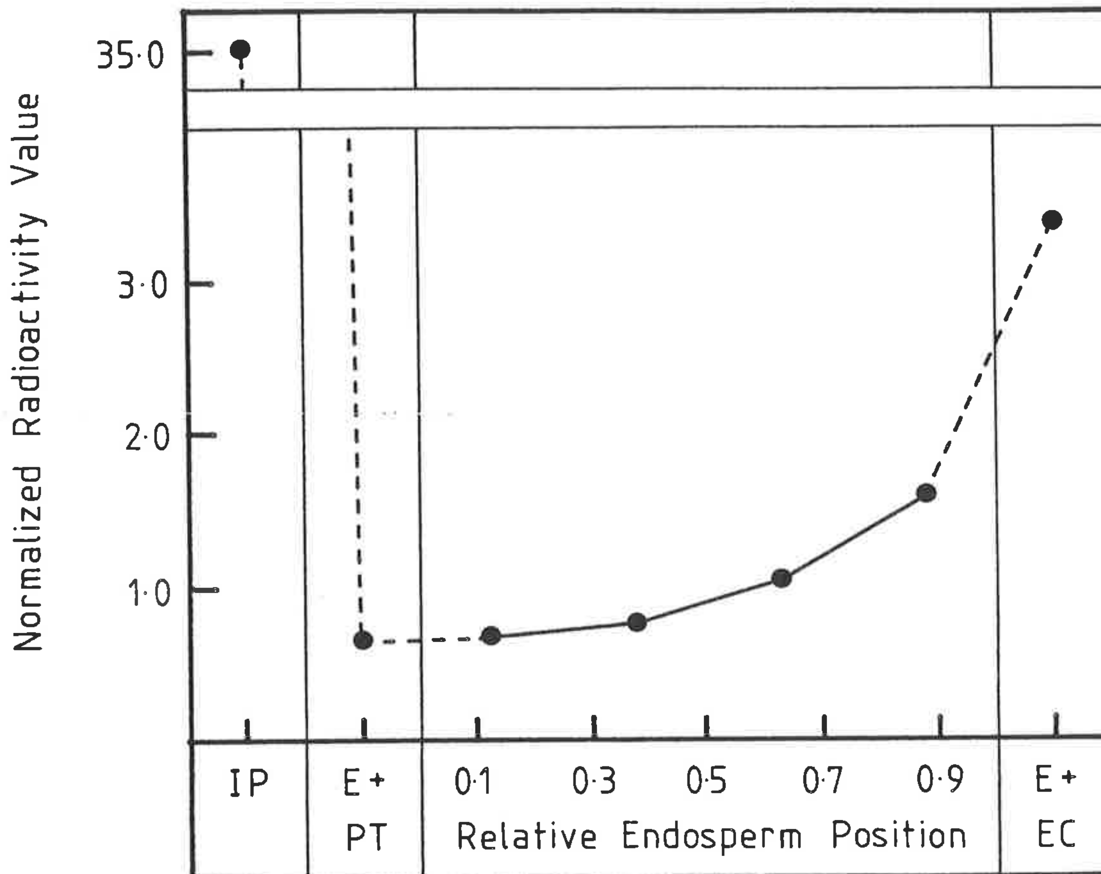

Figure 3.4-3. Pattern of the normalized radioactivity value in the soluble carbohydrate fraction extracted from the sampled region (dorsal side) of a wheat grain detached within an intact spikelet after the ear was exposed for 90 minutes to $^{14}CO_2$ in the light. The notations IP, E + PT and E + EC are as in Fig. 3.4-1A.

tissue at the base of the crease is the primary pathway for longitudinal transport of solutes on route to the endosperm cavity and endosperm. In addition, this demonstrates again (see above) that radioactive carbohydrates do not move from the inner pericarp to the endosperm in an inward radial direction.

5. Effect of Disrupting Transport in the Putative Peripheral Routes

The results so far have shown that the principal route for carbohydrate movement from the main vascular bundle to the endosperm is via the endosperm cavity. The purpose of the experiments reported in this section was to establish whether or not the endosperm is supplied also peripherally by solutes that move in the tissues which surround the endosperm on the inside of the cuticular layer (i.e. the nucellar epidermis and the aleurone layer).

Treatments designed to disrupt any such circumferential movement of solutes around the grain had no effect on either the amount of radioactivity in the sampled region of dorsal endosperm (Table 3.4-3) or the distribution profile of that radioactivity (Figs. 3.4-4A and 3.4-4B). Hence no supply involving a peripheral route was detected.

6. Pattern of Radioactivity in a Ventral Lobe

The region of the grain from which segments for this experient were taken is shown in figure 3.4-5 (inset). After 90 minutes there was a gradient of radioactive soluble carbohydrate extending from the endosperm cavity to the peripheral regions of endosperm (Fig. 3.4-5) which was of similar form to the gradient observed in the dorsal region (Figs. 3.4-1C, 3.4-2B and 3.4-4B). The gradient in the ventral region was more steep however, with a 6-fold difference in the concentration of radioactivity within the endosperm over a distance of 0.875 mm compared to a 2.5-fold difference over 0.500 mm

Table 3.4-3. The concentration of radioactivity in the endosperm tissue sectioned from the dorsal region of grains prepared for experimentation and of grains with transport disrupted in any putative peripheral route after the ears were illuminated in $^{14}CO_2$-rich atmosphere for 60 or 90 minutes. This information is for the same grains referred to in Figs. 3.4-4A and 3.4-4B. Values are the mean, ± S.E. where n = 4.

Treatment	$dpm\ ^{14}C.mgdwt^{-1}$
1. After 60 minutes	
Grains prepared for experimentation (n = 4)	300±98
Grains with putative peripheral transport route disrupted (n = 2)	368
2. After 90 minutes	
Grains prepared for experimentation (n = 2)	821
Grains with putative peripheral transport route disrupted (n = 2)	801

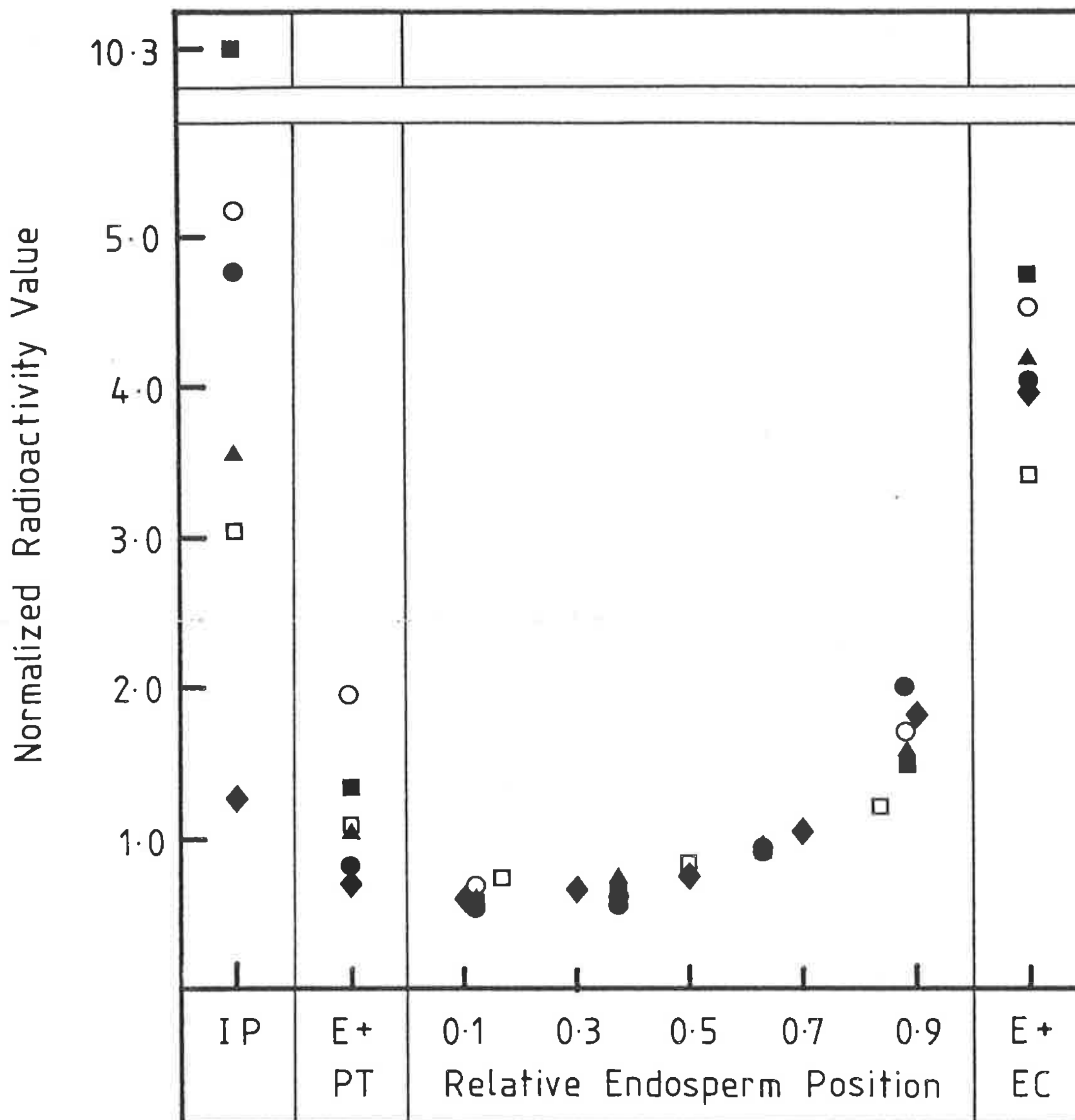

Figure 3.4-4A. Pattern of the normalized radioactivity value in the soluble carbohydrate fraction extracted from the sampled region (dorsal side) of wheat grains after ears were exposed for 60 minutes to $^{14}CO_2$ in the light. The closed symbols are for grains prepared for experimentation (n=4) and the open symbols are for grains with transport disrupted in the putative peripheral routes (n=2). Different symbols denote separate grains. The symbols used here for the prepared grains correspond to the same symbols and grains shown in Fig. 3.4-2A. The notations IP, E + PT and E + EC are as in Fig. 3.4-1A.

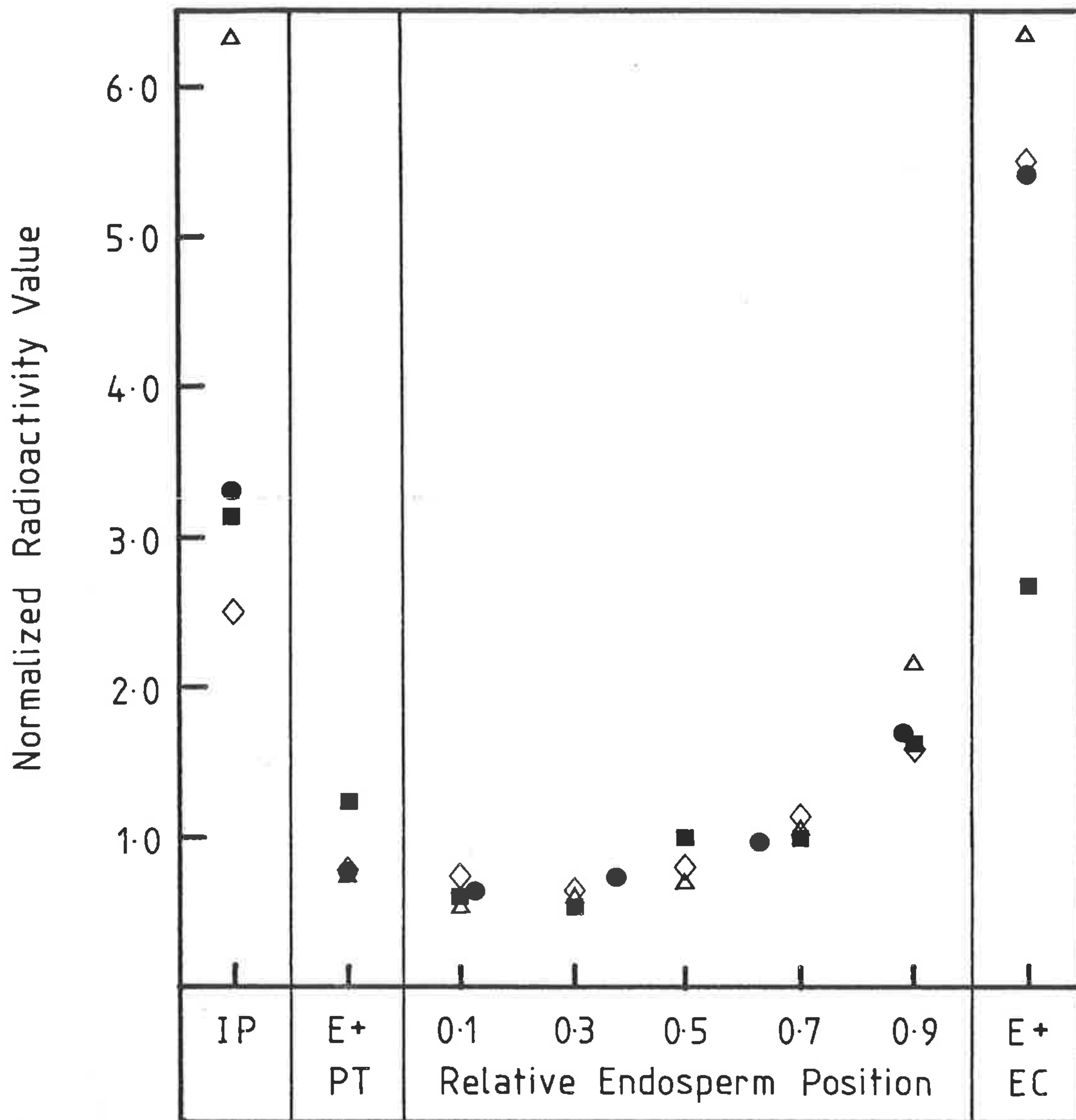

Figure 3.4-4B. As Fig. 3.4-4A, after ears were exposed for 90 minutes. In this case, there were two replicates of each treatment.

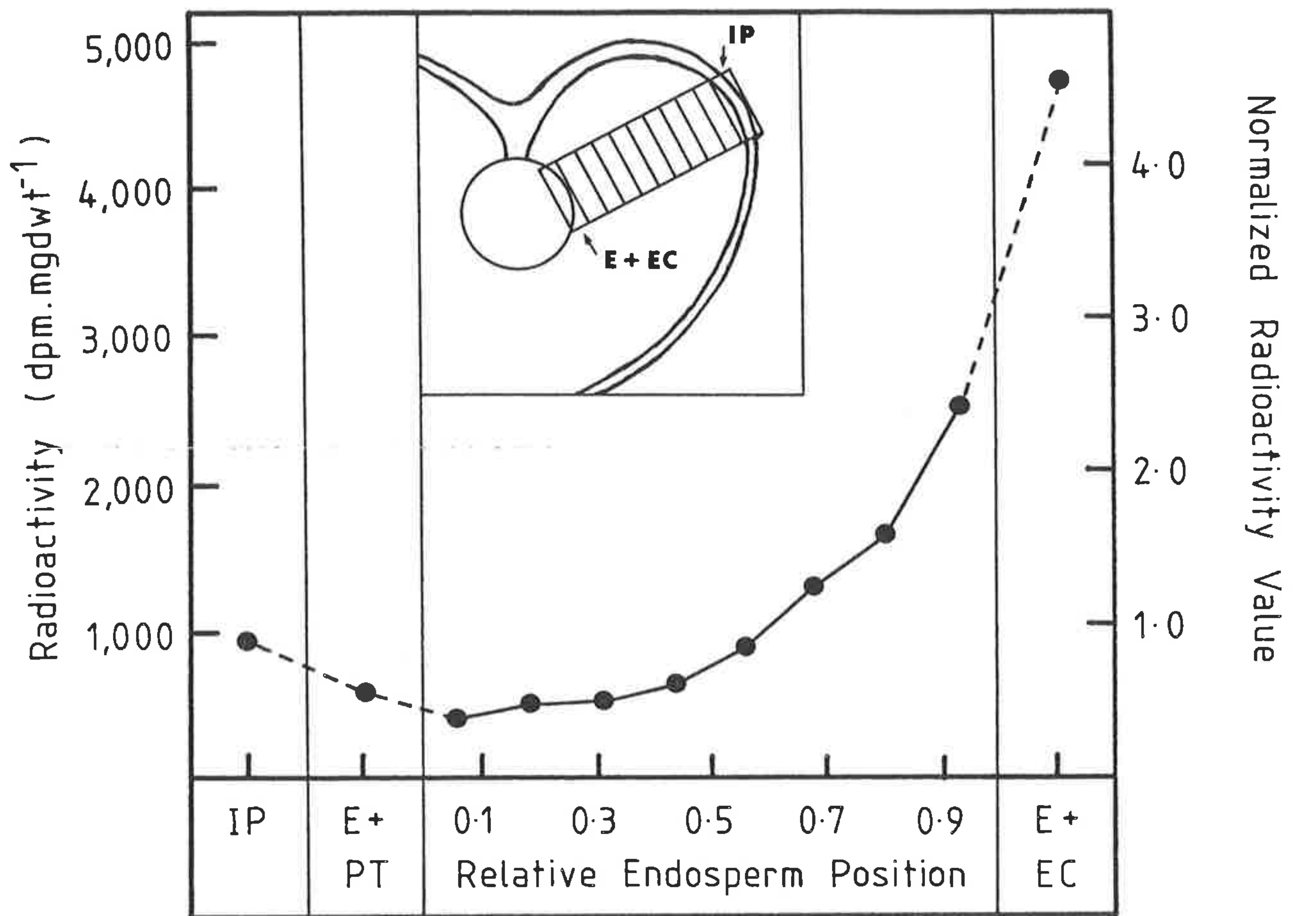

Figure 3.4-5. Pattern of radioactivity and of the normalized radioactivity value in the soluble carbohydrate fraction extracted from the ventral region of a wheat grain after the ear was exposed for 90 minutes to $^{14}CO_2$ in the light, and inset, the region of the grain where these sections were taken. The notations IP, E + PT and E + EC are as in Fig. 3.4-1A.

in the dorsal direction.

DISCUSSION

The study reported here concentrates on the movement of radioactive solutes that separate by cation-exchange chromatography into the 'neutral and anionic' fraction. Essentially all of the radioactivity in this fraction is contained in solutes identified by HPLC techniques as soluble carbohydrates (section 3.2). It is reasonable therefore, to refer to the 'neutral and anionic' fraction as the soluble carbohydrate one.

Preparing a grain for experimentation decreased the total amount of radioactivity extracted from the grains (Table 3.4-2). This procedure also decreased 3-4 fold the amount of ^{14}C fixed by the inner pericarp (see values for detached grains in table 3.4-2) and this alone accounts largely for the difference in total activity. Hence transport from the plant to the grain appears largely unaffected by the act of preparing a grain for experimentation. This procedure did not alter the distribution profile of radioactivity throughout the dorsal region of the grain either (Figs. 3.4-2A and 3.4-2B). Thus any change to the distribution profile resulting from a surgical treatment was due to the treatment itself and not to the preparation procedure.

The vascular bundle that lies within the pericarp tissue at the base of the crease is the main route of longitudinal transport of soluble carbohydrates destined for the endosperm. Disrupting transport in this bundle decreased substantially the total amount of radioactivity extracted from the grain (Table 3.4-3) and no radioactivity was detected in the vascular bundle, the endosperm cavity or the endosperm distal to the point of disruption (results section 4).

The inner pericarp has the photosynthetic capacity to contribute substantially to the overall carbon budget of the grain (results section 3;

see also Cochrane and Duffus, 1979; Duffus, Nutbeam and Scragg, 1985). The amount of radioactivity extracted from a grain with a disrupted stalk is a result of CO_2 exchange by the inner pericarp. However, this value, amounting to between 17% and 36% of the radioactivity extracted from corresponding attached grains (Table 3.3-3) is not necessarily a measure of the proportional contribution by the inner pericarp to the carbon budget of the grain _in vivo_. No allowance was, or could be made for any effect of disrupting the stalk on the photosynthetic rates of the inner pericarp or for differences in specific activity of the solutes arriving at or within the grain (endosperm) from the two sources.

Two lines of evidence lead to the conclusion that soluble carbohydrates do not move from the inner pericarp to the endosperm via a direct inward radial route. (1) In detached grains where the supply of radioactivity is exclusively from the inner pericarp, radioactivity was observed in the endosperm first in the regions adjacent to the endosperm cavity (results section 3; Fig. 3.4-3). (2) When longitudinal movement within the main vascular bundle was disrupted, the levels of radioactivity in the inner pericarp were high, yet there was no detectable movement of radioactivity to the endosperm (results section 4).

The grain is covered almost entirely by the seed coat (Morrison, 1975). Deriving from the inner integument, it is located between the inner pericarp and the nucellar epidermis, appearing firmly joined to both. The seed coat comprises 3 layers; a thick outer cuticle (1.0 um to 1.5 um thick), a 'colour' layer and thin inner cuticle. The two cuticles are conspicuous by 7 days after anthesis and by 3-4 weeks appear closely appressed.

There are no direct measurements on the permeability of the seed coat of developing wheat grains to solute flux, although in aged barley seeds the seed coat does indeed show high resistance to inward flux of aqueous

solutes (Briggs and MacDonald, 1973). From anatomical studies, however, it is expected (Bradbury et al., 1956b, and references therein; Morrison 1976; Zee and O'Brien, 1970b) that the seed coat in wheat would form a barrier of high resistance to the movement of solutes. The current study provides physiological evidence in support of this expectation.

There is a break in the seed coat in the region of the crease, a region called the pigment strand. Hence as also presumed earlier (Cochrane, 1983; Zee and O'Brien, 1970b) this region must be the passage through which move most, if not all of the solutes destined for the developing endosperm and embryo.

Once through the pigment strand the principal route for solute movement to the endosperm is directly across the endosperm cavity with movement through the endosperm in a radial way (Figs. 3.4-1A, 3.4-1B and 3.4-1C).

During the development of the ovule the embryo and endosperm grow within the nucellus. Eventually only the epidermis of the nucellus and a band of cells adjacent to the pigment strand, the nucellar projection, remain. The nucellar epidermis is intact during the grain fill period of development, present over the entire kernel (Krauss, 1933, cited from Bradbury et al., 1965b) except perhaps for a break over the embryo (Bradbury et al., 1965b; Fairclough, 1947). As the grain matures the nucellar epidermis may become compressed and may lose its cellular structure (Bradbury et al., 1965b). The nucellar projection contains transfer cells, suggesting a functional role of this tissue in the transfer of substrate from the maternal symplast to the apoplast (Cochrane and Duffus, 1980; Hughes, 1976, cited from Cochrane and Duffus, 1980). And recently, cells with the characteristic wall ingrowths of transfer cells were observed in the nucellar epidermis (Smart and O'Brien, 1983). Hence the nucellar tissue could provide a continuum around the grain for transport of substrate to the peripheral regions of the endosperm. Indeed

nucellar tissue such as this provides the principal route of substrate supply in the rice grain (Oparka and Gates, 1984).

Anatomical evidence suggests that the aleurone layer also could provide a peripheral route of solute movement. The aleurone layer comprises a double layer of thick walled granular cells lining the endosperm cavity leading onto a single layer around the rest of the endosperm. These cells are rich in endoplasmic reticulum and mitochondria (Evers, 1970; Morrison et al., 1975, 1978). Plasmodesmata are abundant and clearly defined, connecting adjacent aleurone cells and aleurone cells to adjacent peripheral cells of the endosperm (Cochrane and Duffus, 1980; MacMasters et al., 1971, and references therein; Morrison et al., 1975, 1978). Membranous loops develop within the cytoplasm of the cells of the modified aleurone layer (Ayre and Angold, 1979; Cochrane and Duffus, 1980; Morrison et al., 1978; Zee and O'Brien, 1971).

In the current study surgical treatments which undoubtedly would have disrupted any circumferential movement of solutes from the vascular bundle around the endosperm (see Materials and Methods) did not influence in a measurable way the distribution profile of radioactive soluble carbohydrates within the dorsal region. However, while the experimental procedure would have blocked a contribution from a peripheral pathway it was designed not to disrupt the supply of radioactivity via the radial route (Figs. 3.4-4A and 3.4-4B). Accordingly, these results do not discount entirely the possibility of contribution from a peripheral route, but clearly any contribution would be small.

The mean volume of endosperm cells increases continually during grain development, albeit at a decreasing rate, until the rapid decrease during dehydration (Briarty et al., 1979). During grain fill most of this is attributable to growth within the ventral lobes of the endosperm with very little change in cell number and volume in the dorsal region (Evers, 1970,

1974). Conceivably, this pattern of growth may be influenced by substrate supply; indeed substrate supply seems to determine the rate of mitotic activity in the endosperm as a whole (Singh and Jenner, 1984).

The dorsal region of the endosperm, although close to the endosperm cavity is opposite the main vascular bundle and hence is at the extremity of any peripheral route. On the other hand, the endosperm tissue in the ventral lobes is further away from the endosperm cavity (see results section 6) but adjacent to the start of such a peripheral route (see Fig. 3.4-5 inset). The proximity of endosperm in the ventral lobe to the zone where a peripheral route can be surgically disrupted prevents an examination of the profile of radioactivity in this tissue as influenced by this disrupting treatment. However, any contribution from a peripheral route may be more concentrated and hence more obvious in the peripheral zones of the ventral lobes in untreated grains than in the peripheral zones of endosperm in the dorsal region. Contrary to this, the gradient of the radioactivity in soluble carbohydrates was more steep in the direction of the ventral lobe than in the dorsal region reinforcing the view that very little, if any, carbohydrate moves to the endosperm in a peripheral route.

The experimental design was such that the specific activity of substrate entering the grain, and indeed the endosperm itself, was changing with time. Hence, while the results can be used to elucidate the pathway of solute movement into the endosperm, they cannot be used to describe the kinetics of movement or to calculate specific activities of substrate along that pathway as a function of time.

3.5 EVIDENCE FOR TWO TRANSPORT MECHANISMS WITHIN THE ENDOSPERM OF WHEAT

INTRODUCTION

Nitrogen moves to the wheat ear in the transpiration stream primarily as glutamine (Kirkman and Miflin, 1979). Glutamate synthetase (alternatively known as GOGAT) catalyses the reductive transfer of the amide group of glutamine to 2-oxoglutarate, forming two units of glutamate. Transaminases couple the deamination of glutamate with the conversion of α-oxo acids to the α-amino acids for protein synthesis (Hageman, 1979; Miflin and Lea, 1980; Miflin, 1980). In this way radioactivity from carbohydrate becomes incorporated into amino acids (see sections 3.2, 3.4 and 3.5; also e.g. Thorne, 1980).

The pathway by which a ^{14}C-carbohydrate moves within the grain was described in section 3.4. In that study ^{14}C was supplied to the grain by illuminating the ear in a $^{14}CO_2$-rich atmosphere. Cationic exchange chromatography was used to isolate the radioactivity in the soluble carbohydrate fraction. At the end of the chromatography procedure, solutes were recovered from the cationic exchange resin (section 2.11) providing an amino acid fraction (Redgewell, 1980; Thorne, 1980; A.C. Jennings, pers. comm.), and radioactivity therein was measured also.

This section (3.5) describes further the movement of solutes within the wheat grain. The study is based on the radial profile throughout the dorsal region of radioactivity in the amino acid fraction as related to time, as influenced by disrupting putative transport routes and as compared to the corresponding profile in insoluble material and the soluble carbohydrate fraction.

MATERIALS AND METHODS

All materials and methods used in this study were as described in section 3.4.

RESULTS

1. Pattern of Radioactivity in the Amino Acid Fraction Throughout the Dorsal Region as a Function of Time

After 30 minutes radioactivity was found in the amino acid fraction from intact grains in the pericarp tissue at the base of the crease surrounding and including the main vascular bundle, and in the fluid of the endosperm cavity (not shown). It was found also in the endosperm tissue adjacent to the endosperm cavity and in the inner pericarp section on the dorsal surface (see Fig. 3.4-1A). After longer periods of time (60 and 90 minutes) radioactivity was found in the amino acid fraction of all sections extending from the vascular bundle to the inner pericarp on the dorsal surface (see Figs. 3.4-1B and 3.4-1C). The profile of radioactivity in the amino acid fraction throughout the dorsal region differed from the corresponding profile in the soluble carbohydrate fraction on two accounts (Figs. 3.5-1A and 3.5-1B). (1) In the amino acid fraction the lowest level of radioactivity occurred around the midpoint of the endosperm with a rise in radioactivity towards both the peripheral zone and the endosperm cavity, whereas in the soluble carbohydrate fraction there was a continual decline in radioactivity in the direction away from the endosperm cavity. (2) The gradient of radioactivity between the endosperm cavity and the endosperm was shallow in the amino acid fraction, being more steep in the soluble carbohydrate fraction.

These above-mentioned differences were seen in all grains with sufficient radioactivity for a profile analysis and at all times. As

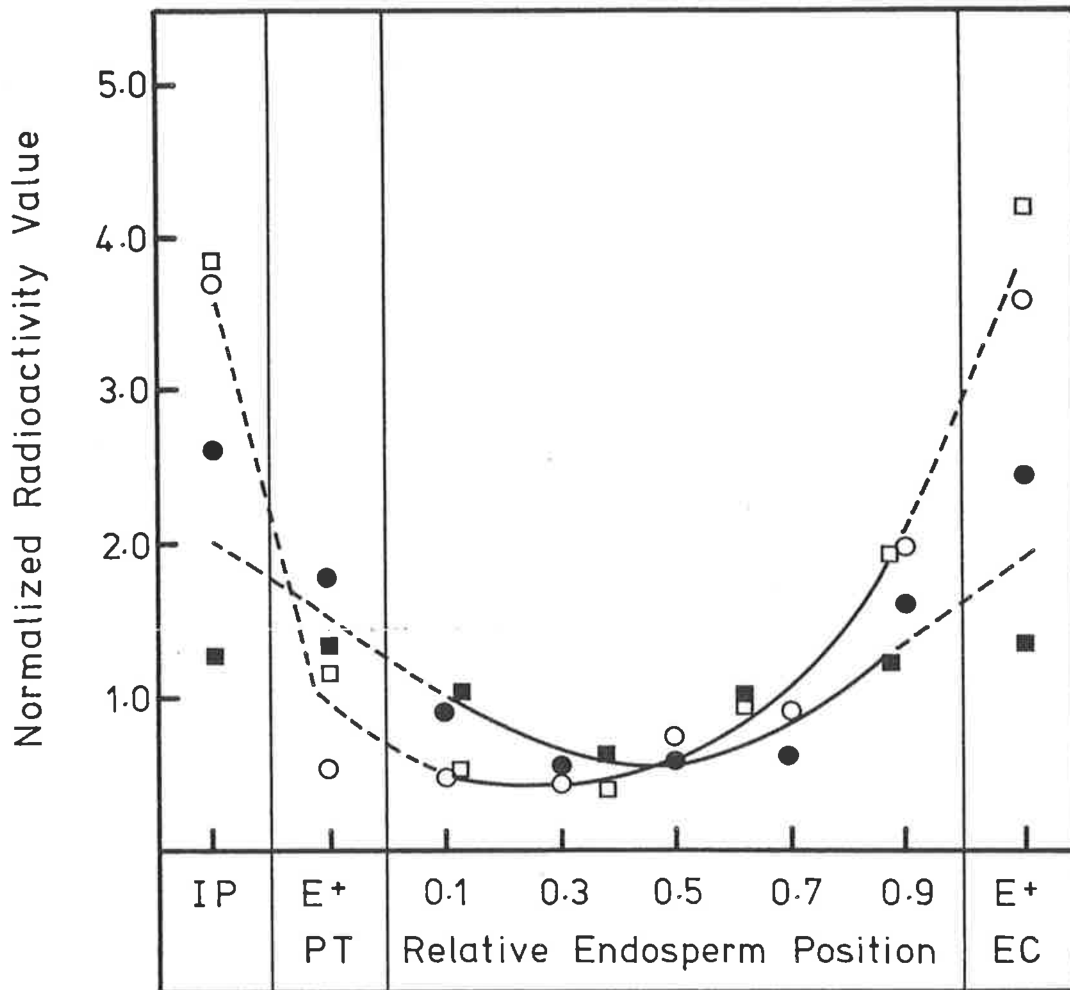

Figure 3.5-1A. Pattern of normalized radioactivity value in the amino acid fraction (closed symbols) and in the soluble carbohydrate fraction (open symbols) extracted from the sampled region (dorsal side) of intact grains after ears were exposed for 60 minutes to $^{14}CO_2$ in the light. Different symbols denote separate grains. The symbols used here correspond to the same symbols and grains shown in Figs. 3.4-1B and 3.4-2A. The lines are estimates of lines of best fit and are drawn to assist with the comparison.

Explanatory Notes.

IP = The section containing mostly inner pericarp tissue (dorsal surface).

E + PT = Mainly endosperm plus a small amount of peripheral tissue.

E + EC = Mainly endosperm plus a small amount of fluid from the endosperm cavity.

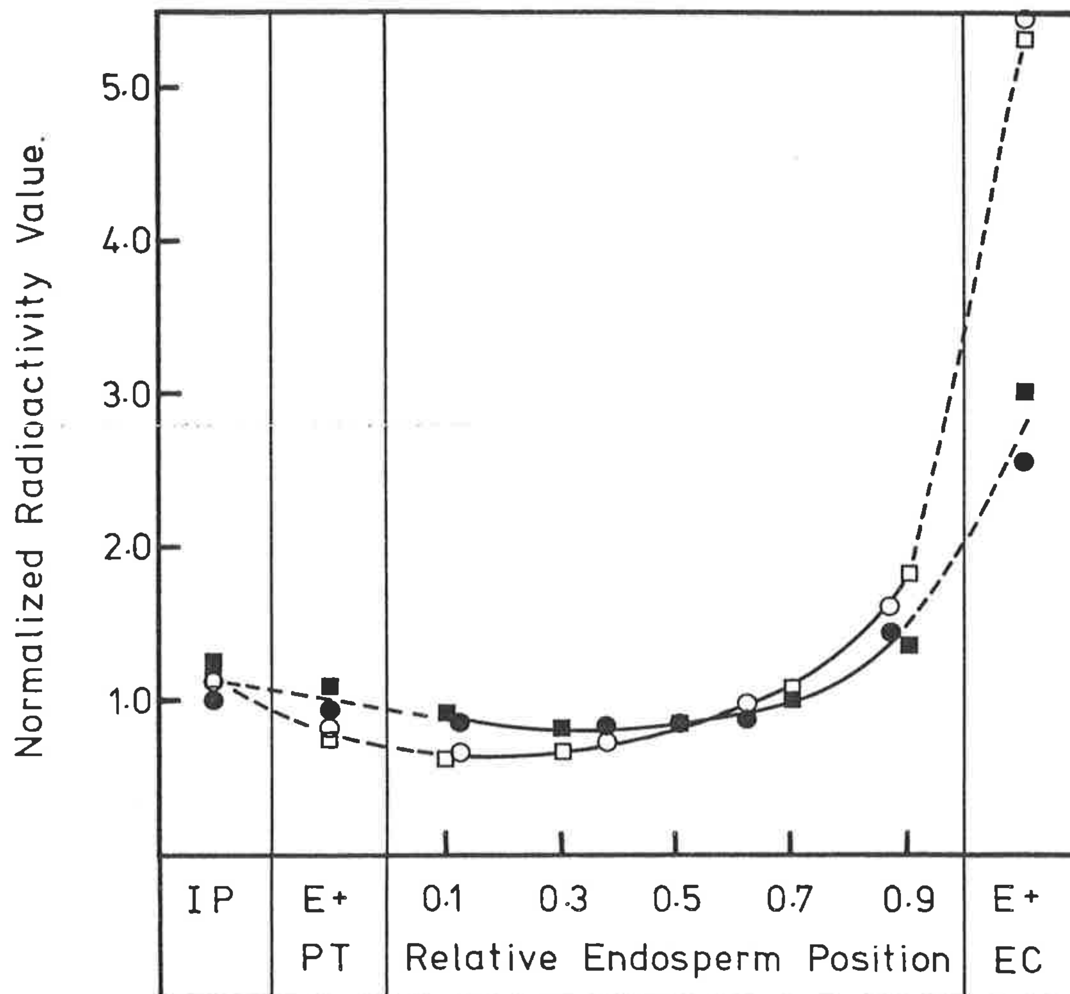

Figure 3.5-1B. As Fig. 3.5-1A, after ears were exposed for 90 minutes. The symbols used here correspond to the same symbols and grains shown in Figs. 3.4-1C and 3.4-2B.

another example, the profile of radioactivity in the amino acid fraction of grains prepared for experimentation is shown in figures 3.5-2A and 3.5-2B and this can be compared with the profile of radioactivity in the soluble carbohydrate fraction previously described for the same grains (Figs. 3.4-2A and 3.4-2B). The ratio of radioactivity in the amino acid fraction to the radioactivity in the soluble carbohydrate fraction (Figs. 3.5-3A and 3.5-3B) shows more clearly these differences. The ratio increased as a function of distance from the endosperm cavity.

Sometimes there was substantial between-grain variation in the observed ratio of radioactivity in the amino acid fraction to radioactivity in the soluble carbohydrate fraction in absolute terms. The plot of this ratio throughout two intact grains after 60 minutes (Fig. 3.5-3A) shows the greatest between-grain variation that was observed within any one treatment over the entire range of intact and treated grains studied. Despite the magnitude of this variation however, the trend, increasing away from the endosperm cavity was the same in both grains and the distribution profile of radioactivity throughout the endosperm appeared to be the same in each of the amino acid fraction (Fig. 3.5-1A) and soluble carbohydrate fraction (Fig. 3.4-2A).

2. Effect of Disrupting Putative Transport Pathways

The effect of disrupting putative transport pathways on the pattern of radioactivity in the amino acid fraction is summarized below. Some of the data are not shown in detail. The responses were the same as those observed in the soluble carbohydrate fraction (section 3.4).

1. Preparing the grain for experimentation. No perceivable effect; the distribution profile was the same in treated grains as in intact grains (Figs. 3.5-2A and 3.5-2B, cf. 3.5-1A and 3.5-1B).

2. Disrupting transport in the stalk. This treatment greatly reduced the

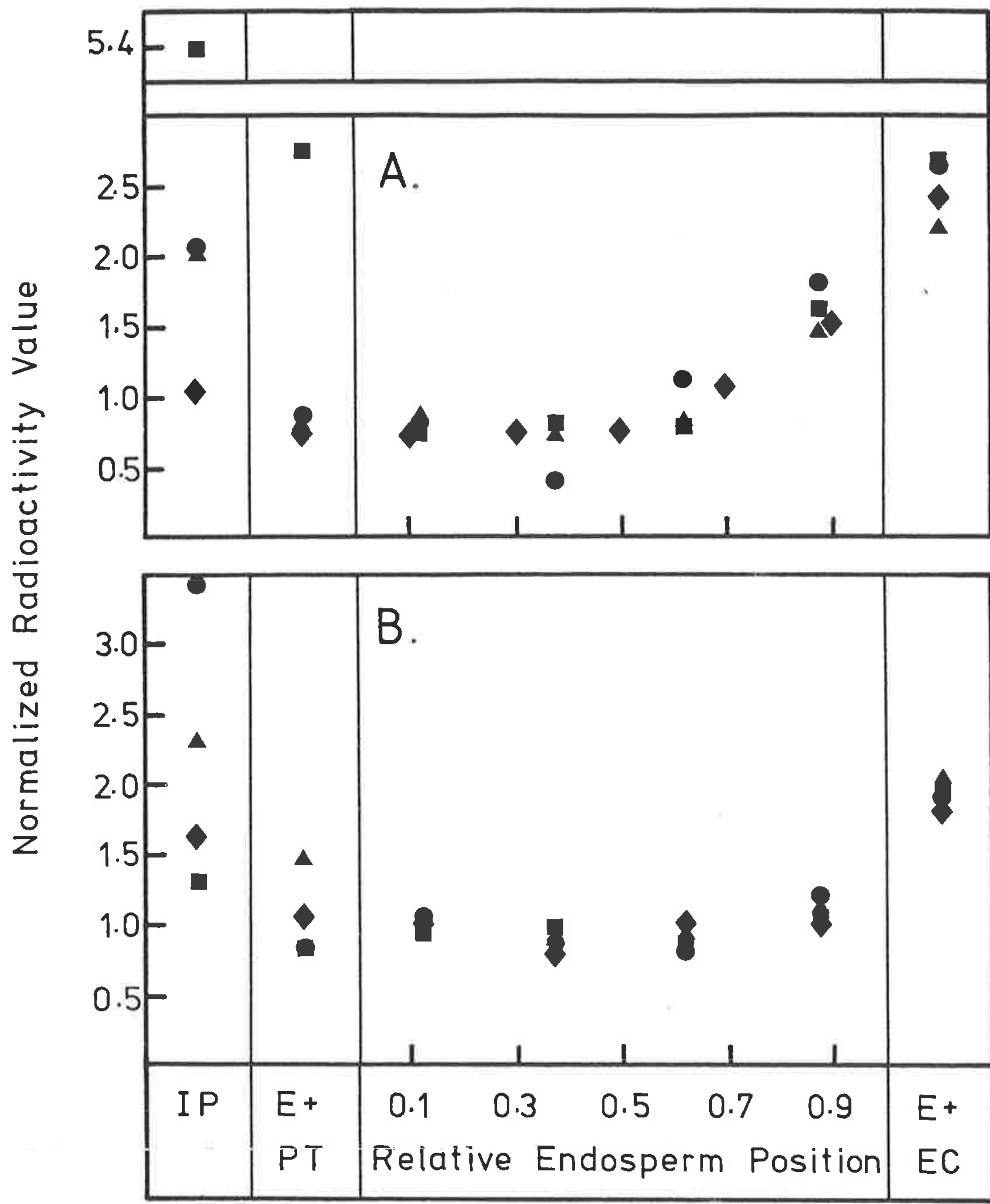

Figure 3.5-2. Pattern of normalized radioactivity value in the amino acid fraction extracted from the sampled region (dorsal side) of wheat grains prepared for experimentation after ears were exposed for 60 minutes (A) and 90 minutes (B) to $^{14}CO_2$ in the light. Different symbols denote separate grains. The symbols used here correspond to the same symbols and grains shown in Figs. 3.4-2 and 3.5-2. The notations IP, E + PT and E + EC are as in Fig. 3.5-1A.

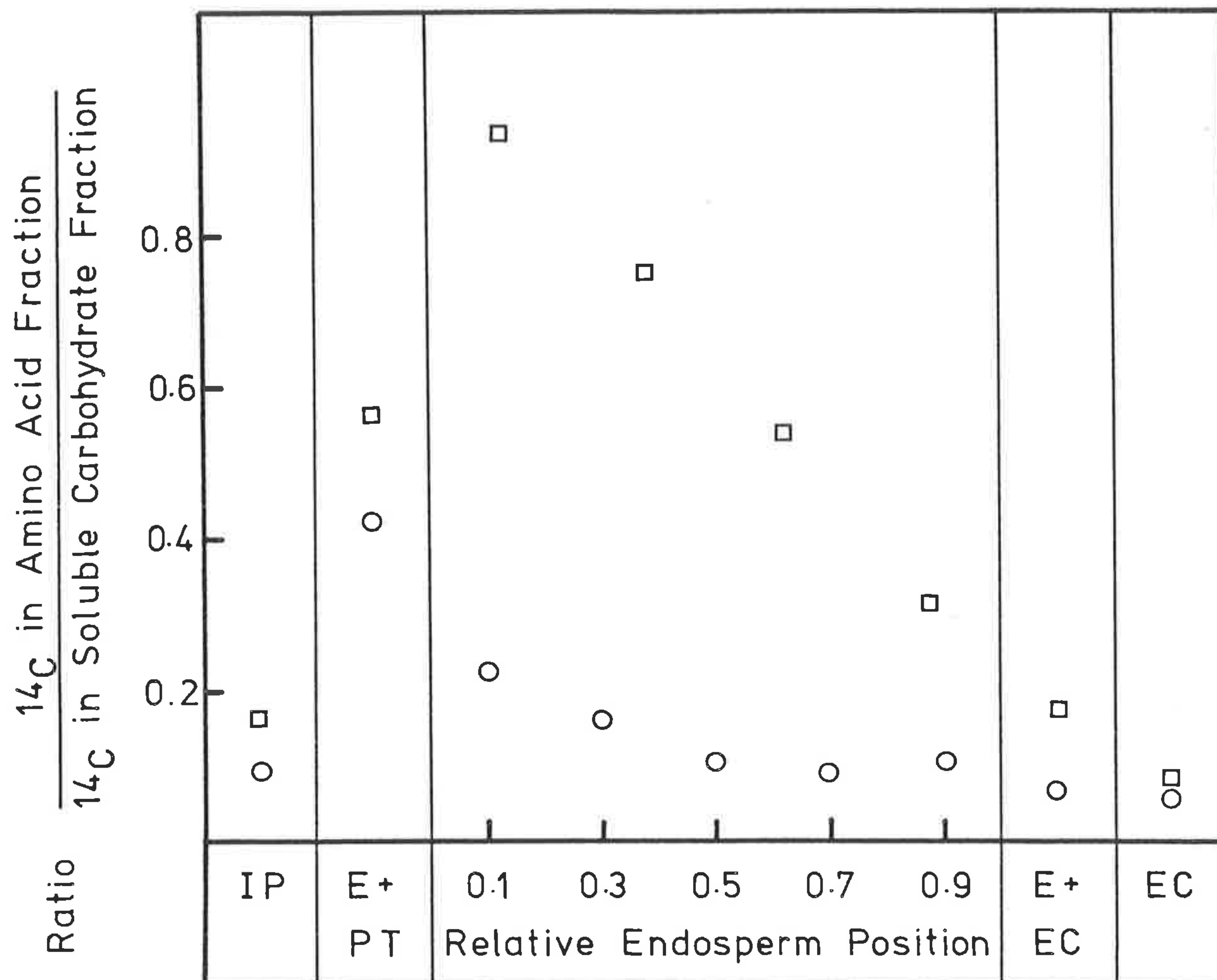

Figure 3.5-3A. Ratio of radioactivity in the amino acid fraction to radioactivity in the soluble carbohydrate fraction of intact grains after ears were exposed for 60 minutes to $^{14}CO_2$ in the light. Different symbols denote separate grains. The symbols used here correspond to the same symbols and grains shown in Figs. 3.4-1B, 3.4-2A and 3.5-1A. The notations IP, E + PT and E + EC are as in Fig. 3.5-1A, while EC = fluid from the endosperm cavity.

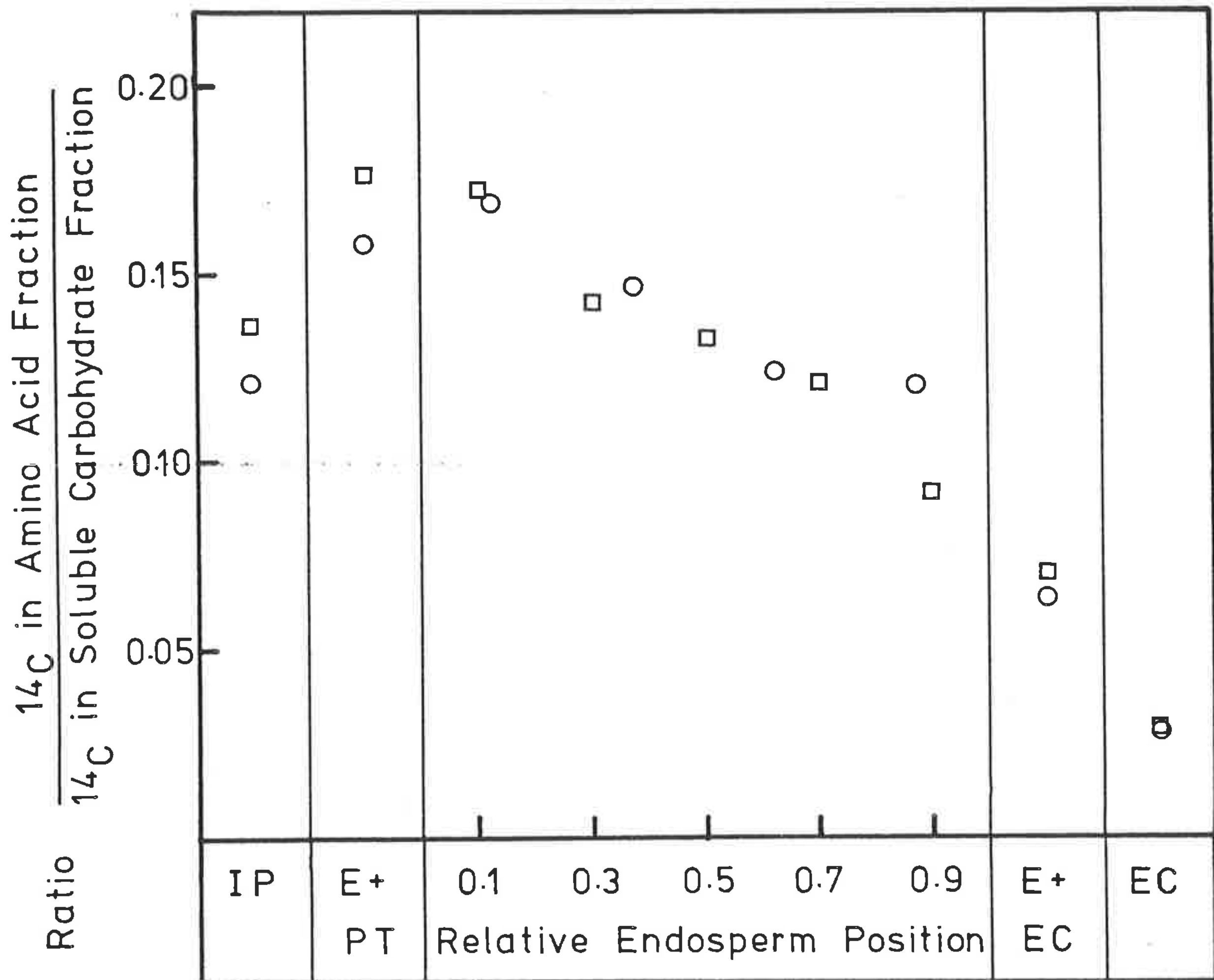

Figure 3.5-3B. As Fig. 3.5-3A, after ears were exposed for 90 minutes. The symbols used here correspond to the same symbols and grains shown in Figs. 3.4-1C, 3.4-2B and 3.5-1B.

level of radioactivity in the main vascular bundle, the endosperm cavity and the endosperm. Within the endosperm itself, the profile of radioactivity in the amino acid fraction appeared the same as in control grains.

3. Disrupting transport in the main vascular bundle. No radioactivity was detected in the sampled region which was distal to the site of disruption, except in the peripheral tissues.

4. Disrupting transport in the putative peripheral routes. No perceivable effect; the distribution profile was the same in treated grains as in intact grains or grains prepared for experimentation (see Figs. 3.5-1A, 3.5-1B, 3.5-2A and 3.5-2B).

DISCUSSION

The pattern of radioactivity in the amino acid fraction throughout the wheat grain, as observed in this study is the product of the interaction between the conversion of radioactivity into amino acid form, the route(s) of substrate supply and the kinetics of movement of radioactive solutes along that route. The results must be interpreted carefully.

First, radioactivity in the amino acid fraction was observed in the endosperm first adjacent to the endosperm cavity (see Fig. 3.4-1A). Thus at least some of the radioactivity extracted from the endosperm in this fraction arrived via the endosperm cavity.

Secondly, essentially all of the radioactivity in the soluble carbohydrate fraction within the endosperm derives from the endosperm cavity and moves within the endosperm in an outward radial direction (section 3.4). In so doing, it creates a characteristic profile of radioactivity throughout the endosperm that changes with time (Figs. 3.5-

1A, 3.5-1B, 3.4-2A and 3.4-2B). Radioactivity in the amino acid fraction also shows a characteristic profile throughout the endosperm that changes with time, but this profile differs from the soluble carbohydrate one in such a way as to suggest that radioactive amino acids may enter the endosperm from both the endosperm cavity and the peripheral zones (Figs. 3.5-1A and 3.5-1B). However, the evidence against a peripheral supply route is overwhelming (results section 2). Taken together the results show that there was only one major route by which radioactivity was conveyed to the endosperm (i.e. via the endosperm cavity), but within the endosperm itself there were differences in the distribution kinetics for each class of solute.

Thirdly, consider now the ratio of radioactivity in the amino acid fraction to the radioactivity in the soluble carbohydrate fraction throughout the sampled region, increasing as a function of distance from the endosperm cavity (Figs. 3.5-2A and 3.5-2B). Consider first the difference in this ratio value between the endosperm cavity and the endosperm taken as a whole. On the average it was about a 5-fold difference. There are three possible explanations.

1. Amino acids move within the transport pathway of the grain (measured here as the endosperm cavity fluid) with a speed 5 times that by which soluble carbohydrates move and in so doing create the ratio as observed in the substrate as a whole (measured by the ratio in the endosperm). This is unlikely however, because both amino acids and soluble carbohydrates move within the transport pathway (measured as longitudinal movement within the main vascular bundle) with similar kinetics (Donovan et al., 1983).

2. A large amount of radioactivity from the soluble carbohydrate fraction (at least 80%) was sequestered in the endosperm. Neither is this explanation possible since the amount of radioactivity in insoluble form is

small (10-20%, see Figs. 3.4-1A, 3.4-1B and 3.4-1C).

3. The most plausible explanation is that most of the conversion of radioactivity from the soluble carbohydrate fraction to the amino acid fraction occurred within the endosperm itself.

The conversion of radioactivity from carbohydrates to amino acids is a cytoplasmic function (see Introduction, also section 1.6), hence the pattern of radioactivity in the amino acid fraction is mainly the distribution profile of solutes that are or at least have been compartmentalized within the symplast of the endosperm. The production of insoluble material is also a cellular process and so in a similar way, the pattern of radioactivity in insoluble material shows the location and to a degree the profile of radioactive compartmented precursors immediately before their conversion into insoluble form. On the other hand, the pattern of radioactivity in the soluble carbohydrate fraction is a measure of the distribution profile of all these solutes be they in a metabolic compartment or not.

Despite a steep gradient of radioactivity in the soluble carbohydrate fraction, decreasing as a function of distance from the endosperm cavity (Figs. 3.4-2A and 3.4-2B), radioactivity in the amino acid fraction and radioactivity in insoluble form became rapidly and evenly distributed throughout the endosperm (Figs. 3.5-1A, 3.5-1B, 3.4-1A, 3.4-1B and 3.4-1C). This is made abundantly clear in the plots of the ratio of radioactivity in the amino acid fraction to the radioactivity in the soluble carbohydrate fraction within the endosperm which was derived from these distribution profiles. The ratio values are greater and increase steeper as a function of distance from the endosperm cavity at 60 minutes than at 90 minutes (Figs. 3.5-3A and 3.5-3B).

There is a high degree of independence between the radioactivity in the metabolic compartment and the radioactivity in the soluble carbohydrate

fraction taken as a whole. Even though there may be substantial between-grain variation in the proportion of radioactivity in soluble carbohydrates that is transferred to amino acids (Fig. 3.5-3A), the distribution of radioactivity throughout the endosperm in the soluble carbohydrate fraction and the subsequent distribution of radioactivity in the amino acid fraction varies little between grains (Figs. 3.4-2A and 3.5-1A).

Consider now mechanisms by which radioactivity could be distributed throughout the metabolic symplast more rapidly than throughout the tissue as a whole. A number of hypothetical schemes fit broadly into 2 models.

Model 1. Radioactivity is conveyed from the cavity fluid directly into a small fast moving transport pool. It is distributed throughout the endosperm and is taken up evenly from this pool into the metabolic symplast. Concurrently, radioactivity is channelled from the small transport pool into larger static pools spread throughout the endosperm in such a way as to create the observed profile of radioactivity in the soluble carbohydrate fraction.

This model seems unlikely due to characteristics of these larger static pools that would be necessary for the system to work. If these pools were the same size (collectively) throughout the endosperm one must invoke a system whereby the pools nearer the endosperm cavity 'fill-up' or exchange more rapidly than the ones further away, even though they would all have access to a transport pool in which radioactivity was dispersed evenly throughout and from which the metabolic symplast was receiving radioactivity evenly throughout the endosperm. To overcome this problem one could suggest that indeed there was equal exchange between the transport pool and the static pools throughout the endosperm, but collectively the static pools are larger nearer the endosperm cavity. This also has flaws however, because the levelling of radioactivity within the endosperm as a function of time would signal a levelling of the size of these pools as a

function of time.

Model 2. The bulk of radioactivity is contained in a large slow moving pool. Some radioactivity is channelled either from this pool or directly from the fluid of the endosperm cavity into a smaller transport pool for rapid distribution throughout the endosperm. This smaller pool could be the metabolic symplast itself or an alternative supply system serving a static metabolic symplast along its route. Either way, this model invokes two transport mechanisms within the endosperm.

The mechanism of solute transport throughout the endosperm could not be pursued further at this time.

3.6 CULTURE OF DETACHED WHEAT SPIKELETS AND THE ROUTE OF MOVEMENT OF AMINO ACIDS WITHIN THE GRAIN

INTRODUCTION

Carbohydrate destined for the endosperm moves along the grain within the vascular bundle at the base of the crease. It then moves across the endosperm cavity and radially throughout the endosperm. Movement of carbohydrate into the endosperm in an inward radial direction from the inner pericarp or any other tissue surrounding the endosperm, if present at all, forms only a very small proportion of total carbohydrate supply (section 3.4).

Essentially all of the carbohydrate moving to the endosperm is sucrose (section 3.2; Ho and Gifford, 1984), and within the endosperm this sucrose becomes distributed with a decreasing gradient along the supply route (section 3.3). In contrast, amino acids, the substrate for protein deposition, become distributed throughout the endosperm more evenly with increases in concentration towards both the endosperm cavity and peripheral tissues (section 3.3).

The differences in the distribution patterns of each class of substrate appear due either to an additional supply rich in amino acids from the peripheral tissues or to different kinetics of movement along the one common route. The purpose of the experiments reported in this section (3.6) was to distinguish between these alternatives by describing the route(s) of amino acid movement to and within the endosperm.

An experimental system based on the culture of detached spikelets seemed the most suitable technique by which to introduce radioactive amino acids to developing grains. From preliminary tests (results not shown) such a system appeared to provide the best balance between minimizing disruption

to the in vivo state of the grain (compared, for instance, to supplying substrate to the cut stalk of an isolated grain, see Jenner, 1985c), and creating a front of radioactivity within the grain sharp enough for the proposed experimentation (in contrast to introducing solutes through an abraded awn, see Lingle and Chevalier, 1984, or culturing detached ears, see for example Donovan et al., 1983). The first part of this report deals with a more comprehensive assessment of grain function under this form of culture.

MATERIALS AND METHODS

Plant Material

Wheat plants of cv. Fransawi (see section 3.1) were grown, and spikelets for experimentation were selected, as described in section 3.4

Manipulative Treatments

Preparing the spikelet for experimentation and disrupting putative transport pathways in the grain are described in section 3.4

Detaching and Culturing Spikelets

Spikelets were detached from the ear and cultured on a solution of ^{14}C-Sucrose ($40 g.l^{-1}$) and ^{3}H-Glutamine ($0.5 g.l^{-1} N$) as described in section 2.4.2.

Dissecting the Floret and Grain into Component Tissues

After the preset time in culture, both the a and b florets were removed from the spikelet. The glumes, the palea and lemma of each floret and the outer pericarp and the grain stalk of each grain was collected separately. Grains were dissected into component tissues as described in section 2.5.1.

Measuring Radioactivity in the Component Tissues of the Spikelet and Grain

Tissue dissected from the a floret was digested *in toto* and radioactivity therein measured as described in section 2.12.3. Tissue from the b floret was extracted with hot (75°C) 90% ethanol (section 2.10). The extracts were dried (Rotovap II* at 50°C) and redissolved in water before separation by ion-exchange chromatography into soluble carbohydrate and amino acid fractions (section 2.11). Radioactivity in each fraction was measured as described in section 2.12.2.

Radioactivity in each of the insoluble residues was measured as described in section 2.12.3, except in the case of endosperm tissue where the residue was separated first into starch and protein fractions. Radioactivity in starch was isolated by chromatographic purification of the soluble product of digestion by α-amylase and amyloglucosidase (section 2.14) and radioactivity in protein was isolated by trichloroacetic acid precipitation of the hydroxide soluble extract (section 2.15).

Sectioning Grains Radially from the Inner Pericarp on the Dorsal Surface to the Vascular Bundle

Grains for this purpose (results sections 4 and 5) were removed from the spikelet and immersed in liquid nitrogen immediately after culture. They were sectioned as described in sections 2.5.2 and 3.4. The procedure to determine radioactivity in a number of fractions extracted from these small pieces of tissue is described in section 3.4.

RESULTS

1. Uptake of Culture Solution by Detached Spikelets

Detached spikelets continued to take up culture solution during the 8 hour period of culture, albeit at a decreasing rate (Fig. 3.6-1). Uptake was influenced by light; under 19 $uE.m^{-2}.sec^{-1}$ uptake was 5 times that under 6 $uE.m^{-2}.sec^{-1}$. On the other hand, EDTA (5mM) in the bathing and washing solutions during preparation had no measurable effect (detailed results not shown).

2. Distribution of Radioactivity Within the Spikelet and Grain

At all times during culture, the palea and lemma contained more than 80% the total amount of radioactivity in the spikelet (excluding the rachis, Table 3.6-1). The grain always (excluding very early in culture, 10 minutes) contained a constant proportion of the total amount of ^{3}H (10%) whereas the proportion of total ^{14}C contained within the grain initially was lower than this (2%) but rose to higher levels (14%) during the culture period (Table 3.6-1). Sucrose and glutamine were taken up into the spikelet system in amounts not proportional to their concentration in the culture solution; sucrose was taken up in proportional terms more rapidly than glutamine (Table 3.6-2). The opposite trend applied for transfer into the grain (Table 3.6-2).

The distribution of radioactivity between the a and b florets was not equal. After 8 hours of culture the a floret (and corresponding glume) contained 58% of the total ^{3}H but only 47% of the total ^{14}C (Table 3.6-1 cf. Table 3.6-3).

Only small amounts of radioactivity were in polymeric form in the glumes and the palea and lemma (Table 3.6-3). A greater proportion of radioactivity was insoluble within the grain as a whole; ^{14}C more so than ^{3}H (Table 3.6-3).

The distribution of radioactivity among the tissues of the grain is shown in table 3.6-4. After 10 minutes, the composite layer (inner pericarp, cuticle, nucellar epidermis) contained 40% the total

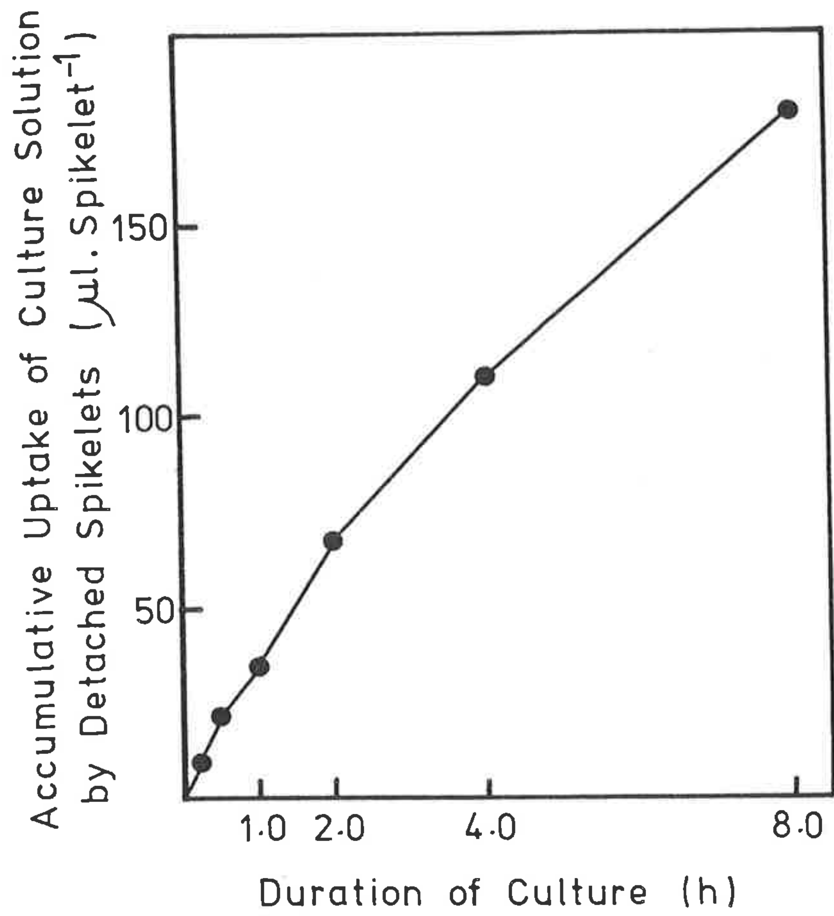

Figure 3.6-1. Uptake of solution by detached spikelets cultured under 19 uE.cm^{-2}. (Values are the mean of 2 replicates for each time period.)

Table 3.6-1. Distribution of radioactivity (^{3}H expressed as ug glutamine and ^{14}C expressed as ug sucrose) among tissues of the a floret and corresponding glume after various times in culture. Ratio of ug sucrose equivalents to ug glutamine equivalents in culture solution at the start of culture was 15.2.

Duration of Culture	^{3}H (ug of glutamine equivalents)				^{14}C (ug of sucrose equivalents)				Ratio ug sucrose equiv. to ug glutamine equiv.			
	Total	Glume	Palea	Grain	Total	Glume	Palea	Grain	Total	Glume	Palea	Grain
10 min	3	19.6	79.2	1.2	85	9.3	90.3	0.4	29	14	33	11
30 min	16	11.3	80.6	8.2	409	9.4	88.5	2.1	25	21	28	7
1 hour	21	6.2	82.1	11.7	639	5.6	91.2	3.2	31	28	35	9
2 hour	54	4.0	85.6	10.4	1372	3.6	91.5	4.9	25	23	27	12
4 hour	91	4.5	85.2	10.2	2156	5.1	86.7	8.2	24	27	24	19
8 hour	160	3.3	86.6	10.2	3037	4.7	81.5	13.8	19	28	18	26

Table 3.6-2. Ratio of sucrose to glutamine entering the _a_ floret, or parts thereof, between various time intervals in culture. These ratios were derived from dpm values (^{14}C and ^{3}H respectively). The ratio in the culture solution at the start of culture was 15.2.

Time Intervals	Whole Floret	Glume	Palea & Lemma	Grain
0 to 10 min	29	14	33	11
10 min to 30 min	24	24	26	6
30 min to 2 hour	25	36	27	14
2 hour to 4 hour	21	31	20	29
4 hour to 8 hour	13	30	10	35

Table 3.6-3. Distribution of radioactivity between the ethanol-soluble and ethanol-insoluble fractions in the tissues of the b floret after 8 hours of culture

	^{3}H (ug of glutamine equivalents)			^{14}C (ug of sucrose equivalents)		
	Glume	Palea & Lemma	Grain	Glume	Palea & Lemma	Grain
Amount	11	93	12	209	2866	308
Percentage Insoluble	12	8	26	6	5	58

Table 3.6-4. Distribution of ^{3}H and ^{14}C between tissues of the grain after various times in culture. Values are % of total in grain.

Durat'n of Culture	^{3}H					^{14}C				
	Crease	Composite Layer	Endosperm	Endosperm Cavity	Embyro	Crease	Composite Layer	Endosperm	Endos-Cavity	Embyro
10 min	28.5	37.7	11.9	17.9	3.0	17.8	40.5	18.7	18.0	4.9
30 min	53.8	9.5	8.1	28.2	0.2	57.3	17.0	6.3	19.1	0.3
1 hour	36.7	10.7	8.0	42.1	2.4	46.9	9.3	6.0	33.8	3.9
2 hour	21.7	16.9	5.7	54.7	1.0	35.0	13.9	4.3	43.7	3.1
4 hour	14.3	20.1	3.6	57.8	0.4	15.4	17.4	2.6	59.1	5.4
8 hour	14.1	29.2	9.7	41.8	5.2	13.7	26.2	6.8	45.6	7.7

radioactivity in the grain. This value subsequentially declined then rose again. The total amount of radioactivity in the composite layer continued to rise throughout, however (Fig. 3.6-2). The proportion of total radioactivity in the endosperm cavity declined throughout, while after an initial rise, the proportion in the crease region declined also (Table 3.6-4).

After 8 hours of culture the proportion of the grain's radioactivity that was within the endosperm had built up to about to 50% (Table 3.6-4). Of this radioactivity, 65% of the ^{14}C but only 21% of the ^{3}H was in the insoluble form (Table 3.6-5). Indeed, the ratio ^{14}C:^{3}H in the insoluble fraction was seven times the corresponding ratio in the soluble fraction, showing that the conversion of ^{3}H into insoluble form occurred much slower than the corresponding conversion of ^{14}C.

3. Form of the Radioactivity in the Ethanol-Soluble Extracts of Tissues of the Floret and Grain, and in the Insoluble Residue of the Endosperm.

The purpose of this part of the study was to test whether or not ^{14}C from sucrose and ^{3}H from glutamine remained confined sufficiently to the pathways of carbohydrate and nitrogen metabolism, respectively, to allow their use as appropriate tracers within the grain. The ethanol-soluble fractions extracted from the component tissues of the floret and grain were separated by ion-exchange chromatography (section 2.11) into neutral and cationic fractions. The distribution of radioactivity between these fractions is shown in table 3.6-6. Also shown is the distribution of radioactivity in the culture solution first as supplied to detached spikelets without extraction, and secondly, after ageing for 8 hours along side spikelets in culture and extracting together with a sample of (non-radioactive) endosperm tissue.

In both samples of culture solution a proportion of ^{3}H separated into

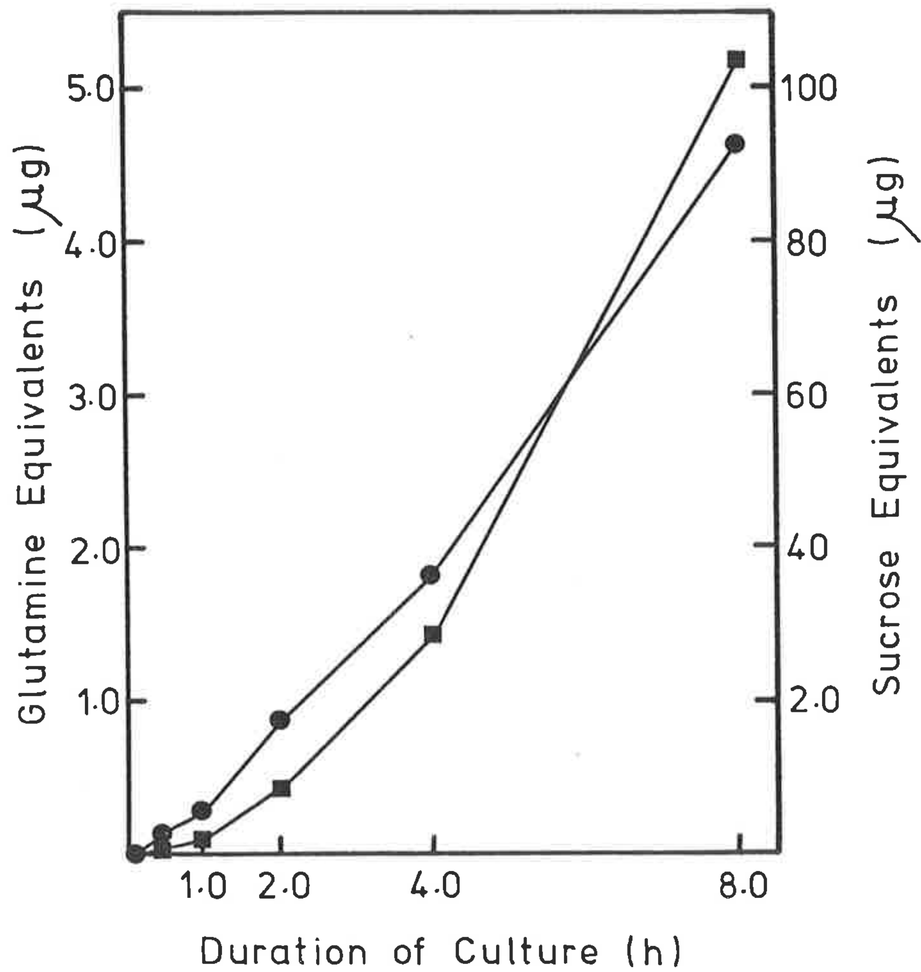

Figure 3.6-2. ^{3}H (● , expressed as glutamine equivalents) and ^{14}C (■ , expressed as sucrose equivalents) in the composite layer of grains during culture. (Values are the mean of 2 replicates for each time period.)

Table 3.6-5. Distribution of radioactivity between the ethanol-soluble and ethanol-insoluble fractions in the tissues of the grain after 8 hours of culture.

	^{3}H (ug of glutamine equivs.)				^{14}C (ug of sucrose equivs.)			
	Crease	Composite Layer	Endosperm Cavity	Endosperm	Crease	Composite Layer	Endosperm Cavity	Endosperm
Amount	2.6	2.9	1.1	5.3	53	66	14	174
Percentage Insoluble	23	50	0	21	29	72	17	65

Table 3.6-6. Distribution of ^{3}H and ^{14}C between the neutral and cationic fractions of the ethanolic extracts of tissues of the floret and grain after 8 hours of culture.

	^{3}H			^{14}C		
	% Recovery After Separat'n	% Distribution of Recovered Radioactivity		% Recovery After Separat'n	% Distribution of Recovered Radioactivity	
		Neutral Fraction	Cationic Fraction		Neutral Fraction	Cationic Fraction
Glumes	94	36	64	100	97	3
Palea & Lemma	97	40	60	99	99	1
Crease	94	36	64	98	91	9
Composite Layer	96	38	62	98	86	14
Endosperm Cavity	97	31	69	96	85	15
Endosperm	97	30	70	98	85	15
Substrate Initially		16	84		100	0
Substrate After Extraction*		36	64		99	1

EXPLANATORY NOTE.

* = See text for details.

the neutral fraction, demonstrating the instability of glutamine and its non-enzymic conversion to neutral forms, one of which is probably pyrrolidonecarboxylic acid (Greenstein et al., 1961; see also section 2.11)

Ethanol-soluble ^3H extracted from the glumes, palea and lemma, tissue of the crease, and composite layer separated into the neutral and cationic fractions in similar proportions as did ^3H from the 'extracted' culture solution. On the other hand, ethanol-soluble ^3H from the endosperm and endosperm cavity separated with a greater proportion in the cationic fraction demonstrating (as in section 3.3) the metabolic transfer of radioactivity from glutamine into other more stable amino acids in or on route to the endosperm.

Ethanol-soluble ^{14}C from the culture solutions, from the extracts of the glumes, and of the palea and lemma separated entirely into the neutral fraction. However, in tissues from the grain appreciable amounts separated into the cationic fraction demonstrating the involvement of carbohydrate in transaminase activity and synthesis of amino acids in these tissues (see e.g. Lewis, 1986).

In the ethanol-insoluble residue of endosperm, the protein fraction contained 93% of the ^3H and 24% of the ^{14}C after 8 hours of culture (Table 3.6-7). This demonstrates again (see Table 3.6-6) the participation in protein metabolism of ^{14}C derived from sucrose. Data in table 3.6-7 show that the conversion of radioactive amino acids into protein is low: While ^{14}C equivalent to 84 ug of sucrose was deposited as starch, ^3H equivalent to only 0.9 ug of glutamine was deposited as protein.

As a result of the metabolic conversion of ^{14}C to amino acids and the non-enzymic breakdown of glutamine (see above), it became clear that chromatographic separation of radioactivity into carbohydrate and amino acid fractions would be essential for all extracts taken from the grain in the experiments, to follow, tracing the route(s) by which radioactive substrate moved (results sections 4 and 5, and indeed chronologically

Table 3.6-7. ^{3}H and ^{14}C extracted as starch or protein from the ethanol-insoluble fraction of endosperm after 8 hours of culture.

	^{3}H (ug of glutamine equivalents)	^{14}C (ug of sucrose equivalents)
Starch	0.07	84.2
Protein	0.93	27.1

sections 3.4 and 3.5 in their entirety). Since most of the degradation of glutamine occurred during the drying of the ethanolic extract before chromatography (Table 3.6-6, additional results not shown), the technique of chromatographic separation was developed further to allow the ethanolic extract _per se_ to be applied to the column (section 2.11), and this modified method was used subsequently.

4. Pattern of Radioactive Carbohydrates and Amino Acids Throughout the Dorsal Region of the Grain as a Function of Time in Culture and as Influenced by Disrupting Putative Transport Pathways

After 30 minutes of culture, tissue taken from the base of the crease containing the main vascular bundle contained high levels of both ^{14}C-soluble carbohydrate (8,600 $dpm.mgdwt^{-1}$, n = 2) and ^{3}H-amino acids (8,300 $dpm.mgdwt^{-1}$, n = 2). Radioactivity was contained also in the inner pericarp (Fig. 3.6-3A) and in the sample of fluid taken from the endosperm cavity (radioactivity on a volume basis in the cavity estimated to be between 10 and 20 times the amount in the adjacent section comprising mainly endosperm but containing also a small amount of cavity fluid, see Fig. 3.6-3A). There were small amounts of radioactivity within the endosperm, but essentially all of it (both ^{14}C-carbohydrates and ^{3}H-amino acids) was located at this time in the region adjacent to the endosperm cavity (Fig. 3.6-3A). Hence, even though the inner pericarp contained much radioactivity, radioactive solutes arriving first in the _endosperm_ came from the direction of the endosperm cavity.

After longer periods of time (60 and 90 minutes, Figs. 3.6-3B and 3.6-3C), ^{14}C-soluble carbohydrates and ^{3}H-amino acids became spread throughout the endosperm, but always with greater amounts nearer the endosperm cavity.

Manipulative treatments exposing the grain in preparation for the surgical treatments (that disrupted putative transport pathways), in

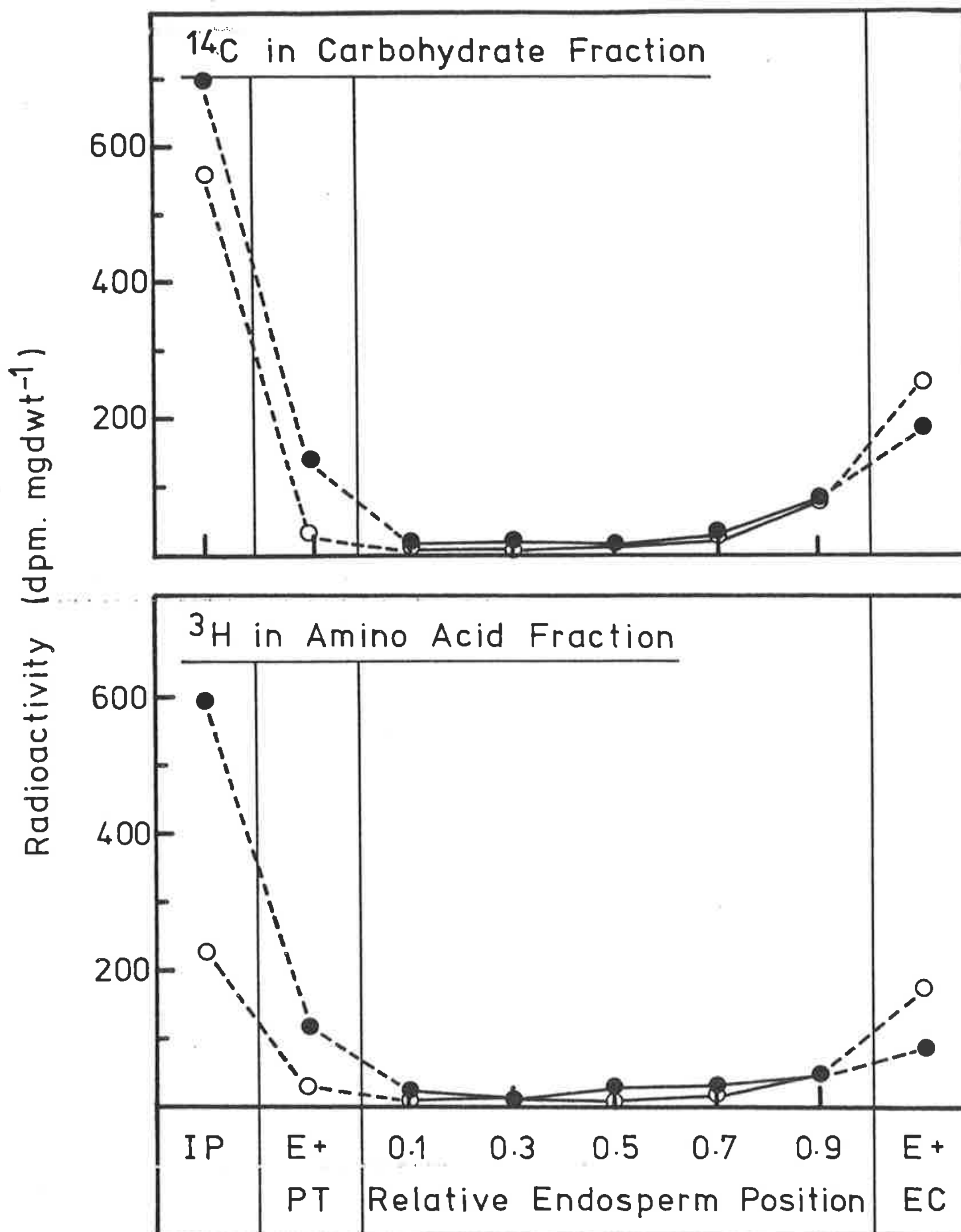

Figure 3.6-3A. Distribution of radioactivity across the sampled region (dorsal side) of wheat grains after detached spikelets were cultured for 30 minutes on radioactive substrate. Different symbols denote separate grains.

Explanatory Notes.

IP = The section containing mostly inner pericarp tissue (dorsal surface).

E + PT = Mainly endosperm plus a small amount of peripheral tissue.

E + EC = Mainly endosperm plus a small amount of fluid from the endosperm cavity.

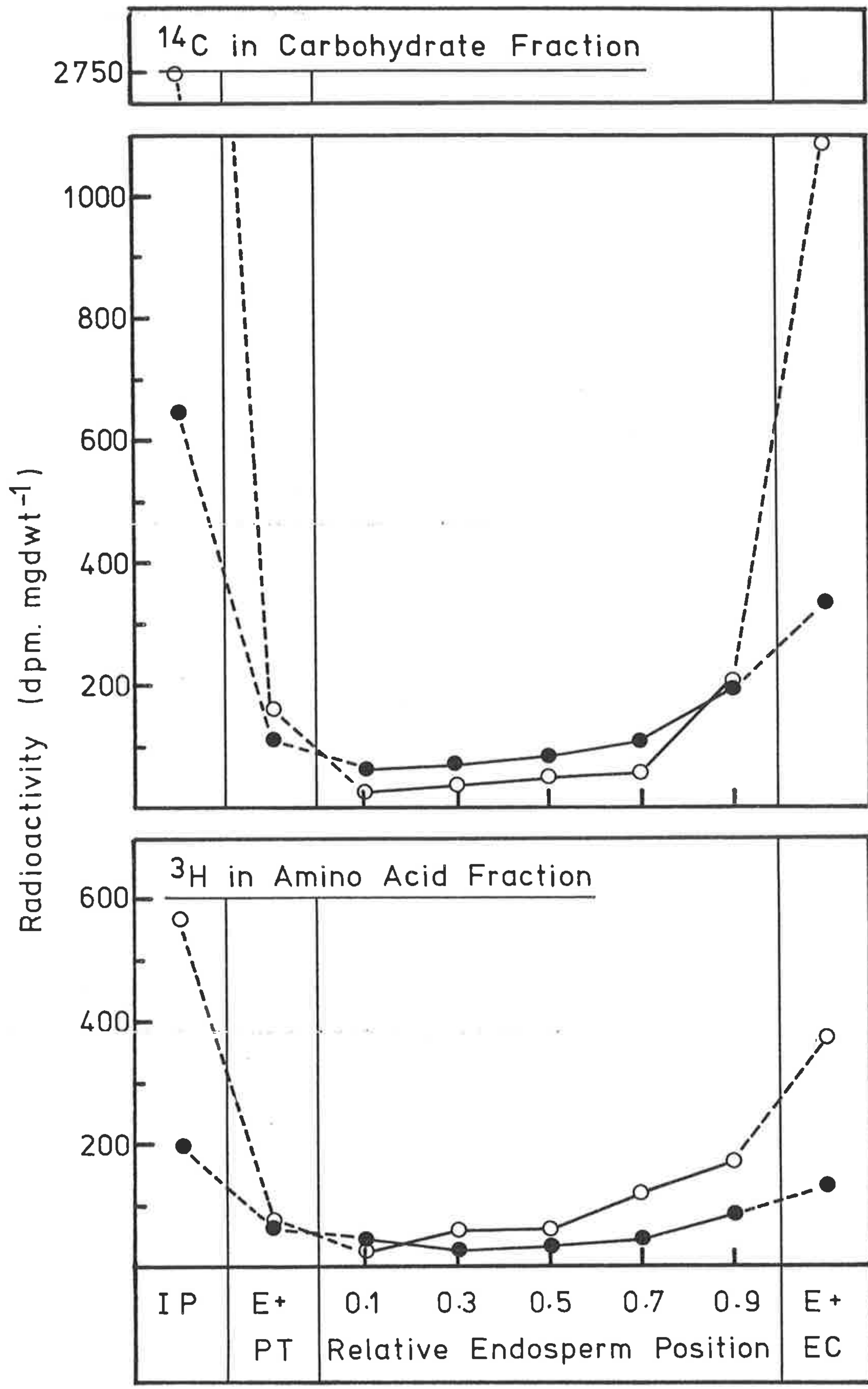

Figure 3.6-3B. As Fig. 3.6-3A, distribution after 60 minutes of culture.

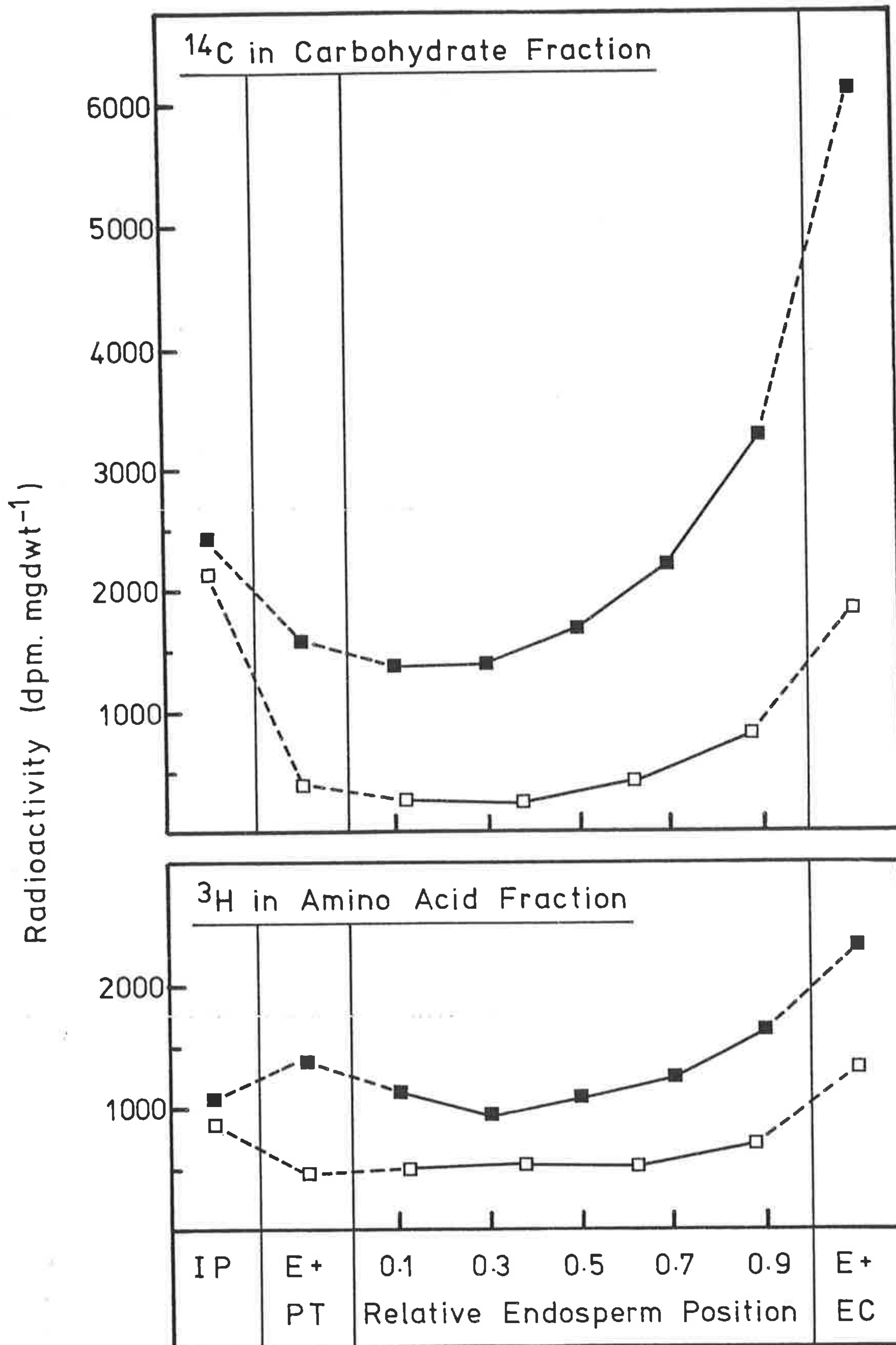

Figure 3.6-3C. As Fig. 3.6-3A, distribution after 90 minutes of culture.

themselves, had no noticeable effect on the distribution profiles of ^{14}C-soluble carbohydrates and ^{3}H-amino acids throughout the dorsal region (Figs. 3.6-4A and 3.6-4B).

Disrupting transport in the stalk prevented entirely the movement of radioactivity to the grain.

Two methods of disrupting transport in the vascular bundle; (1) drilling a hole through it and cauterizing the edges of the hole and (2) placing a highly impermeable barrier across the vascular bundle and endosperm cavity produced similar results. While they had no effect on the amount of radioactivity that moved into the inner pericarp, the levels of radioactivity in the vascular bundle, the endosperm cavity and the endosperm distal to the site of disruption were greatly reduced (by more than 99%, results not shown in detail).

Any circumferential movement of solutes from the vascular bundle around the grain in the tissues of the composite layer (for movement subsequently to the endosperm in an inward radial direction) would have been disrupted by the treatment that cauterized longitudinally the composite layer, leaving a 0.3 mm gap on either side of the vascular bundle. This treatment had no effect on the distribution patterns of ^{14}C-soluble carbohydrate and of ^{3}H-amino acids throughout the dorsal region of the grain (Figs. 3.6-4A and 3.6-4B).

The distribution patterns of ^{14}C-soluble carbohydrates and ^{3}H-amino acids throughout the grain were not identical. Always, ^{3}H-amino acids appeared more evenly distributed compared to the gradient of ^{14}C-soluble carbohydrates decreasing as a function of distance from the endosperm cavity (Figs. 3.6-3A, 3.6-3B, 3.6-3C, and Fig. 3.6-4B cf. Fig. 3.6-4A).

5. Transfer of ^{14}C from Soluble Carbohydrates Into Amino Acid Form

Radioactivity from soluble carbohydrates became incorporated into amino acids, as observed previously (results section 2 and section 3.5). In

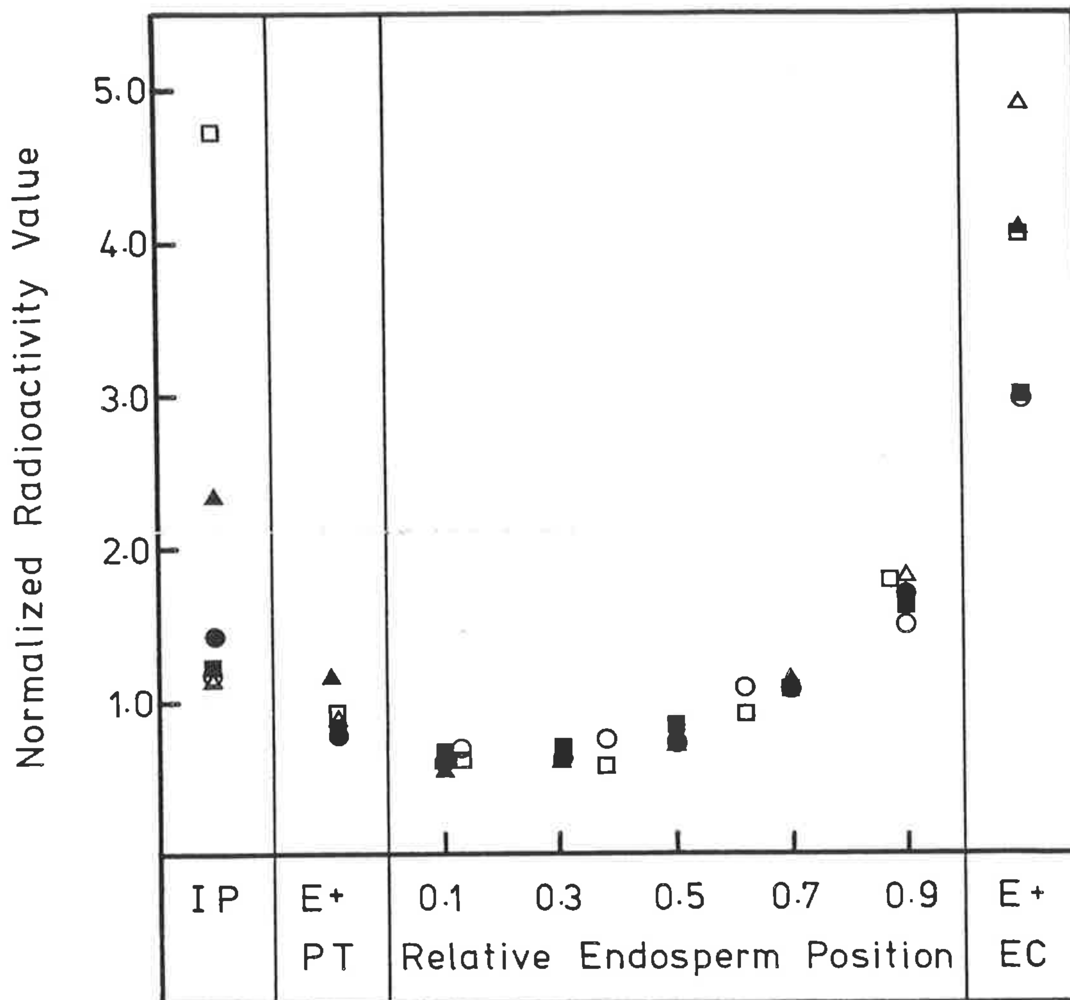

Figure 3.6-4A. Pattern of normalized radioactivity value of ^{14}C in the soluble carbohydrate fraction extracted from the sampled region (dorsal side) of wheat grains after detached spikelets were cultured for 90 minutes. The pattern in two control grains is shown (■ , □) and the symbols used here correspond to the same symbols and grains shown in Fig. 3.6-3C. Also shown is the pattern in two prepared grains (● , ○) and in two grains with circumferential transport disrupted (▲ , △). The notations IP, E + PT and E + EC are as in Fig. 3.6-3A.

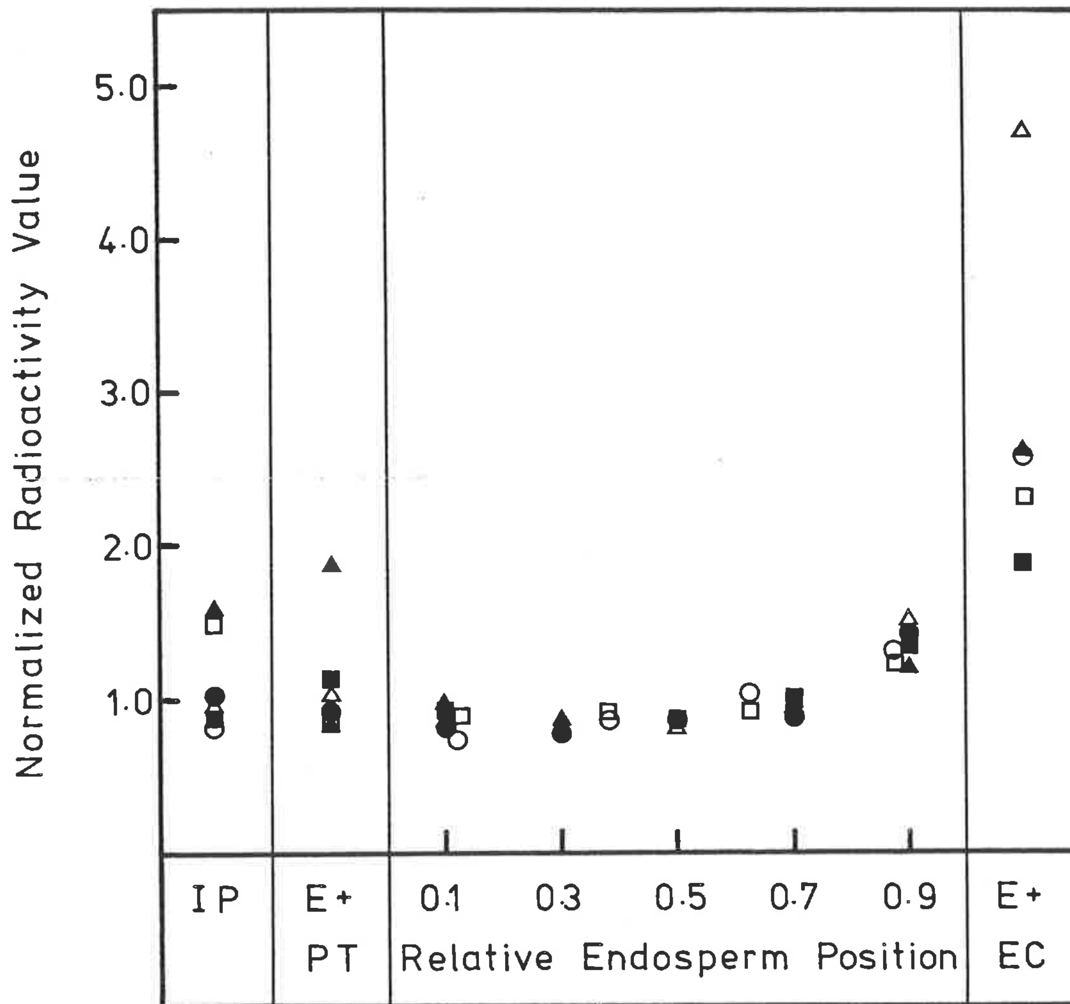

Figure 3.6-4B. As Fig. 3.6-4A, pattern of ^{3}H in the amino acid fraction. The symbols used here correspond to the same symbols and grains shown in Fig. 3.6-4A.

section 3.5 it was argued that the pattern of the ratio ^{14}C in amino acids to ^{14}C in soluble carbohydrates throughout the endosperm can be used to show whether or not most of this conversion occurred within the endosperm itself. The same argument applies here. Although there was substantial between-grain variation in the degree of transfer, the pattern was always the same (Fig. 3.6-5): A low ratio value in the vascular bundle and endosperm cavity and an increasing ratio value throughout the endosperm. This pattern indicates that essentially all of the transfer of ^{14}C from soluble carbohydrates to amino acids occurred within the endosperm. The distribution of amino acids formed within the endosperm (i.e. the ^{14}C ones) was different to the distribution of the amino acids that derived external to it (i.e. the ^{3}H ones): The distribution of ^{14}C-amino acids was even throughout the endosperm while the concentration of ^{3}H-amino acids decreased as a function of distance from the endosperm cavity (Fig. 3.6-6).

6. Circumferential Profile of Radioactivity in the Composite Layer

The rapid build-up of radioactivity in the composite layer followed by a rapid decline in proportional terms (Table 3.6-4) is the type of result expected in tissue forming the pathway of solute transport to the endosperm. Other evidence against a transporting function of this tissue (results section 5) is strong, however. The purpose for examining the circumferential profile of radioactivity in the composite layer is to test in another way whether or not solutes destined for the endosperm move around the grain in this tissue.

Essentially all of the longitudinal movement of solutes destined for the endosperm occurrs within the main vascular bundle (results section 4, see also sections 3.4 and 3.5). Hence the test for transport of these solutes within the composite layer is whether or not there is a time-related lateral gradient around the grain originating from the vascular

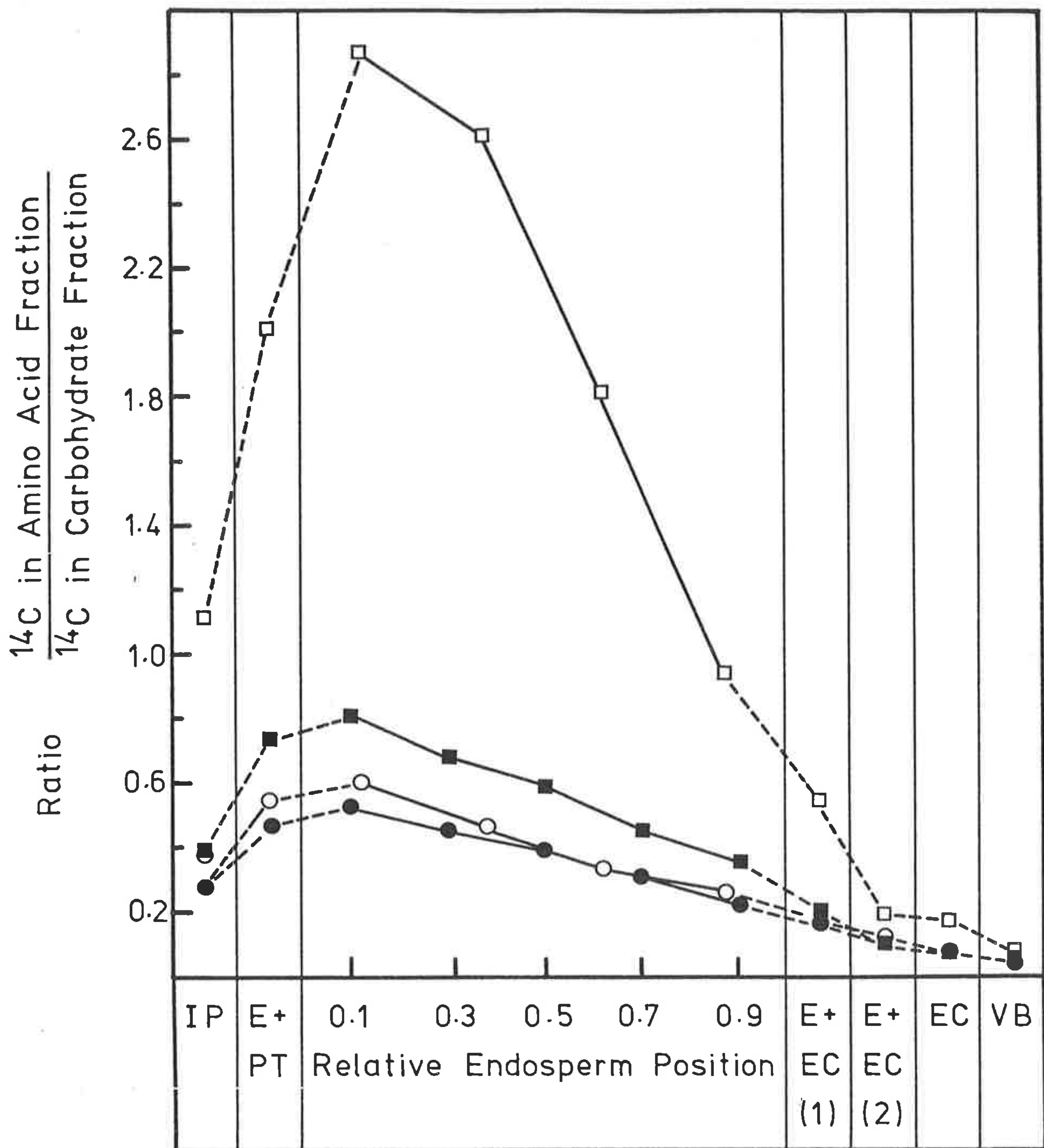

Figure 3.6-5. Ratio of ^{14}C in the amino acid fraction to ^{14}C in the soluble carbohydrate fraction across the sampled region (dorsal side) of wheat grains after detached spikelets were cultured for 90 minutes. The symbols used here correspond to the same symbols and grains shown in Figs. 3.6-4A and 3.6-4B.

Explanatory Notes.
The notations IP and E + PT are as in Fig. 3.6-3A.
E + EC (1) = E + EC in Fig. 3.6-3A.
E + EC (2) = Mainly endosperm but containing a greater amount of fluid from the endosperm cavity than in the adjacent slice (see E + EC (1) above).
EC = Fluid from the endosperm cavity.
VB = Small piece of tissue taken from around and including the main vascular bundle of the grain.

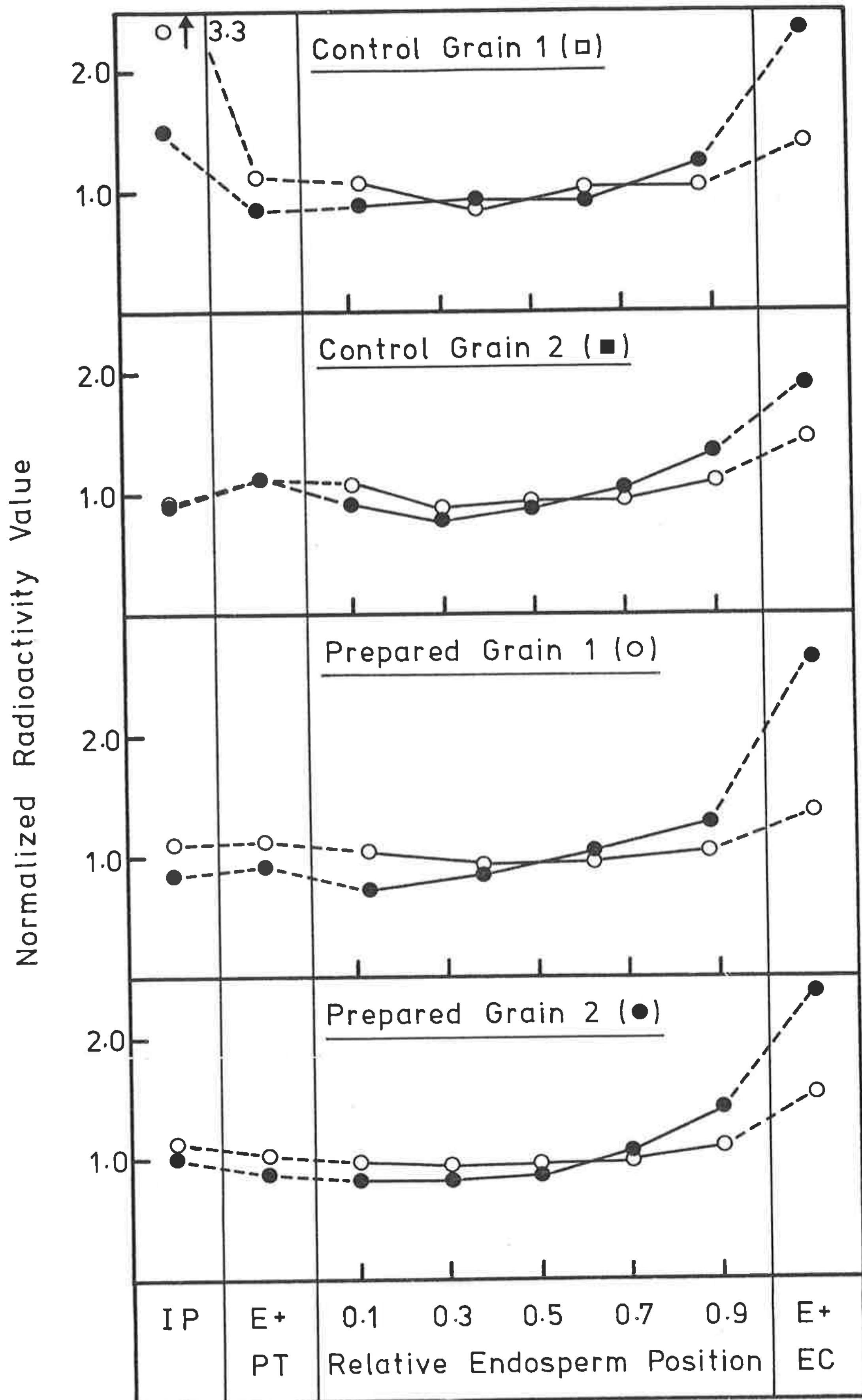

Figure 3.6-6. Pattern of normalized radioactivity value of ^{14}C (O) and ^{3}H (●) in the amino acid fraction extracted from sampled region (dorsal side) of 4 individual wheat grains after detached spikelets were cultured for 90 minutes. The symbol at the top of each graph corresponds to the symbol and grain shown in Figs. 3.6-4A, 3.6-4B and 3.6-5. The notations IP, E + PT and E + EC are as in Fig. 3.6-3A.

bundle.

A band of composite tissue (1.5 mm wide) was peeled from the circumference of the grain in its mid-region. Tissue at the base of the crease was excluded. The distribution of radioactivity along this band was uniform at all times (Table 3.6-8), making unequivocal the conclusion that no more than a very small proportion of radioactivity in the composite layer could be substrate in transit around the periphery of the grain on its way to the endosperm.

DISCUSSION

Assessment of Spikelet and Grain Function in Culture

The purpose for examining the rates of solution uptake by cultured spikelets (results section 1) , the subsequent distribution of radioactivity throughout the spikelets, and the metabolic conversion of this radioactivity into insoluble form (results section 2) was to test whether or not spikelets in culture performed in a way expected of spikelets *in vivo* and hence, whether or not this system was suitable for the proposed experimentation (see Introduction). None of the observed features of spikelet performance disqualify it from further use (see results sections 1 and 2). Although there was conversion of ^{14}C from sucrose into protein metabolism the use of ionic exchange chromatography enabled the tracing of carbohydrate movement and of amino acid movement independently of one another (results section 3, see also sections 2.11, 3.4 and 3.5). Several features of spikelet performance are worthy of further discussion.

The palea and lemma are sites of high rates of transpiration (Blum, 1985), and since most of the radioactivity was channelled first to this tissue (Table 3.6-1) it seems reasonable to presume that most of the

Table 3.6-8. Circumferential profile of radioactivity as a function of time in a band of composite tissue taken from around the grain in its mid-region. Segments 1 and 8 were adjacent to and on either side of the crease, and sections 4 and 5 were located on either side of the dorsal mid-line (mean of 2 replicates).

A. ^{3}H, ng of glutamine equivalents.

Duration in Culture	Segment number							
	1	2	3	4	5	6	7	8
10 min	0.2	0.1	0.3	<0.1	0.4	0.2	0.1	0.5
30 min	0.9	1.3	1.3	1.1	1.7	1.2	0.3	1.1
1 hour	4.0	2.2	2.3	3.6	3.8	2.2	1.2	4.2
2 hour	15.6	6.3	11.4	15.2	20.1	21.4	22.9	25.0
4 hour	42	31	44	51	49	49	49	45
8 hour	93	73	88	123	107	88	75	113

B. ^{14}C, ng of sucrose equivalents.

Duration in Culture	Segment number							
	1	2	3	4	5	6	7	8
10 min	0.5	1.1	5.2	0.5	0.6	8.2	0.9	2.3
30 min	10.7	18.7	21.2	9.8	13.7	15.9	?	10.7
1 hour	25	20	19	20	20	17	15	23
2 hour	128	47	73	93	148	193	399	338
4 hour	511	324	489	640	630	580	450	477
8 hour	1975	1512	1922	2288	1986	1356	928	1557

substrate was conveyed initially in the xylem. However, unexpectedly, the net accumulation of ^3H and ^{14}C from the culture solution was not proportional to their concentration in the solution. Apparently, there was faster uptake of ^{14}C-sucrose (carbohydrate) or a return of some ^3H-glutamine (amino acids) to the culture solution (Table 3.6-2).

On the other hand, at the same time there was preferred channelling of ^3H to the grain; ^3H in the grain at all times (excepting after 10 minutes of culture) accounted for 10% of the total ^3H in the spikelet, whereas the proportion of total ^{14}C contained within the grain was well below this value (Table 3.6-1, at least initially - before appreciable levels of deposition of ^{14}C in starch). This seems to be an expression of the mechanism, not yet understood, whereby the levels of amino substrate within the grain reflect closely the levels of amino substrate external to it, while in contrast, the levels of carbohydrate substrate within the grain do not show the same relationship (see for instance, Jenner, 1985).

Between 4 and 8 hours of culture the grain accumulated radioactivity equivalent to 250 ug of dry matter (Table 3.6-1). This rate would produce a daily dry weight increment of 1.5 mg, not far below dry weight growth _in vivo_ (i.e. 2.3 $mg.day^{-1}$, section 3.3).

The amount of radioactivity (mainly ^3H) incorporated into protein was small, especially in comparison to the amount of ^{14}C incorporated into starch (Table 3.6-7). However this can be attributed first to the lower rate of protein deposition (11% that of starch, section 3.3) and secondly, to the lower specific activity of amino substrate within the endosperm since the turn-over of amino substrate within the endosperm is slower than the corresponding turn-over of sucrose (by a factor of 9, section 3.2).

The pathways of carbohydrate movement throughout the grain have been described (see Introduction) from a study (section 3.4) in which $^{14}CO_2$ was supplied to an illuminated ear; a technique that preserved as much as

possible the _in vivo_ state of the plant. The experiments of section 3.4 were repeated in the current study (3.6) except that radioactive carbohydrate was supplied to the grain by culturing a detached spikelet on ^{14}C-sucrose, primarily to serve as a check that in culture the pathway of carbohydrate movement was the same as _in vivo_.

The patterns of ^{14}C-soluble carbohydrate in the dorsal region of grains cultured in the detached spikelet system, both as a function of time (Figs. 3.6-3A, 3.6-3B and 3.6-3C) and in response to disrupting putative transport pathways (results section 4, including Fig. 3.6-4A) was the same as those obtained when $^{14}CO_2$ was fed to illuminated ears (section 3.4). It seems unlikely that the mechanism of amino acid transport within grains of this system would differ from that _in vivo_ if carbohydrate movement in this system is the same as it is _in vivo_.

Route of Movement of Amino Acids Within the Wheat Grain

The patterns by which ^{3}H-amino acids were distributed throughout the dorsal region, both as a function of time and as influenced by disrupting putative transport pathways were in essence the same as those of ^{14}C-soluble carbohydrate (Figs. 3.6-3A, 3.6-3B and 3.6-3C; Fig. 3.6-4B cf. Fig. 3.6-4A; as in section 3.4). Hence, amino acids move to and within the endosperm along the same route as do carbohydrates (see Introduction). Further evidence against a circumferential and inward radial route is the lack of any lateral gradient of radioactivity in the tissues of the composite layer (Table 3.6-7).

Notwithstanding the above, the patterns of ^{3}H-amino acids and of ^{14}C-soluble carbohydrates throughout the dorsal region were not exactly the same (Figs. 3.6-3A, 3.6-3B and 3.6-3C; Fig. 3.6-4A cf. Fig. 3.6-4B), and indeed with time they each became similar in form to the distribution patterns of amino acids and sucrose _in vivo_ (section 3.3). The differences in distribution must be due to differences in the kinetics of movement of

each class of solute along this common route.

There was substantial conversion of ^{14}C from soluble carbohydrate into amino acids and most of this conversion occurred within the endosperm itself (results section 5, Table 3.6-6). Such conversion is a cytoplasmic function (e.g. Lewis, 1986) and hence the distribution of ^{14}C-amino acids that follows shows the distribution of amino acids that are compartmented within the cells of the endosperm (for detailed argument see section 3.5). On the other hand, the distribution of ^{3}H-amino acids throughout the endosperm represents the distribution of all incoming amino acids whether or not they are compartmented. The difference in the distributions of ^{14}C-amino acids and ^{3}H- amino acids (Fig. 3.6-6) reinforces the argument (sections 3.3, 3.4 and 3.5) that the distribution of solutes (in this case, amino acids) throughout the symplast is different to the distribution throughout the apoplast, or indeed in the tissue as a whole: The distribution throughout the symplast is even whereas there is a gradient of apoplast solute decreasing as a function of distance from the endosperm cavity. This, in turn, may be related to a fast transport (mixing) mechanism throughout the symplast of the endosperm and a slower transport mechanism throughout the apoplast.

Disrupting longitudinal movement in the vascular bundle of the grain did not block entirely the appearance of radioactivity in the vascular bundle, the endosperm cavity and the endosperm distal to the site of disruption (results section 4). However, it seems not unreasonable for small amounts of solutes to pass through other tissues, e.g. the inner pericarp or even the endosperm itself around such a point of disruption.

Role of the Inner Pericarp

The rapid movement of radioactivity into the composite layer (inner pericarp, cuticle, nucellar epidermis) and its subsequent rapid decline in

proportional terms (Table 3.6-4) is characteristic of tissue forming a part of the transport pathway to the endosperm (see response in vascular bundle and endosperm cavity, Table 3.6-4). However, other evidence (1) the subsequent (between 1 hour and 8 hours of culture) increase in radioactivity both in proportional (Table 3.6-4) and absolute (Fig. 3.6-2) terms within the composite layer, (2) the high proportional of this radioactivity in polymeric form and (3) the lack of any inward radial movement of substrate to the endosperm (results sections 4 and 6) suggests that the inner pericarp of wheat serves, as does the maternal tissue of soybean fruit (Thorne, 1979) as a substantial sink providing a pool of substrate for subsequent remobilization.

Such a function of the inner pericarp may be more conspicuous after a prolonged period in the dark as was the case in these experiments (see Materials and Methods).

It seems that solutes moving into the inner pericarp do not derive from the main vascular bundle running the length of the grain. Rather these solutes move longitudinally from the base of the grain in the inner pericarp itself. The evidence for this is as follows.

1. Disrupting longitudinal movement of solutes in the vascular bundle did not interfere with the movement of radioactivity into the inner pericarp (results section 4).

2. Disrupting any circumferential movement from the vascular bundle to the inner pericarp likewise did not interfere (Figs. 3.6-4A, 3.6-4B, other results not shown).

3. Radioactivity in the inner pericarp certainly did not pass from the vascular bundle radially through the endosperm (Fig. 3.6-3A).

4. There was no lateral gradient of radioactivity in the tissues around the grain (results section 6).

It seems not unreasonable to propose that solutes moving in the reverse direction would move by the same route, that is to say, products of photosynthesis or of remobilization in the inner pericarp move predominantly to the base of the grain for transfer there to the main vascular bundle for redirection to the endosperm.

SECTION 4

GENERAL DISCUSSION

SECTION 4 GENERAL DISCUSSION

The yields of wheat under irrigation are high, but mostly there is an inverse relationship between yield and percentage protein of the grain (sections 1.2.3.2.3 and 1.3.3). Protein percentage is the most important parameter of grain quality influencing the saleability of Australia's wheat on world markets (sections 1.1, 1.2.3.2.1 and 1.3.3), yet it is the least predictable of the major quality parameters unable, at present, to be genetically determined (sections 1.2.3.1.3, 1.2.3.1.4, 1.2.3.1.5, 1.2.3.2.2, 1.2.3.2.3 and 1.3.3).

Research programmes attempting to break the inverse relationship between yield and quality need to involve both agronomic and physiological studies. The aims should be to produce both short-term solutions to get the most out of existing systems and longer-term solutions based on a thorough understanding of the agronomic and genetic requirements for efficient production.

The current study investigated the physiological basis of yield and quality. The approach was to divide conceptually the mechanism of growth into a number of individual sections, both external to the grain (sections 1.2.3.2.3 and 1.3) and within (sections 1.4 to 1.10) and to concentrate on the role of one of these, viz. transport of substrate within the grain (see section 1.10).

Most, if not all, the amino-substrate (section 3.6) and carbohydrate-substrate (sections 3.4 and 3.6) destined for the endosperm follow the same route within the grain. That is, longitudinally within the main vascular bundle located at the base of the grain, then radially through the endosperm cavity and radially throughout the endosperm. There was no evidence for an inward radial route for the movement of either class of substrate to the endosperm from the inner pericarp or any other tissue surrounding the endosperm.

Some substrate moving to the grain through the grain stalk may move into the tissue of the inner pericarp longitudinally from the base of the grain (section 3.6, see also sections 3.4 and 3.5). The inner pericarp serves as a site of tempory storage (section 3.6). Remobilized solutes, together with ones synthesized by photosynthetic activity in the inner pericarp, itself (section 3.4), move to the endosperm through the region at the base of the grain for transport along the normal route (section 3.6).

A cavity, continuous with the endosperm cavity was observed adjacent to the embryo (section 3.1), hence, the route of solute movement to the embryo seems to be the same as that to the endosperm.

The most serious challenge to the validity of the tests applied to describe the route of movement may possibly be that solute movement throughout the endosperm was extremely rapid, and as a result radioactivity became distributed quickly into a pattern reflecting the concentration of substrate throughout the endosperm independent of the direction of supply or of treatments that disrupted part of that supply. Such a claim is refuted, however, by the observations that the pattern of radioactivity throughout the endosperm both in control and treated grains changed extensively as a function of time (at 30, 60, and 90 minutes, sections 3.4, 3.5 and 3.6), and that disrupting any circumferential movement did not alter the total amount of radioactivity in the endosperm (section 3.4).

Irrespective of the size of the endosperm cavity, ranging from 0.2ul to 20ul, there was no evidence for any pockets of air within the fluid of the endosperm cavity (section 3.1). Hence in all grains it seems that there would be a pathway of diffusion of low resistance from the nucellar projection to the cells of the modified aleurone layer - or in the case of grains where the modified aleurone layer was ruptured (see section 3.1), to both the cells of the modified aleurone layer and the cells of the inner endosperm that border the endosperm cavity.

Sucrose in the endosperm cavity comprises less than 50% (w/w) the

total amount of soluble carbohydrate (section 3.2), yet sucrose was the only soluble carbohydrate found to contain radioactivity after 90 minutes of spikelet culture on ^{14}C-sucrose (section 3.2, see also Ho and Gifford, 1984 where a similar result was obtained after feeding $^{14}CO_2$ to the flag leaf). Much of the carbohydrate in the endosperm cavity appeared in the form of a fructan series (section 3.2) and may comprise a form of carbohydrate storage as are fructans in the internodes of wheat (Blacklow et al., 1984; Thome and Kuhbauch, 1985). Alternatively, these longer chain oligosaccharides may not be metabolized readily and may remain as a pool creating solute potential in the cavity fluid, in turn, preserving the volume of the fluid and influencing the rate of substrate unloading from the phloem system of the grain to the endosperm cavity. The rate of substrate unloading from the seed coat in legume species appears mediated by an effect of apoplast osmolarity on the turgor of the unloading cells (e.g. Patrick et al., 1986; Wolswinkel and Ammerlaan, 1984).

The kinetics of movement of amino-substrate and of carbohydrate-substrate throughout the endosperm are not the same (sections 3.4 and 3.6) due undoubtedly in part to the slower turn-over of amino acids within the endosperm (section 3.3). However, two types of studies, measuring the concentration profile of solutes throughout the endosperm (section 3.3) and measuring the time-dependent profiles of radioactive substrate (sections 3.4, 3.5 and 3.6) each alluded to differences between amino-substrate and carbohydrate-substrate in their ratios of solute in the apoplast and the symplast throughout the endosperm, and to differences in the kinetics of movement of solute within each zone.

Resistance to movement of substrate along the longitudinal axis of the grain appears lower than the resistance to radial movement (Donovan, et al., 1983). From this it seems reasonable to propose that increasing grain length may be a more effective way of increasing grain weight than

selecting for thicker grains (Jenner, 1985). Underlying this proposal is the expectation that in the grain there would be a distributional gradient of starch deposition , decreasing along radial axes, as inferred in the discussions but not the data (see section 1.7) of Evers (1970) and Kent (1966), and that such a gradient would be determined, at least in part, by the resistance to substrate movement along those radial axes. Results reported in this thesis do not support this proposal:

1. Starch, the major determinant of grain weight, is distributed evenly throughout the inner endosperm of the dorsal region. There is a slight decline in the deposition of starch in the peripheral tissue (section 3.3). Decreasing grain thickness and increasing grain length may increase, both in relative and absolute terms the amount of the lower yielding peripheral tissue.

2. The conversion of radioactive sucrose (section 3.2) to radioactive starch occurred rapidly and evenly along the dorsal (radial) axis of the grain (section 3.4), despite the steep concentration gradients of radioactive (sections 3.4 and 3.6) and non-radioactive (section 3.3) sucrose. Evidently, a mechanism, proposed to be movement within the symplast, conveyed substrate throughout the endosperm along a route of low resistance while much, if not the bulk of the sucrose was moving slowly along an alternative route of higher resistance, a route proposed to be the apoplast in which the substrate was not readily accessible for starch metabolism (sections 3.3, 3.4, 3.5 and 3.6).

3. For whatever reason, during grain fill the ventral lobes grow (measured by expansion) more rapidly than the dorsal region of the grain (Evers, 1974) despite its more remote location from the source of substrate (section 3.4).

The gradients of substrate throughout the endosperm are established

primarily as a result of substrate movement through a zone of resistance. The ruptures in the modified aleurone layer and endosperm observed in some grains in response to reducing the number of grains per ear (section 3.1) would allow the fluid from the endosperm cavity to penetrate directly through the endosperm itself, decreasing the magnitude of the concentration gradient. This has the potential, at least, to provide more substrate to the peripheral areas, but in view of the above discussion, it may not influence markedly the deposition of dry matter therein.

The inference that much, if not most, of the endosperm sucrose is apoplastic (sections 3.3, 3.4, 3.5 and 3.6) implies that intercellar invertase which is present throughout the endosperm in abundant amounts (Jenner, 1974) is not active or only slightly so in vivo.

From both agronomic and physiological studies it seems reasonable to catagorize carbohydrate deposition during grain fill (under conditions of low water stress) as a sink-limited process and conversely protein deposition as a source-limited one (sections 1.3.1, 1.3.2, 1.3.3 and 1.10). Consider now, in view of the results obtained during the current study programme whether or not this is necessarily correct. In other words, is modification to events within the grain itself the only way to achieve an increase in carbohydrate deposition per grain during grain fill, and in a similar context would it be possible to achieve an increase in protein deposition by modifying events within the grain?

Deposition is the integration of rate and duration. Duration is not considered here again (see sections 1.3, 1.7 and 1.10) since no results were obtained that could contribute meaningfully to our understanding of it.

However, consider rate. First, one would expect that the ratio of substrate concentration to rate of deposition of insoluble product would be low for source-limited operations and would be high for sink-limited ones. However the opposite applies within the endosperm; the value of this ratio

for the conversion of amino acids to protein is nearly 10 times the value for the conversion of sucrose to starch (section 3.3).

Consider the processes separately. First the synthesis of starch.

Culturing peeled (and sliced) endosperms introduces substrate directly to the apoplast of the endosperm tissue and hence circumvents any regulatory mechanism that is located within the grain but external to the endosperm. The rates of starch deposition in cultured endosperms are no greater than the rates <u>in vivo</u> (e.g. Bhuller and Jenner, 1986; Rijven and Gifford, 1983; Gifford and Bremner, 1981). In view of the apparent rapid distribution of radioactive sucrose throughout the endosperm once it is compartmented within the endosperm cells and the rapid and even conversion to starch that follows (section 3.4 and 3.6), the transfer of sucrose from the apoplast to the symplast seems to be a prime contender for a/the site of regulation of the rate of starch deposition within the grain. Notwithstanding this, homeostatic levels of sucrose are maintained within the grain despite fluctuations in external supply (section 1.3.1). Hence there must also be a site either within the maternal tissue of the grain or at the modified aleurone layer that maintains the concentration of sucrose in the endosperm apoplast at constant levels, although it may have little or no direct regulation on flux. This view is consistent with starch deposition being sink-limited.

Consider protein: Although the amount of amino-substrate in the endosperm seems high, at least when compared to the amount of carbohydrate-substrate (see above), it seems that the level of amino-substrate in the endosperm needs to increase still further, and substantially so, if there is to be an increase in protein deposition in response to an increase in external supply. This is seen most clearly in the work of Barlow et al., 1983, (NB. Fig. 2d), where increases in the amino-supply to cultured ears 2-fold, 3-fold and 4-fold above the presumed level of <u>in vivo</u> supply increased the rate of protein deposition 1.4-fold, 1.7-fold and 1.9-fold

respectively, but at the same time the levels of non-protein nitrogen within the grain increased 2-fold, 6-fold and 10-fold. This is the type of response one may expect if there was a restriction within the endosperm itself to the rate of the overall process of supply and utilization of amino-substrate. A critical question is whether the rate of movement of amino-substrate into the grain is determined essentially by the level of external substrate *per se* or alternatively by the magnitude of the concentration gradient between external and internal sites. If the magnitude of the gradient is more important, an increase in the efficiency of conversion of amino-substrate to protein within the endosperm itself tending to decrease the level of amino-substrate would have a similar effect on flux as increasing external supply. In so doing, the harvest index of nitrogen and the yield of grain nitrogen may increase. Accordingly, deposition of grain protein during grain fill may not be source-limited entirely.

Much of the discussion throughout this thesis involves assumptions derived indirectly concerning the distribution of substrate within the endosperm between the apoplast and symplast, and the rates of movement of substrate through each zone. Clearly one of the next steps in understanding further the physiological basis of yield and grain quality is to test experimentally the validity of those assumptions.

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