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CARBOHYDRATE METABOLISM AND VIGOUR IN WHEAT

by

Duncan Steven Garrood

A thesis submitted in part fulfilment for the degree of
Doctor of Philosophy of the University of London and for
the Diploma of Membership of the Imperial College.

Department of Pure and Applied Biology
Imperial College of Science and Technology
London, SW7

August, 1983

ABSTRACT

Low vigour in wheat seeds was found to be a serious disorder, the main symptoms of which were poor germination and growth under stress conditions. When these seeds were germinated at an optimum temperature the symptoms were not expressed, except for the presence of an extended quiescent period before germination began, even though the seeds were fully imbibed. Of the agricultural malpractices investigated, post-harvest hot air drying of moist seeds proved to be the most effective elicitor of the disorder. Such damage was easily reproduced in the laboratory.

Several physiological processes proved to be affected by vigour loss, and of these the metabolism of carbohydrates was most closely investigated. A sugar shortage in low vigour seeds proved to be a rate limiting step in their growth, and supplementation with exogenous sugars alleviated the symptoms to a large extent. The seeds also displayed a reduced capability to convert endospermic glucose to sucrose in the scutellum and this was due to the depressed activities of sucrose metabolising enzymes in this organ.

The supply of glucose from the endosperms of low vigour seeds was also deficient; an electrophoretic and immunochemical investigation revealed no change in pattern of *alpha*-amylase isoenzymes, but a severe drop in their abundance. Once antibodies had been raised against these two groups of isoenzymes, immunofluorescent localisation showed that many low vigour seeds had lesions in their aleurone cells which would account for the fall in *alpha*-amylase synthesis. Levels of the phytohormone, and *alpha*-amylase elicitor, gibberellic acid, were found to be reduced in low vigour plants, although this may have been due to increased concentrations of the antagonist abscisic acid. A model for the possible causes of the decreased metabolism of stressed low vigour seeds is discussed.

ACKNOWLEDGEMENTS

I express my sincere thanks to my supervisor, Dr. Andrew Goldsworthy, for his contagious enthusiasm, guidance and patience throughout this work, and in particular for the many useful discussions during the preparation of this manuscript.

Similar thanks are due to my Industrial supervisor, Dr. Dick Ellis of R.H.M. Research Ltd. and for his introduction to the attractions and opportunities offered by industry. His sense of assessment of the importance of research has been of significant influence on my work.

I also wish to thank the many friends and colleagues at R.H.M. Research who in some way have contributed to the success of this project. In particular I would like to thank Jim Sargeant for all his help with the *alpha*-amylase experiments, and in general for his boundless patience and for passing on a small portion of his "green-fingered" abilities at the bench. My gratitude is also extended to Daphne Bennett for help with electron microscopy, Helen Kearns and Gill Lowy for prolific biochemical advice, Chris Fullerton for field trials, Alison Reeves for vigour-testing and to all the members of the graphics unit for aid with photography.

I am indebted to many people at Imperial College for technical help and many fruitful discussions, particularly Melvyn Dover, Malcolm Weir, Dr. Keerti Rathore, Dr. Paul Millner and Dr. David Chapman. My thanks to Linda Williams for advice on immunological techniques.

I am deeply grateful to Tracy Erlam, for her endless patience and sympathies when the work did not go as smoothly as it might have done and for proof-reading this manuscript.

Finally, I gratefully acknowledge the financial support of the Science and Engineering Research Council and Ranks Hovis McDougall Research Ltd.

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ABBREVIATIONS

ABA	ABSCISIC ACID
ADP	ADENOSINE DIPHOSPHATE
ATP	ADENOSINE TRIPHOSPHATE
CoA	COENZYME A
CTP	CYTIDINE TRIPHOSPHATE
DEAE	DIETHYL AMINO ETHYL
°C.	DEGREES CELCIUS
DNA	DEOXYRIBONUCLEIC ACID
EDTA	ETHYLENEDIAMINETETRACETIC ACID
FITC	FLUORESCCEIN ISOTHIOCYANATE
F6P	FRUCTOSE 6-PHOSPHATE
G1P	GLUCOSE 1-PHOSPHATE
G6P	GLUCOSE 6-PHOSPHATE
GA	GIBBERELIC ACID
GTP	GUANOSINE TRIPHOSPHATE
HCl	HYDROCHLORIC ACID
HPLC	HIGH PERFORMANCE LIQUID CHROMATOGRAPHY
IgG	IMMUNOGLOBULIN
KCl	POTASSIUM CHLORIDE
M/C	MOISTURE CONTENT
m-RNA	MESSENGER RIBONUCLEIC ACID
NaCl	SODIUM CHLORIDE
NAD	NICOTINAMIDE ADENINE DINUCLEOTIDE
NADH	REDUCED NICOTINAMIDE ADENINE DINUCLEOTIDE
NADP	NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE
NADPH	REDUCED NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE
PBS	PHOSPHATE BUFFER SALINE
Pi	INORGANIC PHOSPHATE
PPi	PYROPHOSPHATE
POLY(A)-RNA	POLY ADENYLATED RNA
PPP	PENTOSE PHOSPHATE PATHWAY
r-RNA	RIBOSOMAL RIBONUCLEIC ACID
S6P	SUCROSE 6-PHOSPHATE
TCA	TRICARBOXYLIC ACID CYCLE
TRIS	TRIS(HYDROXYMETHYL)AMINOMETHANE
t-RNA	TRANSFER RIBONUCLEIC ACID
UDP	URIDINE DIPHOSPHATE
UDPG	URIDINE DIPHOSPHATE GLUCOSE
UTP	URIDINE TRIPHOSPHATE

1. INTRODUCTION

In any country's economy, agriculture forms a major part; providing food for the inhabitants and a resource which can be traded to earn foreign currency. Wheat is one of the most important crops, being grown from the arctic and antarctic borders to near the equator. During the season 1981/1982 a record 1.66m hectares were grown in the U.K. yielding a further record of 6.18 tonnes/hectare (F.M.B.R.A. 1983; H.G.C.A. 1983).

To grow cereals successfully and profitably requires attention to detail at every stage of crop growth, and none is more important than seedling establishment. To achieve the very high yields sought after in the U.K., the farmer must ensure that all his seed will germinate and grow as required; only after this can he apply chemicals which enhance the growth or keep the crop healthy. The physiology of early seedling growth is, as yet, beyond commercial chemical control.

The Official Statutory Germination Test provides a reliable measure of the ability of a seed to germinate under optimum conditions; however, it has failed in its ability to predict successful field establishment. It does not quantify variations in a hidden seed factor, known as seed vigour. Germinating seeds in the field are subjected to harsh, stressful conditions which cause seeds with weaknesses in their physiology either to die or to germinate but emerge more slowly than their more vigorous counterparts. It is common to observe differences in the rate of seedling establishment between the drill rows of one field and the next; whilst this is often attributed to variable seedbed and weather conditions, it may also be due to vigour variations between seedlots.

If those drill rows which do not produce a vigorous crop are investigated further, we frequently find many small deformed seedlings below soil level as well as completely ungerminated seeds. Even those seedlings which become established grow more slowly than vigorous plants and subsequently yield less (Stormonth 1978). The early slow growth rates of such seedlings suggest that there are some

lesions in their anatomy or metabolism, and are particularly symptomatic of starvation. Perhaps the seedlings die or cannot grow rapidly as they are not being supplied with sufficient respirable substrates from their reserves, or perhaps they are not capable of making full use of what nutrients they do have. Preliminary histochemical investigations have shown that the scutellum (an organ intimately involved in mobilizing carbohydrate nutrient reserves) is visibly damaged in low vigour seedlings (Goldsworthy, personal communication). Before consideration of the possible causes of low vigour, some introduction to the anatomy, physiology, and biochemistry of the wheat and related cereals' seed is necessary.

1.1. STRUCTURE AND COMPOSITION OF THE WHEAT GRAIN

As Briggs has stressed (Briggs 1973), it is vital to have a knowledge of grain anatomy before undertaking a biochemical investigation into seed metabolism. The mature grain is not a true seed but a single-seeded fruit, or caryopsis, where the closely adhering outer layers (pericarp and testa) are composed of dead cells (Percival 1921). This is the characteristic fruit of the grass family (Graminae). It has been suggested that variations in the strength of some pericarp layers may account for differences in germination rates between seeds (Wellington 1956).

A "dry" grain would typically consist of 70% starch, 13% protein, 2% lipid, 2% minerals and between 10 and 15% water. Although the grain is composed of many tissues the ones of primary concern are the embryo axis and scutellum (germ) and the aleurone and starchy endosperm (endosperm). These comprise 9% and 85% respectively of the grain dry weight (Esau 1973). Their positions within the grain are illustrated in Figure 1. The germ is situated at the opposite end of the grain to the brush of fine hairs; running between the two ends is an infolding of the aleurone and outer grain layers, known as the crease. There is a deeply pigmented vascular strand at the bottom of the crease.

The embryo axis is differentiated into a plumule (young shoot) protected by a sheath-like coleoptile, and a

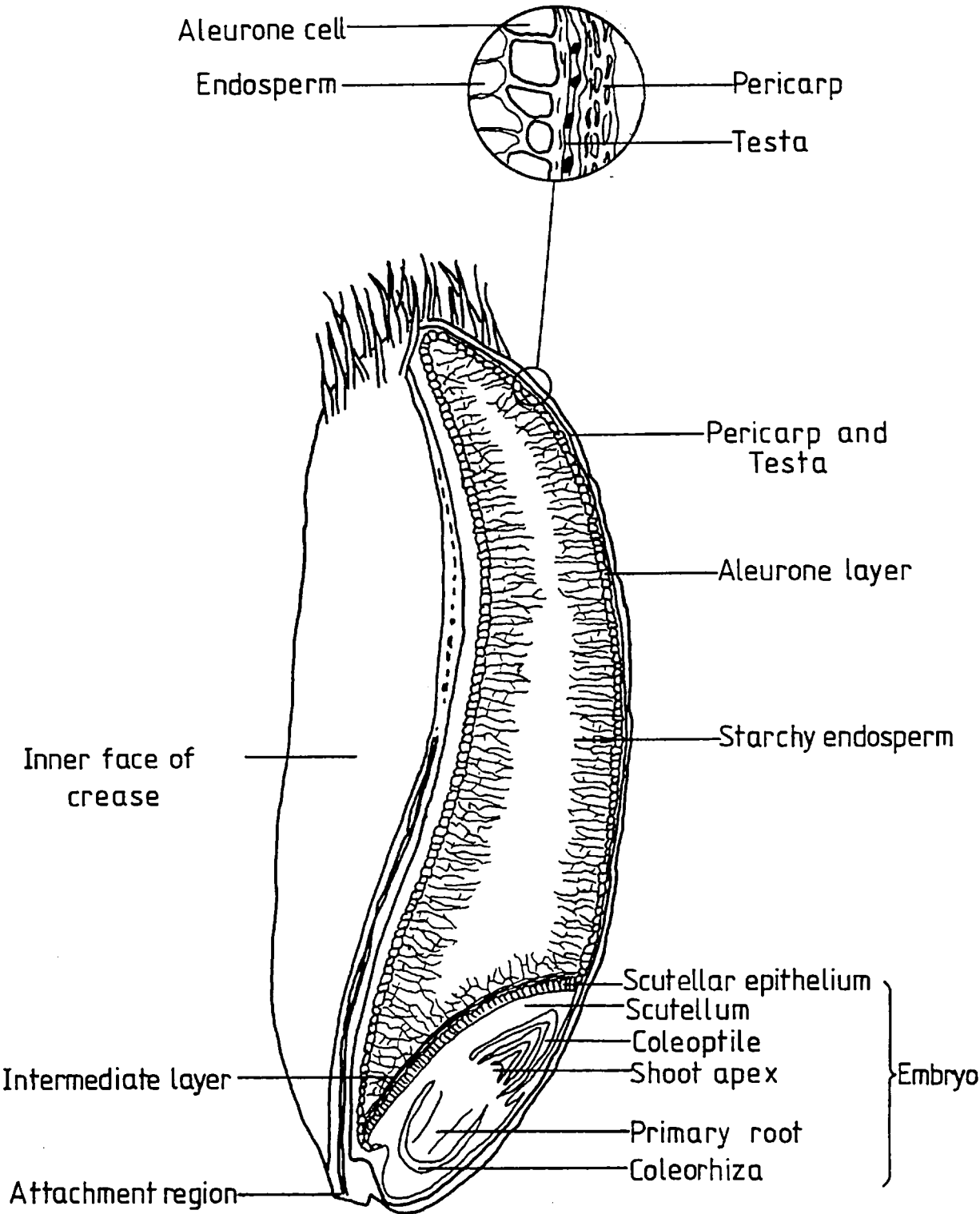


FIGURE 1. Wheat caryopsis: Median Longitudinal Section (adapted from Angold 1979).

radicle (young root) similarly covered with a protective coleorhiza. It is connected to the scutellum by vascular tissue. The scutellum itself is the single cotyledon of the embryo, and whilst it contains some stored nutrients (mostly triglycerides) its main function is as an haustorium transferring mobilized endospermic nutrients to the growing seedling.

The aleurone is composed of a single layer of living, non-dividing, thick-walled cuboidal cells. These cells contain the majority of the grains' mineral stores, as well as substantial protein reserves in the form of aleurone grains (Morrison *et al.* 1975). Numerous plasmodesmata can be seen joining adjacent aleurone cells thus facilitating chemical communication between them. The primary function of the aleurone cells is to secrete the enzymes responsible for the hydrolysis of the starchy endosperm reserves.

In contrast the cells of the starchy endosperm are thin-walled and non-viable. They are filled with starch granules completely embedded in a protein matrix (Barlow *et al.* 1973). The protein consists of salt soluble albumins and globulins as well as glutenins and gluten-forming gliadins. Typically, 80% of the endosperm dry weight consists of starch granules of both the A and B type (Bathgate and Palmer 1972).

Ultrastructural studies have mostly been confined to the aleurone and embryo. Unimbibed rye embryos show short crescent shaped endoplasmic reticulum, few dictyosomes and simple mitochondria with few cristae (Hallam 1972). At the start of germination, these membranes rapidly regain structural organisation and normal biochemical function (Hallam *et al.* 1972), and the numbers of mitochondria increases. Similarly, cells of the scutellum quickly develop into active forms during imbibition, with well developed endoplasmic reticulum and mitochondria (Swift and Buttrose 1972). As in aleurone cells, the cells of the scutellum can be characterised by the presence of specialised microbodies called glyoxysomes, during germination (Colbourne *et al.* 1976; Longo *et al.* 1972).

Many studies of aleurone ultrastructure have been undertaken (Buttrose 1963b, 1971; Colbourne 1974; Jones 1969a,b,c; MacLeod *et al.* 1964; Van der Eb and Nieuwdorp 1967), many of them concentrating on the structure of the aleurone grains. Each grain contains phytin globoids and protein-carbohydrate within a protein matrix, and is surrounded by a single membrane (Altschul *et al.* 1966; Jacobsen *et al.* 1971). Other organelles include sparse mitochondria with few cristae, dictyosomes, leucoplasts and various vesicles.

1.2. IMBIBITION AND GERMINATION

On contact with water a quiescent wheat grain will immediately imbibe and only after ten hours will this uptake slow down (Levari 1960); the embryo completing imbibition before the starchy storage tissues (Ching 1972). Seeds frequently display a triphasic mode of water uptake (Bewley and Black 1978; Moreland *et al.* 1974) which is also noted in isolated wheat embryos (Ginzburg 1978; Marcus 1969) where imbibition and the processes associated with it are much faster than in the intact seed (Bray 1979). The rapid first phase of uptake is a purely physical process, occurring in both viable and non-viable seeds. During the following lag phase, which lasts about four hours in isolated embryos, it is likely that the metabolic systems required for successful germination are developed in viable seeds (Leopold and Kriedeman 1975). It is only the viable and non-dormant seeds which enter the final phase of uptake which is associated with radicle emergence and subsequent true growth.

Many studies have been conducted into the events associated with early imbibition. A common observation is that of solute leakage from the embryo into the imbibition medium (Abdul-Baki and Anderson 1970; Ching and Schoolcraft 1968): this also occurs in isolated aleurones (Eastwood and Laidman 1971b). The leachates usually contain sugars, minerals, organic and amino acids. It was suggested that the rapid water inrush disrupted cell membranes which then became leaky and only when these lesions were repaired would leakage cease (Larson 1968). However, it now seems more likely that the membranes of unimbibed seeds lack

integrity so that some solute leakage is inevitable, but these membranes are quickly re-established during early imbibition thus retaining the remaining solutes (Simon and Raja Harun 1972).

The term germination has come to mean different things to different people. Given favourable conditions of water, temperature, oxygen tension and possibly light, a viable non-dormant seed will germinate. Bewley and Black (1978) suggest that germination begins with water uptake and ends with radicle or hypocotyl emergence. Jann and Amen (1977) extend this definition to include successful establishment of a seedling. For brevity in this treatise, radicle emergence and subsequent seedling growth will both be included under the title of germination, but figures of % germination will not be given. Instead, the % of apparently normal seedlings to grow will be used as this is more applicable when describing variations in vigour (Justice 1972).

1.3. MOBILIZATION OF NUTRIENT RESERVES

Whilst the bulk of the food reserves of cereal grains is in the endosperm, the initial stages of germination can take place using stored nutrients from the embryo itself. Oil droplets in the scutellum rapidly disappear and starch begins to accumulate there within 24 hours of soaking. This is found in both attached and isolated barley embryos (Macleod and Palmer 1966). Such embryos also use their stores of raffinose and sucrose which become depleted after 24 hours (James 1940). Once the soluble reserves of isolated embryos are exhausted, insoluble cellulose and hemicellulose from cell walls are mobilized as an energy source but this naturally leads to death (Bewley and Black 1978). This does not normally occur in the attached embryo where soluble carbohydrates from the hydrolysing endosperm reserves are supplied to the growing regions.

1.3.1. Mobilization of Carbohydrates

One of the earliest events in the utilization of endosperm carbohydrates is the dissolution of the intermediate layer adjacent to and inbetween cells of the absorptive scutellum

epithelium. The latter then elongate to present a larger surface area for subsequent absorption (Briggs 1973). The enzymes responsible for this are mainly endo-*beta*-glucanases (Macleod and Palmer 1966) which degrade glucan components with *beta*-1,3 and *beta*-1,4 links (Palmer 1973).

Before the main cereal carbohydrate reserve, starch, can be mobilized the cell walls of the endosperm must be hydrolysed (Morrall and Briggs 1978). In wheat these walls are simple in composition, containing 15% protein and 75% polysaccharide of which 85% is arabinoxylan (Mares and Stone 1973). A mixture of *beta*-glucanases and arabinoxylanase can attack this polysaccharide (Palmer 1975) and is secreted by the aleurone, thus sufficiently degrading the wall to render the enclosed starch grains accessible to enzymic degradation (Fincher and Stone 1974).

The amylose component of starch can be completely hydrolysed by *alpha*- and *beta*-amylases, but the amylopectin portion is only digested to limit-dextrins by these enzymes. Two debranching enzymes capable of degrading the *alpha*-1,6-bonds in limit-dextrins are known in cereals. The R-enzyme debranches amylopectin and *beta*-limit-dextrin (Palmiano and Juliano 1972), and is synthesized during germination. Limit-dextrinase attacks *alpha*- and *beta*-limit-dextrins, but will not debranch large amylopectin molecules (MacWilliam and Harris 1959; Manners *et al.* 1970), and is also synthesized *de novo* in the aleurone at germination (Hardie 1975). The final products of *alpha*- and *beta*-amylolysis are largely *alpha*- and *beta*-maltose which, following isomerization, are hydrolysed to glucose by *alpha*-glucosidase (maltase); an enzyme found in both embryos and aleurones of germinating barley (Jorgenson 1965). Phosphorylases play little part in the degradation of starch during cereal germination (Murata *et al.* 1968; Nomura *et al.* 1969) although they are important in early pea germination (Swain and Dekker 1966, 1969; Juliano and Varner 1969).

Of all these enzymes, *alpha*- and *beta*-amylase play the major roles with *alpha*-amylase being by far the most abundant (Briggs 1972; Gonzalez and Beevers 1976; Taiz and Jones 1970; Tomos and Laidman 1979). Over 70% of the

protein synthesized by aleurone cells during germination constitutes *alpha*-amylase (Chen and Jones 1974); this is in addition to the *alpha*-amylase secreted by the scutellum (Gibbons 1980). The enzyme is synthesized *de novo* in germinating grains (Filner and Varner 1967) using amino acids released by proteolysis of aleurone grains.

Alpha-Amylase (*alpha*-1,4 glucan 4-glucanohydrolase E.C.3.2.1.1.) catalyses the hydrolysis of *alpha*-1,4 glycosidic linkages at random points throughout the chains of amylose and amylopectin, and shows no preference for either polymer (Palmiano and Juliano 1972). It appears to be the only amylolytic enzyme capable of binding to undegraded starch granules (Dunn 1974). In contrast, *beta*-amylase cannot bind to starch granules; cleaves *beta*-maltose from amylose and amylopectin leaving *beta*-limit-dextrin and is not synthesized *de novo* in aleurone cells (Hardie 1975). Inactive forms of *beta*-amylase are found throughout the barley endosperm (Gibbons 1980) and are bound to gluten proteins by disulphide bridges which are broken by proteases released from the aleurone during germination (Hardie 1975; Rowsell and Goad 1962, 1964). Recent evidence suggests that *de novo* synthesis of *beta*-amylase can occur at the onset of germination in rice scutella although this ceases to be significant in later stages (Daussant et al. 1983; Okamoto and Akazawa 1980).

Once the low molecular weight sugars are released from starch, they are available for uptake by the scutellum. Glucose is absorbed both passively and by active transport (Bewley and Black 1978); maltose may be absorbed too, but is likely to be rapidly hydrolysed. There is an active *alpha*-glucosidase situated at the scutellum surface in isolated maize scutella (Humphreys 1975). Although some glucose is converted to fructose-6-phosphate in the scutellum, neither of these substances accumulate there but they are rapidly converted to sucrose (Edelman et al. 1959; Nomura et al. 1969; Nomura and Akazawa 1973). The pathways of sucrose synthesis in the scutellum have been recently reviewed (Akazawa and Okamoto 1980). Following the conversion of glucose to fructose-6-phosphate (F6P) and uridine di(phosphate) glucose (UDPG), sucrose-6-phosphate

synthetase and sucrose-6-phosphate phosphatase act upon them to release sucrose. This sucrose is then translocated to the embryonic axis where sucrose synthetase, working in the reverse direction, breaks it down to nucleoside diphosphate glucose (usually uridine) and fructose. Chrispeels *et al.* (1973) found that barley aleurone cells would also synthesize sucrose from glucose; the sucrose was released in response to GA. It is not clear how significant this is.

1.3.2. Mobilization of Proteins

Storage proteins in cereals are located in endosperm protein bodies and aleurone grains. Proteases and peptidases released during germination are responsible for the breakdown of these, although the same enzymes may not be involved in both cases (Tomos and Laidman 1979). Free amino acids accumulate in the aleurone within 24 hrs of imbibition, and some are used for *de novo* synthesis of hydrolases (Filner *et al.* 1969; Jacobsen and Varner 1967). The remaining amino acids are released into the endosperm under hormonal control (Chittenden *et al.* 1978).

Endosperm proteins are mobilized at a later stage (Folkes and Yemm 1958; Harvey and Oaks 1974), and the products are very rapidly taken up by the scutellum (Stewart 1971). Increase of gibberellin induced protease activity is paralleled with carboxypeptidase activity, but the latter is not under GA control (Sunblom and Mikola 1972), though it is likely that it is secreted by the scutellum (Mikola and Mikola 1980). Although these enzymes are not able to completely degrade the endosperm proteins, their mixed proteolytic products are actively taken up by the scutellum wherein their hydrolysis is completed by peptidases (Higgins and Payne 1977). The control of germinating cereal protease activity is not clear, but it may be subject to product inhibition as is the case in cucumber cotyledons (Davies and Chapman 1980).

1.3.3. Mobilization of Lipids

During germination the spherosomal triacylglycerol reserves of seeds are broken down to glycerol and fatty acids by

lipase action (Ching 1968; Colbourne 1974; Jelesma *et al.* 1977; Jones 1969; Ory 1969). The glycerol is immediately phosphorylated, and eventually metabolised in the TCA cycle (Stobart and Pinfield 1970). Fatty acids are usually degraded by *beta*-oxidation to acetyl CoA which enters the glyoxylate cycle and hence gluconeogenesis. This is important as the glyoxylate cycle avoids the decarboxylations of the TCA cycle (Lord and Roberts 1980) so that oxaloacetate (produced from succinate which is the end product of the glyoxylate cycle) may be used to produce glucose units for carbohydrate synthesis. Naturally this process, which occurs in the glyoxysomes, is most important in oleagenous seeds (Cooper and Beevers 1969) but it also takes place in cereals (Doig *et al.* 1975a). The increase in glyoxylate enzymes during germination is accompanied by a proliferation of glyoxysomes (Briedenbach *et al.* 1968; Colbourne 1974; Longo and Longo 1970), and there is much evidence that these events are under hormonal control (Doig *et al.* 1975a; Jones 1972; Tavener and Laidman 1972a,b).

1.3.4. Mobilization of Minerals

Phytin is the mixed potassium, calcium, and magnesium salt of inositol hexaphosphate (Liv and Bomarang 1957; Ogawa *et al.* 1977), and forms the major mineral reserve in the aleurone grains of cereals (Tronier *et al.* 1971). During germination phytase acts upon it yielding inositol, inorganic phosphate and the associated cations, which are redistributed to the growing embryo axis (Ergle and Guinn 1959; Hall and Hodges 1966; Macti *et al.* 1978; Okamoto 1962; Sugiura and Sonobe 1962). Phytase activity appears within 24 hours of imbibition (Bukhtoyarova *et al.* 1979; Mukherji *et al.* 1971; Peers 1953), and is dependent on the presence of the embryo, although phytohormones are apparently not involved (Eastwood and Laidman 1971a). Hormones are implicated, however, in the release of the products of hydrolysis (Eastwood and Laidman 1971b; Jones 1973; Laidman *et al.* 1974; Pollard and Singh 1968).

1.4. RESPIRATION

Quiescent seeds have very low rates of respiration and contain many of their enzymes for intermediary metabolism

in an inactive form. Following imbibition these enzymes are rapidly activated and respiration increases manyfold (Mayer and Shain 1974). Seeds synthesize ATP very early in germination (Brown 1965; Ching 1973; Obendorf and Marcus 1974; Pradet *et al.* 1968) and it is likely that it is used for protein synthesis (Marcus 1969). Three respiratory pathways - glycolysis, the pentose phosphate pathway (PPP), and the tricarboxylic acid cycle (TCA) - operate in imbibed seeds, although evidence for all the enzymes involved is patchy (Bewley and Black 1978).

Glycolysis occurs at the outset of imbibition in many species' seeds (Givan 1972; Hatch and Turner 1958; Legatt 1948; Mayer 1977; Mayer and Shain 1974; Mayer and Poljakoff-Mayber 1975; Morohashi and Shimokoriyama 1975a,b; Oata *et al.* 1956; Thomas and Ap Rees 1972), and will operate under anaerobic conditions. This is important as the seed may be subjected to low oxygen tension in early germination, but some ATP may still be produced by glycolysis.

The enzymes of the pentose phosphate pathway are found in dry and freshly imbibed seeds (Chakravorty and Burma 1959; Doig *et al.* 1975b; Mayer 1977; Roberts 1969). Its importance is in the production of NADPH (required for many cellular reductive biosynthesis reactions, especially fatty acids) and pentose sugars which are vital for nucleotide synthesis (Neal and Beevers 1960).

Mitochondrial activity (associated with the TCA cycle) is low in dry seeds but increases as germination progresses (Conn and Young 1957; Kolloffel and Sluys 1970; Mayer 1977; Neal and Beevers 1960; Wilson and Bonner 1971; Yoo 1970), and is associated with structural and biochemical reformation of the organelles. Many studies have been performed on the reactivation and synthesis of mitochondria and mitochondrial enzymes (Mayer and Poljakoff-Mayber 1975; Mayer and Shain 1974; Nawa and Asashi 1971; Nir *et al.* 1969, 1970; Opik 1965; Opik and Simon 1963; Solomos *et al.* 1972; Walk and Hock 1976; Yentur and Leopold 1976), the general conclusion being that complete activity is not regained until the seed is well into the germination process. The actual length of time will vary with species

(Morohashi and Shimokoriyama 1972; Hatano 1963) and environmental conditions.

1.5. PROTEIN AND NUCLEIC ACID SYNTHESIS

Many vital seed enzymes are present in an inactive state in the quiescent seed, so that they are available at very early stages of imbibition. However, many more proteins both structural and enzymatic, must be newly synthesized if a normal seedling is to grow. Whilst DNA synthesis occurs at a comparatively late stage of germination (Mayer and Poljakoff-Mayber 1975), protein synthesis may start within 30 to 60 minutes of water uptake (Bhat and Padayatty 1974; Klein *et al.* 1971; Marcus and Feeley 1966; Spiegel *et al.* 1975). Along with mobilized amino acids, an early supply of ATP is important for polyribosome formation and protein synthesis (Abdul-Baki 1969; Bray 1979; Bray and Dasgupta 1976), as may be a reduction in oxidised glutathione levels (Fahay *et al.* 1980).

Messenger-RNA (m-RNA) is obviously essential for protein synthesis but there is doubt if newly synthesized m-RNA is required during these early periods (Brooker *et al.* 1977; Hammett and Katterman 1975). There is a wealth of evidence pointing to the presence of functional long-lived stored m-RNA (Carlier *et al.* 1980; Dure 1977; Payne 1976; Weeks and Marcus 1971), but whilst it is known that protein synthesis can occur using only existing m-RNA (Klein *et al.* 1971; Walton and Soofi 1969; Waters and Dure 1966) it is not clear whether this is sufficient to code for all the embryo proteins synthesized during early imbibition (Payne 1977; Spiegel and Marcus 1975). Certainly ribosomal-RNA (r-RNA), transfer-RNA (t-RNA), and m-RNA synthesis can be detected within the first hour of imbibition (Bray 1979; Dobrzanska *et al.* 1973; Sen *et al.* 1975; Van Onkelen *et al.* 1974) but this may be more important during later germination. The synthesis of m-RNA in non-proliferating tissues such as aleurone cells (Evins 1971; Evins and Varner 1972; Varty 1975), cotyledons (Hewish *et al.* 1971; Waters and Dure 1966) and endosperms (Marre *et al.* 1965; Payne and Ball 1972) is well established.

Replication of embryo DNA does not usually occur until some hours after RNA and protein synthesis have begun (Chen and Osbourne 1970), although some very early DNA production may occur in exceptional cases (Buchowicz *et al.* 1978). It may be that the delay is due to the slow development of DNA polymerase (Mory *et al.* 1972, 1975). The DNA already present in the dry seed is, therefore, the template for all early RNA transcription and as such it is important that any lesions which have occurred during quiescence are repaired as soon as possible (Osbourne 1977).

1.6. THE INVOLVEMENT OF HORMONES IN GERMINATION

The mobilization of cereal food reserves is clearly under hormonal control, especially by gibberellin (GA), as are several other germinative biochemical processes. The situation is complex and is far from completely understood. Virtually all the work described below has been performed on cereals; the situation in other species is only beginning to be unravelled.

Since the last century it has been realised that endosperm degradation can be prevented by removing the embryo prior to imbibition (Brown and Morris 1890) and it has been proved that exogenous GA can substitute for the embryo in initiating the breakdown of reserves (Paleg 1960; Paleg *et al.* 1962; Yomo 1960). GA synthesis occurs in the embryo axis and scutellum (Macleod and Palmer 1967; Radley 1969) from the stored precursor ent-kaurene (Murphy and Briggs 1973, 1975), possibly stimulated by embryo sugar depletion (Briggs 1978). The mode of release of GA may be via the scutellum vascular strand (Macleod and Palmer 1966; Palmer 1972) or from the entire scutellum periphery (Briggs 1972).

There has been a great deal of attention paid to the enzymes released by the aleurone in response to GA stimulation. These enzymes may be separated into two categories; those whose synthesis and secretion are induced by GA, and those whose synthesis is independent of, but whose release depends on GA (Tomos and Laidman 1979). No enzyme has been more studied than *alpha*-amylase in this respect. The enzyme is synthesized *de novo* in isolated aleurone layers (Filner and Varner 1967) after a 6 to 8

hour lag period following exposure to GA (Chrispeels and Varner 1967a). In the presence of cyclohexamide or abscisic acid (ABA) no enzyme production occurs, and if the GA is subsequently removed no release of *alpha*-amylase will take place either (Chrispeels and Varner 1967b; Ho and Varner 1974).

Events occurring during the lag period mentioned above have given an insight into the mode of action of GA in *alpha*-amylase regulation (Jacobsen 1977; Jones and Jacobsen 1978; Mann 1975). It has been shown that GA induces an increase in poly(A)-containing RNA within isolated aleurone layers, some of which catalyses the synthesis of *alpha*-amylase (Higgins et al. 1976; Ho and Varner 1974; Jacobsen and Zwar 1974; Varty and Laidman 1976). These observations suggest that m-RNA coding for *alpha*-amylase is transcribed within 3-4 hours after GA addition and that translation proceeds over a subsequent 10 hour period. However it is possible that the GA is merely preventing the breakdown of continuously transcribed m-RNA. Undoubtedly, GA acts upon the aleurone cell by directing gene expression in favour of the synthesis of novel proteins.

Alpha-Amylase and protease require GA induction for their synthesis and release but many other enzymes which are synthesized *de novo* in germination only need GA action for their release. Such enzymes include *beta*-1,3-glucosidase (Bennett and Chrispeels 1972; Taiz and Jones 1970), ribonuclease (Chrispeels and Varner 1967a) and acid phosphatase (Ashford and Jacobsen 1974a,b). The mode of release of these enzymes is still a contentious issue. Although most of the evidence points towards a vesicular release (Gibson and Paleg 1972, 1975, 1976; Firn 1975; Laidman 1980), other workers have postulated a soluble release mechanism (Chen and Jones 1974; Jones 1972).

Similar controversy exists over the effects of GA on the development of membranes. Some workers have found that GA stimulates phospholipid and membrane biosynthesis, and the proliferation of endoplasmic reticulum (Evins and Varner 1972; Johnson and Kende 1971; Jones 1969a; Koehler and Varner 1973; Vigil and Ruddat 1973). Others find no effect of GA here (Colbourne et al. 1976; Firn and Kende 1974;

Laidman 1980 : Laidman et al. 1974). The hormone does, however, cause vesiculation of the endomembrane system (Colbourne et al. 1976; Vigil and Ruddat 1973) and this is likely to be connected with the secretory role of the aleurone (Tomos and Laidman 1979).

Far less information has accumulated on the effects of phytohormones other than GA in cereal germination. Auxin is responsible for the development of the scutellar vascular strand which facilitates the transport of nutrients to the growing embryo axis (Macleod and Palmer 1966). It may also be involved in cell extension of the embryo (Cleland 1971; Masuda 1978). Cytokinins originate in the embryo and appear to only have a modifying effect on the action of other hormones rather than a specific action themselves (Khan 1971, 1975; Van Staden and Davey 1979). Such an instance may be in the synergy of GA and a cytokinin in the induction of *alpha*-amylase in wheat aleurone cells (Eastwood et al. 1969). Absciscic acid is a well established GA antagonist (Leopold and Kriedeman 1975) and performs this role in isolated aleurone layers (Chrispeels and Varner 1966, 1967b).

1.7. SEED AND SEEDLING VIGOUR

As most of this thesis is given over to the consideration of the aetiology of low vigour, only a brief introduction to the topic will be presented in this section. Two types of vigour are recognized, genetic and physiological (Roberts 1972; Pollock and Roos 1972). Vigour differences may be encountered between cultivars of a species; some varieties of wheat and french-bean are less tolerant to stress than others (Ching and Kronstad 1972; Kooistra 1971). Alternatively, individual seeds within an identical genetic line may show various degrees of vigour, so that they form a heterogenous population, the overall vigour of which is the average of all the individuals (Yaklich and Abdul-Baki 1975). This second type is physiological vigour, and is the type which will be most extensively examined in this work.

Vigour is less easy to define than viability. Viability is simply the percentage of seeds to germinate under optimal

laboratory conditions but vigour is a far more nebulous term which refers to the ability of a seed to do well under adverse conditions, in the field. In recent years, vigour has been measured as the percentage of seeds which germinate under defined stress conditions (Bray 1979; Heydecker 1972). Whilst these measurements are useful in that they allow easy numerical comparisons to be made between the results of various workers, they take no account of the performance of those seedlings which do emerge and to the farmer this also matters (Ayre 1980). Various workers have attempted to incorporate this factor into their definitions of vigour and these include, response to stress (Abdullahi and Vanderlip 1972); relative stage of seedling development (Adenikinju 1972); relative seedling growth rate as measured by dry weight accumulation (Lowe et al. 1972; Turner and Ferguson 1972); seedling survival (Mukherji et al. 1971b) and length of time a seedling is able to exert an increasing upward force (Jenson et al. 1972). Such definitions all suffer from being too narrow in their terms of reference, as has been pointed out by Bradnock (1975). He considered vigour to be "any attribute of a seed which affects its ability to deliver its potential at any stage of the plant life-cycle". It is under this broad umbrella that vigour will be examined in this treatise.

There are likely to be many agronomical causes of low vigour, including adverse conditions during the development, harvesting and storage of the seed. During grain formation and maturation such factors as nutrient supply, attack by pests, adverse environment, and even its position on the ear of the mother plant may alter the vigour of the mature seed (Austin and Longden 1965; Ching and Rynd 1978; Khan and Laude 1969; Perry 1976). Mechanical damage during harvest and drying damage can be extremely important (Pollock and Roos 1972; Prakobboon 1982), as can deterioration during storage (Maguire 1977). Apart from the well studied effects of storage conditions on viability and vigour (Roberts 1972), few experiments have been performed to attempt to initiate low vigour by deliberate controlled agronomic mishandling.

Several aspects of metabolism have been shown to correlate with physiological vigour, such as rates of glucose utilization (Abdul-Baki 1969; Abdul-Baki and Anderson 1972); respiration rates (Woodstock 1969); membrane permeability (Hallam 1972); rates of RNA and protein synthesis (Bray 1979; Sen and Osbourne 1977; Van Onkelen *et al.* 1974); loss of the elongation factor EF-1 (important in binding amino acyl-tRNA to ribosomes) in embryos (Bray and Chow 1976; Dell'Aquila *et al.* 1976; Roberts *et al.* 1973); enzyme activities of dehydrogenases, decarboxylases and oxidases (Lamkin *et al.* 1983; Koostra and Harrington 1969; Woodstock and Grabe 1967) and the numbers of chromosomal aberrations (Cheak and Osbourne 1978).

Membrane disintegration is also implicated in low vigour (Berjak and Villiers 1970; Delouche 1969; Hallam *et al.* 1973), and is probably the cause of the greater leakage of solutes from low vigour seeds on imbibition (Abdul-Baki and Anderson 1973; Ching and Schoolcraft 1968; Harrison and Perry 1976). Altered membrane permeability may also be the cause of lowered ATP levels in aged soybean axes (Anderson 1977) and ATP content itself has been correlated with vigour loss (Ching 1973). Tocopherol has the properties of stabilising membranes perhaps by functioning as an antioxidant (Lucy 1964, 1970; Tappel 1962, 1965). Membrane oxidation has been cited as a possible cause of vigour and viability loss (Fielding and Goldsworthy 1982; Koostra and Harrington 1969) and it is claimed that applying tocopherol to onion and okra seeds maintains viability (Koloyereas *et al.* 1961). However, natural tocopherol levels did not correlate with loss of vigour in wheat (Goldsworthy and Fielding - personal communication).

Many physiological and biochemical tests have been devised which will predict the vigour of a seedlot (Perry 1976). Some of these are very elegant and show good correlations with growth and yield eg. oxygen uptake by isolated scutella (Ellis and Hanson 1974), glucose utilization in soybean axes (Yaklich and Abdul-Baki 1975) and the activity of glutamine decarboxylase (Kamil 1975). However these are complicated tests and have not found application in commercial seed testing. Other simpler tests have gained popular acceptance though, such as the conductivity of

imbibition leachates in peas and cowpeas (Abedona and Odu 1972; Matthews 1973) and the use of the tetrazolium stain for dehydrogenase activity (Lakon 1949; Moore 1969,1973). Most commercial tests, however, rely on the measurement of germination and growth under stress conditions (Hanson 1973) and can give good predictions of the crop yield from a seedlot (Stormonth 1978). The best measurement of seedling vigour would be to monitor fresh weight changes under stress conditions, as this would provide the closest mimic of growth in the field. An attempt to do this was found to show vigour variations in wheat very accurately (Goldsworthy and Dover 1980) but suffered from its inability to measure the fresh weight for more than a few days. A priority of this work was, therefore, to develop an apparatus which would enable the growth patterns of high and low vigour seed to be monitored over a much longer period.

2. MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1. SEED SOURCES

All wheat seeds (*Triticum aestivum* L.) were produced on the R.H.M. Model Farm in Oxfordshire, under normal agronomical conditions. The main variety used was the winter wheat "Avalon" as this is a popular and currently abundantly grown bread wheat (N.I.A.B. 1982). Each year freshly harvested seedlots were used for experimentation, the % germination and % vigour of which were determined by R.H.M. Research Ltd. In the following experiments all seeds used were "Avalon" unless stated otherwise.

2.2. COMMERCIAL VIGOUR TESTING

The R.H.M. vigour test was employed on all seed samples providing a % emergence figure for seeds germinated under stress conditions. For reasons of commercial confidentiality, the nature of this test may not be revealed. The terms "high and low vigour" used hereafter refer to performance at this test; high vigour seed showing high % germination in optimum conditions and high emergence under stress whereas low vigour shows similarly high % germination but lower emergence under stress.

2.3. MOISTURE CONTENTS

All moisture contents (m/c) are expressed as a % on a dry weight basis. The determinations were done using the constant weight method at 130°C. (Roberts 1972), with the exception of field experiment material which was measured using a calibrated Grainmaster 3 (Protimeter UK).

2.4. FIELD EXPERIMENTS

All trials were conducted at the R.H.M. Model Farm. Each experimental plot was 8 drill rows wide and 15.5m long; seeds were drilled at 325 seeds per square metre. The plots were subjected to normal husbandry practices (eg. fertiliser and pesticide applications) except when special treatments were required. Where possible, duplicate plots were used in experiments but when this was not feasible,

the seed from a single plot was divided into two portions and each half was treated as one out of two replicates.

2.4.1. Induction of Low Vigour Seed in the Field

Various agricultural malpractices were used in order to induce low vigour. Preharvest treatments involved spraying each of 2 plots with a solution of GA every third day for a period of four weeks prior to harvest. The concentrations used were 3 and 300 micromolar GA; 1 litre of solution was applied on each spraying. The control plot was sprayed with 1 litre of water on the same days as the treated plots. No spraying was done on the 5 days immediately before harvest so that the ears were able to dry out sufficiently (harvested at 18% m/c).

The effects of mechanical damage were assessed by harvesting previously untreated seeds with incorrectly set combine harvester blades and, additionally, by harvesting whilst the ears were still moist (31% m/c).

Postharvest treatments solely involved drying damage. Seeds were harvested at 25.8%, 21%, 18.2%, and 15% m/c and then dried at various temperatures in a commercial scale Penney-Porter Grain Drier at the National College of Agricultural Engineering (Bedford U.K.). Control seeds were air dried at ambient temperature in a ventilated glasshouse. The periods of drying were varied in order to bring all the seeds to approximately 15% m/c.

2.4.2. Field Emergence of Low Vigour Seed

High vigour seed was raised to 25% m/c and heated in open trays in the laboratory for 0, 1, 2, 3, and 4 hours at 65°C. After re-equilibration to 14% m/c (section 2.5.), the seed was vigour tested and then sown in October. Emergence was assessed in the following March.

2.5. LABORATORY PRODUCTION OF LOW VIGOUR SEED

During the period of this work no "natural" (ie. not purposely induced) low vigour seed was obtainable! As a consequence, various attempts were made to mimic

agricultural malpractices in the laboratory, the most successful and reproducible being drying damage. High vigour seeds were raised to 25% m/c by agitating them with the required amount of water in a sealed box, followed by one week of equilibration at 10°C. The moist seeds were placed as a single layer in tin trays and dried in a convection oven at 65°C. for various periods. Four hours was the minimum period in which the maximum effect was obtained, and all "low vigour seeds" referred to hereafter were produced in this way, unless stated otherwise. Following cooling, the seeds were scattered on mesh platforms in a running fume cupboard along with the unheated controls (the "high-vigour seeds"). This facilitated equilibration of both types to the same final m/c, thus removing water content as a variable in subsequent experiments.

2.6. SEED STERILISATION

Before seeds were used in any experiment, they were sterilised in sodium hypochlorite solution (1.5% available chlorine) for 15 minutes. To allow penetration of the bleach into the pericarp and testa (where many fungi are found) seeds and sterilent were placed in a buchner flask and subjected to repeated evacuations and their release, via a tap vacuum pump. Following sterilisation, the seeds were transferred to a sterile laminar-flow cabinet and rinsed in sterile distilled water until the smell of chlorine disappeared.

2.7. GERMINATION CONDITIONS

Water saturated vermiculite was scattered to a depth of 4cm in a plastic box (22 x 11 x 8cm from Stewart Plastics Ltd.) and 100 seeds were planted on top using a vacuum counting device. A further 1cm of vermiculite covered them, and an extra 50ml of distilled water was added. The boxes were incubated, with their lids on, in a dark constant temperature room and after the requisite period, the % of normal seedlings was determined. Seedlings required for biochemical investigations were all grown in this way.

2.8. FRESH WEIGHT MEASUREMENTS

A new apparatus was designed enabling fresh weight changes to be monitored without disturbing the growing plants. The manifold consisted of a constant head device feeding tap water to a series of glass 1mm capillary tubes, which in turn slowly delivered water into square plastic troughs (Figure 2.). The height of the constant head device controlled the rate of water flow. Each trough had five equally spaced holes drilled in two opposing sides with a bent 250 microlitre disposable pipette (Clay Adams U.S.A.) lodged through each hole, using plasticine as a sealant (Figure 3.). The five pipettes dripped a constant amount of water onto one end of pre-weighed perspex slide (100 x 150cm) underneath them; the slide being covered with glasshouse plastic shading mesh ("Netlon"). Because these "drip-slides" were sloping downwards into the drainage pipe, an even film of flowing water formed on the entire surface which was then available to the developing seedling. The apparatus was installed in 10 and 25°C. constant temperature rooms. Experiments were usually conducted in the dark, but a portable light bank delivering 90 microEinsteins per square metre could be installed 2 feet from the slides. There was no resultant measurable increase in temperature.

It was necessary to pre-imbibe seeds before growing them on drip slides; typically, 250 seeds were imbibed in shallow water at 4°C. for 12 hours. They were then scattered evenly over the slide surface which was placed under the dripping manifold. The young plants were able to establish normal root networks underneath the netlon thus adequately supporting shoots up to at least 30cm. By removing the slides from the manifold, using a standard draining procedure and weighing them, fresh weight changes could be measured as often as desired.

2.8.1. The "Drip-Slide" Vigour Test

Seeds were germinated on drip-slides at 10°C. in the dark and the increases in fresh weight (expressed as a % of the initial fresh weight) were monitored for 20 days and recorded graphically. From the graphs, the maximum growth

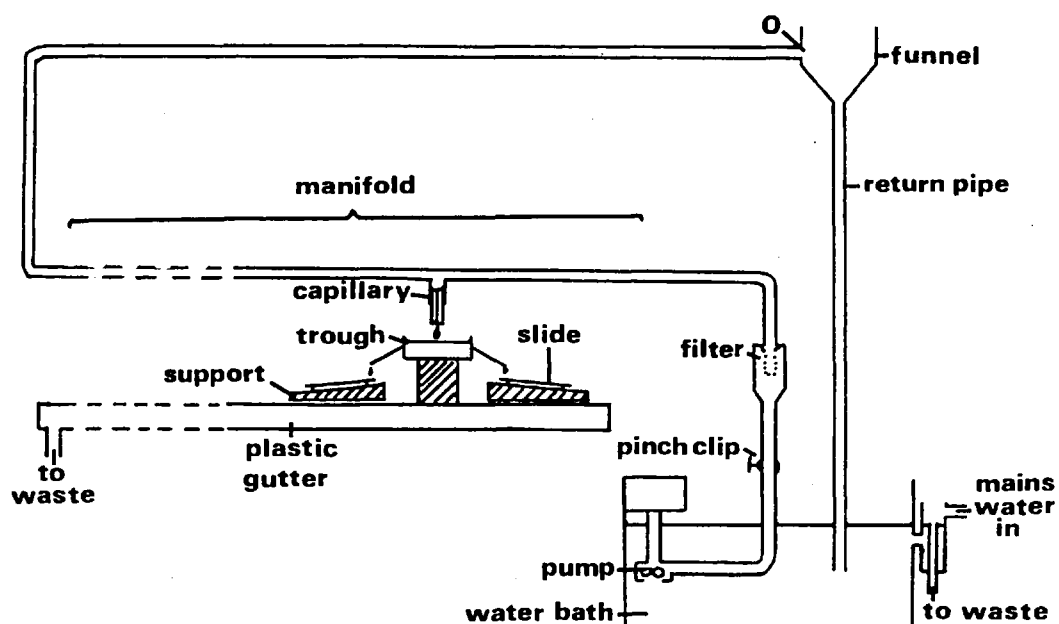


FIGURE 2. Apparatus for delivering constantly dripping water to seedlings grown on slides. Height at O adjusts drip rate. Only 1 trough shown for clarity.

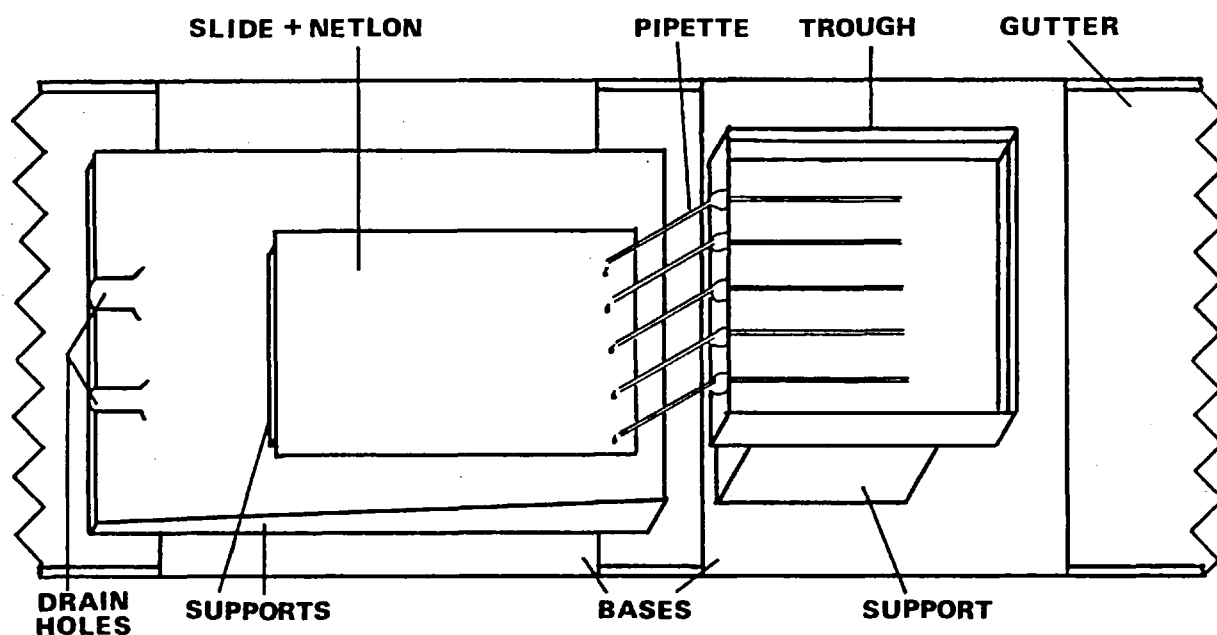


FIGURE 3. Arrangement of trough, pipettes and slide. For clarity, only one set of pipettes is shown on the trough.

rates recorded during this period were determined by taking tangents. These growth rates were then expressed as a % of the growth rate of a control seedlot, the nature of which will be stated when % vigour figures (determined this way rather than by RHM) are given. It is therefore possible for a seedlot to have a vigour status of more than 100%.

2.9. SOURCES OF CHEMICALS

All reagents and solvents were Analar grade and were purchased from BDH. Ltd. or Sigma Ltd. and all water used was distilled, unless otherwise stated.

2.10. PREPARATION OF BUFFERS

Buffers were made using standard recipes (Dawson *et al.* 1969) and the pH was adjusted to within 0.05 pH units (Pye Unicam pH meter).

2.11. OXYGEN UPTAKE

The water jackets of two oxygen electrodes (Rank Brothers U.K.) were connected in series to a circulating constant temperature water bath, and their electrical outputs connected to a dual channel chart recorder. Oxygen saturated water (4ml) was placed in the reaction chamber of each and left to produce a steady full scale trace (full scale was corrected for the varying oxygen solubility at different temperatures). Two plastic mesh "baskets" were constructed so that they held 10 seeds and fitted inside the electrode chambers, thus preventing the seeds from interfering with the stirring action of the "flea". At time 0, the baskets were plunged into the chambers and the electrodes sealed; this allowed the respiration rates of high and low vigour seeds to be measured at the same time. Oxygen uptake was monitored over 15 minutes and the results expressed as nmoles oxygen per minute per seed at the mid point of this period.

2.12. MEASUREMENT OF SOLUBLE SUGARS

Two methods were used, one to specifically assay glucose and the other -high performance liquid chromatography

(HPLC)- to measure any sugar of interest. The extraction procedures were different in each case.

2.12.1. Sugar Extraction for Glucose Assay

50 seeds/seedlings were dissected into embryos (including the scutellum) and endosperms; these halves were treated as separate samples. After freezing in liquid nitrogen they were ground to a powder, which was extracted with 15ml of hot 80% ethanol. Following centrifugation ($8,000 \times g$ for 5 minutes) the pellet was re-extracted with 10ml 80% ethanol and the two extracts combined. 0.5g activated charcoal was added (to remove interfering pigments) and the solution was left stirring for 15 minutes. The solids were removed by further centrifugation and filtration (Whatman no. 50) and the filtrate was reduced to dryness in a rotary evaporator at 50°C . Depending on the glucose concentration, the residue was resuspended in 5-50ml of 0.5M Tris-HCl buffer pH 7.0 containing 0.03% sodium azide. Glucose standard solutions were subjected to the same procedures.

2.12.2. Glucose Assay

The assay is based on the specific reaction between glucose, water and oxygen, catalysed by glucose oxidase, producing gluconic acid and hydrogen peroxide. Peroxidase then causes the peroxide to oxidise a chromoreagent (ABTS) which turns blue, the intensity of the colour depending on the initial glucose concentration.

Glox Reagent was prepared by dissolving 60mg glucose oxidase, 10mg peroxidase (both from Boeringer Mannheim, Grade 2) and 200mg ABTS (2,2,azino-bis(3-ethyl benzthialozine sulphonic acid)) in 400ml 0.5M Tris-HCl buffer pH 7.0 containing 0.03% sodium azide. 2ml glox reagent were placed in a cuvette and at $t=0$ 1ml of sample was added and vigorously stirred. Absorbance at 420nm was read at either $t=7, 10, \text{ or } 15$ minutes. Standards of 0.05mM to 2mM glucose were used to construct calibration curves. Three replicate readings were made per sample.

2.12.3. Sugar Extraction for HPLC

Several methods were investigated, the following being the only one to produce sufficiently clean samples and efficient extraction. Even slight variations from these steps led to poor results. Ten seedlings were rinsed in tap water, blotted dry, weighed and then homogenised in a mortar with 15ml water and a little acid washed sand. The homogenate was transferred to a glass vial and plunged into a boiling water bath; a thermometer was used as a stirrer and the vial was removed when the contents reached 80°C. After capping, the vial was left in an oven at 80°C. for 20 minutes and on removal and cooling the contents were centrifuged for 5 minutes at 8,000 x g. The supernatant was decanted into a measuring cylinder and the pellet re-extracted as before; the two supernatants were combined and acetone was stirred into the extract until it reached a concentration of 75% to ensure complete protein precipitation. This liquid was filtered through Whatman GF/A glass fibre and taken to dryness in a rotary evaporator at 50°C. The residue was resuspended in a solution of 75% acetonitrile (HPLC grade S, Rathburn Chemicals Ltd.) 25% water (deionised and filtered through 0.8 micrometre Millipore mesh); the volume depending on the sugar concentration. After passing the solution through a filter syringe fitted with Whatman GF/C, it was transferred to an HPLC autosampler vial (Kontron Ltd.) and stored in a refrigerator. Standard sugar solutions were subjected to the same procedures.

2.12.4. High Performance Liquid Chromatography

A Kontron U.K. Ltd. HPLC machine fitted with a Laboratory Data Control Refractometer 3 detector and Hewlett-Packard HP3390A integrator was used throughout this work. Three types of column packing were exhaustively evaluated : Spherisorb -5- amino bonded resin; SAX ion exchange resin and HC-75 calcium loaded sulfonic acid resin gel. Of these, the spherisorb resin gave the most reproducible and quickest results under the most convenient conditions. All separations were performed at 30°C. using a Tecon rapid circulation bath, column water jacket and thermal stabilisation of the detector.

Chromatography was conducted using isocratic elution with a mobile phase of 80% acetonitrile and 20% water (for grades see section 2.12.3.) at a flow rate of 1.0ml per minute. Before any samples were analysed, the detector (usually set at an attenuation of x50) was equilibrated with flowing mobile phase. Samples were loaded onto an autosampler fitted with a 100 microlitre sample loop and Rheodyne pneumatic valve injector. At time 0 a programmable microprocessor brought about sample injection, set the chromatographic conditions, started the timer and switched on the integrator. Once eluted, individual compounds were collected in the fraction collector and kept for future identification. Elution continued for 30 minutes after which the integrator reported, was disabled and the HPLC machine was sent through a 5 minute purge cycle. The microprocessor was programmed to perform a duplicate analysis of each sample and then load a new one. Standard solutions were interspersed amongst the unknowns.

The integrator report provided a plot of the run, individual peak retention times and the integrated areas of specified peaks of interest. These areas were converted by a computer into weights of named sugars using the calibration equations obtained for that set of analyses. Individual peaks were identified using co-chromatographic information and specific chemical analyses (eg. enzymatic and colourimetric tests).

2.13. EMBRYO GROWTH IN SUGAR SUPPLEMENTED MEDIA

A manifold was built (Figure 4.) enabling isolated embryos to be grown in aerated sterile solution culture. Surface sterilised seeds were imbibed in the refrigerator for 12 hours, the embryos were then dissected out under sterile conditions and weighed in batches of 20 using a sterile container. The manifold was sterilised by flooding it with 80% ethanol followed with complete drying by blowing air (sterilised by passage through a Whatman "in-line air filter") through the system. The autoclaved medium consisted of 5mM phosphate buffer pH 7.0 containing 0.01% streptomycin sulphate and either 0 or 1% (w/v) glucose or sucrose. Each tube contained 5ml of medium in which 20 isolated embryos were then placed. The tubes and air

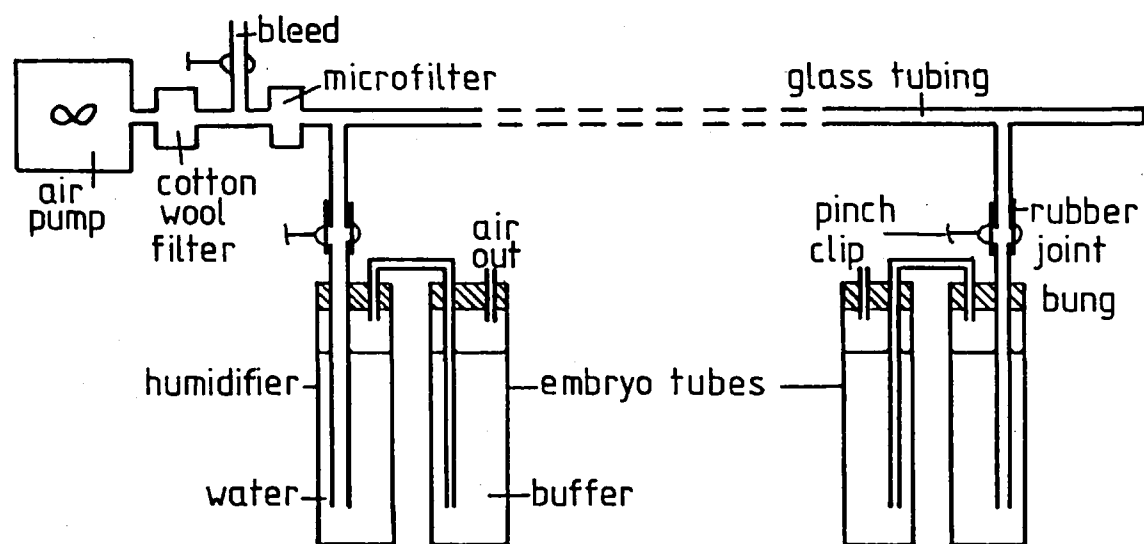


FIGURE 4. Apparatus for culturing isolated embryos in sterile, aerated media.

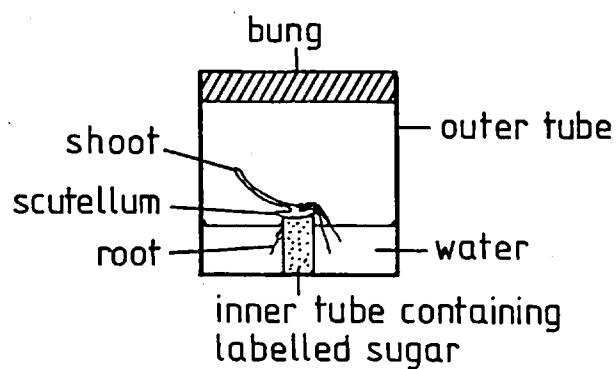


FIGURE 5. Double tube apparatus which enables the scutella of dissected embryos to remain in contact with radio-labelled sugar solutions whilst the roots dip into water.

humidifying vessels were immersed in a water bath and the air pump was switched on. The air flow rate was adjusted using the bleed and pinch clips so that the embryos were gently agitated. After the required period the embryos were removed, blotted and weighed.

2.14. PRODUCTION OF SUCROSE IN THE EMBRYO

Embryos were dissected from germinating seeds, rinsed in cold water and placed in small glass tubes (Figure 5.) so that the scutellum just fitted into the open top making contact with the enclosed liquid, while the roots trailed down into the surrounding water. Inside each tube was 1ml of 20mM tris-HCl buffer pH 7.0 containing 10mM glucose spiked with 2.5 microcuries of d-(U-¹⁴C) glucose (specific activity 0.05 microcuries per micromole - from Amersham U.K.). Embryos were incubated in this way for up to 12 hours; any longer and the liquid lost contact with the scutellum. On terminating the incubations, the embryos were immediately washed thoroughly in iced water and extracted as for glucose (section 2.12.1.) except that the residues were resuspended in 100 microlitres of 80% ethanol.

2.14.1. Paper Chromatography of Sugars

Aliquots (25 microlitres) of extract were streaked onto Whatman no.1 chromatography paper and were analysed by running them in descending fashion using n-propanol : ethyl acetate : water in the ratio 7:1:2 (Smith 1960) for 24 hours. Authentic standards (both radio-labelled and unlabelled) were co-chromatographed with the samples. The chromatogram was dried and the portion containing unlabelled standards was cut away and stained using a modified version of the Hough, Jones and Wadman (1950) reagent :

2g anisidine hydrochloride in 100ml water-saturated n-butanol plus 2 drops conc. HCl; diluted to 50% with acetone.

After dipping in the reagent, the dried chromatogram was heated at 110°C. for 5 minutes. Glucose stained dark brown whilst sucrose was yellow.

2.14.2. Autoradiography

Using the stained portion as a guide, the approximate amount of radioactivity (c.p.m.) in the glucose and sucrose spots of the unstained chromatogram was estimated with a Geiger-Mueller tube connected to an ESI Nuclear ratemeter. The paper was securely fastened to card and inserted with a Kodak Industrex C X-ray film into a folder, in the dark. The length of the exposure was determined using the rule 1,000 cpm per spot = 1 day exposure. The film was developed by normal photographic means. The autoradiographs were then used as an accurate template to visualise the glucose and sucrose spots from the samples.

2.14.3. Determination of Radioactivity

The entire glucose and sucrose spots were cut away from the chromatogram and shredded into small pieces. All of the pieces from one spot were placed in a 22ml scintillation vial and 10ml of PBD-toluene scintillation cocktail (5g 2-(4'-tert-Butylphenyl)-5-(4"-biphenyl)- 1,3,4-oxadiazole in 1l scintillation grade toluene) was added. The radioactivity (dpm) was measured in a Packard 2405 Liquid Scintillation Spectrometer. Label recoveries were calculated to be between 90 and 95%.

2.15. ASSAY OF SCUTELLAR SUCROSE METABOLISING ENZYMES

All the enzymes examined, with the exception of invertase, were subjected to the same initial extraction procedure, but some were further purified. In every case, the enzymes were assayed within 12 hours of the final extraction stage.

2.15.1. Extraction Procedures

Modified combinations of the methods of Bird *et al.* (1974); Gander (1966); Slack (1966) and Tsai (1974) were used. Scutella (2.5g) were dissected from germinating seedlings and ground at 0°C. in a mortar with a little acid washed sand and 4ml extraction buffer (10mM tris-HCl buffer pH 7.0 containing 0.1% (w/v) bovine serum albumin, 10mM DIECA (sodium diethyldithiocarbamate) and 0.25mM PMSF (phenylmethylsulphonylfluoride)). The homogenate was

centrifuged at 15,000 xg for 20 minutes at 2°C. and the supernatant was freed from low molecular weight substances by passing it through a column (1.1 x 24cm) containing Sephadex G25 medium grade gel (Pharmacia Ltd.) previously equilibrated with standard buffer (10mM tris-HCl pH 7.0). A Gilson peristaltic pump connected to an LKB fraction collector was used in conjunction with an LKB UV detector to identify and collect all the proteins. This eluate was called the crude extract.

Sucrose synthetase was further purified by the following steps. Crude extract was subjected to ammonium sulphate fractionation (adjusted to pH 7.0) and the protein precipitating between 20 and 50% saturation was collected by centrifugation at 38,000 xg for 20 minutes at 2°C. The pellet was resuspended in 4ml cold standard buffer and dialysed for 8 hours at 4°C. against 2.5l of the same buffer. The solution was passed down a column (2.5 x 36cm) containing Sephadex G200, eluting with standard buffer; the eluant fraction between 45 and 60ml was collected and immediately applied to a second column (0.6 x 14cm) containing the weak anion exchange gel DEAE Sepharose CL-6B (Pharmacia Ltd.). This column was eluted with 9ml standard buffer containing 0.1M NaCl followed by 8ml buffer containing 0.3M NaCl, the enzyme being collected in the second 4ml fraction of the latter eluent.

To purify UDPG pyrophosphorylase, the crude extract was passed onto a column (0.6 x 14cm) containing DEAE-cellulose. The first elution was with 100ml standard buffer containing 5mM KCl and this eluate was discarded. Secondly, standard buffer containing 10mM KCl was used and the fraction between 20 and 25ml contained the enzyme.

2.15.2. Sucrose Synthetase Assays

Both the forward (sucrose synthesis) and backward (sucrose degradation) reactions were measured :

A. Forward Reaction

This was modified from Tsai (1974). The 1ml reaction mixture contained (in micromoles) 60 of HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer pH 8.4; 3 of UDPG; 2 of fructose; 2 of magnesium

chloride and 250 microlitres enzyme solution. Incubation was at 25°C. and the reaction was stopped, after the desired period, by the addition of 0.5ml acetonitrile. The amount of sucrose was estimated by HPLC (section 2.12). The controls had no UDPG.

B. Backward Reaction

The second method of Avigad (1964) was used with a Perkin Elmer 554 spectrophotometer. The 1ml reaction mixture contained (in micromoles) 50 of tris-HCl buffer pH 8.4; 0.1 of EDTA; 2 of sodium cyanide; 2 of sodium fluoride; 1 of magnesium chloride; 40 of sucrose; 2 of UDP; 1.4 of NAD; 0.1 unit of UDPG dehydrogenase and 250 microlitres of enzyme solution. The rate and amount of UDPG produced were calculated from the NADH formed and measured at 340nm; 2 micromoles NADH corresponds to 1 micromole UDPG. The controls either omitted UDP or sucrose.

2.15.3. Sucrose-6-P Synthase/Phosphatase Assay

The method of Bird *et al.* (1974) was used. The reaction mixture (100 microlitres) contained (in micromoles) 50 of tris-HCl buffer pH 7.0; 1.5 of UDPG; 1 of magnesium chloride; 1.5 of fructose-6-P and 50 microlitres crude extract. Controls either contained no UDPG or fructose instead of fructose-6-P. Following incubation at 25°C., the reaction was stopped by the addition of 100 microlitres acetonitrile. Sucrose was estimated by HPLC.

2.15.4. UDPG Pyrophosphorylase Assay

The method was essentially the same as that of Gander (1966). The 3ml reaction mixture contained (in micromoles) 100 of tris-HCl buffer pH 7.5; 5 of UDPG; 1 of sodium pyrophosphate; 20 of magnesium sulphate; 0.1 of 2,6-dichlorophenol indophenol; 0.3 of phenazine methosulphate; 0.5 of NADP; 1 unit each of phosphoglucomutase and glucose-6-phosphate dehydrogenase and 250 microlitres enzyme solution. The controls contained no UDPG. The velocity of the reaction was estimated by measuring the linear decrease in absorbance (0.8 to 0.2 units) of the blue chromogen at 600nm.

2.15.5. Sucrose Phosphorylase Assay

This involved the measurement of labelled sucrose formed from ^{14}C fructose (Hatch et al. 1963). The 250 microlitre reaction mixture contained (in micromoles) 10 of tris-HCl pH 6.8; 5 of glucose-1-P; 1.3 of d-(U- ^{14}C) fructose (specific activity 0.5 microcuries per micromole, from Amersham U.K.). Incubation was for at least 1 hour at 25°C. and the reaction was stopped by streaking the mixture onto chromatography paper. Separation, detection and quantification of sucrose were as described before (section 2.14.).

2.15.6. Extraction and Assay of Invertase

Scutella were again dissected from germinating seedlings and 2.5g were homogenised with 4ml of 10mM phosphate buffer pH 7.0 containing 20mg poly(vinylpolypyrrolidone) and a little acid-washed sand in a mortar (Purvis and Rice 1983). The homogenate was centrifuged at 30,000 $\times g$ for 15 minutes at 2°C.; the supernatant was used as the enzyme source.

A modified method of Matsushita and Uritani (1974) was used. The 450 microlitre reaction mixture contained 75 micromoles sucrose in 100 mM sodium acetate buffer pH 4.7 and 50 microlitres of enzyme. After incubating at 55°C. the reaction was terminated by the addition of 0.5ml Nelson-Somogyi copper reagent (Nelson 1944). The capped tubes were boiled for 20 minutes, cooled, mixed with 0.5ml arseno-molybdate reagent (Nelson 1944) and diluted with 5ml water. The absorbance at 540nm was determined and reducing sugar concentration estimated from a glucose calibration curve.

2.16. ALPHA-AMYLASE EXTRACTION AND ASSAY

50 seeds/seedlings were washed, blotted dry, weighed and then ground at 0°C. in a mortar with acid-washed sand and 30ml water. The homogenate was centrifuged at 4,000 $\times g$ for 5 minutes at 4°C. and the supernatant was decanted into a conical flask. Each extract was subjected to heating which destroys most enzymes but leaves *alpha*-amylase unaffected, provided the pH is between 5.0 and 7.0 (Briggs 1961; Kneen

et al. 1943). The flasks were plunged into a water bath at 90°C. and their contents were continuously stirred with a thermometer until the temperature reached 70°C. whereupon they were immediately transferred to a 70°C. bath. They were left, with frequent stirring, for exactly 15 minutes, and then plunged into an ice bath and cooled. The precipitated protein was removed by centrifugation at 9,000 xg for 15 minutes and the supernatant was made up to 30ml with water. This was the crude extract.

A modified version of the Greenwood and Macgregor (1965) assay was used. The 2ml reaction mixture contained 1.5mg beta-limit dextrin and 2 units beta-amylase (type 1-B from Sigma) in 20mM sodium acetate buffer pH 5.3 containing 10mM calcium chloride and 0.5ml crude extract. After 3 minutes incubation at 30°C. the reaction was stopped by the addition of 1ml iodine reagent (4g potassium iodide and 0.254g iodine in 1litre 0.02N HCl); the liquid was diluted with 5ml water. Control tubes had the iodine reagent added before the enzyme. The difference in absorbance at 540nm between control and incubated samples was read on a Perkin Elmer 554 spectrophotometer and the enzyme activity was determined from a calibration curve of absorbance difference versus international units of alpha-amylase activity (4 times crystalline enzyme from Sigma).

2.17. ALPHA-AMYLASE pH AND TEMPERATURE OPTIMA

A. Temperature Optimum

A sample enzyme solution was prepared with standard activity (0.1 units/ml) and used in the assay described (section 2.16.) except that the incubations were at a range of temperatures from 20 to 70°C.

B. pH Optimum

The same enzyme solution was used, and the assay was performed as described (at 30°C.) except that the buffer was changed to 50mM dimethylglutaric acid-sodium hydroxide buffer. The pH was varied between 3.5 and 7.5; enzyme activity was determined at 0.5 pH unit intervals.

2.18. THE ALPHA-AMYLASE MICHAELIS CONSTANT (K_m)

The same enzyme preparation was used as in section 2.17.

and was assayed as described (section 2.16.) except that the *beta*-limit dextrin concentration was varied between 0.01mg/ml and 2.5mg/ml. For each substrate concentration, the reaction was stopped after varying lengths of time, so that a graph of decrease in substrate concentration (estimated from a calibration curve of absorbance versus substrate concentration) with time was constructed. The initial velocity of the reaction (V_o) was determined, by taking a tangent, and plotted against the substrate concentration ($[S]$) divided into the initial velocity ($V_o/[S]$); this is the Eadie-Hofstee plot where the slope is the negative Michaelis constant ($-K_m$) (Lehninger 1970).

2.19. ANALYTICAL ISOELECTRICFOCUSSING

Alpha-Amylase isoenzymes in crude extracts were separated by analytical thin-layer polyacrylamide gel electrofocussing based upon the method of Sargeant and Walker (1978). The equipment used was an LKB Multiphor system with LKB "PAG plate" gels containing carrier ampholytes in the pH range 3.5 to 9.5, but contrary to the makers instructions, it was found that cut gels of as little as 4cm in length could be used successfully. The gel and track were pre-cooled to 4°C., then samples were applied using filter paper wicks (1 x 0.5cm) into which 25 microlitres of sample had been absorbed. Each sample solution had previously been adjusted to contain between 0 and 2 units/ml *alpha*-amylase activity. It was found to be important to keep the ionic concentration of the samples as low as possible; every extraction and dilution was therefore with water and not buffer. For every sample, 1cm length of gel was used; electrofocussing was achieved with a constant power of 0.8 watts per cm gel length for 1.5 hours at 4°C. The sample wicks were removed after 30 mins.

After electrofocussing the pH gradient formed across the gel was determined using a surface pH electrode at 1cm intervals. The power was then resumed for 5 minutes to refocus the protein bands. When refocussing was complete, the electrode strips and underlying gel were cut away and the remaining gel (and its plastic base) was removed into a plastic box. Visualisation of amylolytically-active bands was achieved by flooding the gel with a 2% solution of

boiled Merck starch (no other starch will work) for 5 minutes at 25°C. followed by immersion of the gel in several changes of iodine reagent (6.5g iodine and 19.5g potassium iodide in 1l water) diluted to 4% before use. Because the blue colour fades rapidly, the gels were immediately photographed with a Polaroid camera.

2.20. PROTEIN STAINING

Crude extracts were electrofocussed as above except that they were used undiluted i.e. about 20 units/ml. Once the focussed gels were in the plastic box they were flooded with fixing solution for 30 minutes to precipitate the protein, then immersed in staining solution for 10 minutes at 60°C. The gels were destained for 24 hours in several changes of solution and then photographed. The required solutions are :

Fixing solution- 17.25g 5-sulphosalicylic acid and 57.5g trichloroacetic acid in 500ml methanol/water (3:7).

Staining solution- 115mg Coomassie brilliant blue in 100ml destaining solution.

Destaining solution- 500ml ethanol and 160ml glacial acetic acid mixed were made up to 2l with water.

2.21. PURIFICATION OF *ALPHA*-AMYLASE ISOENZYMES

The procedures were mostly those used by Sargeant (1980a) for wheat *alpha*-amylase.

2.21.1. Initial Extraction and Heat Treatment

High vigour seedlings were germinated for 5 days at 25°C., washed and freeze-dried in an Edwardes shelf machine. When 1.5 kg of material was obtained, it was ground in an Atomix blender and slowly stirred into 3l of 10mM calcium acetate; stirring continued for 3 hours. The homogenate was centrifuged at 15,000 xg for 20 minutes and the supernatant was subjected to heat treatment as follows.

The pH of the supernatant was adjusted to 6.5 with dilute ammonium hydroxide solution and 500ml aliquots were placed in conical flasks which were immersed in a 90°C. waterbath. Stirring was continued until the solution reached 70°C.

whereupon the flasks were transferred to a second bath at 70°C. where they remained for 15 minutes. The solutions were rapidly cooled by plunging them into ice baths, then combined and centrifuged at 20,000 xg for 10 minutes to remove the precipitated heat-labile proteins. The volume of the supernatant was reduced to 250ml in an Amicon ultrafiltration cell fitted with a UM10 membrane.

2.21.2. *Alpha*-Amylase-Glycogen Complexing

A 2% solution of rabbit liver glycogen in water was centrifuged at 2000 xg for 10 minutes. Protein and peptide contaminants were removed by adding 100ml of supernatant to 5g Amberlite IRA-400 (anion) and 5g of Dowex-50 (cation) ion-exchange resins. The suspension was stirred for 10 minutes after which the resins were removed by vacuum filtration.

Ethanol was slowly stirred into the 250ml of heat-treated enzyme extract at 4°C., to give a final concentration of 40% (v/v). The precipitate was removed by centrifugation at 15,000 xg for 10 minutes and discarded. The supernatant was decanted into a beaker and, whilst stirring, 4ml of glycogen solution were gradually dropped in; stirring continued for 15 minutes after which the glycogen-*alpha*-amylase precipitate was removed by centrifugation. The pellet was dissolved in 10ml of 5mM tris-HCl buffer pH 6.7 containing 5mM calcium chloride. This precipitation procedure was repeated five times and the combined dissolved precipitates were incubated for 6 hours at 25°C. to enable complete digestion of the glycogen by *alpha*-amylase. Overnight dialysis of the solution at 4°C. against 2.5l of the same buffer removed the products of glycogen hydrolysis.

2.21.3. Preparative Isoelectricfocussing

This was used in order to separate the two groups of *alpha*-amylase isoenzymes and was performed on the LKB Multiphor machine using the preparative attachment. It was found that gel pre-treatment methods and gel bed preparation conditions were critical; deviation from the following steps led to poor resolution.

Sephadex G-75 superfine (40g) was swollen in 1l of water for 10 minutes, then filtered through a sintered glass funnel of 50 micron porosity and washed with 5l water. The gel was dehydrated by washing with 150ml portions of ethanol until no further shrinkage was noted. Drying was completed under vacuum overnight.

A solution was prepared by mixing 5ml Ampholine carrier ampholytes (pH range 3.5 to 9.5), 40 ml water and 50ml enzyme preparation from the glycogen complexing stage; 5g of the washed Sephadex was suspended in this. Six electrode strips were saturated with Ampholine solution (5%) and three layers of strips were placed at each end of the gel bed tray. The gel slurry was immediately poured into the tray (ensuring it was completely level) the full tray weighed and then placed in a running fume cupboard in order to allow even evaporation of water from the gel.

Evaporation was stopped when the weight loss reached 28%. The tray was placed on the cooling plate of the electrophoresis apparatus and precooled to 4°C. Electrofocussing was performed for 18 hours at 4°C. with a constant power of 8 watts. To collect the focussed zones, a special LKB fractionating grid with 30 chambers was pressed into the gel and the pH of each section was determined with a surface glass electrode. The sections containing pH ranges 5.8 to 6.8 and 4.3 to 5.0 were designated as Group 1 and Group 2 isoenzyme fractions respectively. The gel sections corresponding to each fraction were removed with a spatula and placed into a 50ml syringe plugged with a tight fitting pad of Whatman GF/A glass fibre; just enough water was added to completely resuspend the gels which were then left to settle. One complete gel volume (25-30ml) was used to elute the protein, the complete eluate for each fraction was collected and frozen at -18°C. for future use.

2.21.4. Protein Determination

The non-destructive method of Layne (1960) was used. The ratio of absorbance at 280nm and 260nm was measured on a Perkin Elmer 554 spectrophotometer and the concentration of protein was calculated from the following equation :

$$[\text{Protein}] \text{ (mg/ml)} = (1.55 \times A_{280}) - (0.76 \times A_{260})$$

2.22. PRODUCTION OF ANTI- α -AMYLASE ANTISERA

An aqueous solution of 2mg/ml of either Group 1 or Group 2 isoenzyme proteins was prepared; 1ml was emulsified with 1ml of Freund's complete adjuvant (Calbiochem Ltd.). Half of this mixture was used to immunise a rabbit by giving subcutaneous injections in twelve different sites on its back. One month later the injections were repeated and after a further two weeks 30ml of blood were bled from the marginal ear vein. The animal was rested for one month, then boosted with twelve more subcutaneous injections. After two weeks, another 30ml of blood were bled from the ear; the animal was then retired.

Once the blood had clotted and the antiserum removed, the immunoglobulin (IgG) fraction was separated from the antiserum by affinity chromatography using DEAE Affigel-blue (Biorad Ltd.). Antiserum (1ml) was dialysed against 20mM Tris-HCl buffer pH 8.0 containing 28mM sodium chloride for 16 hours. DEAE Affigel-blue (7ml) was packed into a small column (1 x 10cm) and equilibrated with 5 bed volumes of the same Tris-HCl buffer. The dialysed antiserum was applied to the column and eluted with three bed volumes of buffer; the eluate was monitored at 280nm. The unbound protein (IgG) was collected and stored at -18°C . until further use.

2.23. QUANTITATIVE LAURELL ROCKET IMMUNOELECTROPHORESIS

This was carried out, using the LKB Multiphor apparatus, according to the method of Weeke (1973) as adapted by Sargeant (1980b). Agarose (1% -from LKB) was dissolved in hot 20mM barbital buffer pH 8.6 and when the solution cooled to 50°C . 40 microlitres of anti-Group 1 and 30 microlitres of anti-Group 2 antisera were mixed in. The warm gel was rapidly poured onto a levelled scoured glass plate (8.4 x 9.5cm) ensuring that it was evenly spread; the gel was cooled at 4°C . for 2 hours in a humidified box. Sample wells (10 microlitre) were punched out of the gel using the LKB vacuum system and the plate was transferred to the electrophoresis equipment and pre-cooled to 10°C .

The electrode compartments of the apparatus were filled with barbital buffer and contact with the gel was made by resting 7 stacked electrode sheets on each end of the gel and dipping the other ends into these compartments. Samples were loaded into the wells (8 microlitres) along with standards of known concentration of both groups of isoenzymes (5 microlitres). The gels were run at 4.5v/cm for 16 hours at 10°C. Once electrophoresis was completed the gels were pressed, by placing filter paper sheets on the surface with a heavy weight on top, for 30 minutes. They were subsequently dried with a hair-drier on a hot setting. Protein (rocket) visualisation was achieved by immersing the gel in staining solution for 10 minutes (1.25g Coomassie brilliant blue in 250ml destain solution) followed by rinsing in several changes of destain solution (864ml ethanol, 936ml water, 200ml glacial acetic acid) until the blue background colour was lost. The stained gel was dried off with the hair-drier and the amount of each isoenzyme was determined from the height of the rocket, using the standards as a calibration.

2.24. LOCALISATION OF *ALPHA*-AMYLASE ISOENZYMES IN GRAINS

Germinating grains were placed on their sides onto small potato supports, rested on cryostat chucks, embedded in Tissue-Tek 2 OCT Compound (R.Lamb U.K.) for 2 hours and frozen at -20°C. Serial sections (15 micron) were cut in a cryostat and were taken up onto microscope slides subbed with glycerine containing egg albumin (R.Lamb U.K.). After drying overnight, the sections were fixed in 95% ethanol for 15 minutes, then rinsed for 10 minutes in phosphate buffer saline pH 7.4 (PBS- 8g sodium chloride, 200mg potassium dihydrogen phosphate, 2.89g disodium hydrogen phosphate, 200mg potassium chloride made to 1l with water). A tiny drop of anti-Group 1 or anti-Group 2 antiserum, diluted 1 in 8 with PBS, was placed on top of each section and left to incubate in a humid chamber for 30 minutes at room temperature. The slides were washed for 10 minutes in PBS after which a tiny drop of FITC-goat anti-rabbit IgG (Miles Ltd.), diluted 1 in 16 with PBS, was placed on top and incubated for a further 30 minutes. After washing for 15 minutes in PBS, the slides were blotted dry and a drop of phosphate buffered (pH 7.4) glycerol was spotted onto

the sections and a coverslip placed on top.

The sections were viewed on a Reichert-Jung Polyvar microscope with the B1 fluorescence filter in place. The optics were adjusted to give maximum light to the permanently mounted Polaroid camera so that the brightest photographs could be obtained; the fluorescence faded noticeably within a minute of illuminating the section.

Several control reactions were performed to ensure that the specific fluorescence seen was not artifact. These included leaving out, alternately, the anti-amylase IgG and the FITC IgG and using unimmunised rabbit serum instead of either of the usual IgG's.

2.25. MICROSCOPY OF STARCH DEGRADATION

The embryo ends of germinating grains were dissected away from the endosperms. A small portion of the starch from immediately behind the scutellum was removed; such starch from 25 endosperms was combined. This was diluted to 10ml with water and thoroughly mixed with a vortex mixer to wash the starch. Centrifugation at 1,500 xg for 5 minutes recovered the starch, which was resuspended in 10ml water and rewashed. The pellet was finally resuspended in 0.5ml water to make a paste.

For scanning electron microscopy (SEM), SEM stubs were cleaned with acetone and covered with double-sided sellotape; a little paste was evenly spread over the surface and left to dry overnight. Dry stubs were vacuum-coated twice for 7 minutes with a gold-palladium mixture, left to cool and then examined using a Coates and Welter Cwiskscan-100 field emission scanning electron microscope.

Portions of paste were diluted and mounted under coverslips on light microscope slides. These were viewed with a Reichert-Jung Polyvar light microscope using mercury-vapour lamp illumination and interference contrast settings.

2.26. *ALPHA*-AMYLASE SYNTHESIS IN DE-EMBRYONATED GRAINS

Dry grains were cut transversely into two halves with a razor blade and the embryo containing halves were discarded. The remaining halves were surface sterilised and rinsed and placed, in batches of twenty, in sterile 50ml conical flasks. Each flask contained 5ml of sterile medium (10mM sodium acetate buffer pH 5.3 containing 10mM calcium chloride) and a range of concentrations between 1nM and 10mM gibberellic acid (GA). The control flasks had no GA. After capping with sterile aluminium foil, the flasks were placed in shaking water baths at either 10 or 25°C. and left for 5 and 2 days respectively. When incubation was complete, the grains and media were extracted and assayed together for *alpha*-amylase activity (section 2.16.).

2.27. EXTRACTION AND ASSAY OF GIBBERELLIC ACID

Germinating seeds (50) were homogenised with ethyl acetate (50ml) in a mortar, then centrifuged at 8,000 xg for 10 minutes; the supernatant was reduced to dryness in a rotary evaporator. The residue was taken up in 500 microlitres of absolute ethanol; this was the GA extract.

Half seed incubations were set up as before (section 2.26.) using only high vigour grains. The incubation media were prepared as described, except that some flasks contained various volumes of GA extract instead of standard GA solutions; control flasks contained either no standard GA solution or a volume of absolute ethanol equivalent to the volume of GA extract used. Incubations were at 25°C. for 2 days, after which *alpha*-amylase was extracted and assayed. The concentration of GA in the extracts was estimated by interpolation on the GA:*alpha*-amylase dose:response curve obtained for the standard GA solutions.

2.28. EXTRACTION AND ASSAY OF MALTASE

Extraction was the same as for *alpha*-amylase (section 2.16.) except that no heat treatment was used. For the assay, the 2ml reaction mixture contained 20mM sodium acetate buffer pH 5.3; 5mM maltose and 1ml maltase extract.

After various incubation periods at 25°C., the reaction was stopped by the addition of 8ml absolute ethanol and the quantity of glucose formed was determined enzymically (section 2.12.).

2.29. DETERMINATION OF GERMINATING SEED SURFACE POTENTIALS

These were measured using silver/silver chloride (Ag/AgCl) electrodes, which were constructed as following. 4cm long pieces of 1mm diameter silver wire were chloridized by an electrolytic process. These were inserted into glass capillaries containing 3M KCl in 2% agar (Oxoid Ltd. London) and sealed at the insertion end with epoxylite resin; the open ends were kept moist by storing them in a 3M KCl solution.

Two Ag/AgCl electrodes were fixed onto micromanipulators (Prior, England) and positioned so that the reference electrode was sited at the brush end of the seed, whilst the recording electrode was on the embryo. Contact between the electrodes and the seed surfaces was made with small drops of 10mM KCl. To avoid vibration affecting the positions of the electrodes, all measurements were performed on a marble bench. Both electrodes were connected to a high input impedance amplifier with a gain of X10, as used by Rathore (1981), and the output from this was connected to a chart recorder. Zero was set by short circuiting the electrodes with a 10mM KCl bridge.

2.30. STATISTICAL ANALYSES

Where applicable, all data was subjected to significance tests. For graphically displayed data the 95% confidence limits were calculated and indicated as bars for each point; these confidence limits were determined by multiplying the standard error of the mean by the value of Student's t for $P=0.05$ and $n-1$ degrees of freedom. This indicates the range around the mean within which 95% of the results should fall if these results were replicated indefinitely. Two points can be assumed to be significantly different, with 95% confidence, if the plotted bars do not cross.

For tabulated data, the Student's t test was used which indicates whether the mean of one set of replicates is significantly different from the mean of another set, usually high versus low vigour seeds. Calculations were performed with the established formula using only raw data i.e. even though in many tables % figures are given, the significance was determined before the percentages were calculated. The value of t was used (with the appropriate number of degrees of freedom) to ascertain the probability that the difference between the two means could have arisen by chance. These probabilities are indicated using a star system viz. $*=P<0.05$ $**=P<0.01$ $***=P<0.001$.

3. RESULTS

3.1. FIELD EXPERIMENTS

A number of experiments were conducted in an attempt to induce low vigour in the seed of field crops and to discover if some varieties of wheat were more susceptible than others. Additionally, the field emergence of artificial low vigour seed, induced by laboratory drying damage, was investigated to see if a vigour test could predict this field performance.

3.1.1. The Influence of Wheat Variety on Vigour

Identical field plots were sown with eight currently available winter wheat varieties. The 1,000 grain weights of the initial seedlots were recorded, as were the % emergences and final yields of the plants. After harvest, the % germinations (i.e. the % of normal seedlings to grow under optimum conditions) and % vigours of the newly harvested seeds were determined (Table 1.). Although the % germinations were all similar (hence dormancy was not a factor), some varieties were apparently of lower vigour than others. This effect did not seem to be related either to the initial size of the grain sown, as indicated by the 1,000 grain weight, or to the emergence and yield of the plants.

3.1.2. Induction of Low Vigour Seed in the Field

Plots of "Avalon" wheat plants were sprayed with GA solutions in order to induce pre-harvest sprouting. After harvest, the grain was examined for signs of sprouting but none were found even on the grain obtained from plots sprayed with the highest GA concentration. This was probably due to the weather conditions which were not conducive to sprouting during the period of experimentation, a point noted by other workers (Ellis and Clayton 1976). The grain was then vigour-tested, not by the RHM emergence method but by the drip-slide technique using seeds from plants which had not been treated in any special way in the field as the 100% controls. Although there were no visible aberrations in the treated grains,

VARIETY	1,000 GRAIN WEIGHT (g)	% FIELD EMERGENCE	YIELD (tonnes/ha)	% GERM- INATION	% VIGOUR
AVALON	57.0	74	6.81	96	91
BOUNTY	55.2	80	6.23	93	89
COPAIN	48.2	85	5.63	94	83
FLANDERS	47.3	88	6.12	95	82
HUNTSMAN	55.9	75	6.42	93	84
MARDLER	54.2	74	7.35	92	86
NORMAN	56.3	80	7.18	99	74
TIMMO	50.4	84	6.54	97	90

TABLE 1. The effect of wheat varieties on crop field performance and on the quality of the resultant harvestable grain. Germination and vigour results are the average of three replicates; variation between replicates was never more than 9%.

there was a slight loss of vigour (Table 2.) at the highest GA concentration, suggesting that had conditions favoured sprouting, a more pronounced loss of vigour might have occurred.

Two treatments were devised to test the effects of mechanical harvesting damage to grain on its vigour. The first involved setting the combine harvester blades too high, which had the effect of shearing and shattering many grains; there were approximately 30% damaged grains estimated by inspection of visible cracks and cuts. The second treatment had the combine harvester blades set correctly but the grain was still very moist at harvest (31% m/c); cracks and cuts were not so common here as mishapen seeds produced by squashing and it was noted that the embryos were often split. It was not possible to estimate a % damage figure for this treatment. Both treatments had a marked effect on seed vigour (Table 2.) but both also reduced the % of normal seedlings produced; it appears that mechanical damage can be potent in destroying viability of the embryos. It should be noted that the growth tests were conducted under semi-aseptic conditions so that seeds with a cut in the non-embryo portion were hardly disadvantaged; in the field, pathogenic microorganisms are more likely to infect and kill those seeds which sustained small cuts, as the exposed endosperm may act as a microbial food source.

A gift, from RHM, of some seed harvested from plants which had been deliberately heavily infested with aphids throughout the growing season, was also tested for germination and vigour. No loss in either parameter was noted even though the grain size was small (1,000 grain weight = 45.2g).

3.1.3. The Effect of Postharvest Drying on Seed Vigour

Grain was harvested at different moisture contents and dried at various temperatures in a farm-scale commercial grain drier and also at ambient temperature in a glasshouse. The seeds were vigour-tested, by comparing their growth rates at 10°C. with those of an untreated control harvested at 15% m/c which was not dried.

TREATMENT	% GERMINATION (NORMAL SEEDLINGS)	% VIGOUR (F.WT.)
CONTROL (NO TREATMENT)	93	100
CONTROL (WATER SPRAYED)	98	105
SPRAYED (3 MICROMOLAR GA)	94	96
SPRAYED (300 MICROMOLAR GA)	92	* 86
MECHANICAL DAMAGE TO DRY GRAIN	*** 65	*** 53
MECHANICAL DAMAGE TO MOIST GRAIN	*** 54	*** 39
APHID INFESTED	96	93

TABLE 2. The effects of agronomical mishandling on the germination and vigour of the harvested grain. Vigour was measured by the drip-slide method using the untreated control plants as the 100% standards. No results were significantly different from those of the untreated control except those with asterisks : * = $P < 0.05$
 ** = $P < 0.01$ *** = $P < 0.001$

Only grain with the highest m/c was affected; it seems that initial m/c is more critical than the temperature at which the grain is dried within the ranges tested. High moisture grain was harmed by drying at both high (80) and moderate (50) temperatures but grain of lower m/c was unaffected at any temperature (Table 3.). Typical growth curves for four types of treated grain are shown in Figure 6.; the low performance of the high m/c seed is significantly different ($P < 0.05$) from the growth of control or lower m/c seeds.

3.1.4. Field Emergence of Low Vigour Seed

Because postharvest grain drying affected seed vigour, an experiment was conducted to find out whether laboratory dried grain would also lose vigour and whether any correlation existed between % vigour and % field emergence. Moist grain was dried in a convection oven for various periods (section 2.4.2.), vigour-tested (using the drip-slide method) and scored for field emergence. The grain obtained from these plants was harvested normally and was vigour-tested to find out if any vigour loss could be transferred genetically. The results (Table 4.) showed that low vigour was induced by laboratory drying damage and that the vigour-test could predict field emergence, although it somewhat underestimated the severity of the damage. Since low vigour plants gave rise to high vigour grains, this form of low vigour is not inherited and must be of physiological origin.

3.2. LABORATORY INDUCTION OF LOW VIGOUR SEED

The effects of laboratory drying damage were investigated further by drying seeds for various periods at a single temperature (65°C.) and then testing their germination, vigour and growth performance under different conditions.

3.2.1. Effect of Drying Duration on Seed Vigour

Following drying and re-equilibration of m/c, seeds were germinated on drip-slides at 10°C. in the dark and the fresh weight changes were monitored (Figure 7.).

INITIAL % M/C	DRYING TEMP. (C)	DURATION (MINS.)	FINAL M/C	% GERM- INATION	% VIGOUR (F.WT.)
15.0	CONTROL	-	15.0	96	100
18.2	AMBIENT	-	14.9	94	93
18.2	50	70	14.5	95	97
18.2	65	50	14.0	97	103
18.2	80	30	14.7	93	92
21.0	AMBIENT	-	14.8	98	93
21.0	50	80	14.5	97	99
21.0	65	65	14.6	93	94
21.0	80	45	14.6	97	97
25.8	AMBIENT	-	14.3	96	101
25.8	50	135	18.0	89	*** 58
25.8	80	60	17.4	88	*** 47

TABLE 3. Effects of grain moisture content and postharvest drying temperature on seed germination and vigour. Germination is expressed as the % of normal seedlings to grow under optimum conditions and vigour was determined by the drip-slide method. Significant results are indicated as before.

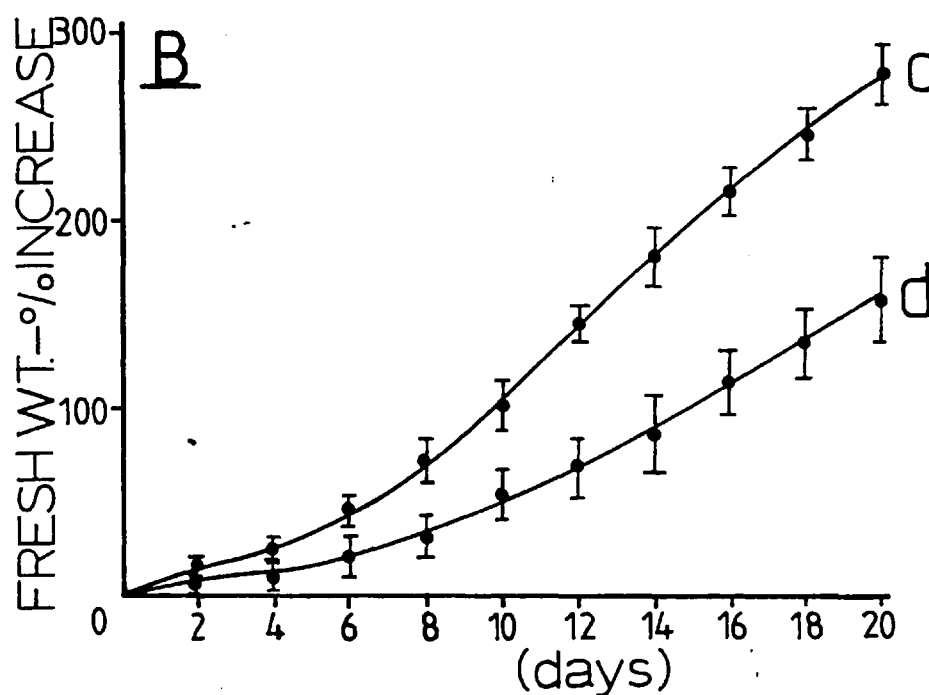
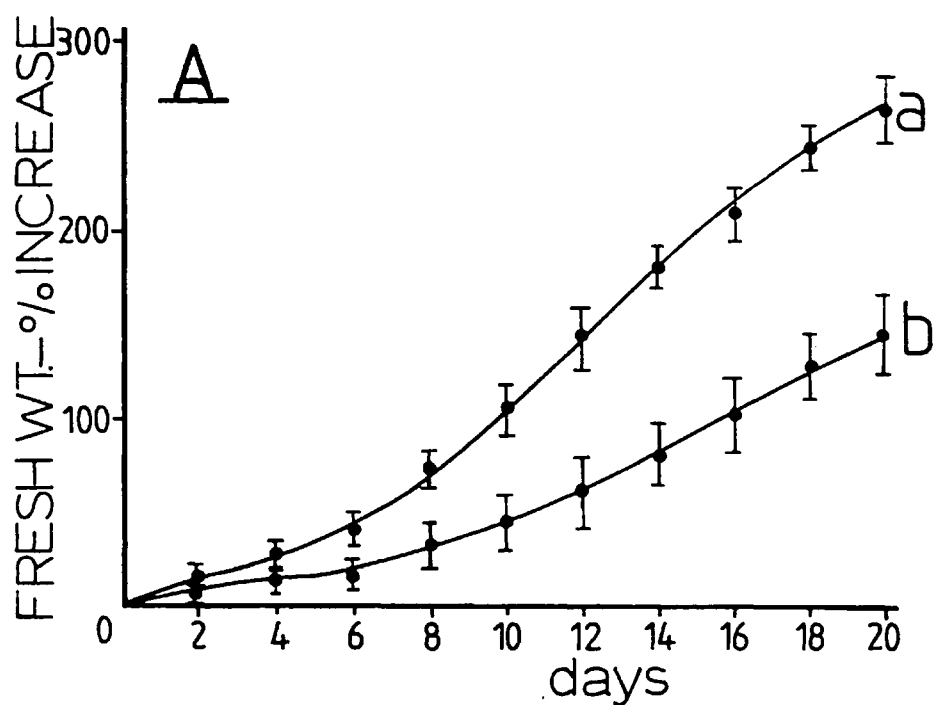


FIGURE 6. (A&B) % Increase in fresh weight with time from imbibition for hot-air dried seeds, germinated on "drip-slides" at 10°C. in the dark. Each point is the mean of 4 replicates and the bars represent 95% confidence limits. (a)=15% m/c undried control grain (b)=26% m/c grain dried at 80°C. (c)=21% m/c grain dried at 80°C. (d)=26% m/c grain dried at 50°C.

HOURS OF DRYING	% GERMINATION	% VIGOUR	% EMERGENCE	% VIGOUR OF GRAIN
0	97	100	86	100
1	90	* 88	** 71	91
2	94	** 80	*** 60	92
3	96	*** 56	*** 37	96
4	93	*** 40	*** 22	93

TABLE 4. Effect of drying 25% m/c seeds at 65°C., for various periods, on germination, vigour, field emergence and vigour of the grain which they yielded (final column). Germination, vigour and significance levels are expressed as before.

An increasingly deleterious effect of drying duration on growth rates under cold stress was noted for up to 4 hours heating but after this no further reduction was apparent. No effect of heating on the % of normal seedlings produced under optimum conditions was found as all seedlots had between 91% and 98% germination. Once these seeds had grown for 20 days at the stress temperature, the shoot lengths were measured and grouped into categories. Figure 8. shows that the numbers of shoots in the longest category decreased dramatically with length of heating. Conversely, as the duration of heating increased so did the proportion of small shoot plants and non-viable (i.e. no sign of radicle protrusion) seeds. There was no major difference in the proportions of plants occurring in the various categories, for seeds dried for more than 4 hours.

These results clearly indicated that low vigour seed could be generated in the laboratory and that heating 25% m/c seed for 4 hours at 65°C. would produce the maximum effect in the shortest time. Although this 4 hour drying time was longer than that required in the commercial drier, the final m/c was not dissimilar - 13.8%. Laboratory drying damage is clearly a realistic mimic of the situation on the farm if over-moist seed is used. For this reason, all low vigour seed used in subsequent experiments was generated in this way. "High vigour" seed was taken from the same 25% m/c stock but was dried at ambient temperatures in a fume cupboard (section 2.5.).

3.2.2. Germination and Growth of Low Vigour Seed

Germination temperature was found to have a marked effect on the performance of high and low vigour seed (Figure 9.). As expected, both germinated equally well at optimum temperatures (between 20 and 25°C.) but whereas the germination of high vigour seed was only marginally affected by increasing stress, the low vigour seed was devastated by such conditions. The difference in germination was significantly different ($P < 0.001$) at 30°C. and at 15°C. and below. Germination at 10°C. was markedly different and as this is a typical soil temperature that winter wheat seeds would experience during field

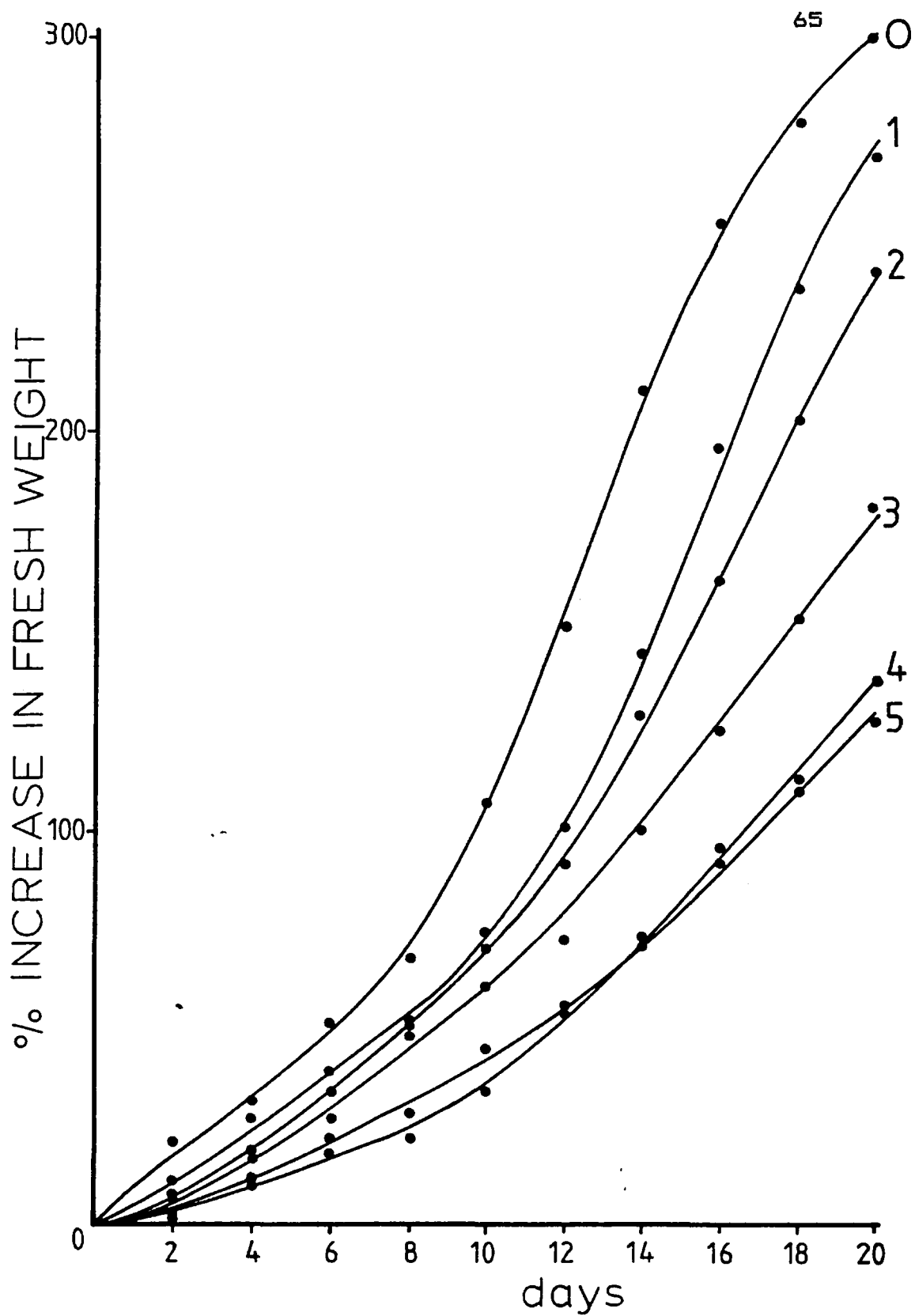


FIGURE 7. The effect of drying duration (hours) at 65°C. on subsequent increases in seedling fresh weight at 10°C. Each point is the mean of 3 replicates; maximum variation between replicates = 12%.

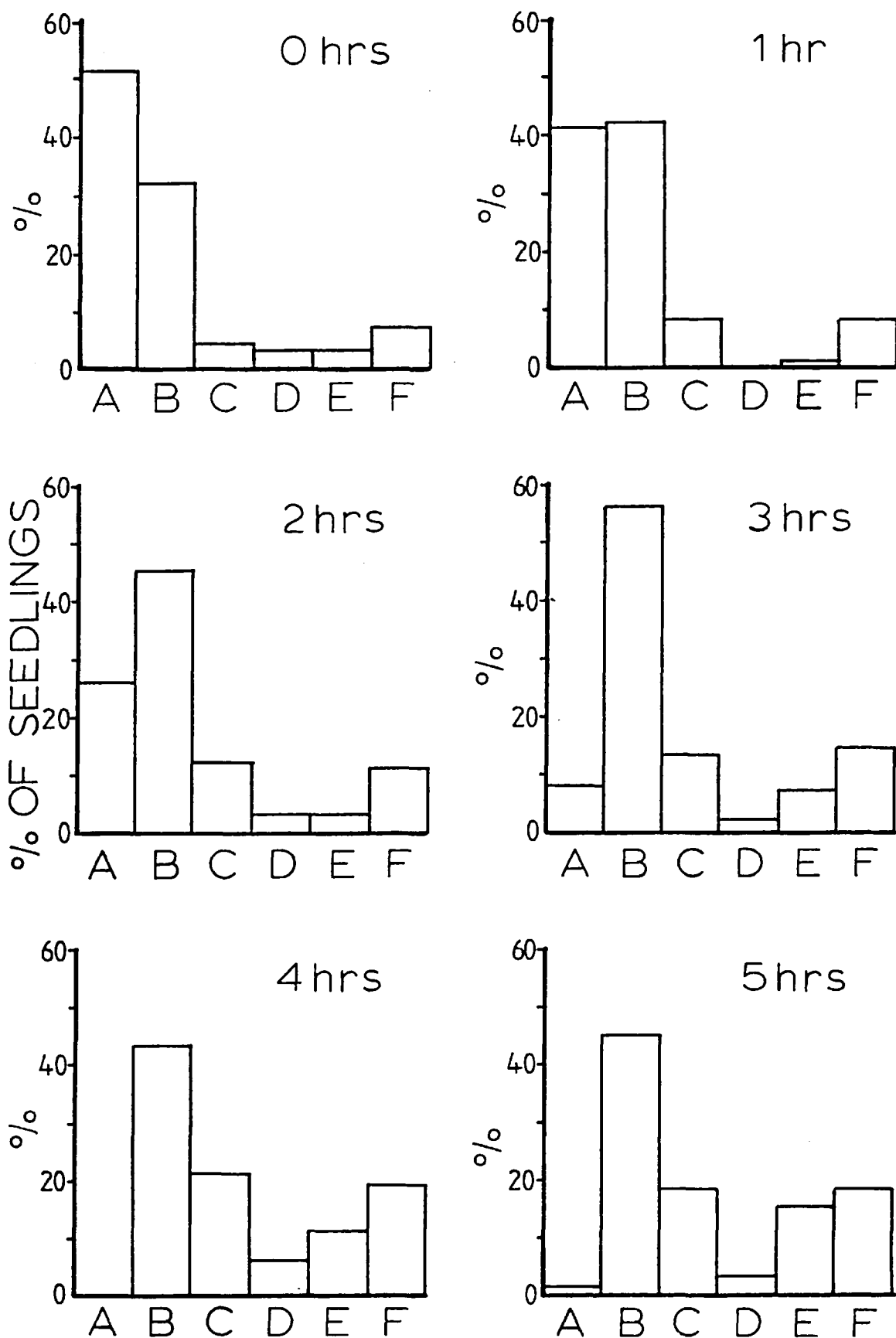


FIGURE 8. The effect of grain drying duration on the heights (cm) of resultant seedlings grown for 20 days at 10°C. A=>20 B=15-20 C=10-15 D=5-10 E=<5 F=non-viable
Each bar = the mean of 3 replicates with a maximum variation of 8%

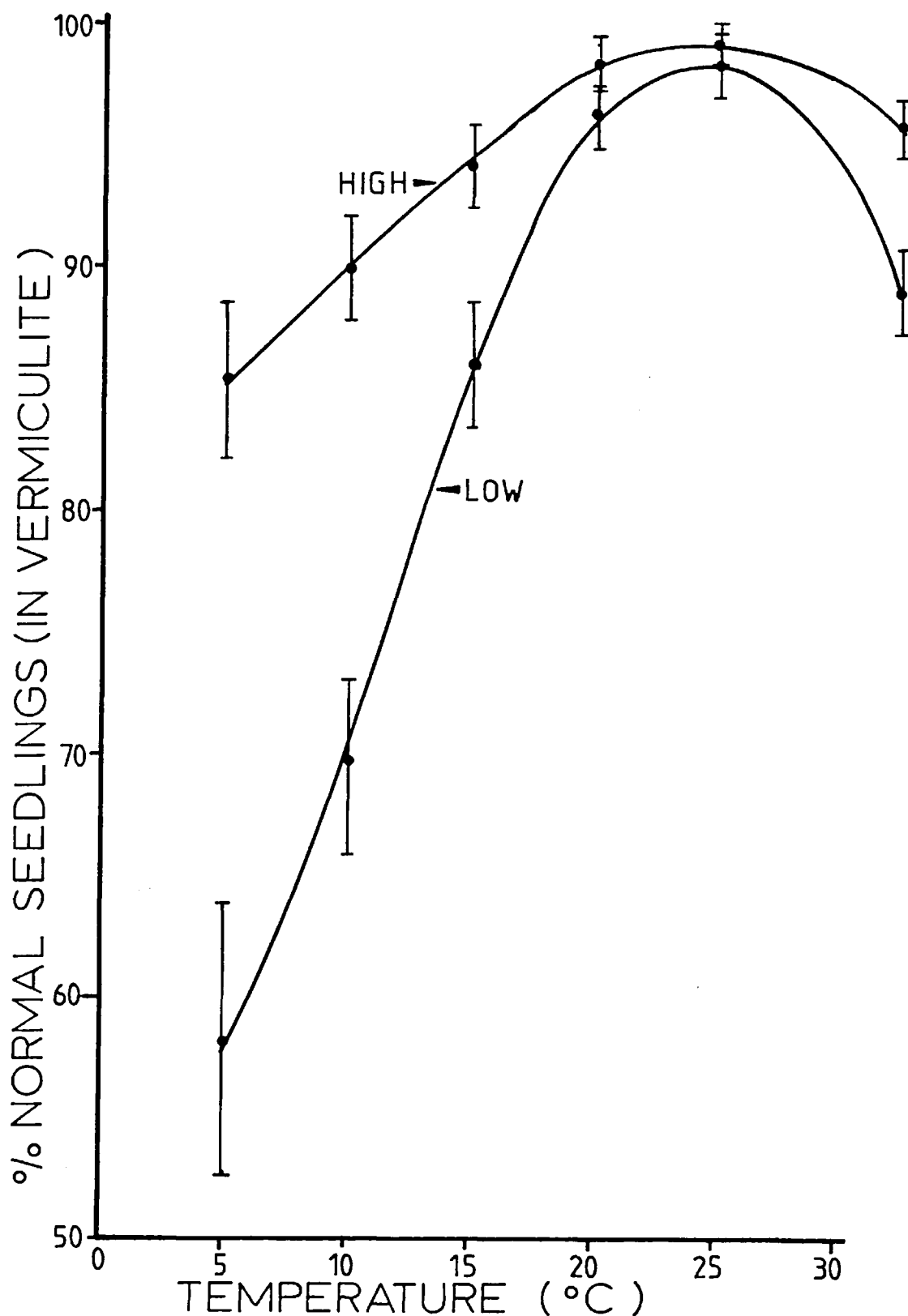


FIGURE 9. The effect of germination temperature on the % of normal seedlings to grow from high and low vigour seed. Germination was in vermiculite, in the dark. Bars = 95% confidence limits.

germination, it was used henceforth in all experiments to compare seed performance under stress.

The differences in germination noted above were reflected in the growth rates measured at optimum and stress temperatures. At 25°C., in the dark, the maximum growth rates of both seedlots were similar (Figure 10.) but at 10°C. the low vigour rate dropped to 50% of that of the high vigour seed. Although the maximum growth rates were higher for both types in the light (Figure 11.), there were still significant differences between them under stress. It is interesting to note that whilst high and low vigour maximum growth rates were similar under optimum conditions, they were not expressed at the same time. There was a noticeable lag period of about 1 day before the low vigour seeds started to grow at equivalent rates. This lag was increased at 10°C. to 3 or 4 days and similar growth rates were not achieved.

Table 5. provides a summary of the maximum growth rates (i.e. the rates of increase in fresh weight at the steepest portions of the growth curves) obtained for high and low vigour seed under each growth condition. These data were used to calculate the % vigour (using the high vigour seed as the 100% control) for seed grown in the light and dark. Vigour was also estimated by the RHM emergence method and whilst this test showed that the vigour of the low vigour seeds was indeed reduced, it was not as large a decrease as that indicated by the drip-slide method.

It can be seen that when this low vigour seed is cold stressed, it only grows at about 50% of the rate of high vigour seed but this can be alleviated by about 20% if the seeds are grown in the light. This suggests that low vigour may in part be due to a lack of respiratory substrate from the endosperm, which can be partially made good by photosynthesis.

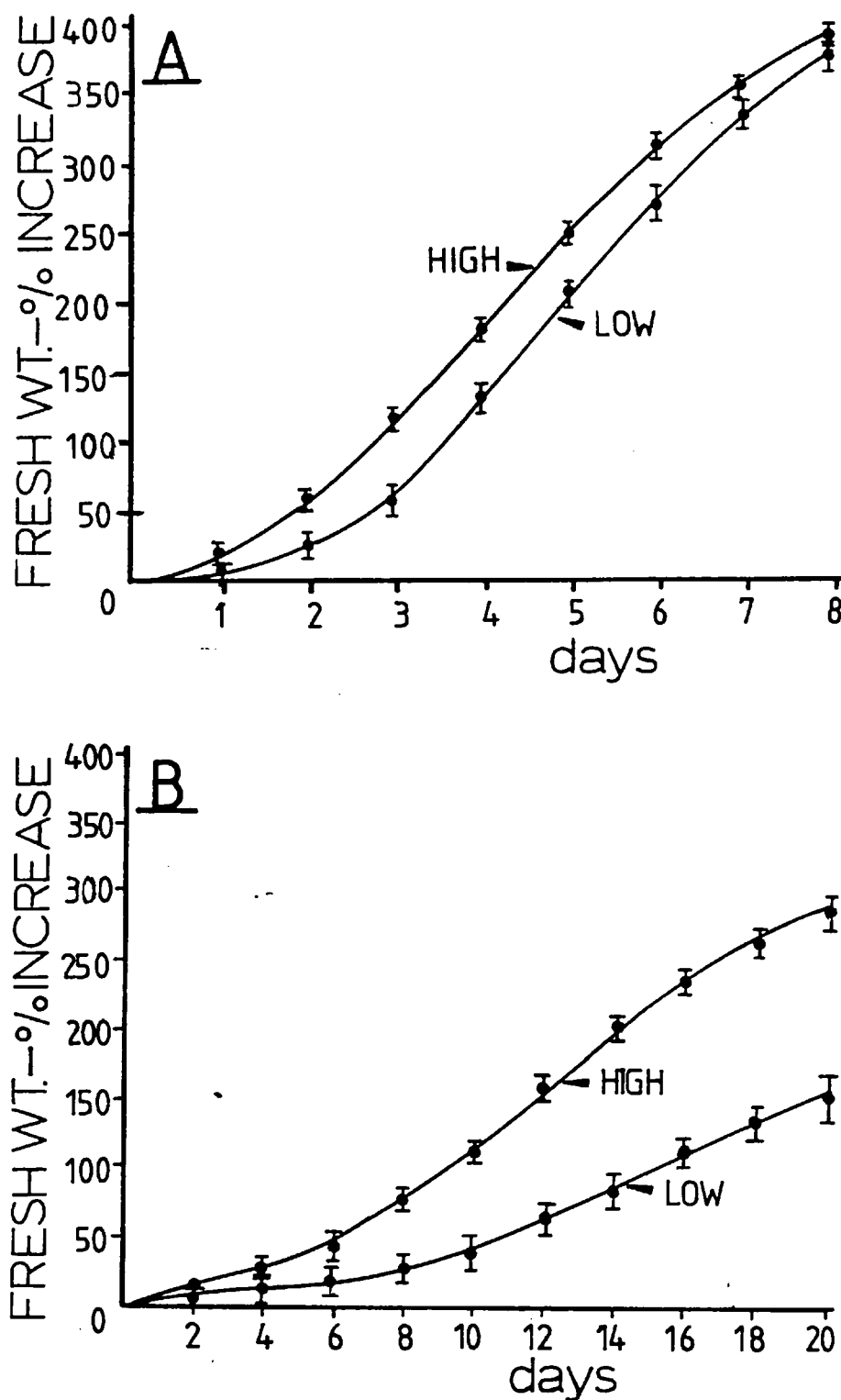


FIGURE 10. Increase in fresh weight with time from imbibition for high and low vigour seeds germinated in the dark on drip-slides at (A) 25°C. and (B) 10°C. Bars = 95% confidence limits.

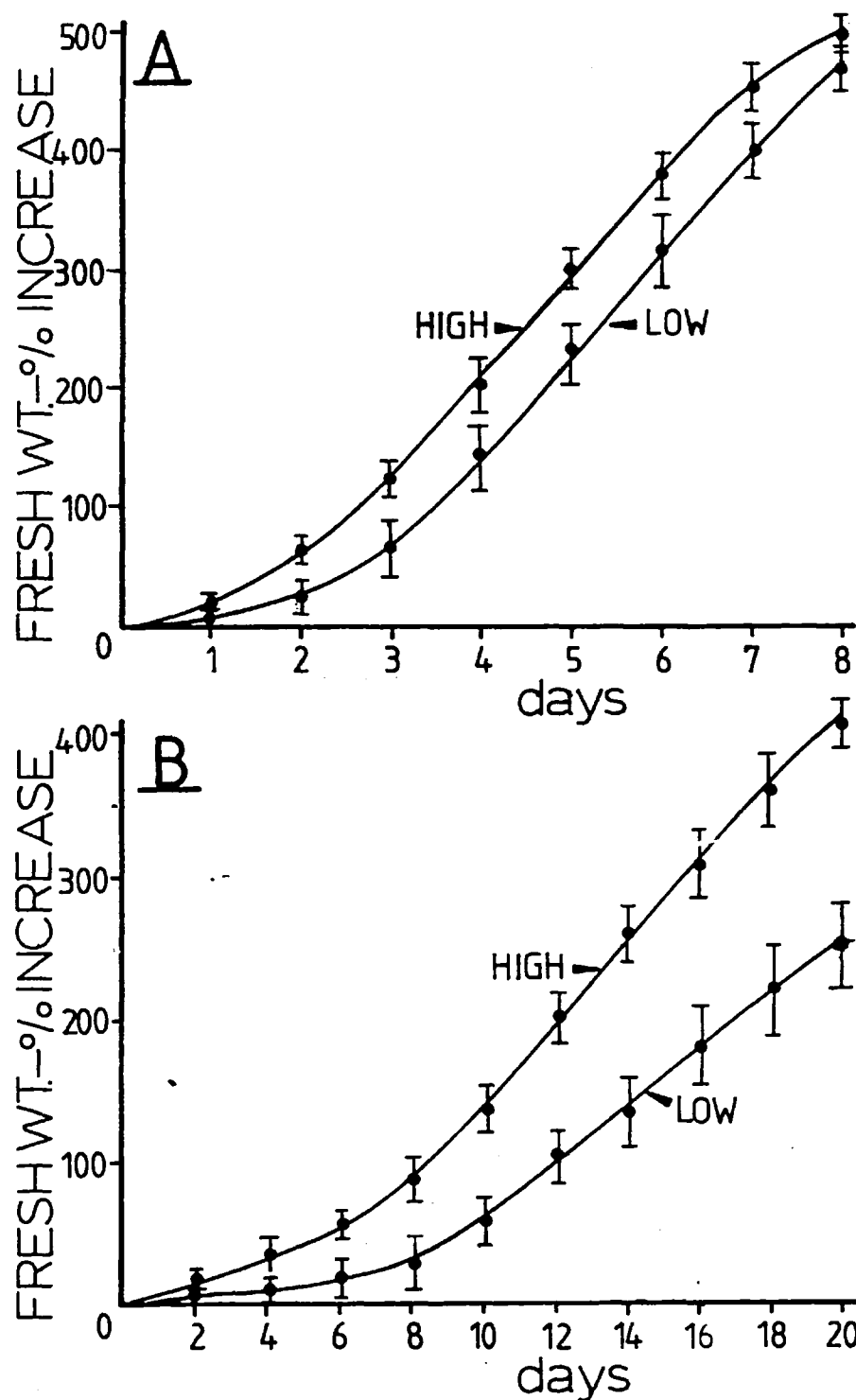


FIGURE 11. Increase in fresh weight with time from imbibition for high and low vigour seeds germinated in the light on drip-slides at (A) 25°C. and (B) 10°C. Bars = 95% confidence limits.

PARAMETER	TEMP. (C.)	HIGH VIGOUR	LOW VIGOUR
% NORMAL SEEDLINGS	25	99	98
% NORMAL SEEDLINGS	10	90	*** 70
MAX. GROWTH RATE - DARK	25	60	62
MAX. GROWTH RATE - DARK	10	23	*** 11
MAX. GROWTH RATE - LIGHT	25	84	86
MAX. GROWTH RATE - LIGHT	10	31	*** 21
% VIGOUR (F.WT.) - DARK	10	100	*** 48
% VIGOUR (F.WT.) - LIGHT	10	100	*** 68
% VIGOUR (RHM EMERGENCE)	-	89	** 74

TABLE 5. Effect of temperature and light on the germination and maximum growth rates (% increase in fresh weight per day) of high and low vigour seeds grown on drip-slides. Vigour was determined by the RHM emergence and the drip-slide method (high vigour = 100% controls). Low vigour results significantly different from high vigour ones are expressed as before.

3.3. RESPIRATION IN GERMINATING SEEDS

The rates of respiration and levels of respirable substrate in germinating high and low vigour seeds were investigated under optimum and stress conditions.

3.3.1. Oxygen Uptake of High and Low Vigour Seedlings

Oxygen uptake was monitored over the early parts of imbibition and germination (Figure 12.) and graphs were plotted by the use of computer based best-fit analyses. At both 10°C. and 25°C. the high and low vigour uptake rates were significantly different ($P < 0.05$).

Both seedlots showed similar triphasic patterns of oxygen uptake with an early burst, then a lag phase followed by a steady linear increase. The lengths of these phases varied with temperature and with vigour. At the optimum temperature, the low vigour linear uptake rate equalled that of the high vigour (although there was an extended lag phase) but under cold stress the linear rate for the low vigour seed was considerably smaller. This implies that the efficacy of the respiratory machinery is reduced in low vigour grains, or that there is an inadequate source of substrates such as glucose.

3.3.2. Glucose Concentrations in Germinating Seeds

As glucose is the main respirable substrate utilized in germinating wheat, levels in its source (endosperm) and main sink (embryo) were determined for high and low vigour seeds, germinated under both optimum and stress conditions. Germinating seeds were dissected into embryo and endosperm portions and each part was assayed for glucose using the specific enzymic method. This technique gave very reproducible results, and absorbance readings could be taken after 7, 10, and 15 minutes, giving easy triplicate checking for each sample. Calibration lines for each time interval are given in Appendix A.

At 25°C. the embryos of both high and low vigour seeds showed similar rising glucose levels as germination progressed, until a peak was reached after which glucose

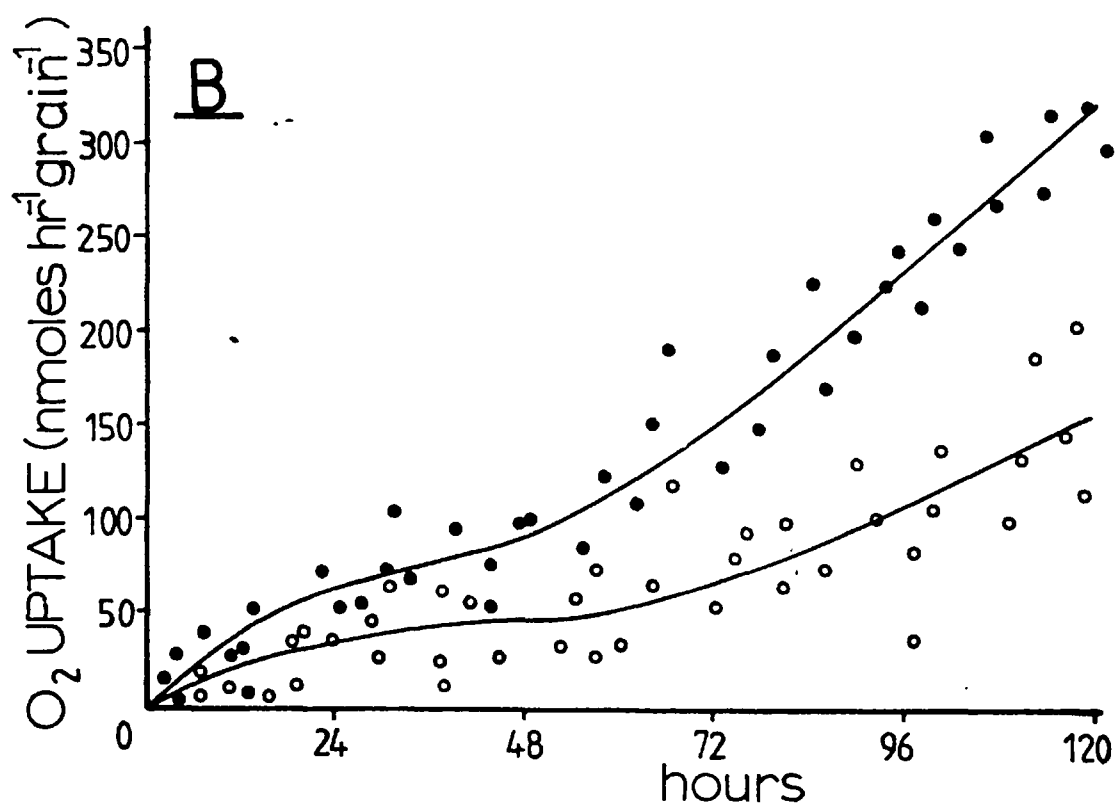
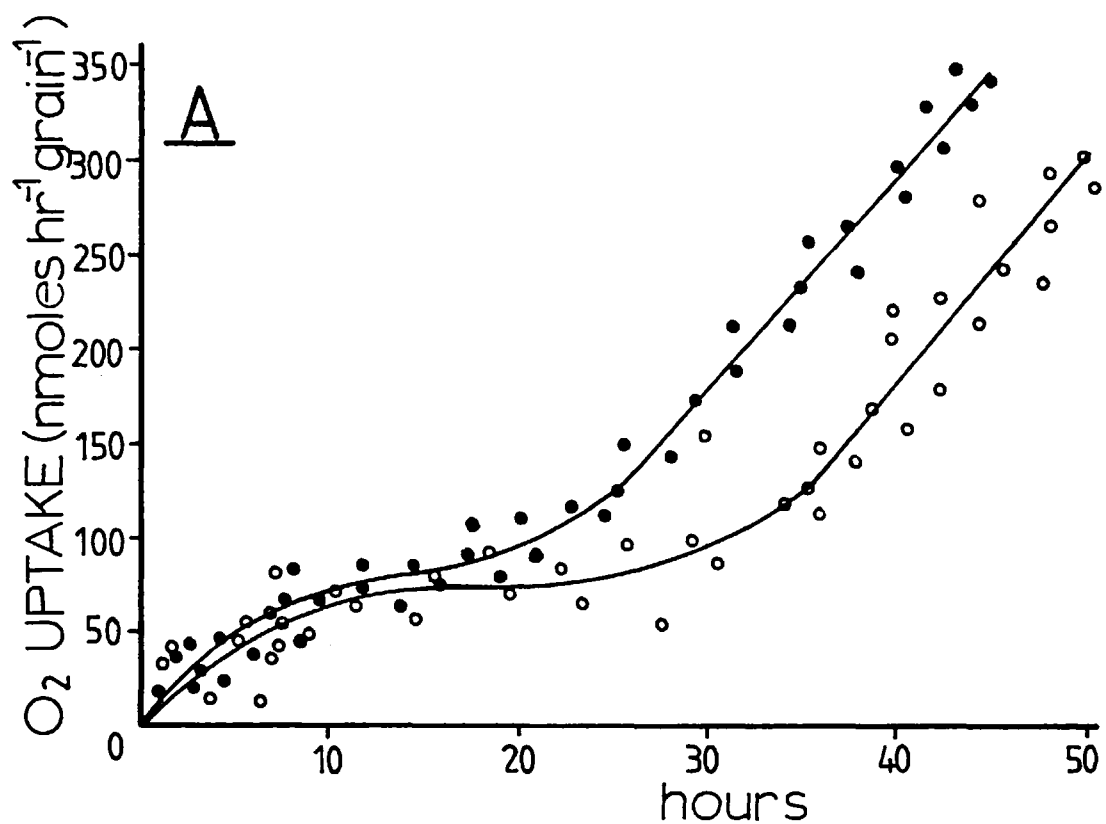


FIGURE 12. Rates of oxygen uptake for germinating high and low vigour grains at (A) 25°C. and (B) 10°C. Lines were fitted by non-linear regression analysis.

concentrations fell (Figure 13.). As before, the low vigour seeds lagged about 1 day behind the high vigour seeds, so that they peaked at 7 days rather than 6 days; this could account for the extended lag in respiration rates. The decline in glucose concentrations in the later stages of germination was probably due to the near exhaustion of the endosperm reserves, caused by a lack of replenishment from photosynthesis. Seeds germinated in the light would probably not show such drastic declines. The high vigour endosperm portions had similarly rising glucose levels but low vigour ones had much lower levels. It appears that even under optimum conditions, low vigour plants suffer from a short supply of sugar.

Not surprisingly, the low vigour seeds performed worse under stress (Figure 14.). The decreased endospermic glucose concentrations noted above were reduced even further; and we might expect the growth of low vigour plants to be severely checked by this sugar shortage. This could also explain the inability of low vigour seeds to exhibit respiration rates equivalent to those of high vigour seeds, when germinated under stress. It is possible that there is a specific lesion in the glucose metabolising system of low vigour seeds but other sugars might also be involved. To test whether vigour loss is associated only with alterations in glucose supply, a study of the levels of other sugars normally present during germination was undertaken.

3.4. SUGAR LEVELS IN GERMINATING SEEDS

High Performance Liquid Chromatography (HPLC) proved to be a valuable tool in studying plant sugars. Many extraction techniques and numerous chromatographic conditions were used, the most successful of which have been described (sections 2.12.3. and 2.12.4.). Typical traces for standard sugars and a plant extract are shown in Figure 15. and it is clear that there is no apparent interference from other substances. Once the nature of the sugar peaks had been chemically verified, they were routinely identified by their retention times. The technique was made quantitative by constructing calibration curves of peak areas against weights of injected sugars (Appendix B.); the relationship

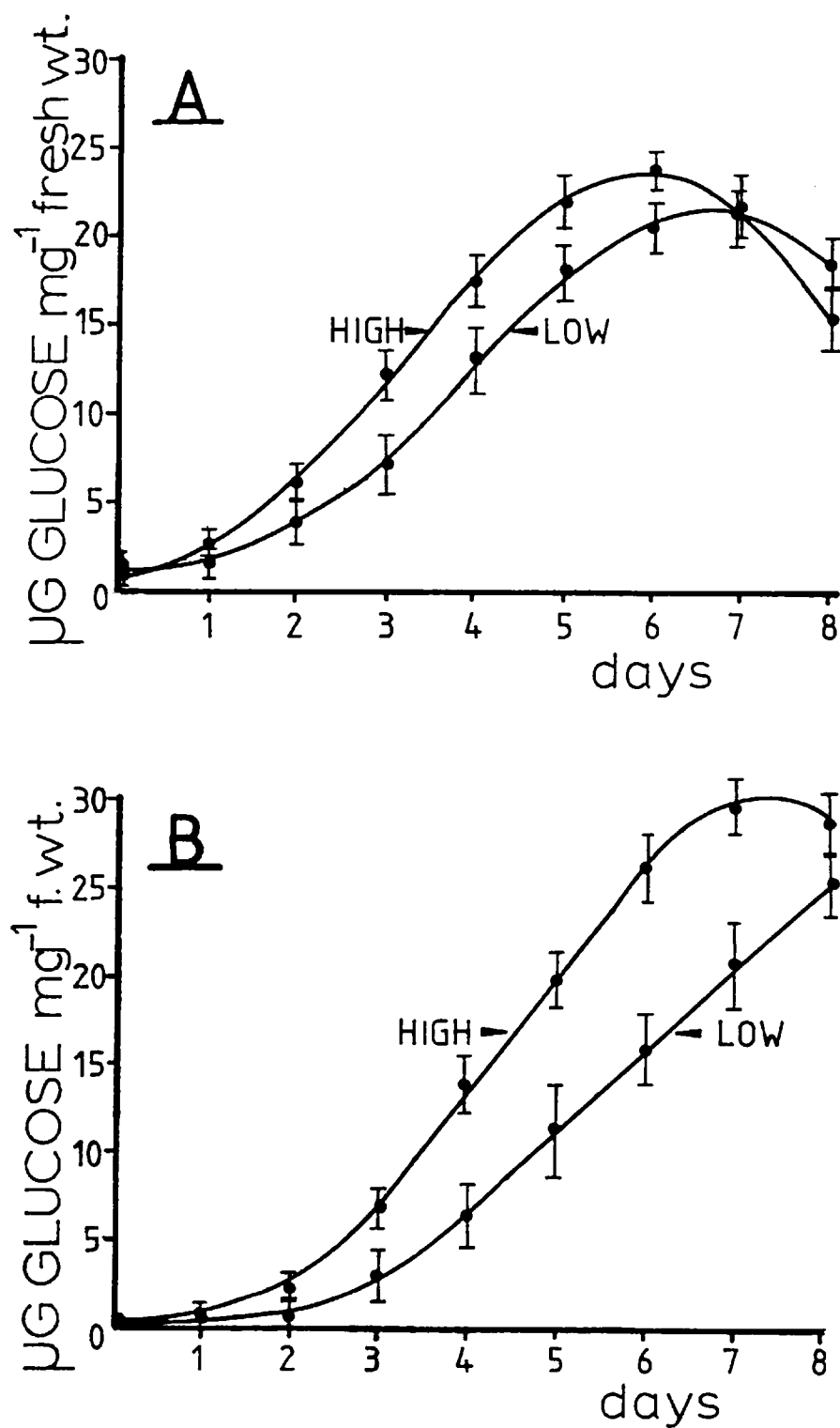


FIGURE 13. Changes in glucose concentration during germination at 25°C. in the dark for dissected high and low vigour seedlings. A = embryo portions (including scutella). B = endosperm portions.

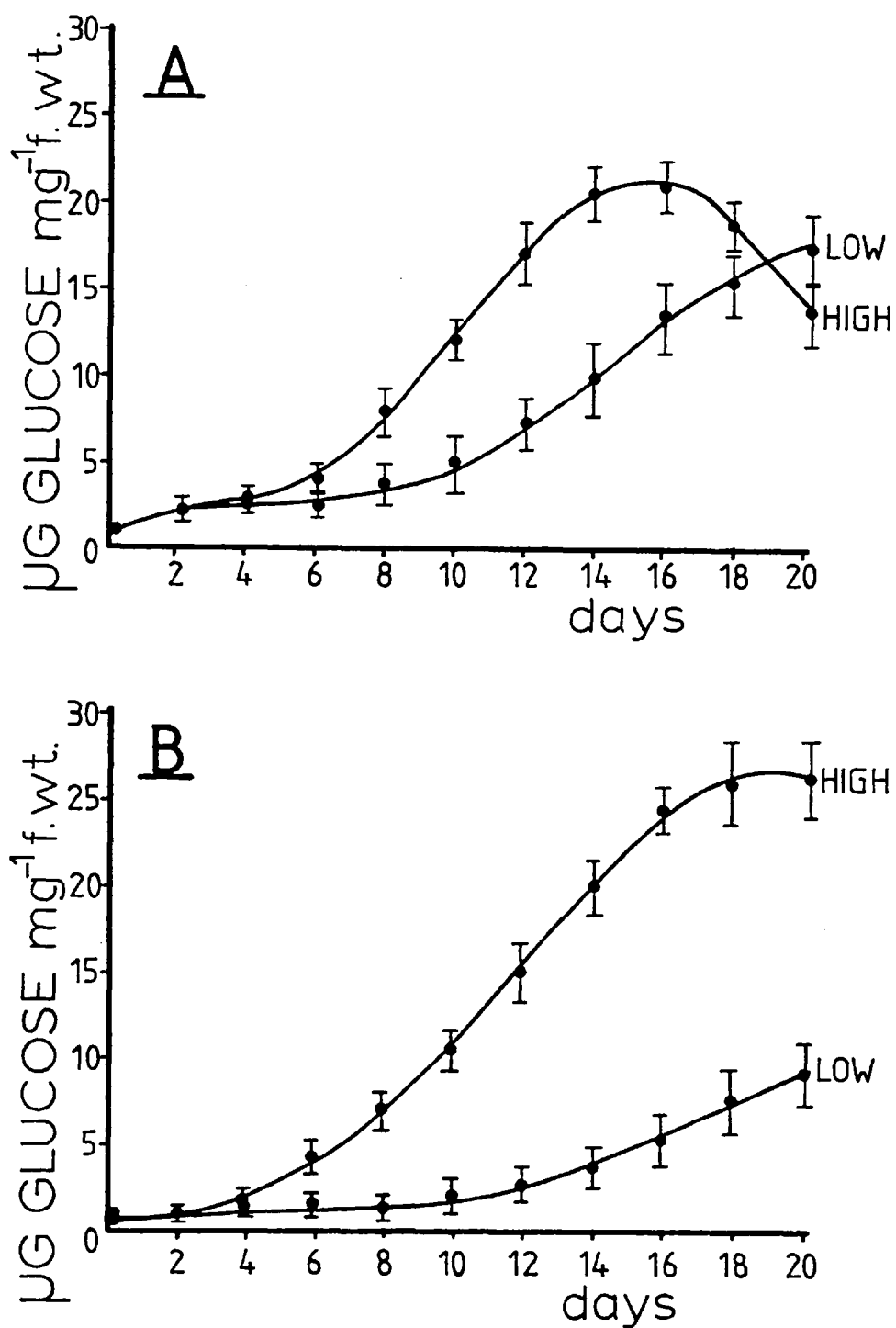


FIGURE 14. Changes in glucose concentration during germination at 10°C. in the dark for dissected high and low vigour seedlings. A = embryo portions (including scutella). B = endosperm portions.

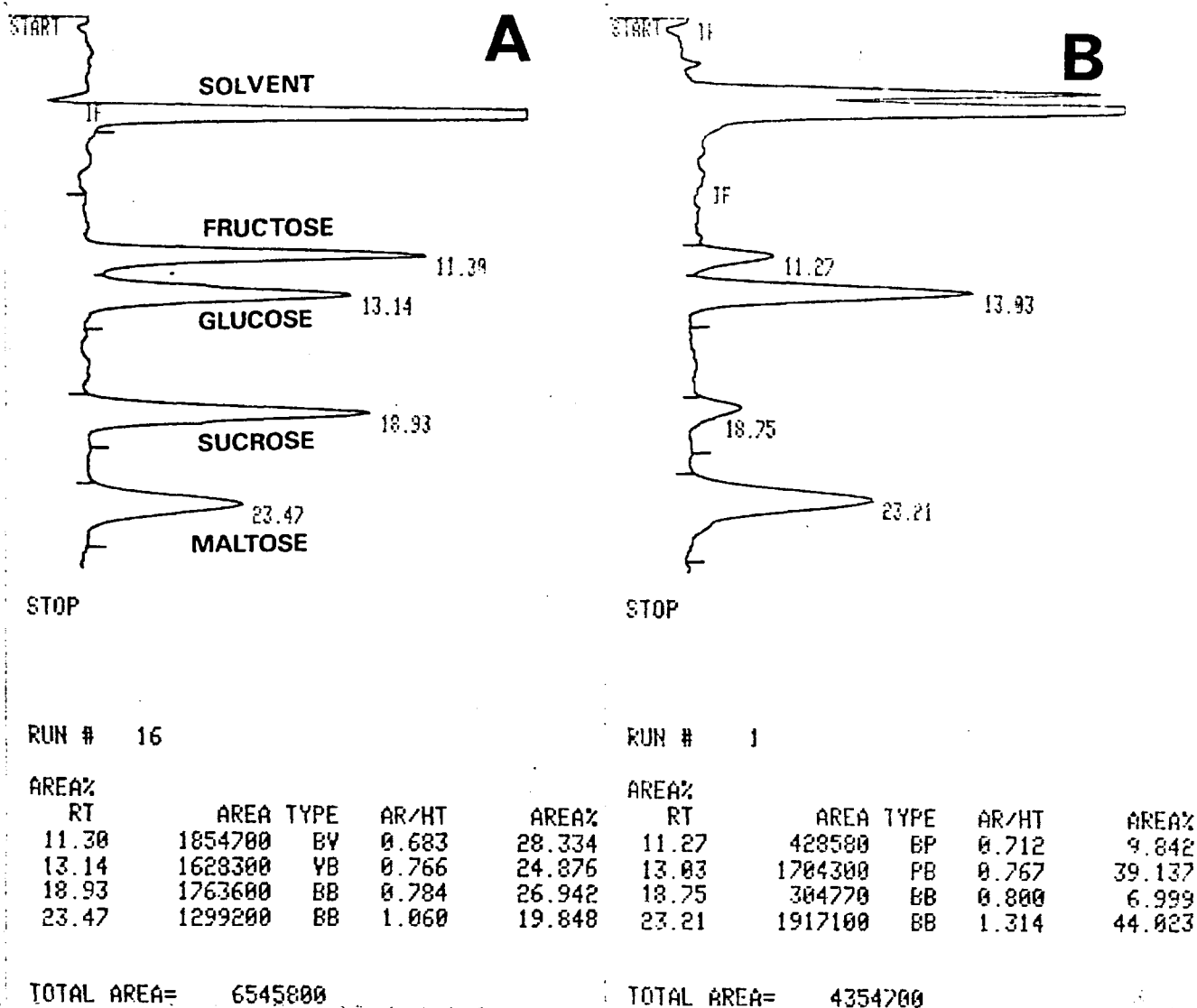


FIGURE 15. Examples of HPLC traces for (A) a 1mg/ml standard sugars solution and (B) an extract of germinating seeds. The identities of the four sugar peaks in the extract are the same as the standards.

was linear for up to 300 micrograms of sugar, and variation between replicates was never more than 8%.

The sugars from germinating seeds were extracted and assayed by HPLC. The five most abundant sugars were studied, namely glucose, sucrose, fructose, maltose and raffinose. In no case was the level of any of these significantly less in unimbibed low vigour seed than in high vigour seed so sugar concentrations in "dry" seed could not be used as a simple vigour test. The trisaccharide raffinose was present in the embryo of the dry seed but was quickly broken down, presumably for respiration and growth, and never reappeared (Figure 16.). The rate of breakdown varied with temperature, as expected, and also with vigour; low vigour seeds took three days longer than high vigour ones to utilize raffinose at 10°C. which ties in with the low respiration rates during early germination. However, if embryonic raffinose is an adequate early source of respirable substrate, then this result suggests that it is a deficiency in the respiratory mechanism, and not a sugar shortage, that is responsible for reduced respiration rates of low vigour seeds during the earliest stages of germination. Once the raffinose store is exhausted the embryo must rely on a supply of glucose from the endosperm for respiration, and this may not be forthcoming in low vigour seed.

The only other sugar to show declining levels during the first days of germination was sucrose. Like raffinose it is probably used as an early respiratory substrate by the embryo until glucose is supplied from the endosperm. In all cases, sucrose levels fell to nearly zero from an initially high concentration of about 150micrograms per grain; they subsequently rose during the later stages to reach much higher levels (Figure 17.). As was found with raffinose, low vigour seeds did not use up the early sucrose stores as quickly as the high vigour ones and again the differences were greater at 10°C. There were similar differences in the magnitude and speed of the sucrose rise; at 10°C. the maximum rate of sucrose accumulation in low vigour plants was approximately one third of that found in high vigour plants which compares with a difference in growth rates of only one half which suggests that sucrose

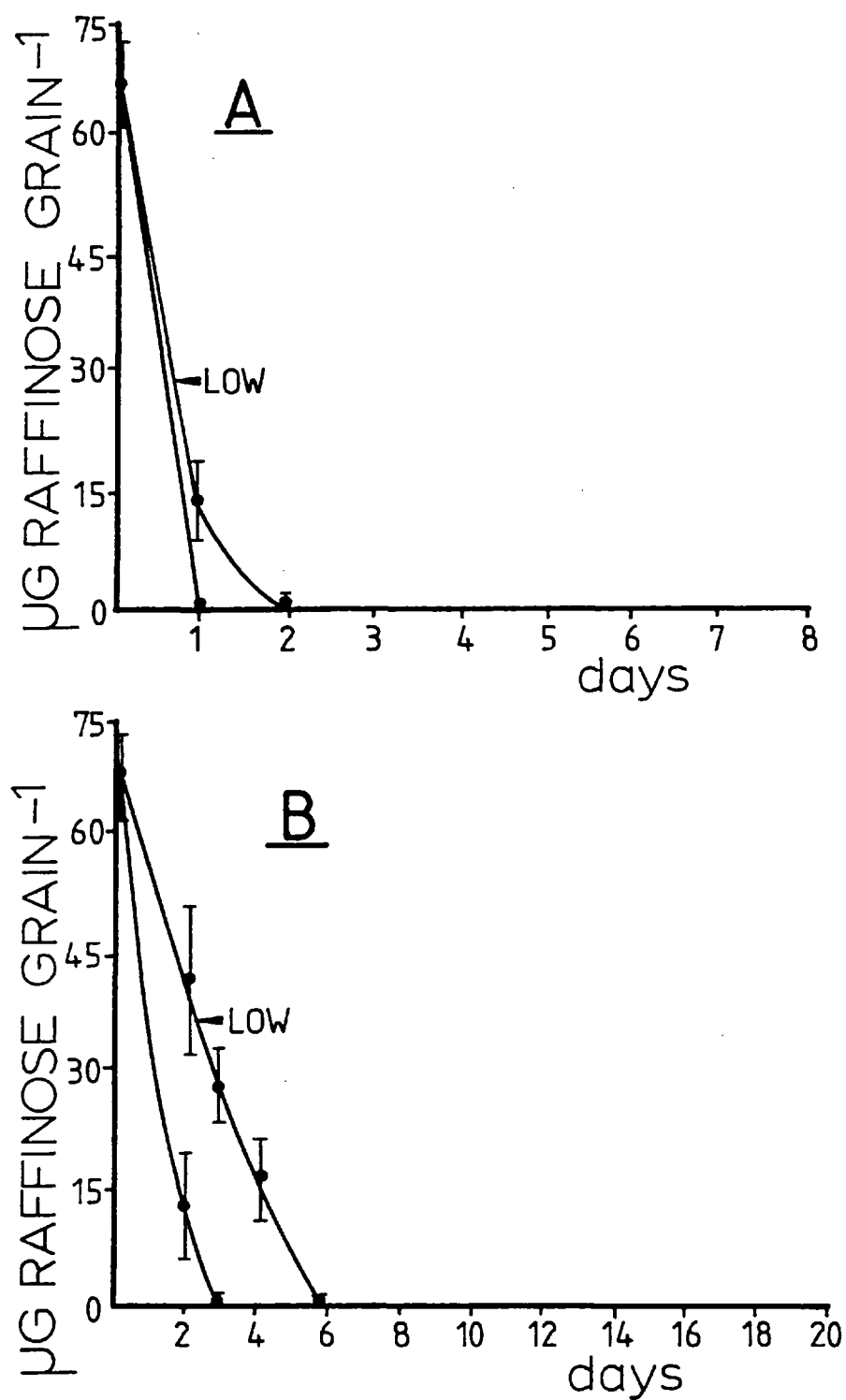


FIGURE 16. Changes in raffinose concentrations of high and low vigour grains germinated in the dark at (A) 25°C. and (B) 10°C. Bars = 95% confidence limits

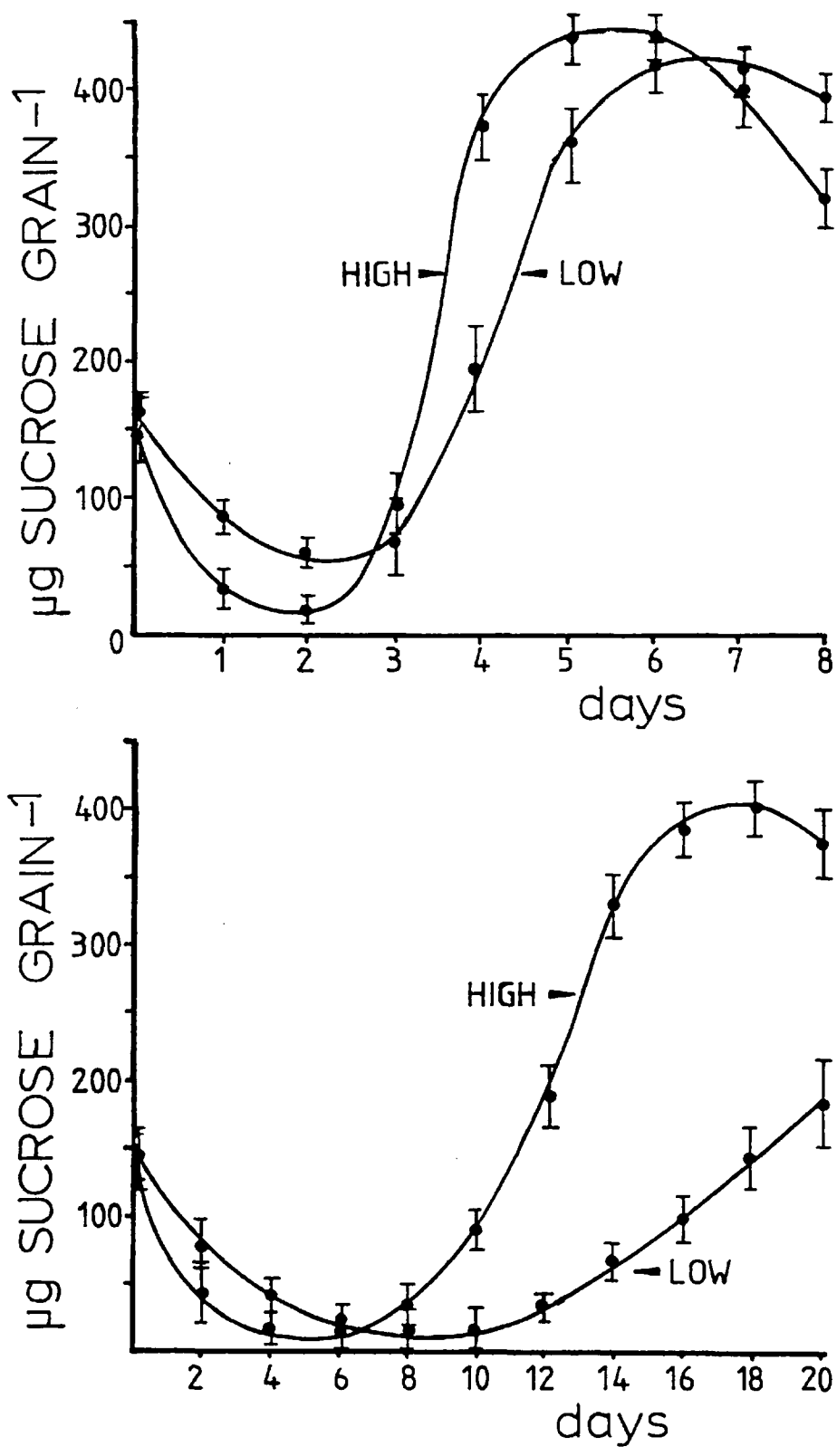


FIGURE 17. Changes in sucrose concentrations of high and low vigour grains germinated in the dark at 25°C. (Top graph) and 10°C. (Lower graph). Bars = 95% confidence limits

availability could be an important factor limiting growth in the later stages of germination, when the initial supply is exhausted.

Glucose showed similar patterns of accumulation to those found using dissected seedlings (section 3.3.2.) and as before the low vigour seeds were extremely deficient in free glucose when grown at 10°C. (Figure 18.). The three phases of the "S" shaped graphs are probably due to the initial lack of starch degrading enzymes, followed by a period of rapid enzyme synthesis and consequent endosperm breakdown, and the final peak and decline due to the exhaustion of the starch. This "S" pattern was similar for all of the sugars which were generated during germination.

Maltose is mostly confined to the endosperm where it is one of the first products of starch hydrolysis. As expected maltose concentration increased as germination progressed (Figure 19.) following a similar pattern to that shown by glucose, but it preceded the glucose by a short period, the length of which depended on temperature. This is probably due to the fact that glucose is only released in large quantities by the action of maltase on maltose, and it is therefore impossible for glucose release to precede that of maltose. The magnitude of this "phase difference" did not vary with vigour i.e. when maltose was released from starch hydrolysis there is sufficient maltase to convert it to glucose even in low vigour plants. However, the amount of maltose released did vary drastically with vigour, especially under stress, again in a similar way to the glucose. This implies that the shortage of glucose is due to a lack of maltose, suggesting that the starch mobilizing system in low vigour plants is faulty in some way.

The final sugar to be studied was fructose, the concentrations of which were usually about half those of glucose. Fructose accumulation substantially followed that of glucose and sucrose (Figure 20.), probably because fructose, like glucose, is the direct product of sucrose hydrolysis. Like all of the other sugars, fructose concentrations were depressed by low vigour, especially at 10°C., although not quite as severely.

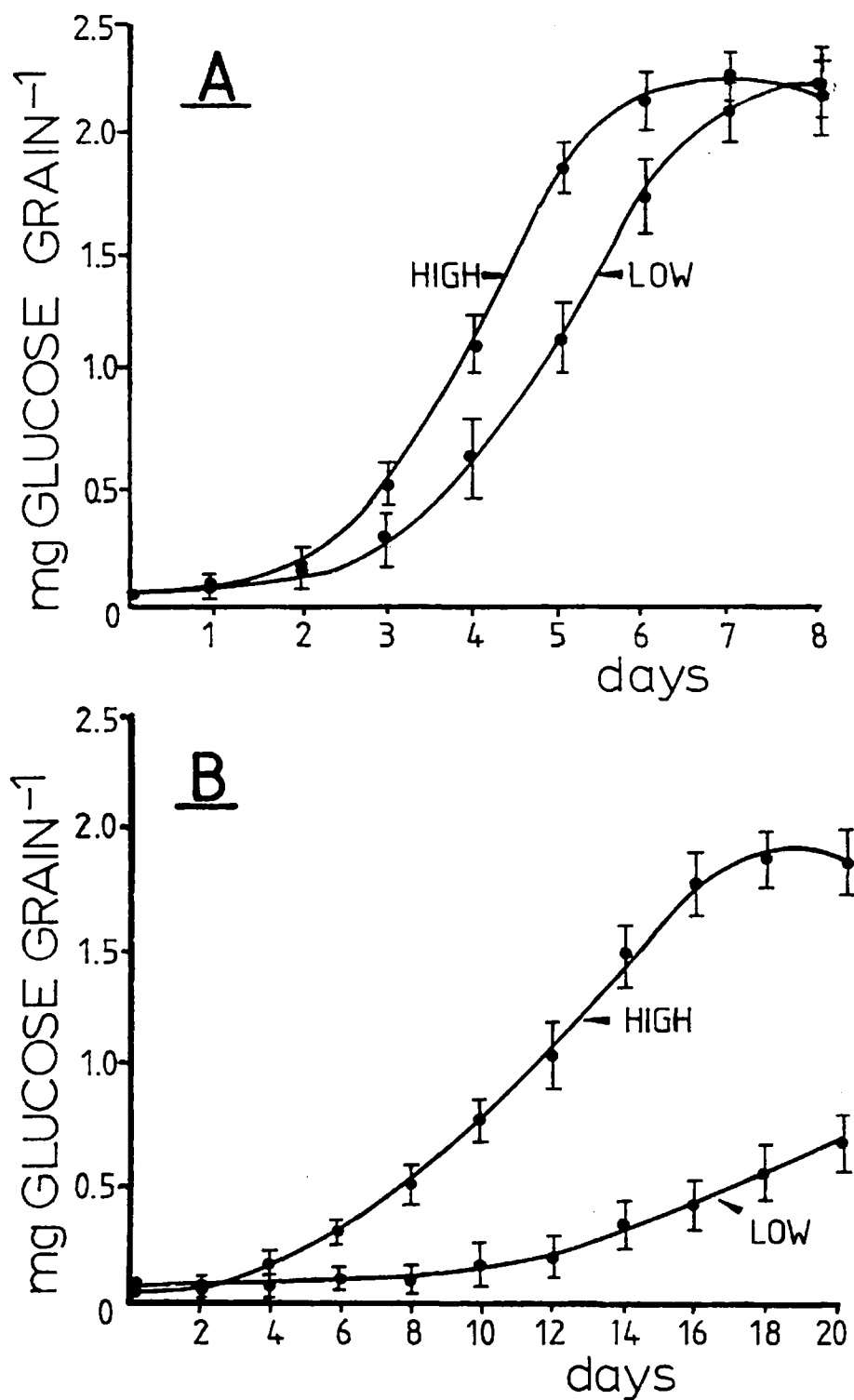


FIGURE 18. Changes in glucose concentrations of high and low vigour seeds germinated in the dark at (A) 25°C. and (B) 10°C. Bars = 95% confidence limits

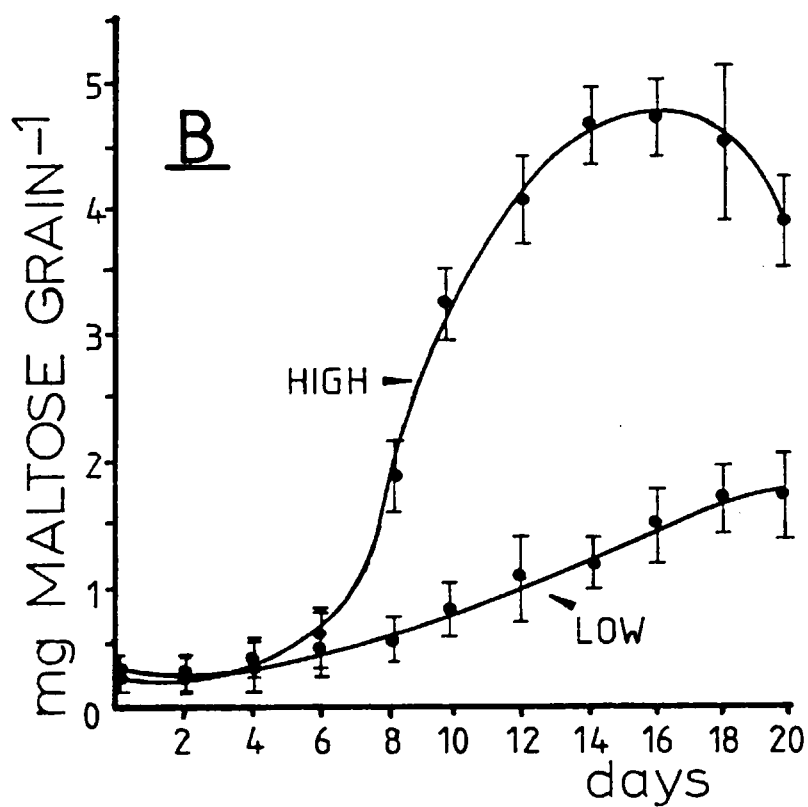
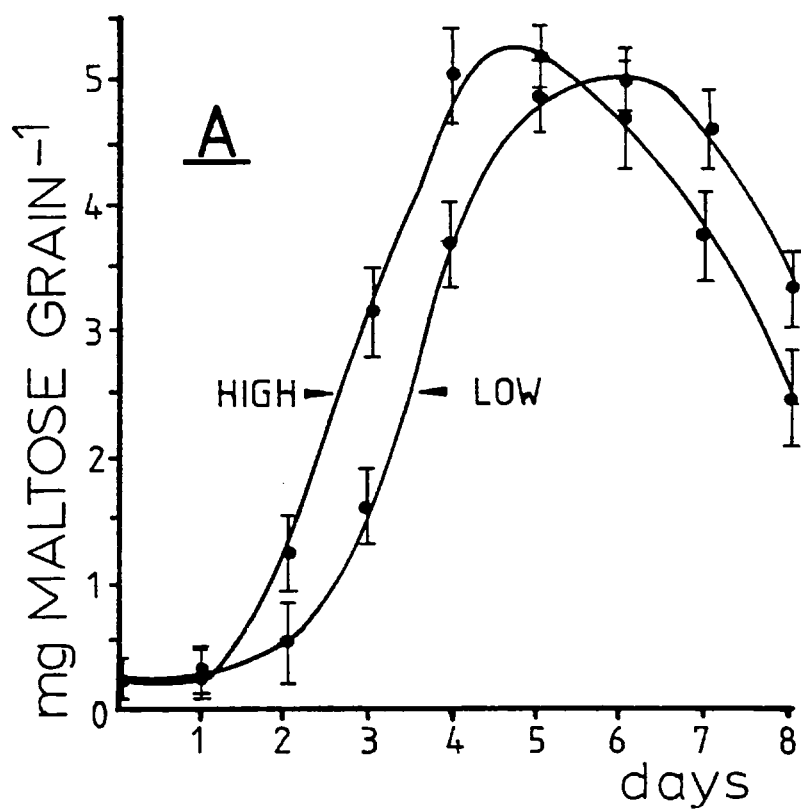


FIGURE 19. Changes in maltose concentrations of high and low vigour seeds germinated in the dark at (A) 25°C. and (B) 10°C. Bars = 95% confidence limits

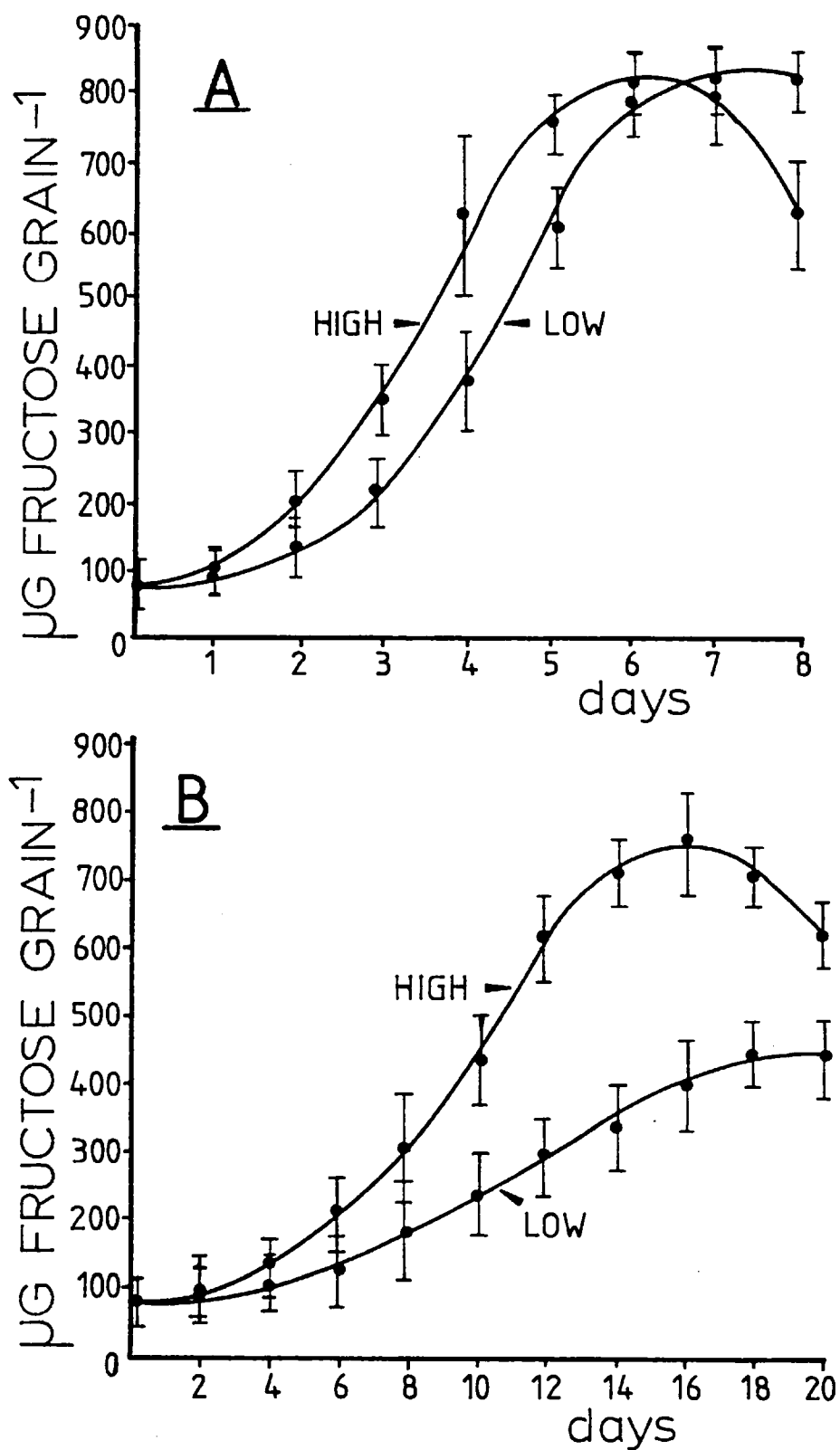


FIGURE 20. Changes in fructose concentrations of high and low vigour seeds germinated in the dark at (A) 25°C. and (B) 10°C. Bars = 95% confidence limits

These results suggest that low vigour could be concerned, at least in part, by sugar deficiency. If this is the case it should be possible to alleviate the symptoms by artificially supplying the necessary sugars.

3.5. EMBRYO GROWTH IN SUGAR SUPPLEMENTED MEDIA

Ungerminated wheat embryos were dissected from grains and were incubated in the dark in phosphate buffer pH 7.0 with or without 1% glucose or sucrose. They were left for various periods after which they were weighed and discarded; new batches of embryos were used for each incubation condition and duration. Infection was not usually a problem as extreme care was taken to keep the whole incubation system sterile. Germination was equivalent to that found in undissected grains. Some preliminary experiments were carried out using the method of Johnson and Stern (1957) to isolate embryos *en mass*, but this was discontinued as the embryos, whilst maintaining high viability, were discovered to have lost the scutellum during the extraction process.

The results were expressed as the % increases in fresh weight from the initial weights measured at $T=0$. When embryos were incubated without sugar the maximum weight increase was only 40%; this represents the amount an embryo can grow without obtaining nutrient supplies from the endosperm. At 25°C. the observed maximum weight was reached in 75 hours by both high and low vigour embryos (Figure 21.A), although it is impossible to say at exactly what time the real maximum was reached as readings were only taken every 25 hours. The decline after these maxima were reached is likely to be due to the degeneration of the tissues following sugar starvation. As was discovered with whole seeds, the low vigour embryos had a lag period in which growth increases were small, before they started to show more substantial changes; the lag was more pronounced at 10°C. (Figure 21.B). Again, as in whole seeds, the low vigour embryos exhibited much worse growth at the stress temperature with an observed maximum weight increase of 20% which is about half of the high vigour maximum at 10°C. This compares with their achievement of 48% of the maximum high vigour growth rate at 10°C. for whole seeds (Table 5).

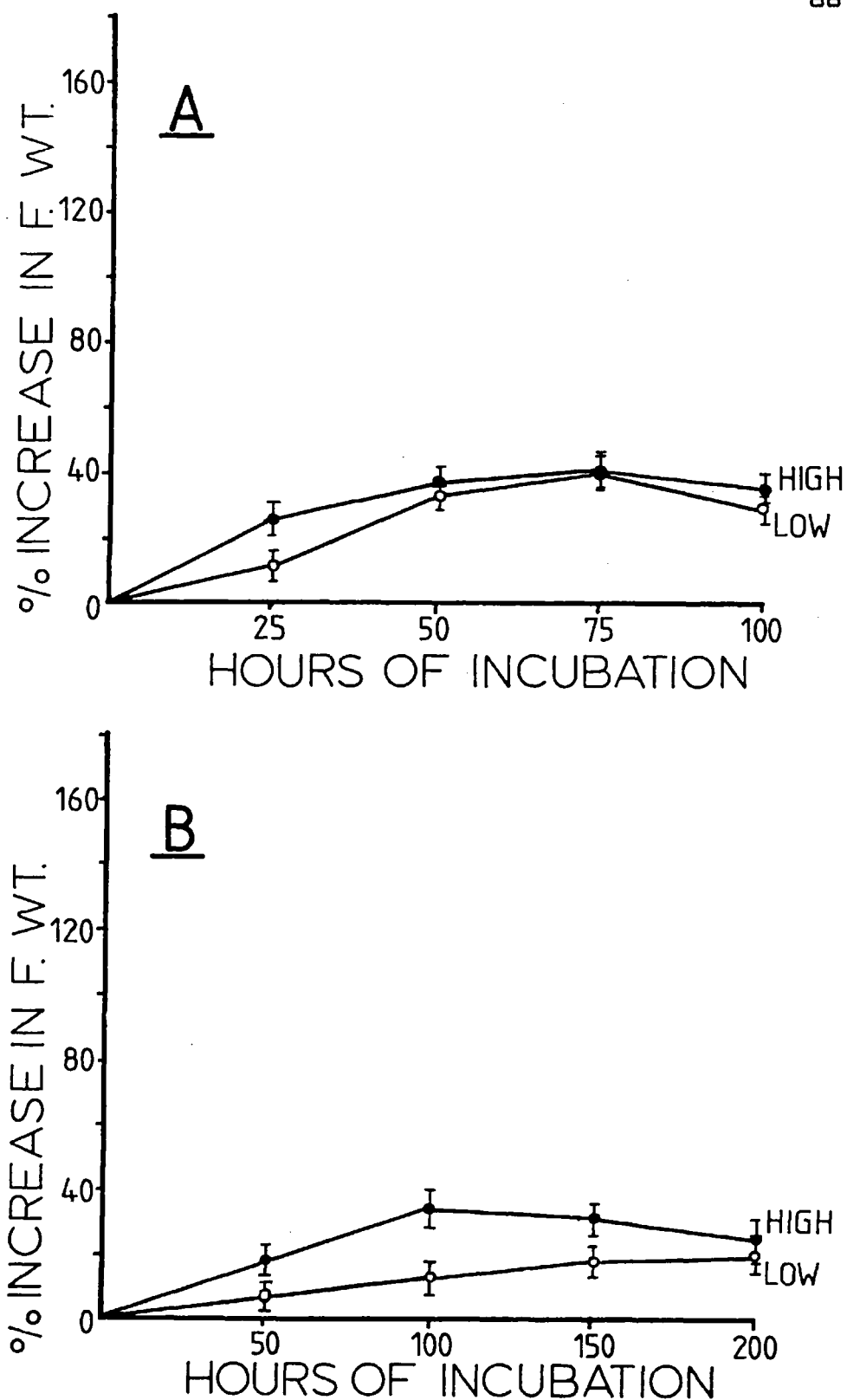


FIGURE 21. % Fresh weight changes of isolated high and low vigour embryos incubated in aerated buffer at (A) 25°C. and (B) 10°C. Low vigour results which are significantly different from high vigour ones are expressed as before.

Once the embryos were supplied with sugar their growth rates increased dramatically, with a highest increase of nearly 160%. In fact, at 25°C. high vigour embryos reached double the weight in 25 hours that they did throughout the whole period when starved of sugar. Figure 22. indicates that when glucose is supplied to the embryos, no tailing off of growth occurs as the embryos do not have to digest their own tissues in order to survive; this situation is changed neither by temperature or vigour. However, when the incubations were continued for longer than those reported here the fresh weights reached a maximum and then declined, probably due to the exhaustion of the sugar in the medium perhaps coupled with a lack of minerals and other essential metabolites.

The measurement of the low vigour embryos' growth in glucose solution provided some interesting results. The lag phase was still present at 25°C. but by 50 hours of incubation the low vigour growth was not significantly different from that of the high vigour embryos (Figure 22.A), as was also the case when sugar was omitted. This was similar at 10°C. where the lag, as usual, was more pronounced (Figure 22.B). However, if the maximum fresh weight increase of the low vigour embryos at 10°C. is expressed as a % of that of the high vigour plants, it is 79% which may be compared with 38% for growth in the sugar-free media and 48% for whole seeds (Table 6). There is apparently a considerable alleviation of the poor growth potential of low vigour embryos when they are supplied with exogenous glucose although they still show a highly significant 21% deficit compared with their high vigour counterparts.

These patterns of growth were similar, but not identical, for embryos grown in sucrose solution. The lag phase at 25°C. was yet again apparent, but this time the low vigour embryos did not equal the increase in weight of the high vigour ones until 100 hours of incubation (Figure 23.A). This was also the case at 10°C. where the % increases in weight for both high and low vigour embryos were slightly lower than with glucose. This is possibly because the enzymes which are required to metabolise sucrose are more scarce during early germination than the enzymes involved

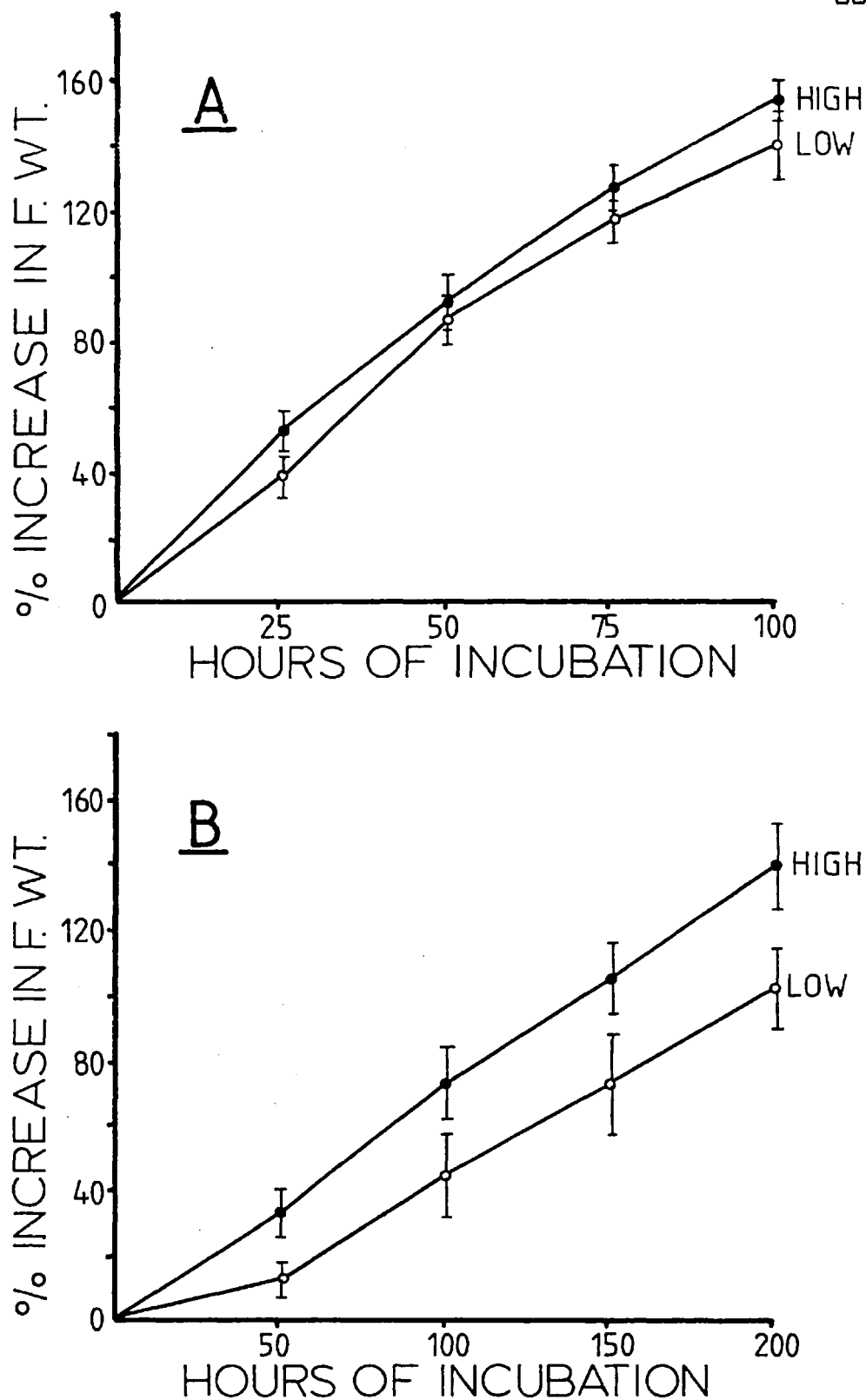


FIGURE 22. % Fresh weight changes from T=0 of isolated high and low vigour embryos incubated in aerated glucose solution at (A) 25°C. and (B) 10°C. Significant results are indicated as before.

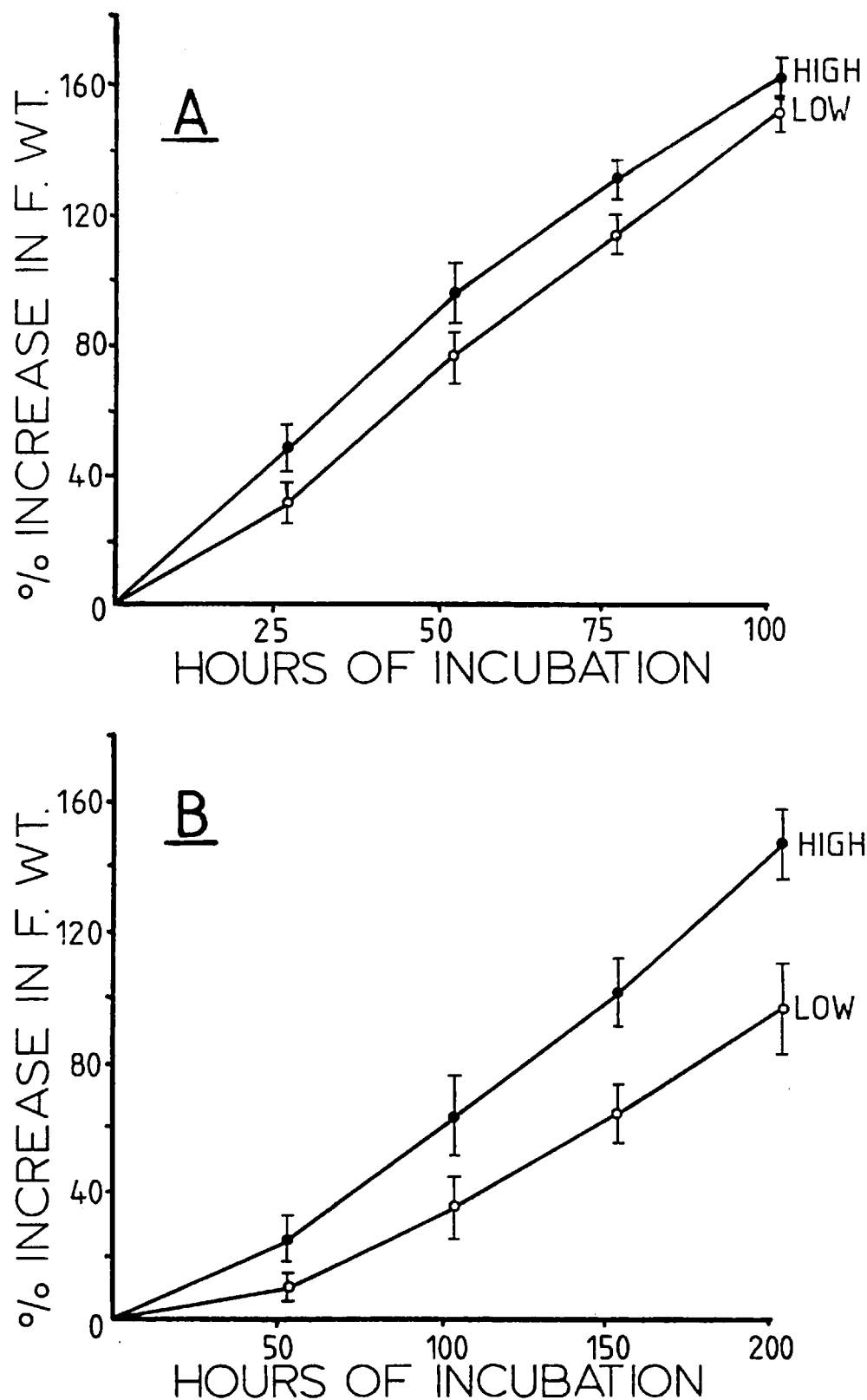


FIGURE 23. % Fresh weight changes of isolated high and low vigour embryos incubated in aerated sucrose solutions at (A) 25°C. and (B) 10°C. Significant results are indicated as before.

INCUBATION CONDITION	HIGH VIGOUR	LOW VIGOUR
WHOLE SEEDS (DRIP-SLIDES)	100%	48%
EMBRYOS IN BUFFER ONLY	100%	38%
EMBRYOS IN GLUCOSE SOLUTION	100%	79%
EMBRYOS IN SUCROSE SOLUTION	100%	71%

TABLE 6. Comparison of the maximum rates of fresh weight increase of high and low vigour seeds and embryos when grown in the dark at 10°C. Results are expressed as a % of the high vigour rates.

glucose metabolism. As with glucose, low vigour embryos showed some relief of their symptoms when supplied with exogenous sucrose. The maximum rate of fresh weight increase at 10°C. of the low vigour embryos was 71% of the high vigour rate, which is less than the 79% obtained with glucose, but still represents a significant improvement (Table 6.).

These results undoubtedly implicate sugar availability in the manifestation of the low vigour syndrome, but do not indicate which portion of the seed is primarily responsible. The embryo shows a significant alleviation of its poor growth potential when supplied with exogenous glucose and sucrose, but the relief is not complete. The scutellum is the organ which is most directly involved with sugar metabolism during germination; possibly it is not functioning correctly in low vigour seeds. To test this, a study of the metabolism of the scutellum in high and low vigour wheat seeds was undertaken.

3.6. THE CARBOHYDRATE METABOLISM OF THE SCUTELLUM

The primary function of the cereal seed scutellum during germination is the uptake of glucose, mobilized from the endosperm reserves, and its conversion to sucrose which can be translocated to the embryo axis. As has been shown, sugar starvation is strongly implicated in low vigour plants and this could be due to a lesioned scutellum.

The histochemical stain 2,3,5, triphenyl tetrazolium chloride is colourless until it is reduced when it turns bright red; it may be so reduced by the dehydrogenase enzymes of biological materials. The intensity of the colour can be related to the amount of reduction and has consequently been used as an indication of the viability of tissues (Moore 1973). Low vigour seeds were germinated at 10°C. for 20 days, after which the embryos were dissected from the endosperms and plunged into an aqueous solution of 1% tetrazolium. After 20 minutes incubation the embryos were removed and the scutella were examined for staining. It was apparent that those embryos which had grown least well had a much paler intensity of red coloration than did those which had produced much larger shoots.

Seeds which had not germinated at all never showed any redness, indicating that the scutellum was dead. This technique is open to criticism in that the presence of dehydrogenase enzymes does not necessarily mean that the cells are viable or vigorous, as the enzymes may still be active long after the cells have died. However, it does indicate that unstained cells are almost certainly dead and that the death of all or part of the scutellum could be a major contributory factor to low vigour.

3.6.1. Production of Sucrose By the Scutellum

Seeds were germinated at 25°C. and 10°C. for various periods after which the embryos were dissected out. These were placed inside special tubes so that the scutellum was in intimate contact with a solution containing [^{14}C] glucose whilst the roots trailed into water. The embryos were left in these tubes for up to 12 hours and on removal they were assayed for the production of [^{14}C] sucrose.

The rate of accumulation of sucrose was found to be linear throughout the 12 hour incubation but it tailed off if the embryos were left for much longer due to the scutellum losing contact with the underlying glucose solution. Consequently, the incubation duration could have been safely varied between 1 and 12 hours; 8 hours was the chosen length. Paper chromatography showed that many compounds other than sucrose became labelled during the incubation, amongst which fructose, galactose and mannose were tentatively identified by co-chromatography, in addition to the label which was lost as carbon dioxide. The results are expressed as the average (of 30 embryos) amount of labelled sucrose produced as a % of the total amount of radioactivity present in the embryo.

At 25°C. the embryos were assayed for sucrose synthesis after 50 or 100 hours of germination. By 50 hours, the average uptake of label per embryo per hour was 32,000 dpm, approximately equivalent to 290 micromoles of glucose and was similar in both high and low vigour embryos. After 100 hours this had increased to 160,000 dpm, or about 1,350 micromoles of glucose, again with little difference between the high and low vigour specimens.

(A) GERMINATION AT 25°C.

GRAIN TYPE	AGE OF SEEDLING (HOURS)	
	50	100
HIGH VIGOUR	14%	38%
LOW VIGOUR	* 6%	34%

(B) GERMINATION AT 10°C.

GRAIN TYPE	AGE OF SEEDLING (HOURS)	
	100	200
HIGH VIGOUR	9%	22%
LOW VIGOUR	** 1%	*** 4%

TABLE 7. [^{14}C] Sucrose concentration as a % of the total radioactivity in high and low vigour embryos whose scutella had been fed with [^{14}C] glucose. Seeds were germinated at (A) 25°C. and (B) 10°C. Significant results are expressed as before.

Although there was no measurable difference in the uptake of the glucose, there were significant differences in the amount of sucrose synthesized, as the low vigour embryos produced only 6% sucrose after 50 hours of germination compared with 14% in high vigour ones (Table 7.A). By 100 hours the amount of sucrose had increased to about 35% in both types of seed suggesting that the initial poor performance of the low vigour seed was again due to a lag period.

When the seeds were germinated under stress less sucrose was formed, but the glucose uptake was lower too. At 100 hours of germination the average uptake of label per hour per high vigour embryo was 27,000 dpm and this rose to 97,000 dpm by 200 hours. The low vigour embryos took up slightly less glucose than the high vigour ones at 100 hours (22,000 dpm) but considerably less at 200 hours (43,000 dpm). Thus sucrose synthesis was apparently impaired in stressed low vigour embryos as at both time intervals chosen there was a highly significant difference between the production of labelled sucrose in high and low vigour embryos (Table 7.B).

This experiment strongly suggests that the scutella of low vigour seeds are not functioning to the same extent as in healthy seeds. However, care must be taken in the interpretation of these results as a low concentration of labelled sucrose does not necessarily mean that the enzymic pathways for sucrose synthesis are impaired. It may, for example, mean that there is a higher turnover of sucrose in low vigour plants or that the glucose is used to synthesize some other important metabolite that is in short supply, before sucrose can be made. The most sensible way to discover if the sucrose metabolising machinery is indeed lesioned is to see if all the enzymes required are present and, if so, whether they are fully functional.

3.6.2. Activity Of Sucrose Synthetase

The first enzyme to be assayed was sucrose synthetase (UDP-D-glucose: D-fructose 2-glucosyl transferase E.C.2.4.1.13.) which catalyses the reaction:



The reaction is freely reversible and the direction of operation, i.e. sucrose synthesis or degradation, may depend on the intracellular site, pH and the concentrations of several cellular metabolites (Davies 1974). For this reason, the forward and back reactions were both assayed; the same enzyme extract was used for each. Seeds were germinated at 25°C. and 10°C. for various periods, following which the scutella were dissected out and the enzyme was extracted from them.

Sucrose synthetase activity was found in these scutella extracts and appeared to decrease slightly with seedling age. The maximum rate of synthesis was 25.4nmol sucrose per minute per 100mg scutella, shown by high vigour plants grown at 25°C. and, suprisingly, the low vigour plants showed hardly any difference (Table 8.A). By 100 hours of germination the "high vigour" enzyme activity had dropped to 21.0nmol sucrose per minute per 100mg scutella; the low vigour one also dropped, to 16.2nmol, but the difference between these was not significant. For seedlings grown at 10°C. the enzyme activities were noticeably less than for those grown at 25°C. but the falls in those of low vigour seeds were considerably greater. This low activity was significantly different from that for high vigour seeds (Table 8.B) and may be a partial explanation of the low sucrose synthesizing activities noted before (section 3.6.1.). The fall in sucrose synthetase activity with seedling age was not noted at 10°C., possibly because there was an insufficient period between the two sampling times. Slowly decreasing activity with time has been noted by other workers (Akazawa and Okamoto 1980) when cereals are germinated under optimum conditions.

The back reaction of sucrose synthetase was assayed by measuring the amount of UDPG released when the enzyme was incubated with a high concentration of sucrose; one nmole of UDPG corresponded to one nmole of degraded sucrose, according to the reaction given above. The pattern of enzyme activity was very similar to that of the synthesizing reaction (Table 9.), which is not surprising

(A) GERMINATION AT 25°C.

GRAIN TYPE	AGE OF SEEDLING (HOURS)	
	50	100
HIGH VIGOUR	25.4	21.0
LOW VIGOUR	23.7	16.2

(B) GERMINATION AT 10°C.

GRAIN TYPE	AGE OF SEEDLING (HOURS)	
	100	200
HIGH VIGOUR	16.7	16.2
LOW VIGOUR	** 6.2	** 6.4

TABLE B. Sucrose synthesizing activities (nmoles sucrose synthesized per minute per 100mg scutella) of sucrose synthetase extracted from the scutella of high and low vigour seeds germinated at (A) 25°C. and (B) 10°C. Each result is the mean of 3 replicates; significant results being expressed as before.

(A) GERMINATION AT 25°C.

GRAIN TYPE	AGE OF SEEDLING (HOURS)	
	50	100
HIGH VIGOUR	16.6	12.2
LOW VIGOUR	13.2	11.6

(B) GERMINATION AT 10°C.

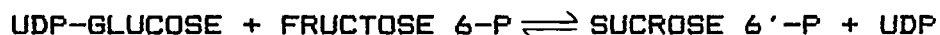
GRAIN TYPE	AGE OF SEEDLING (HOURS)	
	100	200
HIGH VIGOUR	10.7	10.6
LOW VIGOUR	* 3.8	* 3.5

TABLE 9. Sucrose degrading activities (nmoles UDPG synthesized per minute per 100mg scutella) of sucrose synthetase extracted from the scutella of high and low vigour seeds germinated at (A) 25°C. and (B) 10°C. Each result is the mean of 3 replicates; significant results being expressed as before.

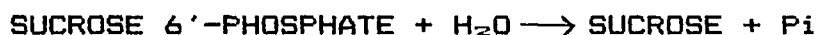
as the same enzyme extract was used for both assays. However, there was a constant difference in the reaction rates with the synthesizing direction showing between 1.4 and 1.9 times the activity of the reverse direction. This factor was the same for both seed types and both temperatures indicating that this is a fundamental property of the enzyme and it suggests that the overall direction of the reaction would slightly favour sucrose synthesis in the scutellum, if the conditions of pH, substrate and product concentrations were similar to those used in the assays.

3.6.3. Activity of Sucrose Phosphate Synthase/Phosphatase

Another, and less reversible, route for sucrose synthesis in plants is via sucrose 6'-phosphate. The reaction is in two stages, and is mediated by two enzymes but because of the intimate relationship between these, the rate of the overall reaction was determined experimentally. The first reaction is catalysed by sucrose phosphate synthase (UDP-D-glucose: D-fructose 6-phosphate 2-glucosyl transferase E.C.2.4.1.14.):



The second enzyme is sucrose phosphatase (sucrose 6'-phosphate phosphohydrolase E.C.3.1.3.24.) which catalyses the hydrolysis of sucrose phosphate by the essentially irreversible reaction:



Unlike sucrose synthetase, the activities of these enzymes increased substantially over the period of germination in which samples were taken. Whether this is due to an increase in the amount of enzyme or a decrease in an inhibitor is not known. There are differences in the relative activities of these enzymes and those of sucrose synthetase.

After 50 hours of germination at 25°C. the activities were nearly double those of sucrose synthetase (Table 12.A) for high vigour scutella, but the low vigour enzymes were significantly lower than the corresponding

(A) GERMINATION AT 25°C.

GRAIN TYPE	AGE OF SEEDLING (HOURS)	
	50	100
HIGH VIGOUR	42.7	62.3
LOW VIGOUR	** 18.3	58.1

(B) GERMINATION AT 10°C.

GRAIN TYPE	AGE OF SEEDLING (HOURS)	
	100	200
HIGH VIGOUR	29.6	41.2
LOW VIGOUR	** 6.1	** 14.3

TABLE 10. Combined activities (nmoles sucrose synthesized per minute per 100mg scutella) of sucrose phosphate synthase/phosphatase in scutella of high and low vigour seeds germinated at (A) 25°C. and (B) 10°C. Each result is the mean of 3 replicates; significant results being expressed as before.

(A) GERMINATION AT 25°C.

GRAIN TYPE	AGE OF SEEDLING (HOURS)	
	50	100
HIGH VIGOUR	1.7:1	3.0:1
LOW VIGOUR	0.8:1	3.6:1

(B) GERMINATION AT 10°C.

GRAIN TYPE	AGE OF SEEDLING (HOURS)	
	100	200
HIGH VIGOUR	1.8:1	2.5:1
LOW VIGOUR	1.0:1	2.2:1

TABLE 11. Ratios of sucrose phosphate synthase/phosphatase activities to those of sucrose synthetase (forward reaction) for seedlings germinated at (A) 25°C. and (B) 10°C.

sucrose synthetase activity. This implies that during early germination of high vigour seeds the sucrose phosphate pathway is predominant but in low vigour seeds this route is less important than the sucrose synthetase mediated one. By 100 hours of germination at 25°C. the low vigour sucrose phosphate enzyme activities had reached those of the high vigour ones and for both high and low vigour these enzyme activities were about three times greater than the sucrose synthetase levels (Table 11.A).

This increase in the sucrose phosphate synthase/phosphatase activities with advancing germination was also found for seeds germinated at 10°C., but the overall levels were lower (Table 10.B). The picture of a dominant sucrose synthesizing pathway directed through sucrose phosphate was confirmed for high vigour seeds at both time intervals, but again the pattern was different in low vigour plants. Here, at 100 hours of germination, the activities of these enzymes were the same as those of sucrose synthetase. By 200 hours of germination though, the ratio had increased, in favour of the sucrose phosphate pathway (Table 11.B). Perhaps the low vigour seeds actually have the same pattern as their high vigour counterparts but because they are slower to develop, the stages where sucrose synthetase is dominant last for a longer period. High vigour seeds may go through this period too, but this has not been detected due to the inadequate number of samples taken.

3.6.4. Activity of UDP-Glucose Pyrophosphorylase

Once glucose is released from the endosperm reserves and then taken up by the scutellum epithelium, it is converted to glucose 6-phosphate by hexokinase which in turn is converted to glucose 1-phosphate by phosphoglucomutase. Before sucrose may be synthesized, the glucose 1-phosphate must be coupled with uridine triphosphate (UTP) to yield uridine diphospho-glucose (UDPG). This reaction is catalysed by UDPG pyrophosphorylase (UTP: α -D-glucose 1-phosphate uridyl transferase E.C.2.7.7.9.):



(A) GERMINATION AT 25°C.

GRAIN TYPE	AGE OF SEEDLING (HOURS)	
	50	100
HIGH VIGOUR	0.34	0.41
LOW VIGOUR	* 0.26	0.37

(B) GERMINATION AT 10°C.

GRAIN TYPE	AGE OF SEEDLING (HOURS)	
	100	200
HIGH VIGOUR	0.28	0.39
LOW VIGOUR	* 0.18	* 0.29

TABLE 12. Activities of UDP-glucose pyrophosphorylase (decrease in Absorbance at 600nm per minute per 100mg scutella) in scutella extracted from high and low vigour seeds germinated at (A) 25°C. and (B) 10°C. Each result is the mean of 3 replicates; significant results being expressed as before.

Like the sucrose phosphate metabolising enzymes, the activities of this enzyme increase with advancing germination which is not surprising, as if sucrose synthesis increases as germination continues then an increased supply of UDPG is required. As has been noted before, the low vigour seeds show a significant lag during early germination at 25°C. which disappears by 100 hours, when the enzyme activity equalled that of the high vigour enzyme (Table 12.A). There were similar increases in this enzyme's activity at 10°C. for low vigour seed but this time they did not catch up with the high vigour ones, being significantly different from them at both time intervals (Table 12.B). The decreased activity is in proportion to the lowered activities of the sucrose synthetase and sucrose phosphate synthase/phosphatase enzymes, implying that none of these presented a single rate-limiting step in sucrose synthesis, but that there was a general decrease in the activity of all of these important enzymes.

3.6.5. Activity of Sucrose Phosphorylase

The final sucrose synthesizing enzyme that is known is sucrose phosphorylase (D-glucose 1-phosphate: D-fructose 2-glucosyl transferase E.C.2.4.1.7.) which catalyses the reaction:



Although many attempts were undertaken, no enzyme activity could be found from any samples. Incubation lengths were varied, as were temperatures, pH, substrate concentrations and extraction procedures, but no activity was detected.

3.6.6. Activity of Invertase

The second sucrose cleaving enzyme to be assayed was invertase (sucrose hydrolase E.C.3.2.1.26.) which catalyses the following reaction:



The activities of this enzyme were extremely low in comparison to those of the sucrose synthesizing enzymes;

(A) GERMINATION AT 25°C.

GRAIN TYPE	AGE OF SEEDLING (HOURS)	
	50	100
HIGH VIGOUR	4.2	4.9
LOW VIGOUR	4.4	4.6

(B) GERMINATION AT 10°C.

GRAIN TYPE	AGE OF SEEDLING (HOURS)	
	100	200
HIGH VIGOUR	4.0	4.4
LOW VIGOUR	* 2.6	* 2.9

TABLE 13. Activities of invertase (nmoles sucrose hydrolysed per minute per 100mg scutella) in the scutella of high and low vigour seeds germinated at (A) 25°C. and (B) 10°C. Each result is the mean of 3 replicates; significant results being expressed as before.

this is not surprising in that the scutellum is primarily concerned with sucrose synthesis so that it would be futile for much sucrose degradation to occur there. At 25°C. there were no differences between high and low vigour seeds (Table 14.A) and there was little evidence that invertase levels altered with advancing germination. However, at 10°C. the low vigour scutella did contain less invertase than the high vigour ones, but as the levels were so low anyway, this is unlikely to be of much significance *in vivo*.

3.7. ALPHA-AMYLASE IN HIGH AND LOW VIGOUR SEEDS

The investigation into sugar levels in germinating seeds revealed that there was a severe shortage of maltose in low vigour seeds grown under stress conditions. Maltose is released, during germination, by the action of various enzymes on the starch which is stored in the endosperm. These hydrolases are therefore crucial for effective mobilisation of the endosperm reserves and hence for the establishment of a healthy seedling. As was shown in the introductory section, *alpha*-amylase is by far the most abundant hydrolytic enzyme released during germination, so that a study of the activity and characteristics of high and low vigour seeds' *alpha*-amylase may reveal why there is such a discrepancy in the maltose levels between the two seed types.

The assay for *alpha*-amylase proved to be accurate only over the range of 0.05 to 1.8 International units of activity so that many of the samples had to be diluted by large amounts before the activity could be measured. A sample calibration curve is shown in Appendix C. Unlike many other assays for *alpha*-amylase this one was specific, in as much as *beta*-amylase, *alpha*-glucosidase and R-enzyme had no apparent effect on the rate of reaction when added to the incubation mixture.

3.7.1. Alpha-Amylase Activity During Germination

Seeds were germinated in the dark at 10 and 25°C. and sampled at intervals for *alpha*-amylase activity.

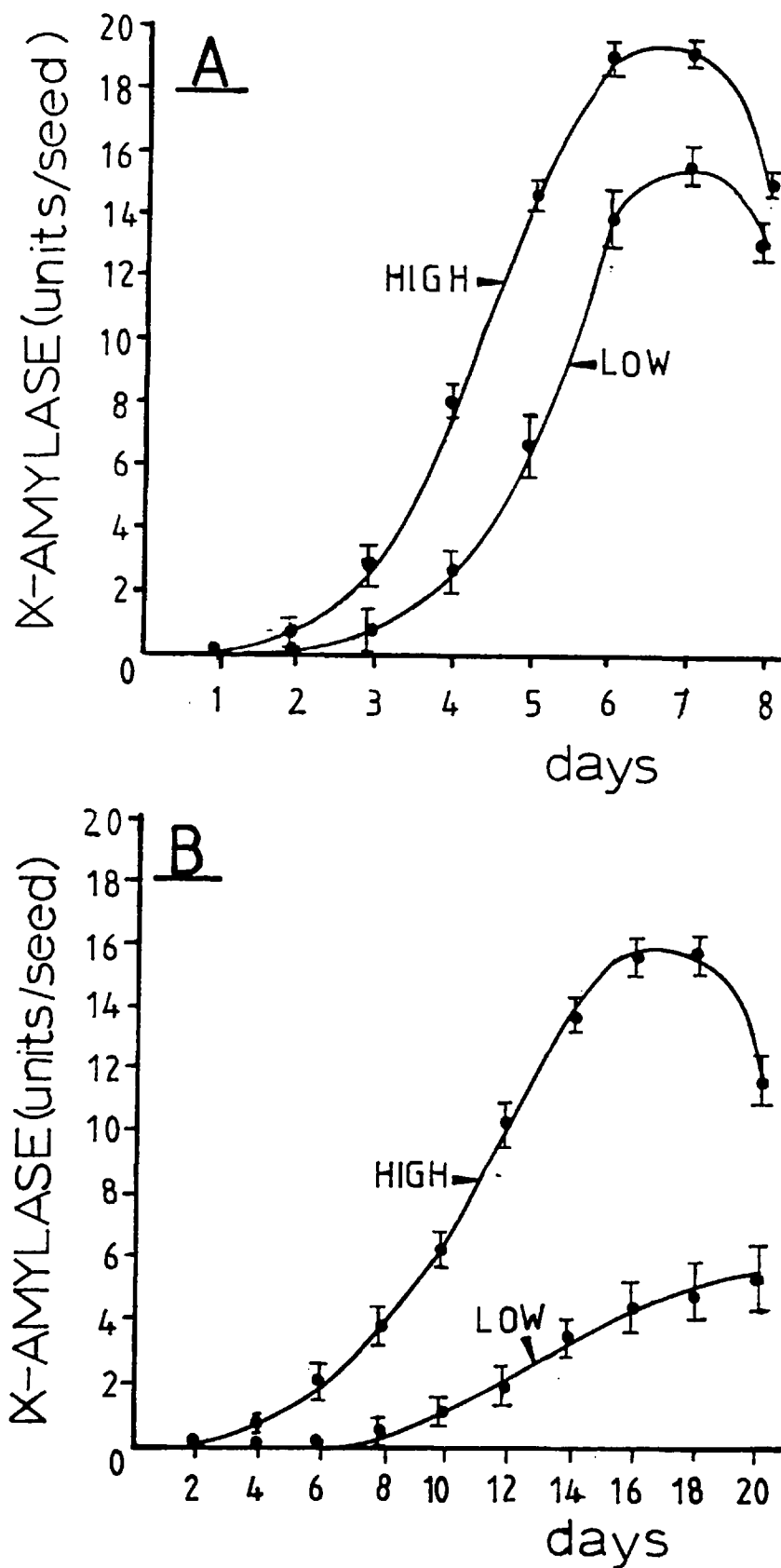


FIGURE 24. Changes in *alpha*-amylase activity in high and low vigour seeds germinated in the dark at (A) 25°C. and (B) 10°C. Each point is the mean of 11 replicates; the bars represent 95% confidence limits.

At 25°C. the pattern of enzyme activity was clearly "S" shaped (Figure 24.A), in that initially there was no enzyme, followed by a period of increasing activity until a maximum rate was reached, and finally a plateau of activity with a subsequent decline. This was true of both the high and low vigour seeds, but the latter lagged about one day behind the former which is consistent with previous data. Although the low vigour plants had similar patterns of enzyme accumulation, they did not reach the same final plateau and there was significantly less enzyme in the low vigour seeds at all stages after 2 days germination.

These differences were considerably more pronounced at 10°C. High vigour seeds again displayed an "S" shaped curve (Figure 24.B), but the maximum rate of increase in activity was lower than it had been at 25°C. Low vigour seeds did not show anything like the same pattern; *alpha*-amylase activity did not appear until one weeks' germination had passed and even then the accumulation was extremely slow. This would explain why there is such a shortage of maltose in stressed low vigour seeds.

These reduced enzyme levels at 10°C. cannot be explained by poor growth rates alone. It could be argued that the deficit of *alpha*-amylase is merely due to the slow growth of the low vigour seedlings i.e. that the low enzyme level is a direct reflection of the size of the plants. If the amount of enzyme per plant is plotted against the weight of that plant, then a linear relationship is found up to 120mg of seedling weight (Figure 25.). However, the slope of that line depends on the temperature at which the plants were grown and on the vigour status of the seeds. It seems that low vigour seedlings will produce less *alpha*-amylase per unit fresh weight than will high vigour ones and that the situation is much worse when the seeds are germinated at a stress temperature. Although these experiments showed that there was low *alpha*-amylase activity in low vigour seeds, it did not tell us whether this was due to a shortage in the amount of *alpha*-amylase protein or due to some biochemical lesion in the enzyme itself which lowered its specific activity. In order to ascertain which of these was most likely, three fundamental biochemical characteristics were investigated.

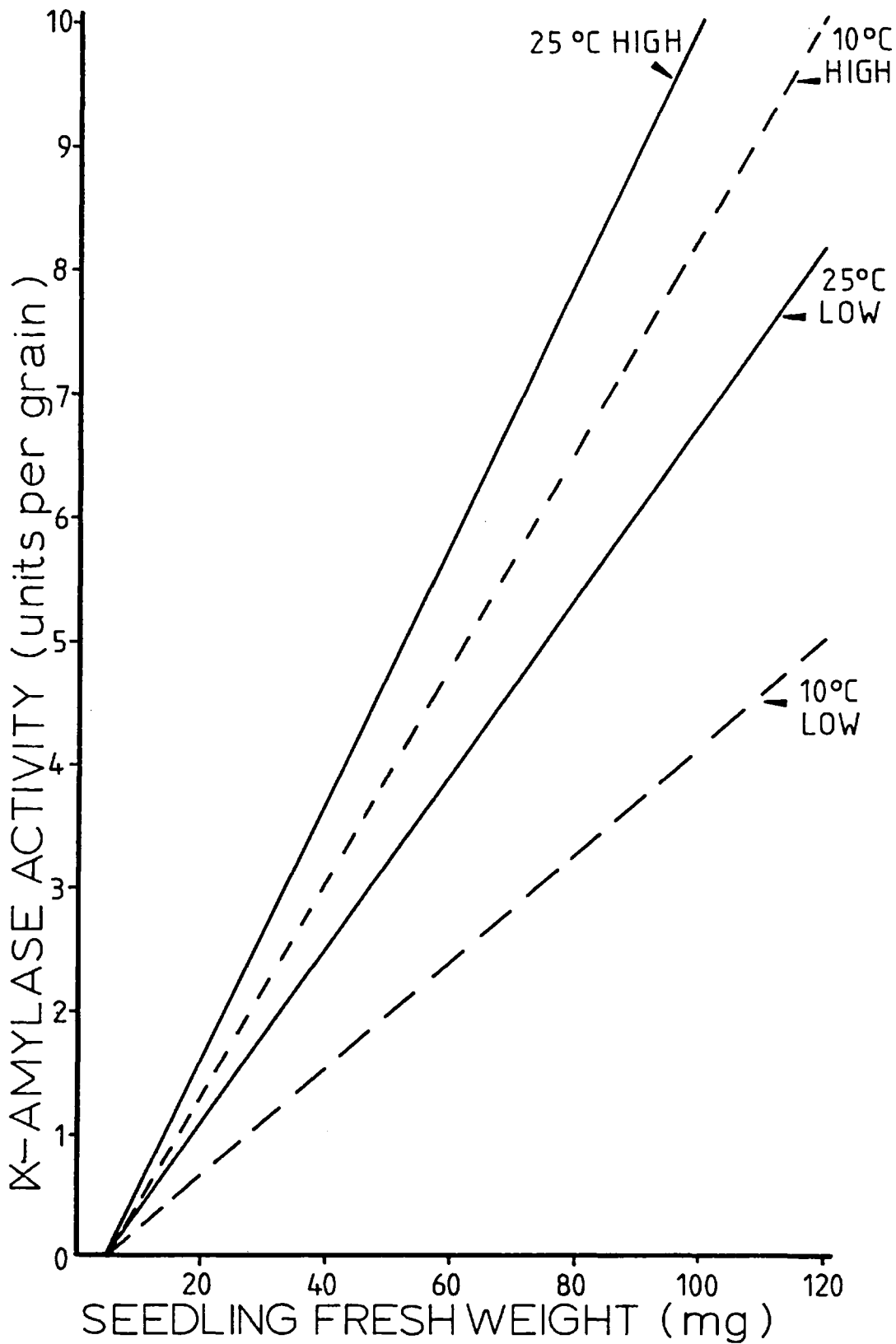


FIGURE 25. *Alpha*-Amylase activity per grain (seedling) as a function of seedling fresh weight. The lines were plotted by linear regression; points are not shown for clarity. Each line was significantly different from the others at the 95% confidence level.

3.7.2. The *Alpha*-Amylase K_m

The Michaelis-Menten constant (K_m) of an enzyme is a measure of its affinity for a particular substrate, under the conditions in which it is assayed. To determine this, *alpha*-amylase was extracted from germinating high and low vigour seeds and the two extracts were adjusted so that they contained approximately the same number of units of activity per ml. A calibration curve of substrate (*beta*-limit-dextrin) concentration versus absorbance at 540nm was constructed (Figure 26.A), so that the initial velocity of substrate utilization (V_o) for a given initial substrate concentration ($[S]$) could be determined for a fixed concentration of enzyme. The V_o was estimated for many substrate concentrations. These data were plotted using the Eadie-Hofstee method, and the K_m was found by calculating the slope of the resultant line by linear regression analysis (Figure 26.B). The K_m for high vigour *alpha*-amylase was 0.315 mg/ml *beta*-limit-dextrin and for the low enzyme it was 0.307 mg/ml; there was no significant difference between them. This proves that the reduced activities of *alpha*-amylase in low vigour seeds is, at least, not due to a lowered affinity for the substrate.

3.7.3. The *Alpha*-Amylase Temperature and pH Optima

The same enzyme preparations as were used to determine the K_m 's, were employed to investigate the temperature and pH optima.

Alpha-Amylase had a high optimum temperature of 55°C. which was identical for both high and low vigour enzymes (Figure 27.A). Between 20°C. and the optimum, the increase in activity was approximately linear, and there was a rapid decline in activity at temperatures above this optimum; again the pattern was the same for high and low vigour enzymes.

The pH optimum for both high and low vigour *alpha*-amylases was 5.5. The activity declined to about the same extent above and below the optimum pH, and there was no difference between the high and low vigour enzymes (Figure 27.B).

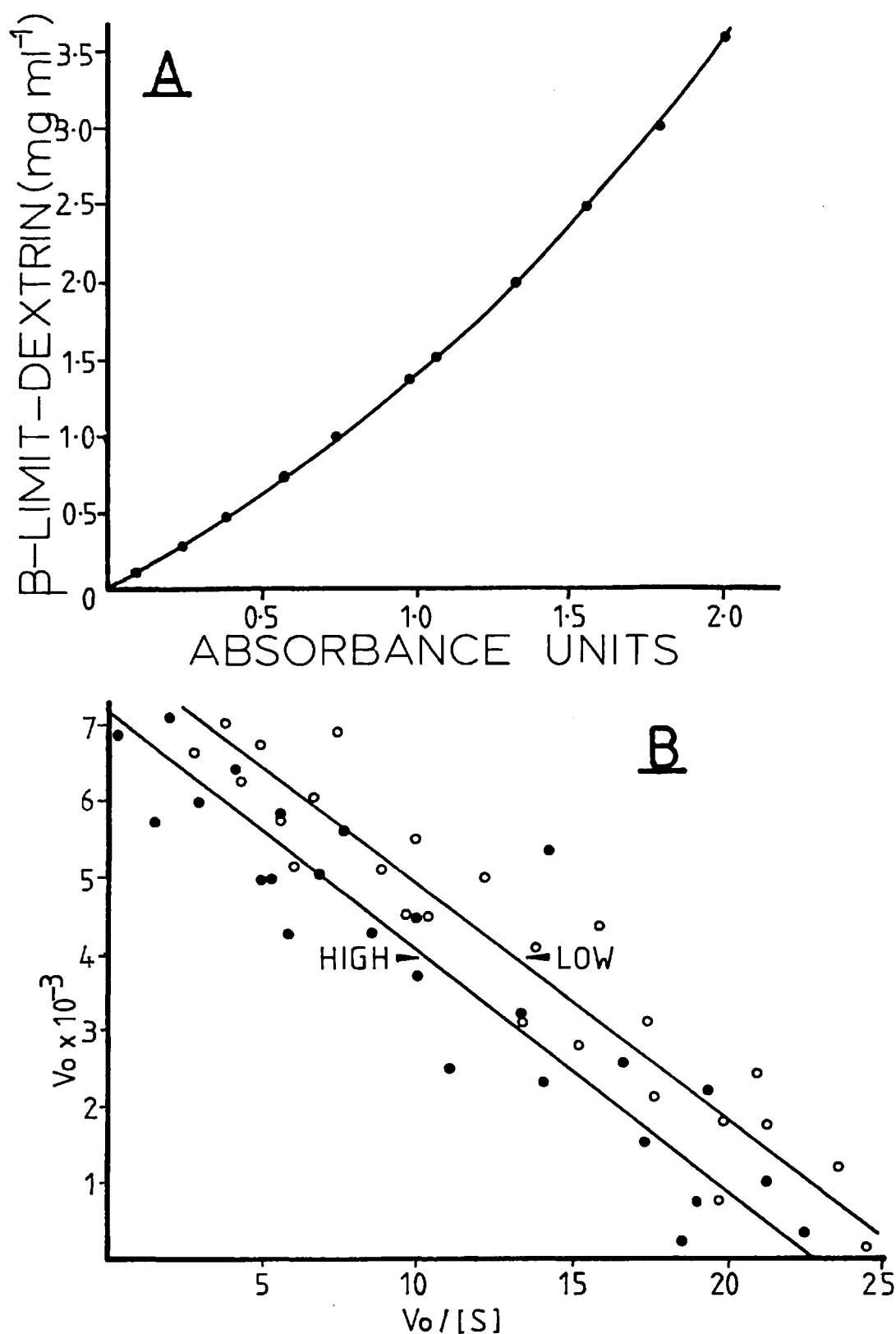


FIGURE 26. (A) Calibration curve of α -amylase substrate (β -limit-dextrin) concentration versus absorbance at 540nm, after it had been mixed with iodine solution. (B) Eadie-Hofstee plot for high and low vigour α -amylase where the slopes represent the negative K_m 's.

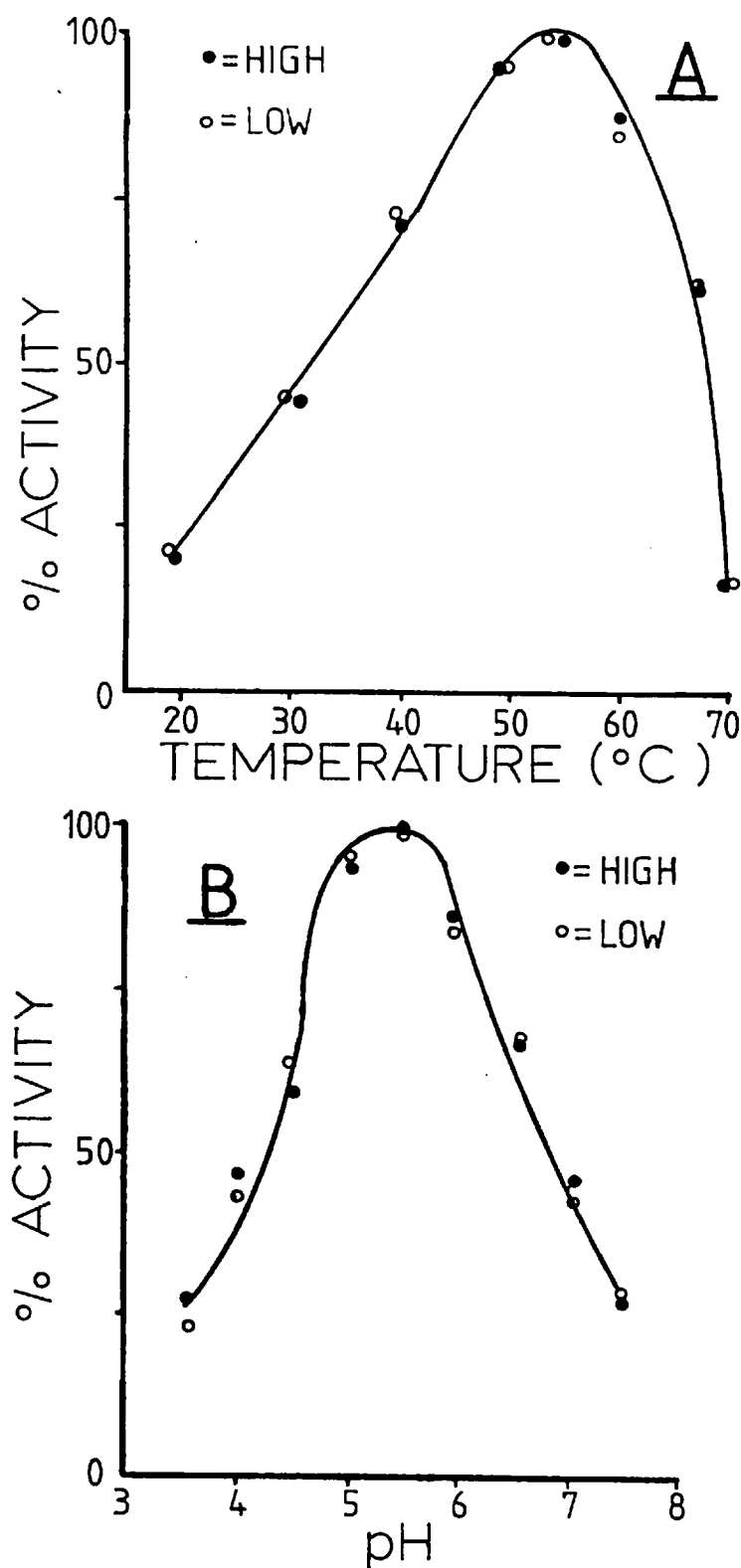


FIGURE 27. (A) Temperature-Activity curve for high and low vigour α -amylase at pH 5.5. (B) pH-Activity curve for high and low vigour α -amylase at 30°C.

All three characteristics investigated, Km, pH and temperature-activity curves, showed that there was no difference between those of the *alpha*-amylase found in high vigour seeds and those in low vigour seeds. To test this similarity further, an investigation into the isoenzyme complement of *alpha*-amylase from germinating high and low vigour seeds was undertaken using analytical isoelectric focussing.

3.7.4. Analytical Isoelectric focussing

Alpha-Amylase was extracted from germinated grains and examined by isoelectric focussing; in both high and low vigour seeds two groups of amylolytically active isoenzymes were identified. The group 1 isoenzymes focussed between isoelectric point (pI) 6.0 - 6.5, and consisted of 4 major and 5 or 6 minor bands (Figure 28.A.). The group 2 isoenzymes focussed between pI 4.5 - 4.8, and consisted of 3 major and 2 minor bands. The fact that these were *alpha*-amylase isoenzymes and not those of any other amylase was verified by developing the gels in *beta*-limit-dextrin rather than soluble starch. Precisely the same pattern of isoenzymes was found, but the staining was not intense enough to photograph. Other proof was obtained by focussing seed extracts which had not been heated; these showed many more bands than the heated ones, the lost bands being *beta*-amylase and debranching enzymes. That the bands were proteinaceous was indicated by the observation that the same pattern of bands arose when gels were developed using coomassie blue, as were when starch was used (Figure 28.B.). In both heated and unheated extracts the bands were identical for high and low vigour seeds.

When seeds are germinated in the laboratory, there is an inevitable infection by saprophytic fungi and bacteria. Such microorganisms are likely to contain active *alpha*-amylases which might be responsible for some of the bands seen on the gels. To check this, *alpha*-amylase extracts from bacterial and fungal sources were focussed along with barley and wheat enzymes. Additionally, a *beta*-amylase extract was focussed, as well as an extract of unknown amylolytic enzymes washed from wheat starch granules. Only the wheat extract produced the two groups

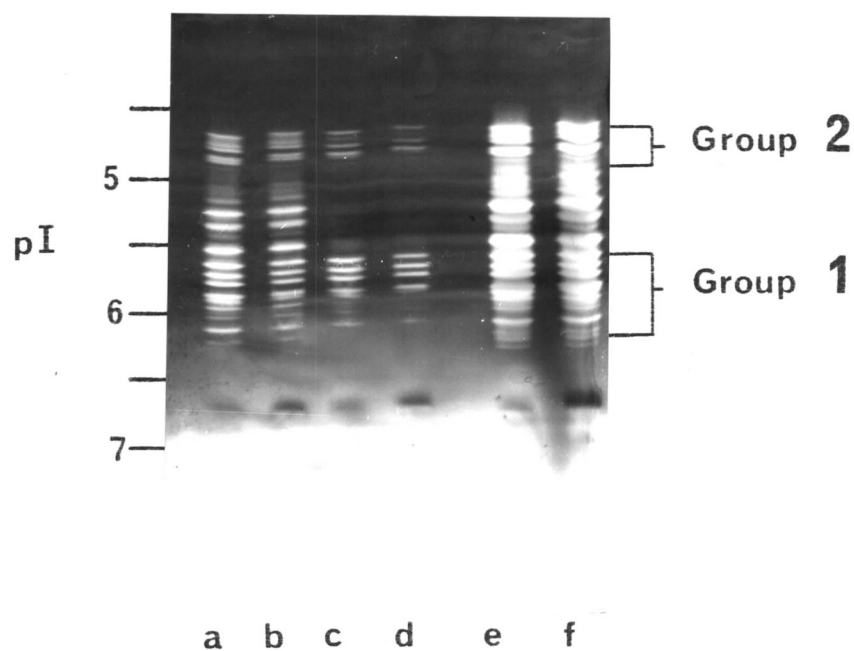


FIGURE 28.A. The isoenzymes of 6 days germinated wheat.
(A) - Zymogram of 6 extracts: a = unheated high vigour; b = unheated low vigour; c = heated high vigour; d = heated low vigour; e = unheated high vigour (undiluted); f = unheated low vigour (undiluted).

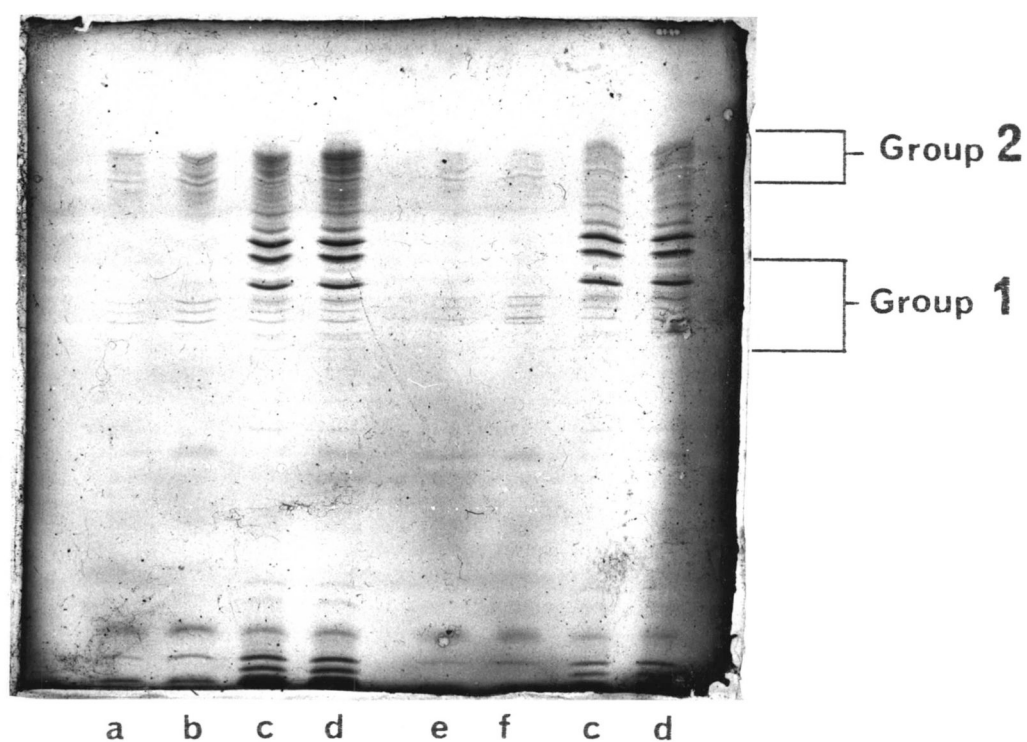


FIGURE 28.B. The isoenzymes of 6 days germinated wheat.
(B) - Protein staining of 6 extracts: a = heated high vigour; b = heated low vigour; c = unheated high vigour; d = unheated low vigour; e = heated high vigour (double strength); f = heated low vigour (double strength).

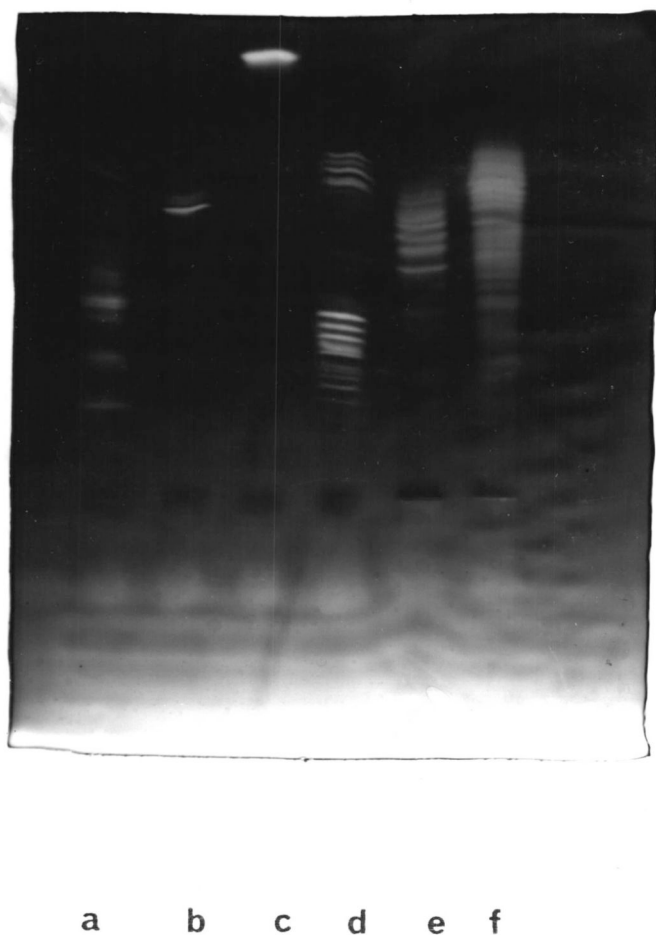


FIGURE 29. Zymogram of amylases from various sources: a = barley *alpha*-amylase; b = bacterial *alpha*-amylase; c = fungal *alpha*-amylase; d = wheat *alpha*-amylase; e = wheat *beta*-amylase; f = amylases washed from wheat starch granules.

of isoenzymes described above, indicating that contaminating proteins were not responsible for any of the isoenzymes observed in wheat (Figure 29.). It is interesting that the wheat and barley zymograms were so different, as these two species are so closely related. When two different varieties of wheat were tried, the zymograms were identical.

During germination, the two groups of isoenzymes did not appear at the same time. In high vigour seeds the group 1 isoenzymes appeared after 1 day of germination at 25°C., but the group 2 isoenzymes had only just begun to appear on day 2 (Figure 30.A.). Dissection of germinating seeds into scutellum and aleurone showed that both groups were detectable in both tissues, so that the origin of each group was not tissue specific. Low vigour seeds had exactly the same complement of isoenzymes as high vigour seeds; there was no loss of any particular *alpha*-amylase isoenzyme associated with loss of vigour. They did differ, however, in the rate at which the groups appeared. Whereas high vigour seeds had visible group 1 isoenzymes after 1 days' germination, these isoenzymes were not apparent until 2 days in the low vigour seeds (Figure 30.B.). Similarly, the group 2 isoenzymes were not observed until 4 days of germination. The lag phase that was observed in the activity of *alpha*-amylase during germination was reflected by the delay in the appearance of the isoenzymes.

At 10°C. the group 2 isoenzymes apparently appeared before the group 1 isoenzymes (Figure 31.A.). This would seem to be a reversal of the trend found at 25°C. However, careful inspection of the zymograms shows that some *beta*-amylase bands are present too, which suggests that the heating process may not have been sufficient in these extracts to destroy the interfering enzymes. Consequently, these bands that appear to be group 2 isoenzymes may be nothing more than contaminants; this was borne out by subsequent immunochemical analyses. Apart from this, the pattern is the same as that observed at 25°C., in that the group 1 isoenzymes increased in intensity and then faded with advancing germination whilst the group 2 isoenzymes did not increase until a later stage and did not fade. Although this was the same in the low vigour seeds, the time scale

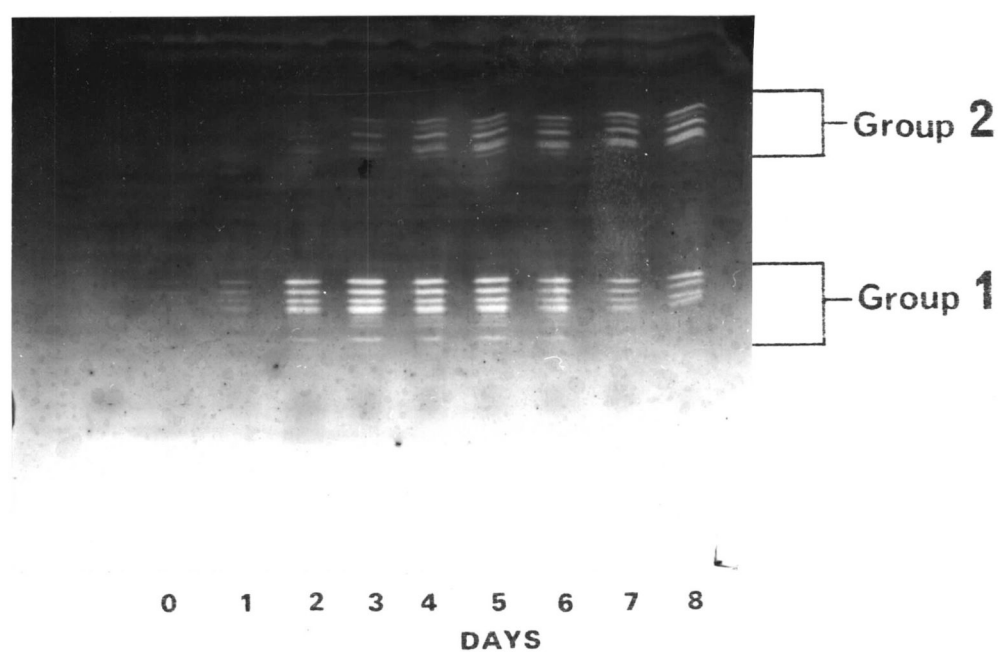
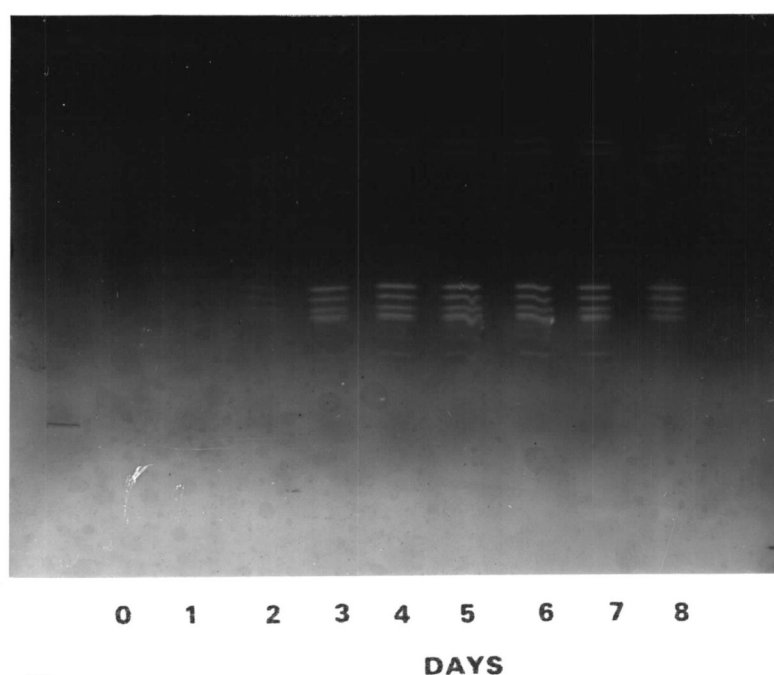
**A****B**

FIGURE 30. Zymograms of α -amylase extracted from wheat germinated at 25°C. in the dark. (A) High vigour seeds. (B) Low vigour seeds.

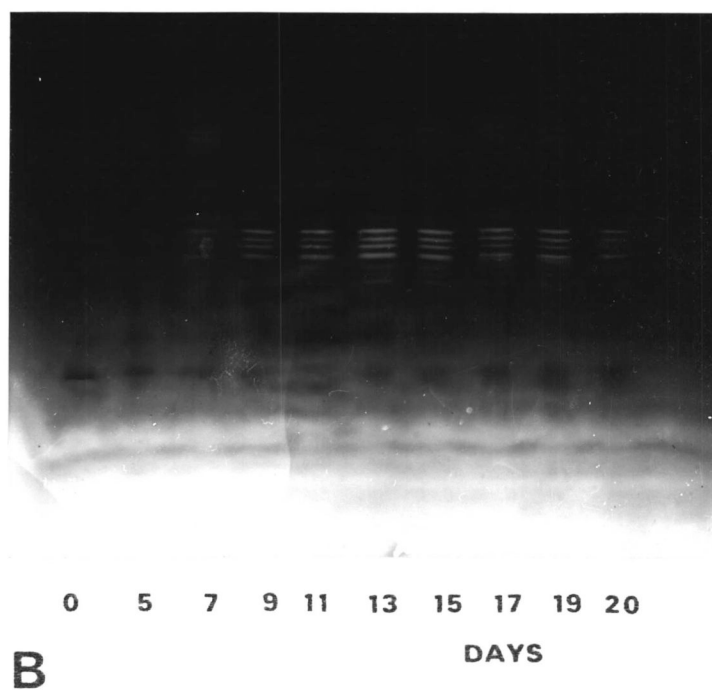
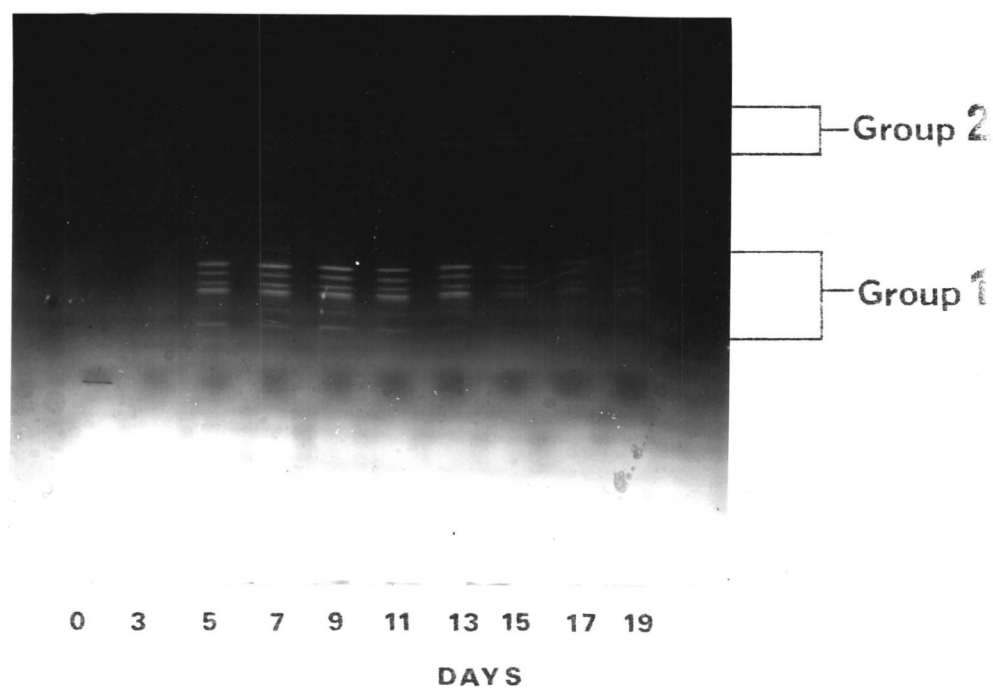


FIGURE 31. Zymograms of *alpha*-amylase extracted from wheat germinated at 10°C. in the dark. (A) High vigour seeds. (B) Low vigour seeds.

was quite different so that no isoenzymes were observed until 7 days of germination (Figure 31.B.). These results confirm those of the measurement of *alpha*-amylase activities, which indicated an extended lag phase of about 7 days in low vigour seeds germinated at 10°C.

3.7.5. Purification of *Alpha*-Amylase Isoenzymes

The purification procedures resulted in the homogenous preparations of the two groups of isoenzymes. In the initial crude extract the specific activity of *alpha*-amylase was approximately 4 units per mg protein, which rose to 18 units/mg after heat treatment. As has been mentioned before, the heat treatment was of primary advantage in destroying *beta*-amylase and other interfering enzymes. The glycogen-complexing stage was a far more powerful technique for the purification of the enzyme. It relied on the fact that in 40% v/v ethanol, *alpha*-amylase was at the limit of its solubility but upon addition of glycogen the enzyme-substrate complex that formed was insoluble. After the five complexing stages the specific activity of the extract had increased to 440 units/mg. The inherent specificity of the method was indicated by the fact that after this step the extract apparently contained only three contaminating proteins, as revealed by isoelectric focussing and protein staining.

The final purification stage, and separation procedure for the two groups of isoenzymes, was preparative flat-bed isoelectric focussing. This technique had the advantages of a high loading capacity (100mg of protein could be applied); a high degree of resolution; easy elution of the focussed zones and easy identification of the isoenzymes by measurement of the pI by a surface electrode. Following elution from the gel, 53mg of group 1 and 17mg of group 2 isoenzymes were obtained. The specific activities of the group 1 and group 2 isoenzymes were 492 units/mg and 469 units/mg respectively, indicating the possession of similar activities on the *beta*-limit-dextrin substrate. The homogeneity of the isoenzymes was confirmed by analytical isoelectric focussing; each group of isoenzymes was free from contamination by the other group or by non-*alpha*-amylase proteins. Additionally, SDS-polyacrylamide

gel electrophoresis indicated that each group consisted only of a single protein band.

3.7.6. Quantification of the *Alpha*-Amylase Isoenzymes

The homogenous preparations of group 1 and group 2 isoenzymes were used to raise immunoglobulins (IgG) against them, by immunising rabbits. Once these IgG's were obtained they were tested for specificity; both lots of antisera were found to be monospecific for the appropriate group of isoenzymes. This was shown by the use of Ouchterlony double-diffusion plates: no cross-reaction was obtained with bovine serum albumin, non-*alpha*-amylase wheat proteins, an extract of ungerminated wheat or the wrong group of isoenzymes i.e. group 1 antisera did not cross-react with group 2 isoenzymes and vice-versa. A single line of precipitation was only achieved when group 1 isoenzymes and antisera were mixed or group 2 isoenzymes and their antisera.

By mixing antisera against both groups of isoenzymes into an agarose gel, the quantity of isoenzymes in any seed extract could be determined by quantitative Laurell rocket immunoelectrophoresis. This technique is based on the fact that as the protein moves across the gel by electrophoresis it will become complexed with the appropriate antibody. When the proportion of antigen to antibody is correct, the complex will precipitate out and will no longer move by electrophoresis. In this way, the advancing protein leaves a trail of precipitate behind it, until there is no more uncomplexed protein left hence leaving a rocket shaped trail. The more protein there is, the further the trail of precipitate will extend so that the height of the rocket is directly proportional to the amount of protein. If standard preparations of antigenic protein are run at the same time as the unknowns then a calibration curve of rocket height versus mg of protein may be constructed, and the amounts of protein in the extracts may be determined by interpolation.

Seeds of high and low vigour were germinated at 10°C. and 25°C. in the dark. Crude extracts of *alpha*-amylase were made, without heat inactivation of the other enzymes,

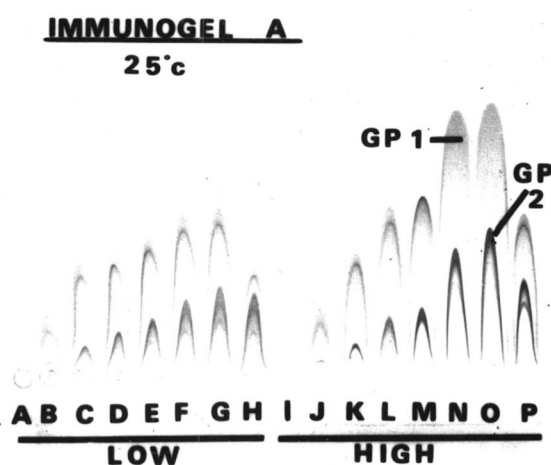


FIGURE 32.A. Immunogel A. Quantitative rocket immunoelectrophoresis of *alpha*-amylase isoenzymes from high and low vigour seeds. A & I = 1 day of germination; B & J = 2 days; C & K = 3 days; D & L = 4 days; E & M = 5 days; F & N = 6 days; G & O = 7 days; H & P = 8 days.

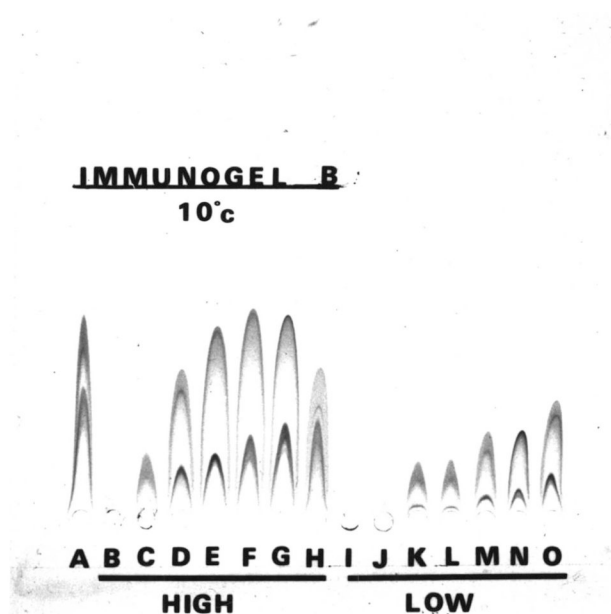


FIGURE 32.B. Immunogel B. Quantitative rocket immuno-electrophoresis of *alpha*-amylase isoenzymes from high and low vigour seeds. A = standard solution of isoenzymes; B & I = 3 days of germination; C & J = 5 days; D & K = 9 days; E & L = 12 days; F & M = 16 days; G & N = 18 days; H & O = 20 days.

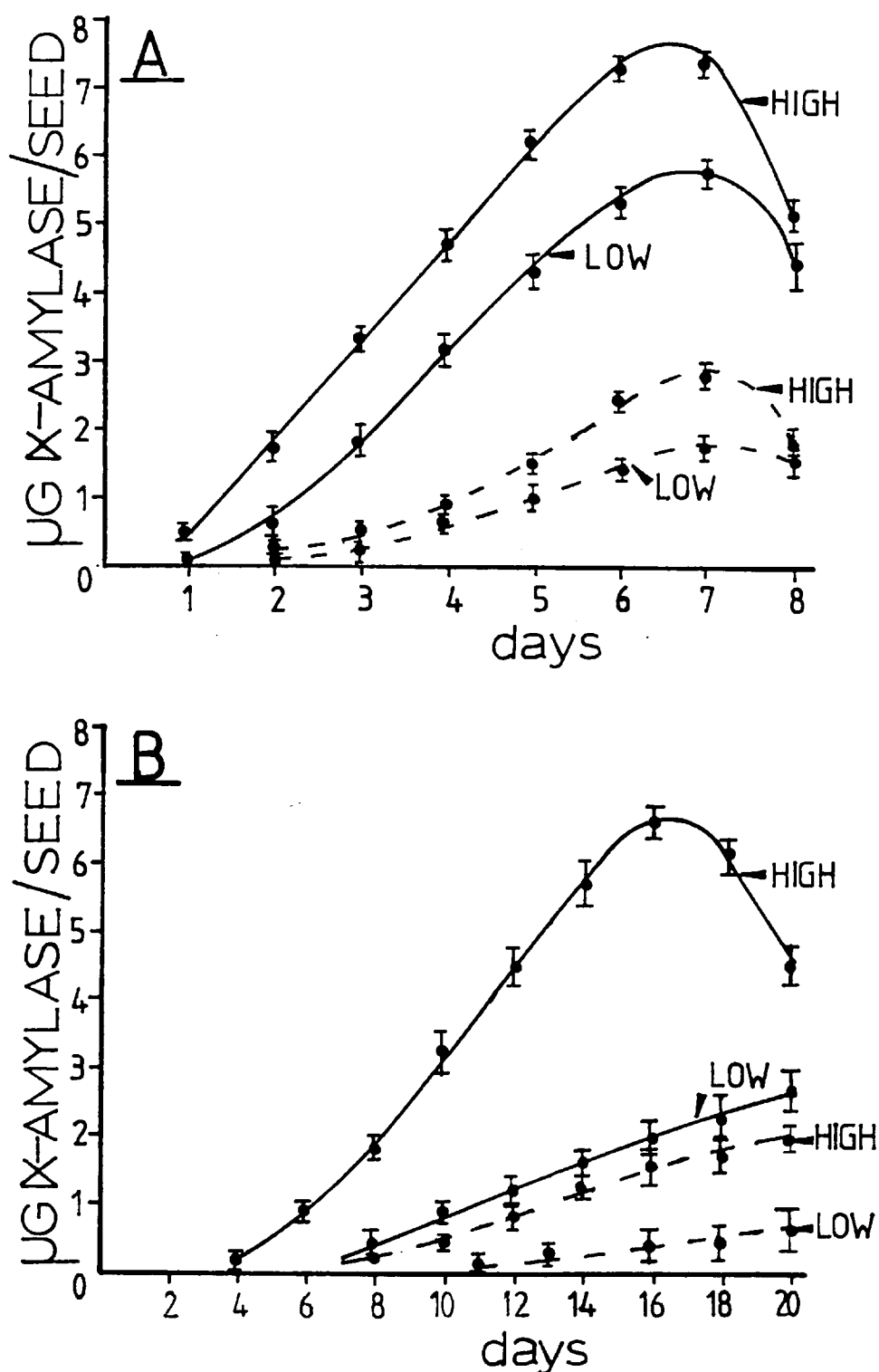


FIGURE 33. Changes in the quantities of group 1 and group 2 α -amylase isoenzymes during germination of high and low vigour seeds at (A) 25°C. and (B) 10°C., as determined by immunoelectrophoresis. Solid lines = Group 1 isoenzymes. Broken lines = Group 2 isoenzymes. Bars represent 95% confidence limits.

and subjected to rocket immunoelectrophoresis, along with four standards of purified isoenzymes. Sample gels are shown in Figure 32. but it should be noted that the standards were not included on these gels. This was because both high and low vigour extracts were run on the same gel to allow easy comparison; such gels were not used for quantification.

When the seeds were germinated at 25°C. the increases in the quantities of the isoenzymes was similar to that found for the increases in *alpha*-amylase activity. Group 1 isoenzymes were always more abundant than the group 2 isoenzymes, and appeared at an earlier stage of germination (Figure 33.A.). The same was true for low vigour seeds, but they never reached the same amount of total amylase as the high vigour seeds. The ratio of group 1 to group 2 changed during germination, so that by 8 days of germination there was about 25% group 2 compared with 0% at day 1. Although there was less amylase overall in low vigour seeds, the ratio of group 1 to group 2 was approximately the same as in high vigour seeds, suggesting that there is no lesion in the enzyme synthesizing machinery specifically affecting one group of isoenzymes.

At 10°C. the high vigour seeds showed a very similar pattern of isoenzyme changes as they had at 25°C. The ratios of the two groups were similar although the total weight of *alpha*-amylase proteins was slightly less than at 25°C. (Figure 33.B.). The low vigour seeds had very much less amylase in total, but again the ratios of group 1 to group 2 were similar to those at 25°C. The poor showing of the low vigour seeds is in agreement with that found by measuring enzyme activities. It is obvious that when grown under stress conditions low vigour seeds do not secrete an adequate amount of *alpha*-amylase to supply sugars to the growing embryo. This lack of enzyme is not due to a shortage of one particular group of isoenzymes, but due to an overall decrease.

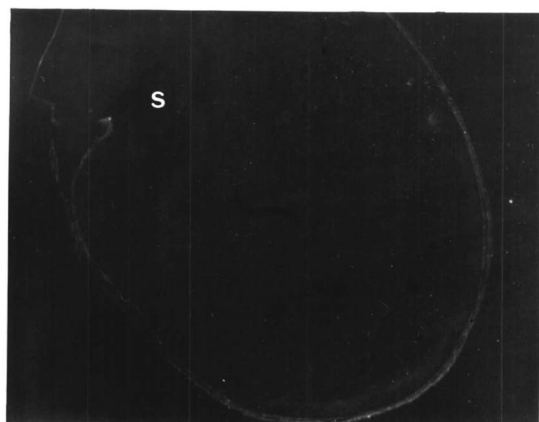
3.7.7. Localisation of *Alpha*-Amylase Isoenzymes in Grains

The short supply of *alpha*-amylase in low vigour seeds could be due to one of two factors. Either all of the cells

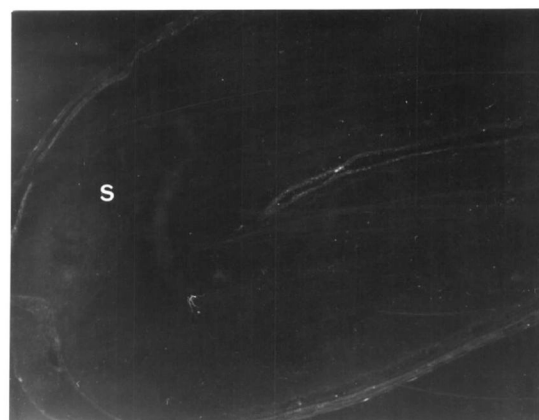
which synthesize and secrete the enzyme are doing so to a lesser extent than is normal, or some of these cells are not yielding enzyme at all. In order to discover which of these was the most likely, a study of the localisation of both groups of isoenzymes in germinating grains was undertaken. This study utilized the antisera which had previously been prepared against the two groups of isoenzymes. Thin sections of germinating grains were treated with antisera which bound to the isoenzymes, washed, and then treated with FITC-goat anti-rabbit IgG. The latter IgG bound to the original rabbit IgG, so that the isoenzymes were fixed with two IgG's of which the latter had a fluorescent label attached. Thus when the sections were viewed with a fluorescence microscope, the areas where the isoenzymes were originally located showed up as heavily fluorescent patches.

Before any interpretation of the fluorescence could be made, the specificity of the various IgG's was assessed, as described (section 2.24.). All of the control reactions gave negative results, indicating that any fluorescence seen was attributable to the presence of one of the groups of isoenzymes. When "dry" seeds were examined, no fluorescence was found with either group 1 or group 2 antisera, in either high or low vigour seeds (Figure 34.A. & B.). Only the yellow-green autofluorescence of the pericarp and testa was visible (Figure 34.C.).

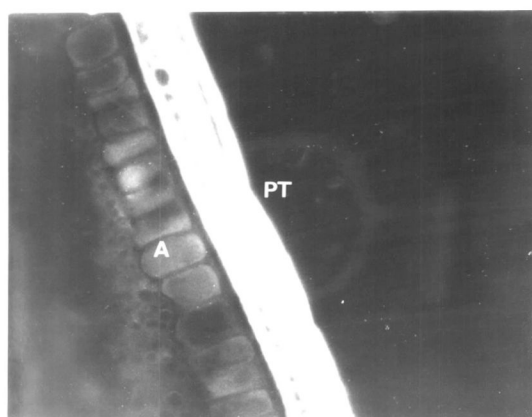
After 1 days' germination at 25°C., bright green fluorescence was apparent around the periphery of the scutellum of high vigour seeds (Figure 35.A.), suggesting that *alpha*-amylase was being produced by this organ. The scutellum epithelium was fluorescent, but the aleurone was not, which substantiates the conclusion that the enzyme was released from the scutellum and not the aleurone at this stage. The fluorescence was only just visible with anti-group 2 staining (Figure 35.B.) which agrees with the findings of the immunoelectrophoresis where the group 2 isoenzymes did not reach the threshold of detection until after the group 1 isoenzymes. There was no fluorescence with either IgG in low vigour seeds. By 2 days of germination the group 1 fluorescence around the scutellum of high vigour grains had increased in intensity and area,



A

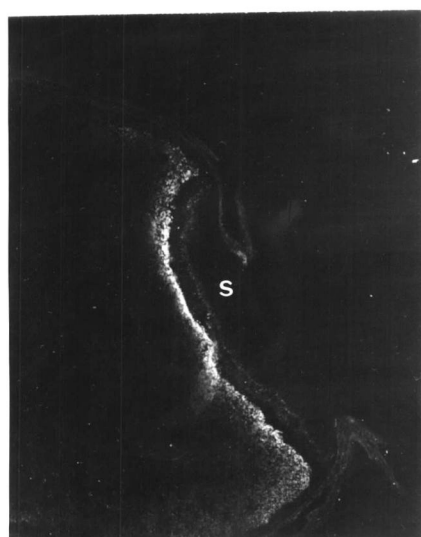


B

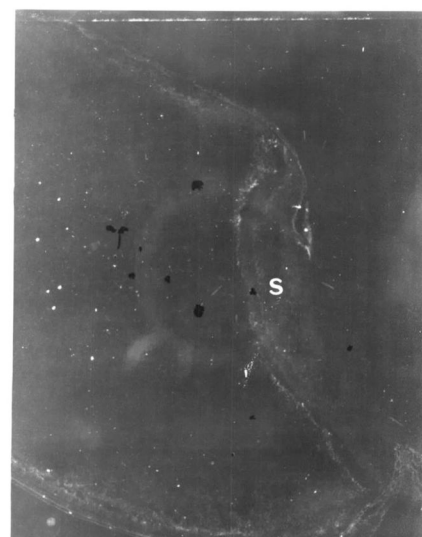


C

FIGURE 34. Immunofluorescence of "dry" seeds stained for group 1 and group 2 α -amylase isoenzymes. (A) = high vigour seed (x12); (B) = low vigour seed (x12); (C) = Close-up (x100) of periphery of high vigour seed (autofluorescence). S = scutellum A = aleurone PT = pericarp & testa.



A

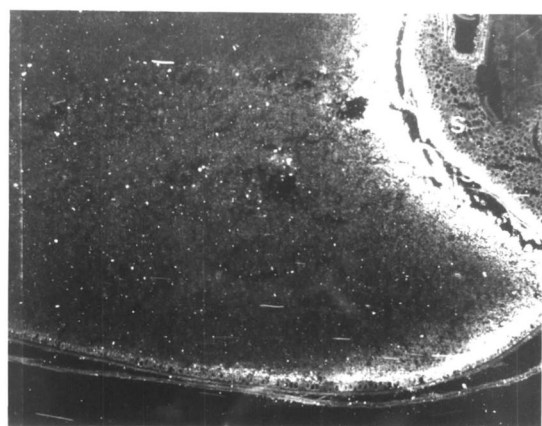


B

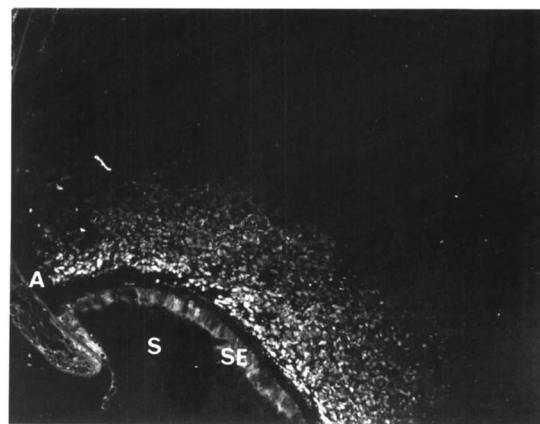
FIGURE 35. Immunofluorescence of *alpha*-amylase in high vigour seeds germinated for 1 day at 25°C. (x14).

(A) = group 1 isoenzymes; (B) = group 2 isoenzymes.

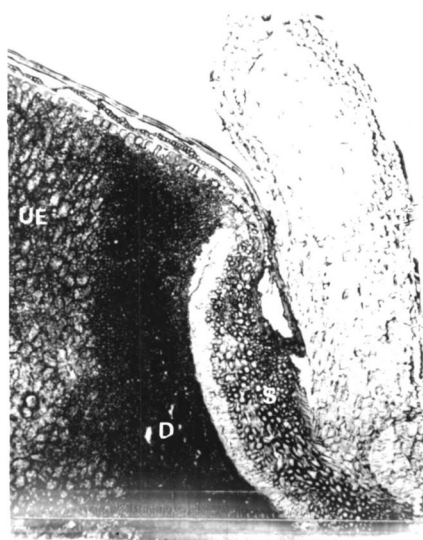
S = scutellum.



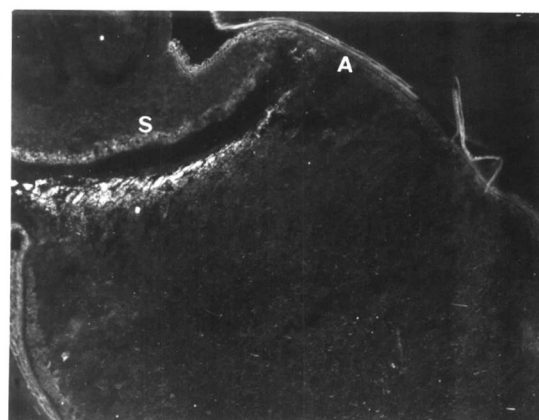
A



B



C



D

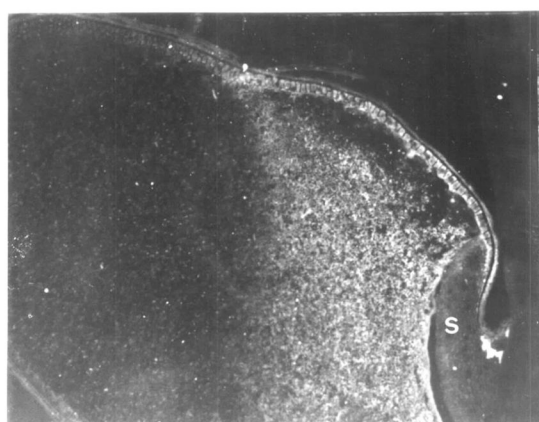
FIGURE 36. Immunofluorescence of *alpha*-amylase in seeds germinated for 2 days at 25°C. (A) = high vigour, group 1 (x12); (B) = high vigour, group 2 (x14); (D) = low vigour, group 1 (x14). (C) = high vigour, light micrograph (x11). S = scutellum SE = scutellum epithelium A = aleurone D = degraded endosperm cell walls UE = undegraded endosperm.

and now the aleurone was just beginning to release the enzyme (Figure 36.A.). This photograph shows beyond doubt that the group 1 isoenzymes were produced in the scutellum epithelium as well as the aleurone. In contrast, the group 2 isoenzymes were still only being released by the scutellum epithelium (Figure 35.B.), although a very tiny portion of the aleurone behind the scutellum had just begun to fluoresce. It was interesting to note that the areas of group 1 fluorescence corresponded to the areas of breakdown of the endosperm cell walls. The breakdown of the walls was clearly visible, using a light microscope, as a dark patch adjacent to the scutellum (Figure 35.C.). Such areas are likely to be due to the action of endo-*beta*-glucanases, whose release precedes that of *alpha*-amylase. There was still no sign of group 2 isoenzymes in low vigour seeds after 2 days, but the group 1 isoenzymes were just appearing (Figure 36.D.), illustrating the lag phase between these and the high vigour seeds which has been so apparent in many experiments. Again, secretion was from the scutellum epithelium alone.

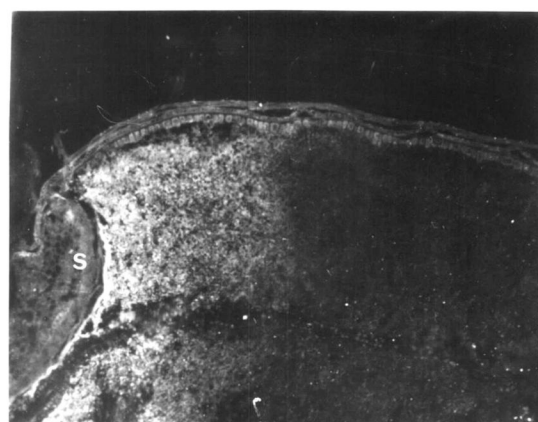
Three days after imbibition, the fluorescent fronts of both groups 1 and 2 had advanced in high vigour seeds, and it was clear that the aleurone cells, as well as the scutellum epithelium, were secreting the isoenzymes (Figure 37.A & B.). Low vigour seeds also showed an advance of the group 1 front, and for the first time group 2 fluorescence was observed (Figure 37.C & D.). As with the high vigour seeds, the aleurone was found to be of no importance in *alpha*-amylase secretion during the early stages.

Degradation of the endosperm cell walls in high vigour grains was seen to have accompanied the advancement of the *alpha*-amylase front (Figure 38.A.), as was found after 2 days of germination. The starch granules immediately behind the scutellum were beginning to be well degraded in high vigour seeds, and the intermediate layer between the scutellum and the endosperm had disappeared (Figure 38.B.).

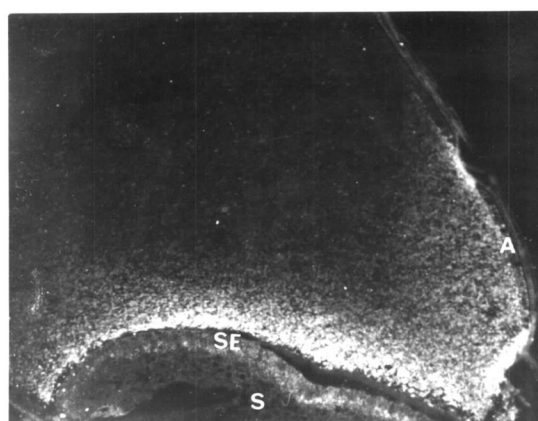
On close inspection, all the fluorescence in the endosperm adjacent to the scutellum was observed to be on, or in, the starch granules (Figure 38.C.) indicating that the enzyme was bound to them. No fluorescence was observed in the matrix between the granules suggesting that the enzyme has a very high affinity for the starch granules. This was true for both groups of isoenzymes in both seed types.



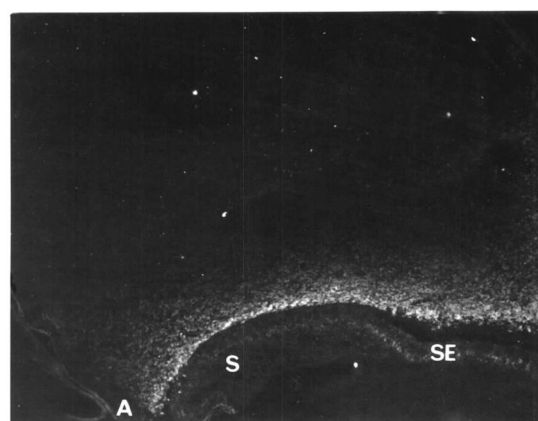
A



B

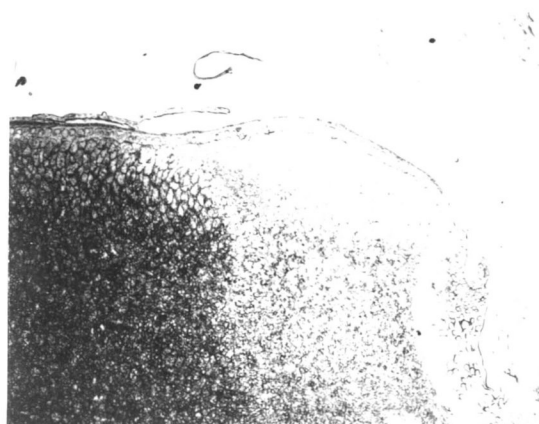


C

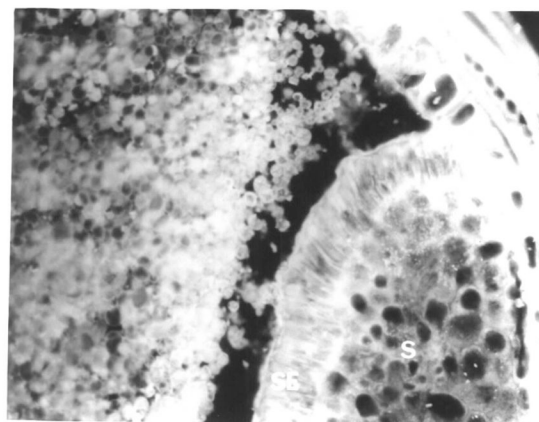


D

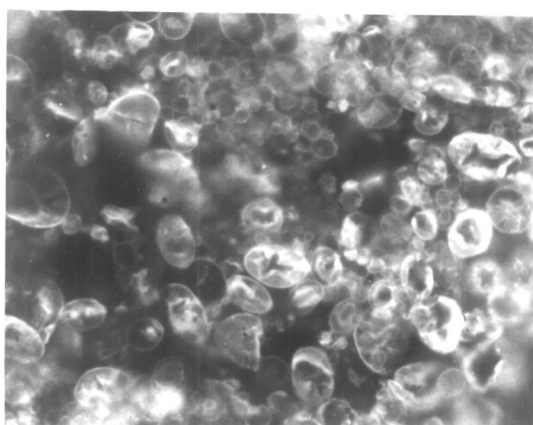
FIGURE 37. Immunofluorescence of *alpha*-amylase in seeds germinated for 3 days at 25°C. (A) = high vigour, group 1 (x12); (B) = high vigour, group 2 (x11); (C) = low vigour, group 1 (x13); (D) = low vigour, group 2 (x13).
S = scutellum SE = scutellum epithelium A = aleurone.



A

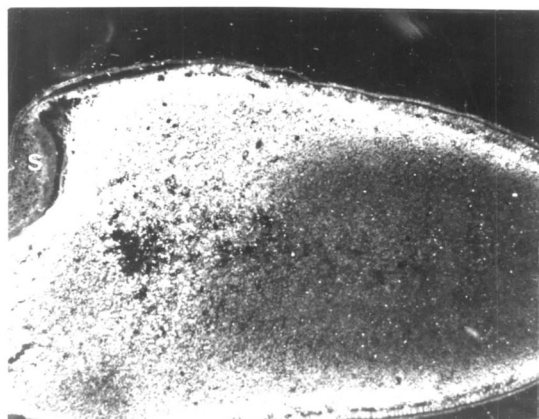


B

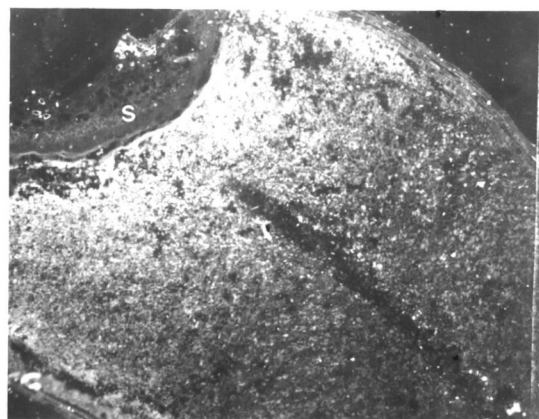


C

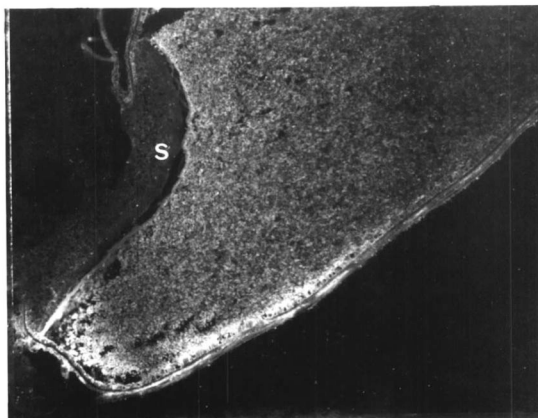
FIGURE 38. Endosperm degradation in high vigour seeds germinated for 3 days at 25°C. (A) = light micrograph of seed section (x12) (B) = immunofluorescence of group 1 isoenzymes at the scutellum periphery (x80) (C) = magnified view (x220) of immunofluorescently-stained starch granules; group 1 isoenzymes stained.



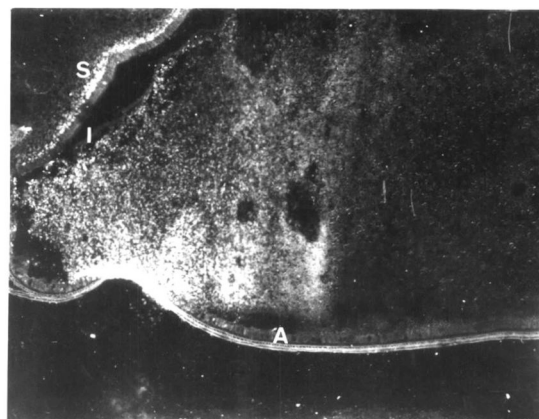
A



B

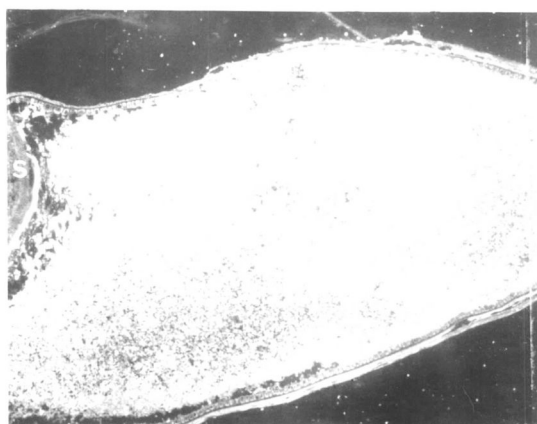


C



D

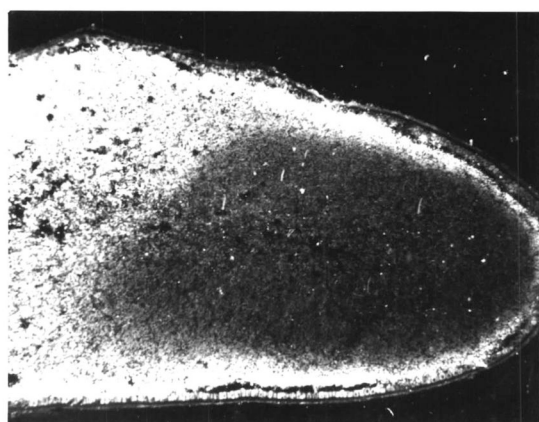
FIGURE 39. Immunofluorescence of *alpha*-amylase in seeds germinated for 4 days at 25°C. (A) = high vigour, group 1 (x12); (B) = high vigour, group 2 (x13); (C) = low vigour, group 1 (x11); (D) = low vigour, group 2 (x12).
S = scutellum A = aleurone I = intermediate layer.



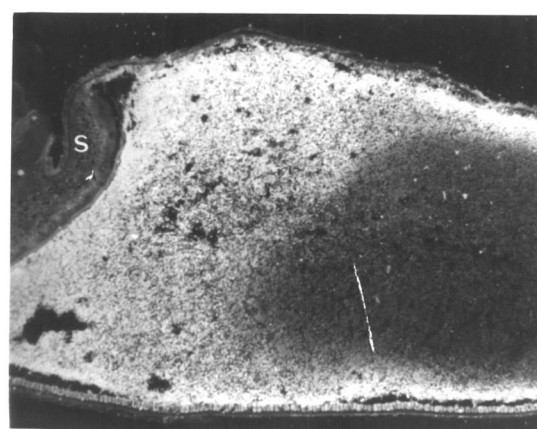
A



B

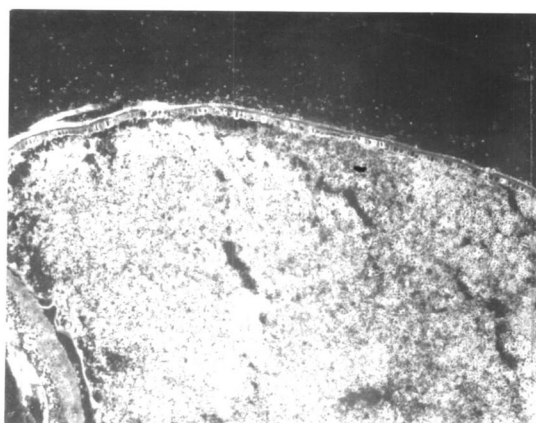


C

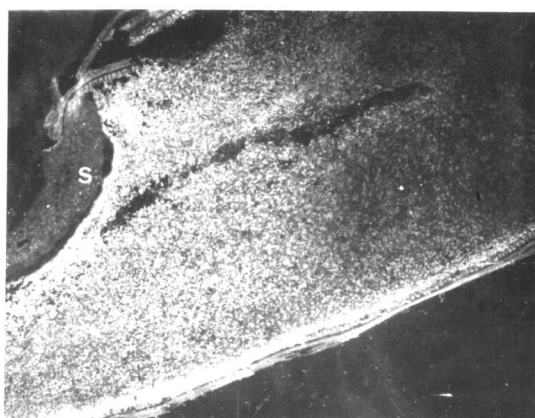


D

FIGURE 40. Immunofluorescence of *alpha*-amylase seeds germinated for 5 days at 25°C. (A) = high vigour, group 1 (x12); (B) = high vigour, group 2 (x14); (C) = low vigour, group 1 (x12); (D) = low vigour, group 2 (x12).
S = scutellum



A



B

FIGURE 41. Immunofluorescence of *alpha*-amylase in seeds germinated for 6 days at 25°C.. (A) = low vigour, group 1 (x12); (B) = low vigour, group 2 (x12). S = scutellum

A day later saw a further increase in the areas of both groups of isoenzymes in both seed types (Figure 39.), with most of the new protein apparently arising from the aleurone. At this stage, virtually all of the aleurone cells in the high vigour seeds were fluorescing, indicating that they had all been "switched-on" by the hormone messenger, GA. On the fifth day of germination the whole endosperm was fluorescent with either group of isoenzymes, in high vigour seeds (Figure 40.A & B.). Although the low vigour seeds showed significant increases over the areas of fluorescence the previous day, they had not reached the point of filling the entire endosperm (Figure 40.C & D.). This did not occur until the sixth day (Figure 41.).

There were few differences between the patterns of accumulation, and the localisation, of the two groups of isoenzymes in high and low vigour seeds germinated at 25°C. The only significant difference was the delay of about one day before the low vigour seeds began to synthesize the isoenzymes. When germinated at 10°C., high vigour seeds showed exactly the same patterns as they did at 25°C. except that everything was slowed down. The same was not true of low vigour seeds.

When photographs of enzyme localisation are shown they are obviously only a representative of the range of results observed. With both seed types at 25°C. and high vigour seeds at 10°C. the selection of representative photographs was not too difficult as the variations were relatively small. However, when low vigour seeds were germinated at 10°C. a considerable range in germinability and enzyme production was observed, making it impossible to find a single representative photograph. Consequently, a photograph of 15 typically ungerminated or abnormal low vigour seedlings, collected from a sample of low vigour seeds germinated for 10 days at 10°C., was taken (Figure 42.) and the immunofluorescence of the group 1 isoenzymes was determined for each. Photographs of some of these immunofluorescence patterns are shown here, and may be related back to the actual seeds from which they were produced.

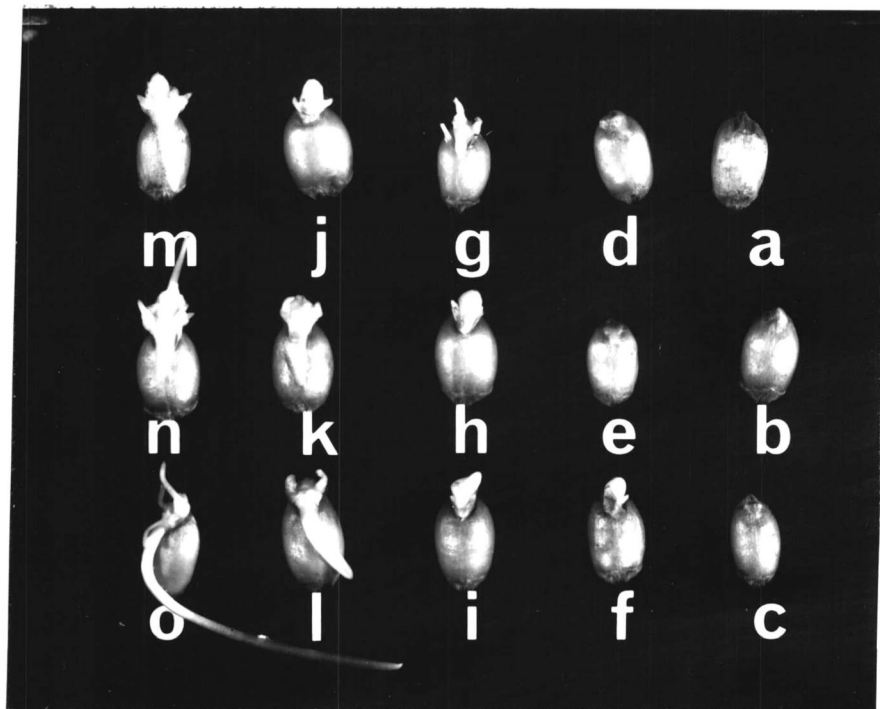
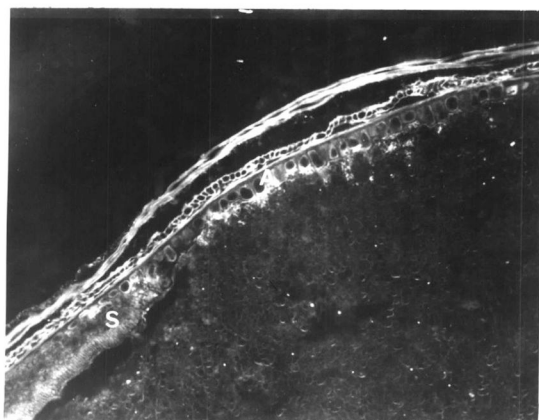


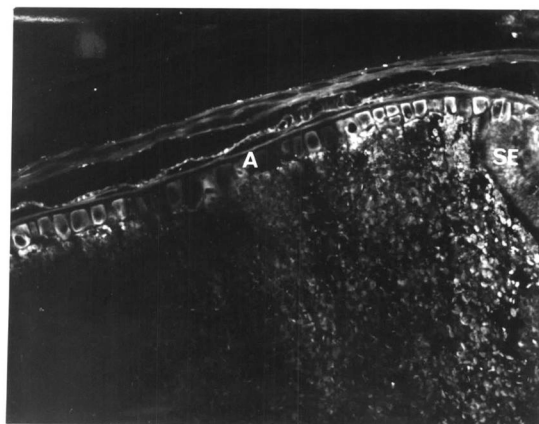
FIGURE 42. Ungerminated and abnormal low vigour seedlings found after 10 days germination at 10°C. These seeds were used for immunofluorescence staining, shown in subsequent photographs.

Those low vigour seeds which germinated to produce normal, but small, seedlings tended to show normal patterns of immunofluorescence, although they often contained a few aleurone cells which did not fluoresce. Completely ungerminated seeds eg. seeds a - e (Figure 42.) never showed any fluorescence. Examples of the fluorescence in these two types of "low vigour seed" are therefore not provided. The intermediate seeds, such as those illustrated in Figure 42. had varied patterns of immunofluorescence, but there was one phenomenon common to all of them, which was that where aberrations were found using group 1 localisation, they were also found with group 2 staining. Only group 1 staining is shown in the figures.

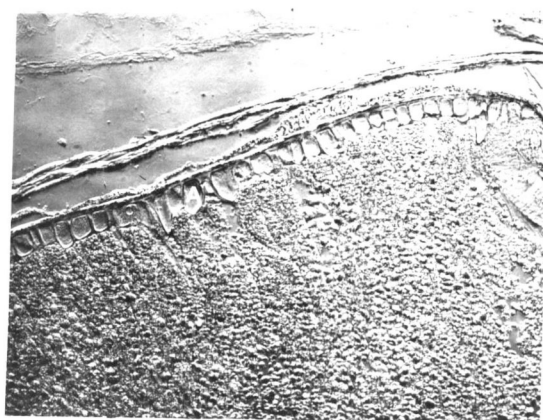
A frequently observed pattern was that of all aleurone cells secreting a little protein, but never producing enough to degrade a significant amount of starch (Figure 43.A.). However, the most common observation was that of groups of non-fluorescent aleurone cells interspersed amongst fluorescent ones, giving the impression of complete non-function (Figure 43.B.). It could be argued that the lack of fluorescence in these cells was due to the loss of their contents during sectioning, and hence the loss of the antigenic proteins. This was not true, as the presence of cell contents was verified using interference microscopy. Figure 43.B. shows a section containing some non-fluorescent aleurone cells. Figure 43.C. shows exactly the same section, unmoved, using interference settings on the microscope. The non-fluorescent cells clearly have contents. A larger magnification of the aleurone cells is given in Figure 43.C., where the cell contents are obvious. Examples of other low vigour seeds with non-fluorescing aleurone cells are shown in Figure 44.A, B & C. Another aberration observed, but far less frequently, was that of a non-fluorescent scutellum epithelium. Figure 44.D. shows a seed in which the aleurone fluoresced, albeit weakly, but the scutellum epithelium did not suggesting that this tissue was not able to secrete *alpha*-amylase. These observations suggest that one of the major causes of the poor supply of *alpha*-amylase in low vigour seeds must be damage to individual cells or groups of cells, which causes them to die or at least become unable to synthesize any *alpha*-amylase.



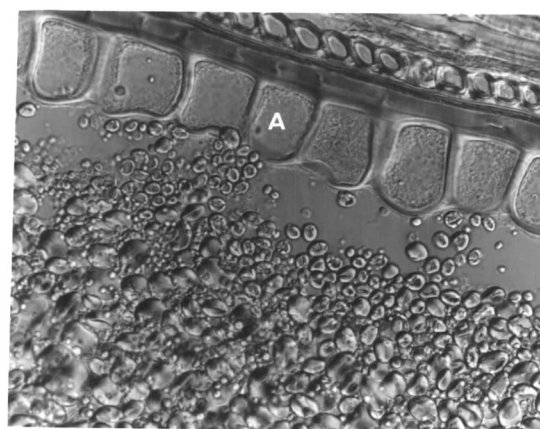
A



B

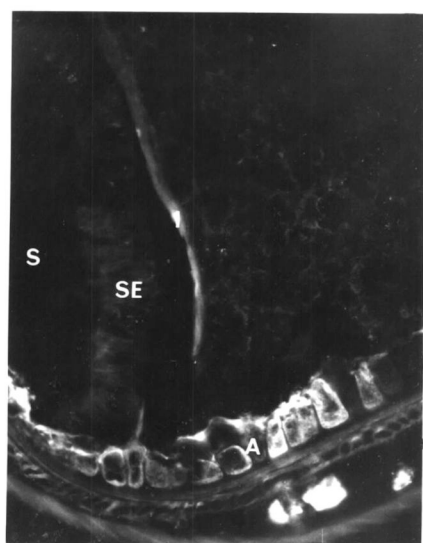


C

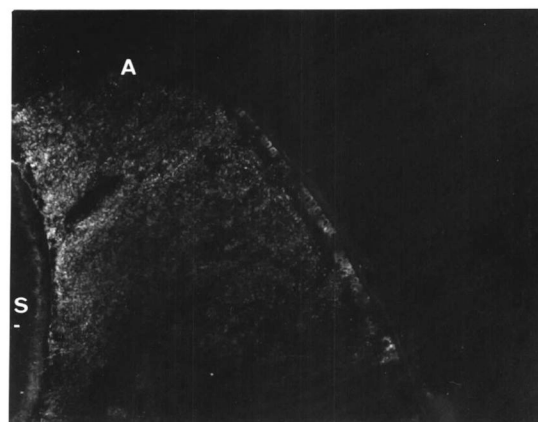


D

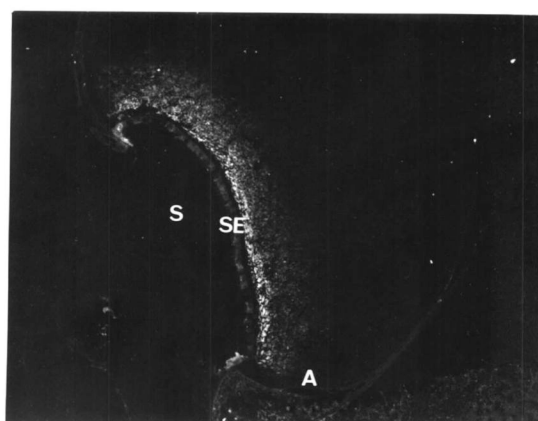
FIGURE 43. Immunofluoresence of *alpha*-amylase in low vigour seeds germinated for 10 days at 10°C.. (A) = Seed F (x40). (B) = Seed K (x40). (C) = Seed K, using interference microscopy (x40). (D) = Seed K, magnified view of aleurone with interference microscopy (x160). S = scutellum A = aleurone.



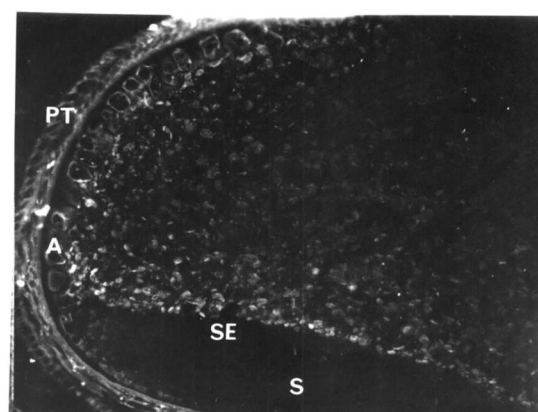
A



B



C



D

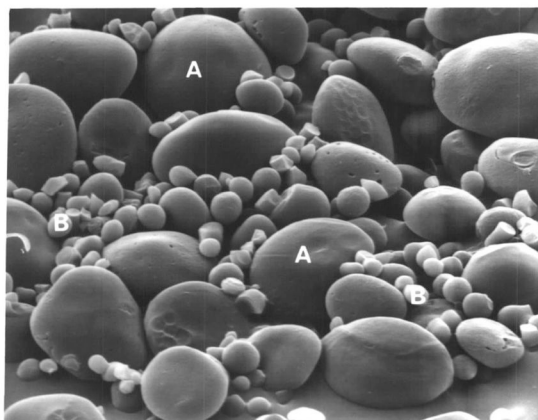
FIGURE 44. Immunofluoresence of α -amylase in low vigour seeds germinated for 10 days at 10°C. (A) = Seed L (x40). (B) = Seed N (x14). (C) = Seed O (x12). (D) = Seed M (x18). S = scutellum SE = scutellum epithelium A = aleurone PT = pericarp & testa.

3.7.8. Microscopy of Starch Degradation

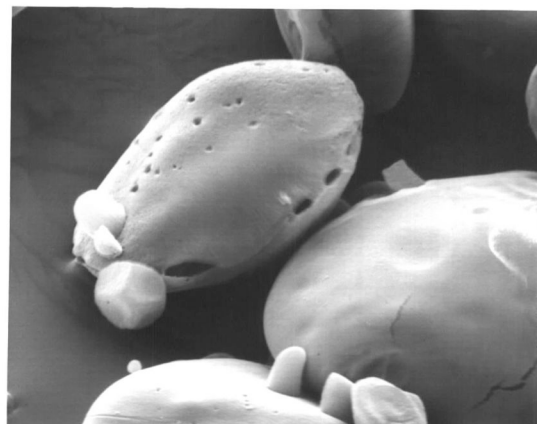
Previous experiments had determined that there was no difference between the *alpha*-amylase enzymes of high and low vigour seeds in terms of *K_m*, pH and temperature optima, and isoenzyme complement. These measurements did not give any indication of how effective the enzymes were on their real substrate, solid starch granules. It could be that although the enzymes were equally effective on *beta*-limit-dextrin they were not equal in their abilities to bind to and degrade starch granules *in vivo*. To ascertain whether there was any difference or not, a study of the degradation of starch granules immediately adjacent to the scutellum was undertaken, using scanning electron microscopy.

Careful examination of degrading starch granules showed that there was no difference between those in low vigour seeds and those in high vigour ones. The only factor that distinguished the two was the time at which certain types of decay were found. The following sequence is described for high vigour seeds but applies equally to low vigour seeds if a delay of one day is allowed for. There was also no difference at 10°C., although the time delay between high and low vigour seeds was considerably greater. It appeared that this fundamental property of the enzyme, that of binding to and degrading starch granules, was not altered on vigour loss.

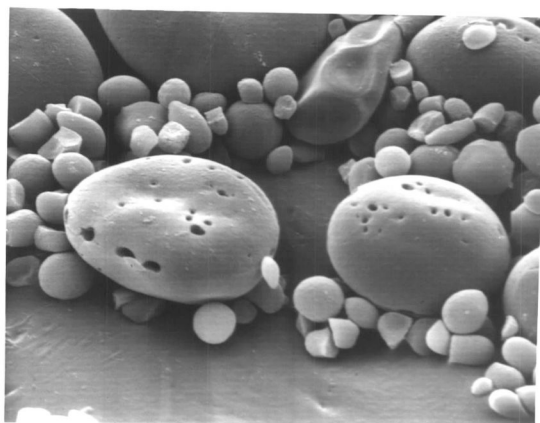
After one day of germination, most of the A-type elliptical granules were undamaged (Figure 45.A.), but a few were just beginning to show surface pitting (Figure 45.B.). The equatorial groove was particularly liable to initial attack. The B-type granules were unaffected at this stage. A day later saw the extension of this early degradation, with enlarging of the surface pits (Figure 45.C.) and the first signs of internal decay (Figure 45.D.). More granules were beginning to show pitting but many, including the B-granules, were still unblemished. Scanning electron microscopy can only show surface features and so suffers from the disadvantage of shielding internal structures. When starch granules from 2 days germinated wheat were examined by interference microscopy, both internal and



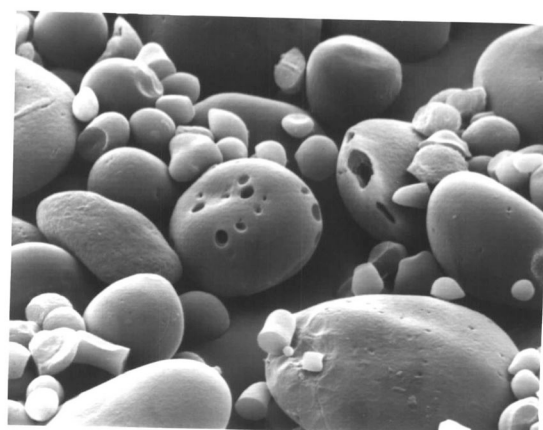
A



B

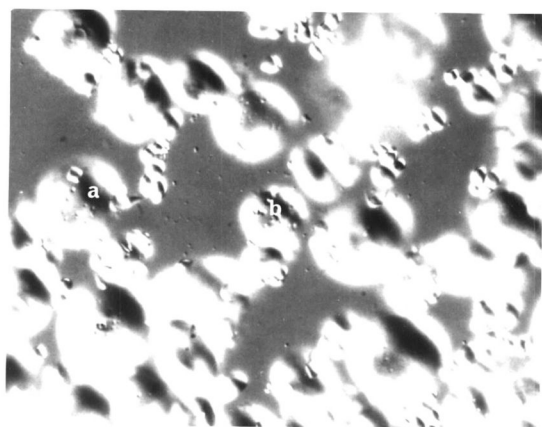


C

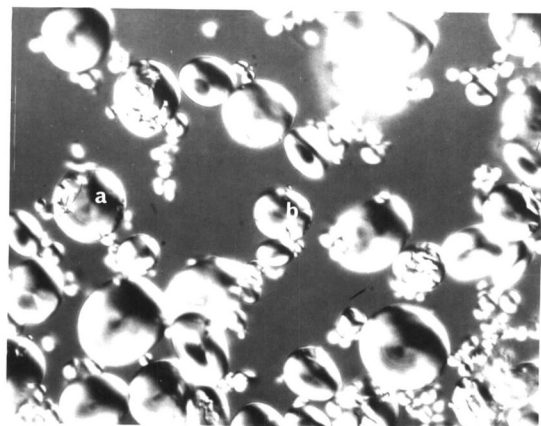


D

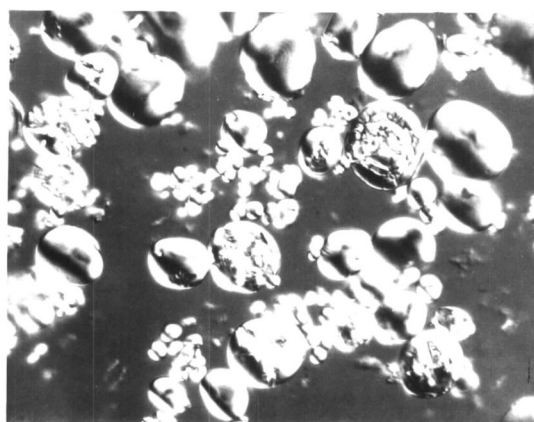
FIGURE 45. Starch degradation in wheat germinated at 25°C., by Scanning Electron Microscopy. (A) = 1 day of germination (x600); (B) = 1 day (x1500); (C) = 2 days (x1060); (D) = 2 days (x1090). A = A-type starch granules, B = B-type granules.



A



B



C

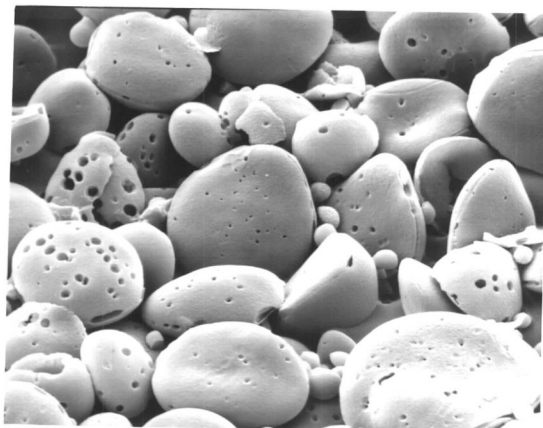
FIGURE 46. Starch degradation in wheat germinated for 2 days at 25°C., by Interference Contrast Microscopy.

(A) = top focussed granules (x325); (B) = middle focussed granules (x325); (C) = middle focussed granules (x325).
a = granule a; b = granule b.

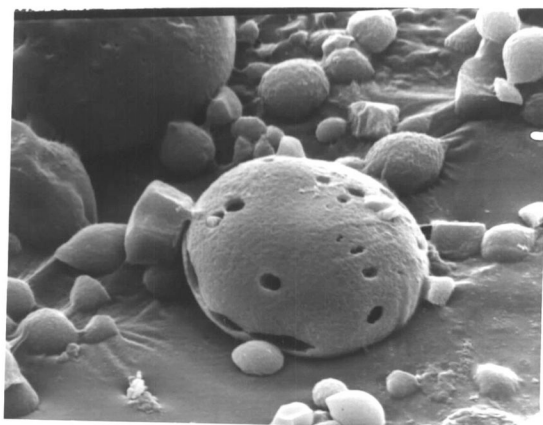
external features were visible. The surface pitting was clear when focussing on the outermost regions of the granules (Figure 46.A.), but when the focussing was lowered there were conspicuous signs of internal decay (Figure 46.B & C.). By looking at granules a & b in Figure 46. it is easy to see this external pitting and also the more severe internal decay. Such internal destruction has not been noted before, for granules in these early stages of degradation.

By the third day of germination most of the A-granules were showing signs of decay (Figure 47.A.). In many granules the areas of degradation on the equatorial groove were close to coalescence (Figure 47.B.), which would split the granule into two halves. The B-granules were also beginning to undergo degradation but not by the same mechanism as the A-granules. Only a general surface pitting, without the formation of deep holes was noted (Figure 47.B.), giving the granules a rough appearance on their periphery. After another day, the A-granules were further decayed, many showing signs of incipient breakup (Figure 47.C & D.). The now enlarged, and frequently coalesced, surface holes revealed the exact nature of the enzymic attack (Figure 47.D.). This consisted of ridging, that is certain layers of the starch granule were further decayed than others giving a ridged appearance.

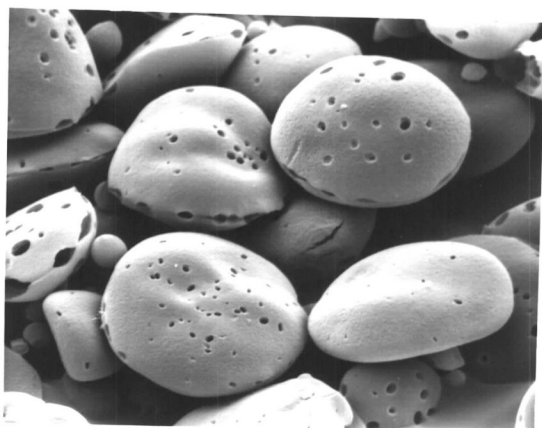
After 5 days of germination virtually all of the A-granules were decayed to some extent, many of them had completely lost their integrity (Figure 48.A.), whilst the B-granules only showed more extensive surface pitting. The holes in the A-granules were coalescing to form large expanses of decay (Figure 48.B & C.), which frequently made the granules split into two halves. The ridging decay was very clear in these half-granules (Figure 48.D.), and it was obvious that whilst all of the internal starch had been degraded much of the surface starch was left undecayed. This may be because the outer layers are more resistant to *alpha*-amylase attack than the internal starch. The same may be true of the B-granules which appeared to retain their integrity for a longer period than the A-granules, and never showed signs of severe decay. It may be that *alpha*-amylase is less able to bind to the B-granules.



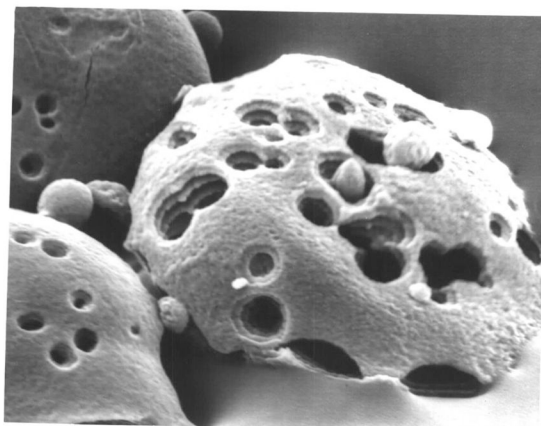
A



B



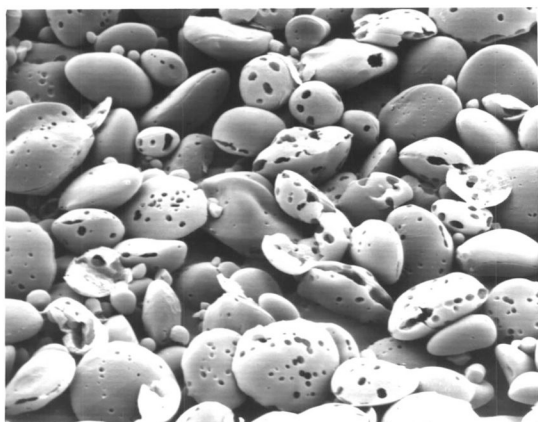
C



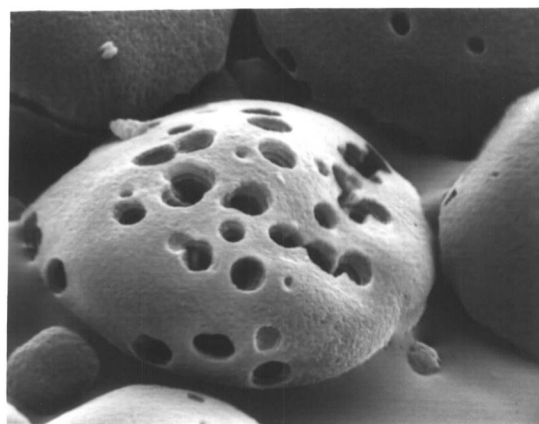
D

FIGURE 47. Starch degradation in wheat germinated at 25°C.

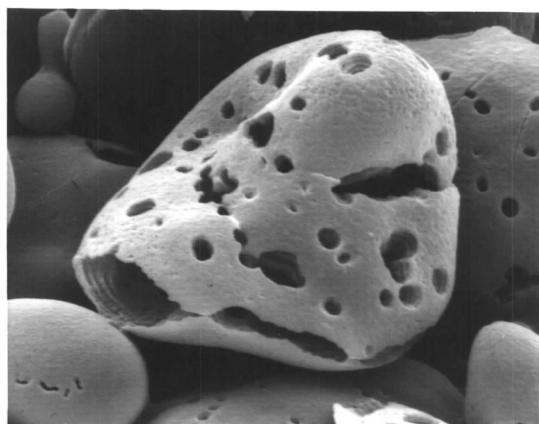
(A) = 3 days of germination (x660); (B) = 3 days (x1430);
(C) = 4 days (x910); (D) = 4 days (x2200).



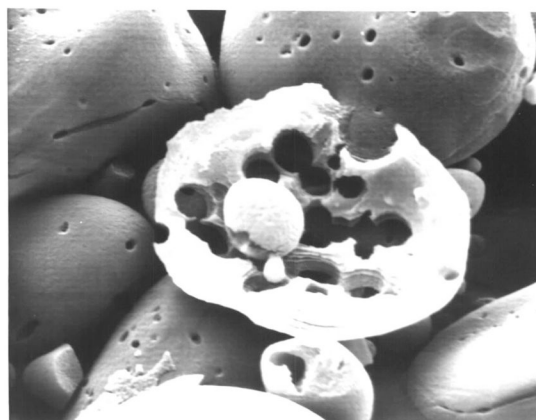
A



B



C



D

FIGURE 48. Starch degradation in wheat germinated for 5 days at 25°C. (A) = x500; (B) = x2120; (C) = x1630; (D) = x1370.

3.8. ALPHA-AMYLASE SYNTHESIS IN DE-EMBRYONATED GRAINS

There is no doubt that in low vigour seeds a shortage of *alpha*-amylase is responsible for the low glucose concentrations in the endosperms. It is also clear that some of the cells, both in the aleurone and scutellum epithelium, are not able to synthesize the enzyme to the required extent. This weak performance of the cells may be due to their inherent inability to undergo normal metabolism or it may be that the hormonal signal from the embryo is not strong enough. Thus the reduced secretion of *alpha*-amylase could be due to the inability of the aleurone to respond to a GA signal or to the fact that they could respond adequately but that the amount of GA secreted by the embryo is reduced, or both.

Seeds of high and low vigour were de-embryonated so as to remove their source of GA. These endosperm halves were incubated at 10°C. or 25°C. in buffer solutions containing various concentrations of GA. By assaying the activity of *alpha*-amylase following incubation, a dose-response curve of GA concentration versus units of *alpha*-amylase activity per grain was drawn up. At 25°C. both high and low vigour seeds showed maximum response with 10 micromolar GA or higher (Figure 49.A.). However, the low vigour seeds had a maximum response of less than half that of the high vigour seeds, indicating that their aleurone cells were not capable of producing the same amount of enzyme in the same length of time as their high vigour counterparts. When these extracts were examined by isoelectric focussing, it was noted that both groups 1 and 2 were present in high vigour extracts but only group 1 was visible in the low vigour extracts. When the seeds were incubated for a further day, the group 2 isoenzymes appeared in the low vigour extracts and the maximum level of *alpha*-amylase activity equalled that of the high vigour seeds which had been incubated for a day less. It thus seems that the response of the low vigour endosperms was due to their still being involved in a lag phase i.e. they were about a day behind the high vigour seeds in their response to GA.

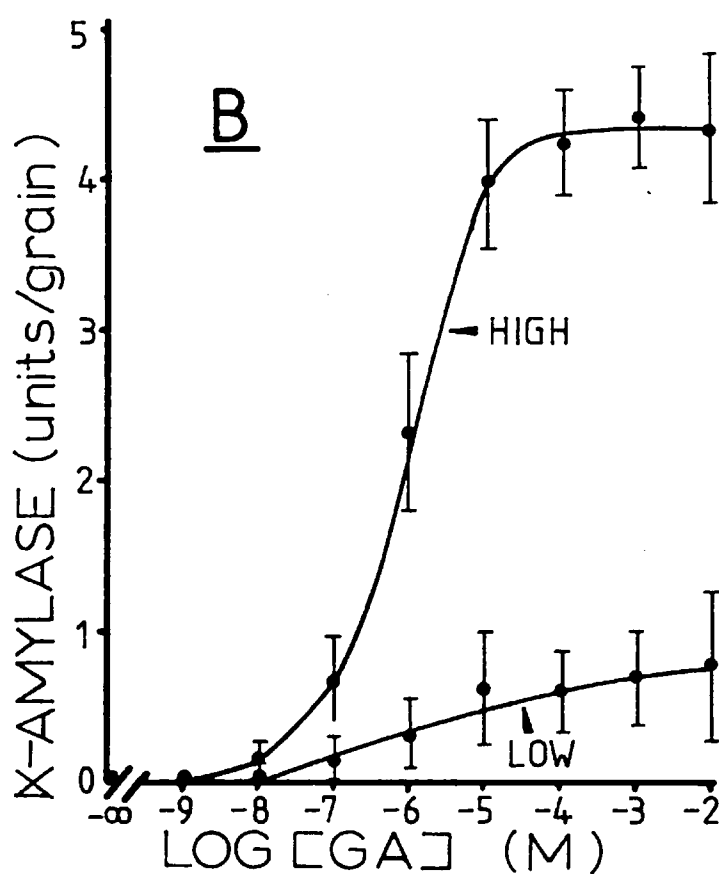
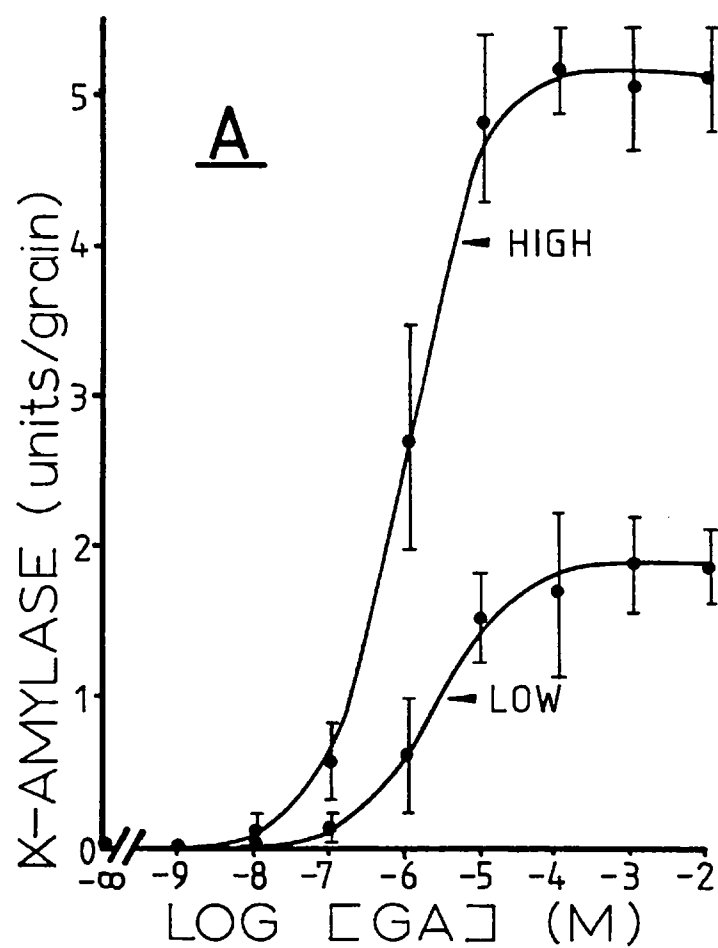


FIGURE 49. Dose-Response curve of GA concentration versus amount of α -amylase produced in de-embryonated high and low vigour grains, incubated at (A) 25°C. and (B) 10°C. Bars = 95% confidence limits.

At 10°C. the high vigour seeds exhibited the same response that they had at 25°C., in that 10 micromolar GA was the smallest concentration to elicit the maximum response (Figure 49.B.). However, the low vigour seeds showed hardly any response at all and again, on investigation by isoelectric focussing, the group 2 isoenzymes were found to be absent. Low vigour aleurone cells are clearly unable to respond adequately to the hormonal signal when incubated under stress conditions.

3.8.1. The Production of GA by High and Low Vigour Embryos

Although it had been shown that low vigour aleurone cells were inherently unable to give an adequate response to exogenous GA, this did not preclude the possibility that in the whole seed the production of GA by the embryo is also reduced. The amount of extractable GA was assayed in high and low vigour seeds germinated at 10°C. and 25°C. In the dry seeds the amount of GA was low but this rapidly increased as germination began (Figure 50.). After reaching a peak the GA concentration fell rapidly and then levelled off; the latter levelling was possibly due to a combination of decreasing synthesis in the scutellum but increasing synthesis in the newly arisen shoot. Both high and low vigour seeds showed the same pattern at 25°C., although a lag in the response of the low vigour seeds was apparent. This lag was of the same length as that noted in the response of the endosperms to exogenous GA, so that there is a combination of the unresponsiveness of the aleurone and the lack of GA from the embryo that causes the lag in *alpha*-amylase output.

At 10°C. the high vigour seeds showed the same pattern of changing GA concentrations as they had at 25°C., but the low vigour seeds were quite different. The GA concentration hardly rose until about 12 days of germination, and then it did so very slowly. It appears that low vigour seeds are highly deficient in the production of GA when grown under stress conditions. However, care must be taken in the interpretation of these results. Any bioassay that is to measure a promotion in a biochemical event by GA is likely to be affected by the presence of GA antagonists. In this case it is possible

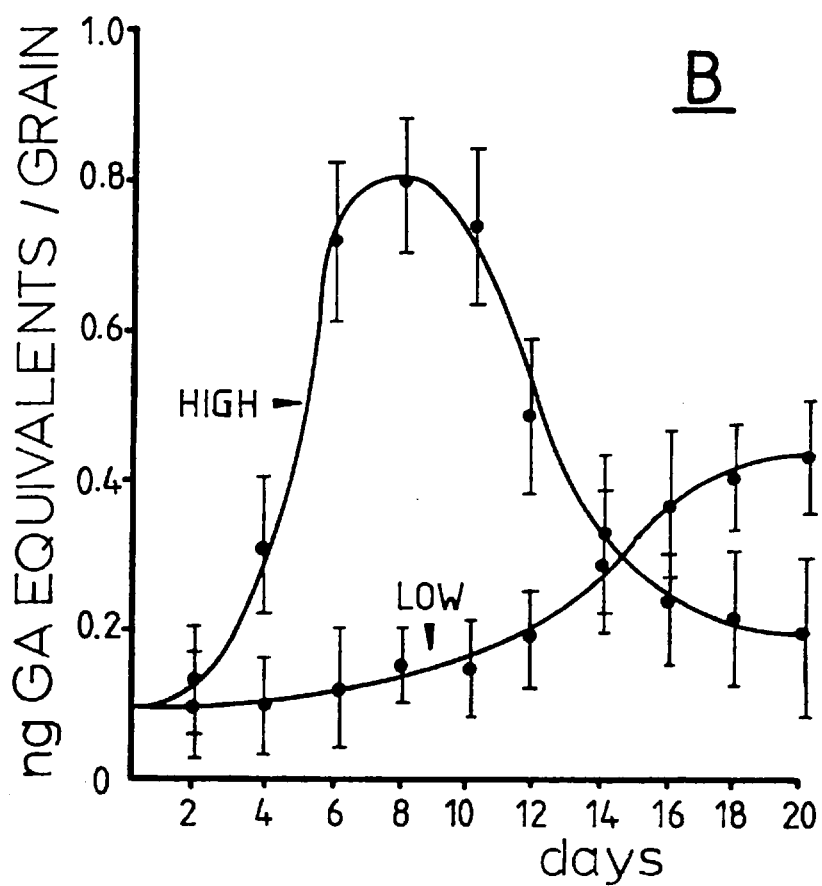
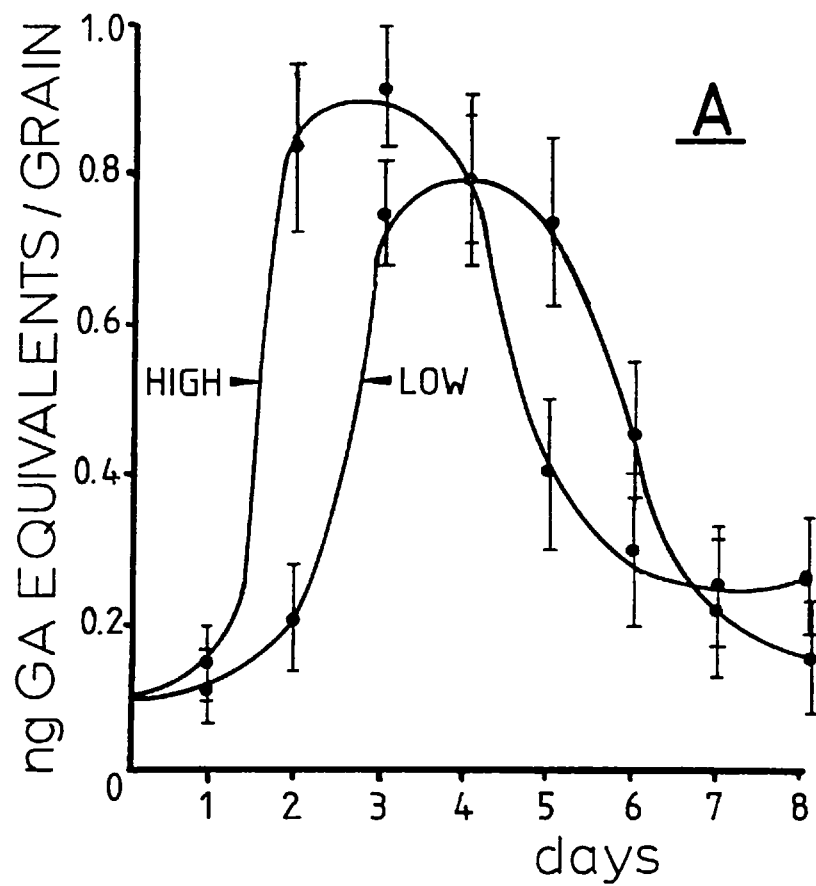


FIGURE 50. Changes in GA equivalents in high and low vigour seeds germinated at (A) 25°C. and (B) 10°C. Bars = 95% confidence limits.

that ABA interferes with the assay, so that any measure of GA is really a measure of the balance of GA and ABA, hence the use of "GA equivalents". Thus, although there appeared to be a small amount of GA in the low vigour seeds this could be due to an elevated amount of ABA. As ABA is a recognised wound or stress hormone, this possibility may be very probable in these injured seeds.

3.9. CHANGES IN MALTASE CONCENTRATIONS DURING GERMINATION

Maltase (*alpha*-glucosidase) is another enzyme which is important in the degradation of starch, and like *alpha*-amylase it is also secreted by the aleurone in response to GA. A study of the changes in concentration of this enzyme during germination therefore provided a useful comparison to that of *alpha*-amylase, in order to test whether the effects found in low vigour seeds were unique to this latter enzyme. The activity of maltase increased very slightly during early germination, then decreased, rose sharply and finally decreased dramatically (Figure 51.). The increase during the early stages was possibly for the digestion of the embryo starch, and the sharp rise probably due to the synthesis and release of the enzyme by the activated aleurone cells, in response to embryonic GA. This pattern was the same for high vigour seeds at 10°C. and 25°C. and for low vigour seeds at 25°C. When low vigour seeds were germinated at 10°C. they hardly produced any maltase; the shape of the graph bears a distinct resemblance to that of the concentration of GA equivalents released by the embryo during germination at the same temperature (Figure 50.). It is likely that the reduced secretion of maltase is due to the low concentration of GA equivalents. The release of maltase also closely follows that of *alpha*-amylase indicating that the effects described before are not specific for *alpha*-amylase, but may be due a general decline in the functioning of the aleurone and embryo cells.

3.10. THE SURFACE POTENTIALS OF GERMINATING SEEDS

It has been established that the carbohydrate metabolism of low vigour seeds is slower than that of high vigour seeds and that the differences are exaggerated during germination

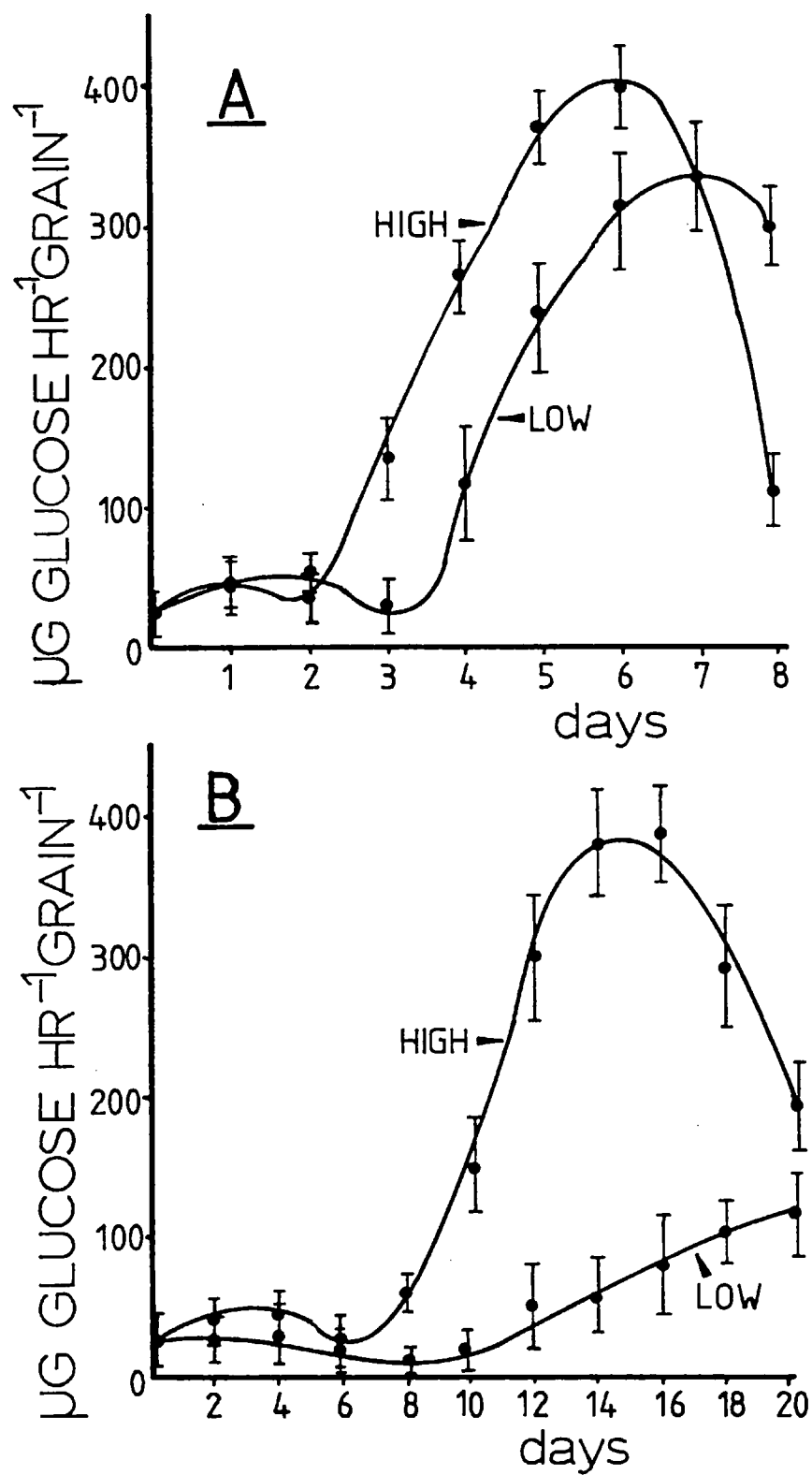


FIGURE 51. Changes in maltase activity during the germination of high and low vigour seeds at (A) 25°C. and (B) 10°C.

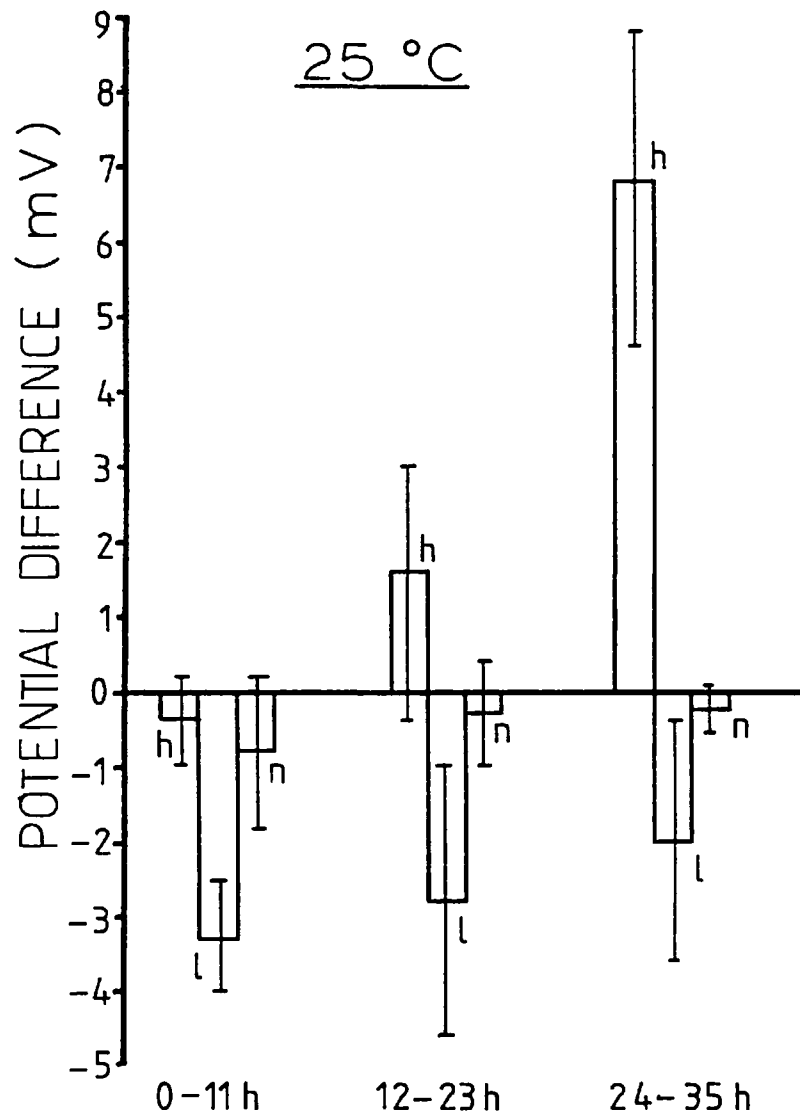


FIGURE 52.A. The potential difference between the embryo and non-embryo ends of high (h), low (l) and non-viable seeds (n) following imbibition at 25°C.

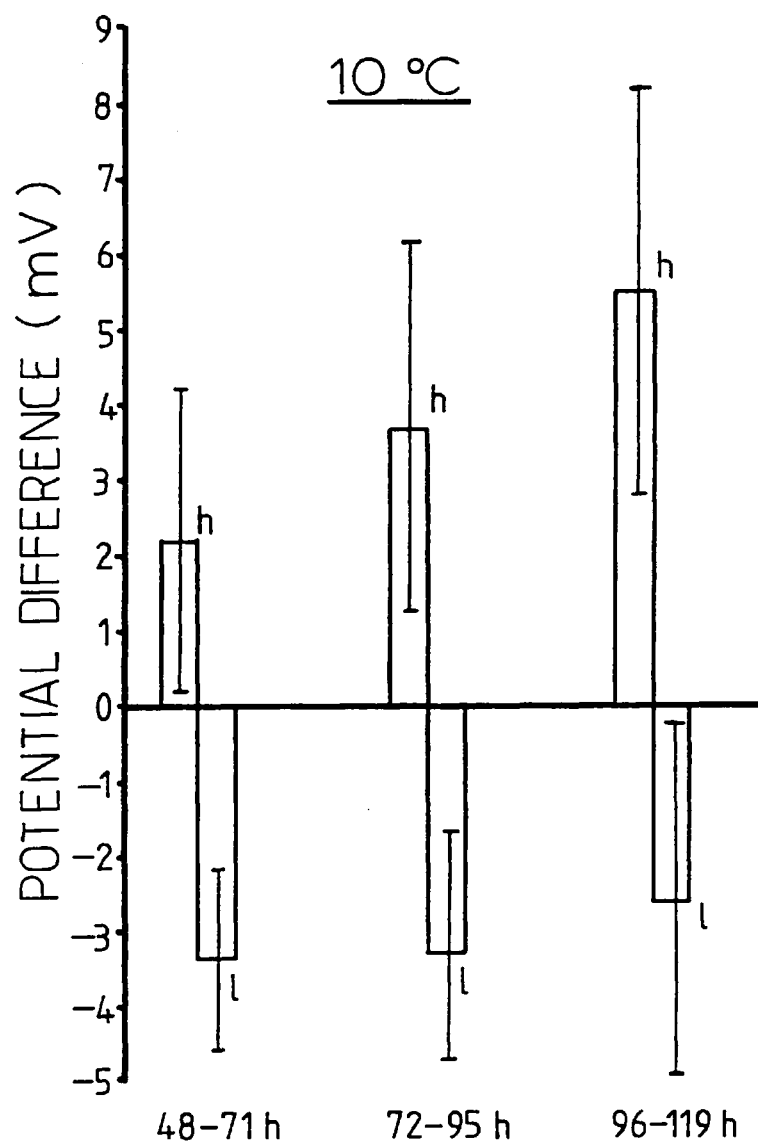


FIGURE 52.B. The potential difference between the embryo and non-embryo ends of high (h) and low (l) vigour seeds following imbibition at 10°C.

at 10°C. Another indicator of active metabolism is the seeds' surface potential. Differences in electrical potential between different regions of the seed surface are a reflection of the ion-transport activity of seeds; their exact nature will be elucidated in the discussion section. Surface potentials were measured in germinating high and low vigour seeds as well as in non-viable seeds as the potential difference between the embryo and brush ends. Seeds with a negative potential therefore had an embryo with less charge than their brush end, whilst those with a positive potential had a greater charge at the embryo than at the brush. Figure 52.A. shows that non-viable seeds had a potential of virtually zero, and that this did not change during imbibition. High vigour seeds began with a low potential, but after 12 hours of germination this became much greater and by 35 hours it had increased manyfold. Low vigour seeds did not show the same increase in potentials, which suggests that there may be a lag phase before they would begin to exhibit increasing potentials. At 10°C. high vigour seeds again exhibited increasing potentials as germination progressed (Figure 52.B.), but the low vigour seeds still had negative potentials. The possession of a negative potential does not mean that the seeds are dead, but indicates that they have not undergone the same pattern of germination as those seeds which do germinate vigorously and have rising positive potentials. The connection between cellular activity and surface potentials will be discussed at a later stage, although it can be stated here that the low potentials in the low vigour seeds suggest a generally slow metabolism.

4. DISCUSSION

The results presented in this thesis indicate that low vigour is a serious disorder of wheat which can be induced by agricultural mishandling, especially post-harvest drying damage. The metabolism of carbohydrates is severely lesioned in low vigour plants and this may be a major contributory factor to the syndrome.

4.1. THE INFLUENCE OF SEED SIZE AND VARIETY ON VIGOUR

The maximum possible vigour of a seedlot is ultimately determined by the genotype, although environmental and developmental factors are likely to prevent the expression of this ideal. Thus it is not surprising that different varieties of a species will vary in their vigour as each has been bred for different attributes, such as yield, stress tolerance and disease resistance. Although the field experiments reported here were limited in their scope and in replication, the results do indicate that some wheat varieties are more likely to give rise to low vigour seed than others when grown under seemingly identical conditions. This phenomenon is unrelated to the vigour of the parent plants or to their yield. It is interesting to note that the vigour of the parents, as indicated by field emergence, was not related to their ultimate yield; the variety with the lowest emergence had the highest yield. This is at variance with the findings of other investigations (Perry 1980; Stormonth 1978).

Varietal differences in vigour have been found in several crops such as wheat, french bean, maize and groundnut (Carter 1973; Ching and Kronstad 1972; Gubbels 1974; Kooistra 1971) and these may be due to heterosis, or hybrid vigour (McDaniel 1973). A commonly cited cause of hybrid vigour is seed size; it is often assumed that the larger the seed the more vigorous will be the resultant seedling. The results from the eight varieties tested here do not support this, as the seedlot with the highest 1,000 grain weight showed the poorest field emergence. Conversely, the seedlot with the highest field emergence also had the smallest 1,000 grain weight. Reported correlations of seed size with vigour are highly contradictory (Perry 1976).

Seed size in millet had such a significant effect on seedling vigour and crop yield that it was suggested that selection for high yield be done on seed size alone (Chhina and Phul 1982). Similar results are reported for pasture grass (Lahiri et al 1982). However, seed weight in Italian ryegrass was correlated neither with vigour nor yield (Naylor 1971), as was also the case in maize (Shieh and Macdonald 1982), broad bean (Salih and Salih 1980), bird's-foot trefoil (McKersie and Tomes 1982), clover (Gaspar et al 1981) and winter wheat (Don et al 1981). The variability of the effects of seed size may be partly due to species specific attributes and also to the maturity of the seed. Small seed may have arisen from the harvesting of immature grain and this will reduce vigour (Pinthus and Sar-Shalom 1978; Singh and Gupta 1982); the wheat varieties used in this study were all harvested at maturity and this may be why no size effect was noted. Perhaps Jethro Tull was correct when he said that "Small, thin seed produced as large ears of full-bodied corn as the full-bodied seed selected with the greatest care." (Tull 1733).

4.2. INDUCTION OF LOW VIGOUR SEED

In the last twenty years more attention has been directed towards the causes of seed quality variations than ever before. The concept of vigour, as distinct from viability, has emerged as an important one and many investigations into its fundamental aetiology have been made. Most of these investigations have used deteriorated seed derived from poor or lengthy storage, or from the laboratory mimic of this - "artificial-ageing". It is now established beyond any doubt that storage conditions are a major factor in the induction of low vigour, and mathematical relationships between these conditions and the resultant seed quality have become accepted (Roberts and Ellis 1982). However, many seeds, especially cereals, harvested in one year are used as seedstock for crops to be sown in that same year; the period of storage is thus minimal and may have less influence on the vigour of the seed than other factors. Some of these factors may be beyond the control of the farmer, such as weather and pest incidence, but others are directly affected by his actions and these include harvesting, drying and chemical applications.

Whilst the effects of storage conditions on vigour, and the biochemical parameters that control it, are well documented (Roberts 1972) the influence of these other agronomical factors is less well appreciated. The field experiments reported here were an attempt to redress a small part of this imbalance.

The range of pre-harvest conditions which influence vigour is immense and has been the subject of several review articles, although the literature from which the reviewers could draw their conclusions was limited (Austin 1972; Heydecker 1972; Perry 1980). Amongst these conditions, the following feature most frequently; weather during sowing, emergence, growth, ripening and harvest; fertiliser applications, especially nitrogen and potassium; presence and activity of microbes and insects; wise or unwise use of chemical treatments and the stage of maturity at harvest. One of the best documented causes of spoilage in cereal seeds is pre-harvest sprouting, which in the U.K. alone costs around £90m per annum (Mitchell *et al* 1980). The ability of a wheat seed to sprout in the ear will depend on whether it has dried out at the 18-20 day post-anthesis stage, whether there is subsequently sufficient moisture to enable germination and whether there is a balance of chemicals inside the grain which does not favour dormancy - usually high GA levels (Adam 1980; Mitchell *et al* 1980).

Field experiments designed to induce pre-harvest sprouting were not successful, even though all of these conditions were apparently met. This could have been due to insufficient moisture in the grain during the periods between spraying since the season was particularly dry that year. Although there were no outward signs of sprouting, treatment of ears with frequent applications of 300 microMolar GA did significantly reduce the vigour of the seeds; it would appear that incipient pre-harvest sprouting could be a cause of low vigour. This is not surprising, as when a seed sprouts it has effectively begun germination and consequently the tissues will be sensitive to dessication like any growing plant. The further advanced the germination, the more severe the damage when the seed eventually dries at harvest. It would be expected that if the GA treatment had been more potent in initiating

sprouting, then the loss of vigour would have been more prominent.

The other pre-harvest treatment investigated was aphid infestation. Wheat plants were protected from insecticidal sprays throughout the season and aphids were encouraged to settle on them. It was anticipated that the aphids would drain the plant of carbohydrates and amino-acids so that less of these would be available for grain filling. Indeed the 1,000 grain weight of the harvested seed was very low but there was no effect on seed vigour. This substantiates the case for the nil effect of seed weight, and hence amount of reserves, on seed vigour. Perhaps it is the way the seed uses its reserves, and not how much it has, that is the determining factor.

Mechanical damage, caused by the ill use of a combine-harvester, was very effective in reducing both viability and vigour. An inspection of the seeds showed that for those harvested at a "normal" moisture content the commonest forms of damage were cracks and cuts in the embryo. Gregg (1954) and Moore (1972) also noted this in cereals, and found that often even the most minor injuries led to delayed germination, a reduction in vigour, encouragement of infection and a hastened loss of viability; the same has been reported for soybeans (Prakobboon 1982). If mechanical damage occurs only in certain parts of the seed, then the seedling may be able to recover. For example, if the seminal roots are severed from cereals then they can compensate by producing adventitious roots (La Rue 1935). Mechanically damaged seeds are also prone to microbial infection, hence any vigour test on such seeds which does not take this into account is likely to overestimate the vigour of those seeds. Experiments using laboratory infection have given inconsistent results (Harper and Lynch 1981; Kulik and Schoen 1982).

Mechanical damage to seeds was found to be more severe when they were harvested at a higher moisture content. The grains appeared to be squashed between the combine-harvester blades which ruptured the embryos. The opposite result has been found with maize and soybeans (Bunch 1960) and mung beans (Herath et al 1981). Both

reports indicate that the lower the seed moisture content, the more likely the seed was to fracture and shatter; it was an effect of brittleness. However, neither investigations used seed of such a high moisture content as was used in the experiments described here, and it is likely that there is a biphasic damage-moisture content curve. Below a critical moisture level, seeds will be shattered by the impact of the blades the incidence of which would be related to the moisture content, but above this level damage would increase as the seeds became squashed. It should however be borne in mind that farmers would not normally harvest seeds at the high moisture levels used in the experiment, but great care should nevertheless be taken during mechanical harvesting as even the slightest damage can lead to losses in seed vigour.

Whilst mechanical damage induced decreases in vigour and viability, post-harvest drying affected only the former. It has long been realised that low vigour is far more common during wet seasons with wet harvests (Wellington and Bradnock 1964); this could be due to increased pre-harvest sprouting, as discussed above, or due to heat damage inflicted during essential grain drying. Experiments using a commercial large-scale grain drier on seedlots of wheat at three moisture contents showed that only those seeds with the highest moisture content of 26% were affected by hot air drying. The loss in vigour was substantial, with seeds dried at 50°C. having 58% vigour and seeds dried at 80°C. having only 47% vigour compared to the seeds dried at ambient temperatures. Not only was the grain quality related to initial moisture content and drying temperature but the duration was important too. Laboratory experiments using 25% m/c seed dried at a constant 65°C. showed that there was an increasingly deleterious effect of drying up to 4 hours, after which further drying gave no apparent increase in damage. Within this four hours, the longer the drying period, the more ungerminated seeds and abnormal seedlings appeared during germination at a stress temperature. This loss of vigour was reflected in the field performance where seeds dried for 4 hours at 65°C. had less than 25% of the emergence of the seeds dried at ambient temperatures with seeds dried for shorter periods having correspondingly higher emergence. Whilst the drying

at high temperatures had a clear effect on vigour, in no case was viability significantly reduced and this drying damage would not be detected using the official germination tests. Similar effects of rapid drying damage have been found in sugar beet seeds (Austin and Longden (1968).

As was stated above, the intensity of the damage was governed by the relationship between moisture content, temperature and duration. Unfortunately, only a restricted number of seed moisture contents could be used for the commercial scale experiments, and only the wettest seeds were affected; it would have been interesting to have found the precise range of moisture contents over which such damage would occur. However, other workers have investigated similar relationships and their findings are completely compatible with those reported here. If "dry" wheat seeds are heated at 100°C., then no loss in quality is observed for up to one hour (Siegel 1950). A mathematical approach has been used by Nellist (1980, 1981) to predict loss of viability in commercial grain driers. He found that at a constant temperature and moisture content seed deaths are normally distributed with time, so that deaths can be predicted by the integral of the normal distribution. He then extended this relationship for variable temperatures and moisture contents, and found that his predictive equations were highly correlated with experimental results and that his relationships were similar to those described by Ellis and Roberts (1980) for seed viability under various storage conditions. Although these equations are very efficacious for predicting viability losses during grain drying, they take no account of the decreased vigour, which will precede any viability losses. Similarly, the British Ministry of Agriculture, Food and Fisheries leaflets concerning grain drying (M.A.F.F. 1970, 1971) recommend a temperature of 49°C. as the maximum for drying wheat if it is to be used as seedgrain; whilst this temperature will not usually reduce viability, the experiments described above indicate that vigour may well be impaired.

The discussion above has been concerned with drying damage that is brought about when seeds are dried to a "normal" moisture content, that is agriculturally typical. If seeds

are over-dried then a further type of damage may occur on imbibition, reputedly caused by the rapid inrush of water (Simon and Raja-Harun 1972). This has been found in broad beans and spinach (Orphanos and Heydecker 1968), soybeans (Obendorf and Hobbs 1970), cowpeas (Ellis et al 1982) and peas (Ellis and Roberts 1982). In most cases, rehumidifying the seeds before imbibition prevented the occurrence of damage. Although there are no documented cases of over-drying lesions in cereals, care was taken to rehumidify all artificially dried seeds used in the experiments described to ensure that this type of problem was not responsible for the induction of low vigour.

Little attention has been paid to the physiological or biochemical causes of drying damage in seeds. An early investigation by Iljin (1957) suggested that cell membranes tend to stretch excessively and break during rapid drying, and that during imbibition such seeds took up water too rapidly and thus lost membrane integrity. An interesting discovery by Wellington and Bradnock (1964) was that the inner tissues of the embryo were most likely to be affected by drying damage and of these the scutellum was the most significantly affected. This topic will be discussed in greater detail at a later stage.

4.3. GERMINATION AND GROWTH OF LOW VIGOUR SEED

Following the realisation that low vigour could be induced most potently by drying damage, and that this could be reproducibly performed in the laboratory, the remainder of the work was aimed at elucidating the characteristics of this low vigour seed as opposed to the unheated, and hence high vigour, seed. The statutory germination test requires cereal seeds to be germinated under optimum conditions of moisture and temperature and when this was done for the seeds described above, no difference was noted between them. Germination was always higher than 92%, whether seeds were germinated in moist vermiculite, on blotting paper, in agar or on drip-slides. Both types of seed were, therefore, of equally high germinability. As the germination temperature was raised above or lowered below this optimum, the germination of both seed types was reduced as a result of exposure to stress. Under these

circumstances the low vigour seeds performed conspicuously less well, so that at 5°C. the high vigour seeds had about 85% germination but the low vigour ones had only 55%. This relatively poor performance under stress is a sign that a seedlot is of low vigour. Similar responses to germination temperature were found by Stormonth (1978) for high, median and low vigour winter wheat seeds. These results confirm the opinions of Delouche and Baskin (1973) that loss of germinability is preceded by a loss of vigour. It could be argued that the high temperature drying treatment enforced secondary dormancy on the seeds, which would then give the impression of loss of vigour. This can probably be discounted for two reasons, firstly that the seeds had high germination under optimum conditions i.e. dormancy was not apparent then, and secondly that low germination temperatures usually have the effect of breaking dormancy (Maguire 1972) so that a higher % germination would be expected at 10°C. than at 25°C. which was not the case.

4.3.1. Drip-Slides: A New Vigour Test

The germination of low vigour seed has been shown to decrease with decreasing temperatures, but this does not give any indication of the performance of those seeds which do germinate. To find this out, some estimate of growth rate is required; commonly used parameters are shoot or coleoptile length (Perry 1980), or hypocotyl extension rates (Matthews and Hayes 1982), both of which have been shown to correlate with field emergence. Whilst these are easy to measure they are extremely time consuming, as many plants must be used in order to gain a statistically significant sample; additionally, plants must be disturbed during measurements. A better method is to monitor fresh weight changes of large numbers of seeds during germination and the subsequent growth period, under sub-optimum conditions. Such methods have been attempted in the past with some degree of success (Goldsworthy and Dover 1980; Hegarty 1973; Tekrony and Egli 1977).

In order to measure fresh weight changes in germinating high and low vigour seeds, a new apparatus was designed which enabled the seeds to germinate and grow to a considerable height upon perspex slides. These

"drip-slides" have several advantages over previous methods for fresh weight determinations. Firstly, they are non-destructive so that the same plants can be sampled time after time. Secondly, the fresh weight changes can be easily monitored for up to 300 plants at the same time. Thirdly, plants may be grown at any temperature in the presence or absence of light. Fourthly, because the seeds are supplied with a continuous stream of water, any water soluble germination inhibitors are washed away, as are leachates which would otherwise encourage the growth of interfering microorganisms. In this respect, there is no reason why solutions other than water should not be used to feed the seedlings; this would be useful for studies concerning the effects of mineral deficiencies or various pesticide treatments, for example. Finally, the seedlings produced are uniform, in that they have all been subjected to similar conditions especially of water supply; in blotting paper tests some seeds are placed in areas of higher free water content than others, which can lead to erroneous differences in germination rates.

In order for a vigour-test to be acceptable it must fulfill certain conditions (Matthews 1981). The results must be repeatable and relate to field emergence; the test must be simple and give better predictions of field performance than the germination test. The drip-slide technique fulfils all of these and as such is recommendable as a vigour-test. However, care must be taken in any claim for such a test, as history has shown. Many vigour-tests have been devised (Ching and Danielson 1972; Ghosh *et al* 1981; Moore 1972; Woodstock 1973) which have been useful for limited numbers of species or environments. Unfortunately, the results of one test do not necessarily correlate with those of another when applied to the same seedlots (Don *et al* 1981; Goldsworthy *et al* 1982). For these reasons it is suggested that several measurements of vigour be used if accurate predictions are required (McKersie and Tones 1982). Such variations are probably due to the different forms of stress that a seed encounters. Low vigour may only be expressed in stress conditions (Abdul-Baki and Anderson 1972) and what one defines as stress depends on the species; peas may be tolerant to low temperatures but maize may die under the same conditions (Heydecker 1977;

Perry 1972). Differences between cold stress tests and osmotic stress tests, noted in the experimental work here, may be due to the seeds being partially imbibed under osmotic stress so that some repair to lesioned tissues can occur before germination begins; this cannot occur under cold stress (Hegarty 1977).

Using the drip-slide apparatus the growth rates of high and low vigour seedlings were investigated, at two temperatures in the light and dark. The maximum growth rates for both high and low vigour at 25°C. were identical, in both light and dark, which reflects the situation with germination i.e. at the optimum temperature there is no difference in the viability of either seed type. The low vigour seeds, however, whilst showing the same maximum rates, lagged about a day behind the high vigour ones suggesting that some essential processes had to occur before germination could begin. Lag phases in the activity of seeds with reduced vigour have been noted by other workers (Smith and Bray 1982). Such a lag probably allows repair to lesioned parts of the seed so that it may successfully germinate; this will be discussed in greater detail later. Under stress conditions it is likely that this vital repair does not occur to a sufficient extent, and this would explain both the extended lag phase and the poor growth of low vigour seeds at 10°C. Whereas the germination of low vigour seeds was 70% at 10°C. their maximum growth rate was less than half that of their high vigour counterparts, in the dark. It is clearly the case that those low vigour seeds which do germinate do not grow as well as high vigour ones; drying damage does not only affect viability but growth performance too. Measurement of fresh weight changes at 10°C. makes a very sensitive vigour-test. The maximum growth rate was increased to about 70% of the high vigour rate when seeds were germinated in the light, implying that a supply of embryonic photosynthate can alleviate the poor growth symptoms of low vigour seedlings.

4.4. RESPIRATION IN GERMINATING SEEDS

Measurements of oxygen uptake of germinating seeds have shown that at both 25°C. and 10°C. the rates of uptake in low vigour seeds are significantly lower than in high vigour seeds. Correlations between oxygen uptake and vigour have been found in several other studies (Abdul-Baki and Anderson 1972; Ellis and Hanson 1974; Harrison and Perry 1976; Kittock And Law 1968; Matthews and Collins 1975; Woodstock 1969; Woodstock and Grabe 1967). The reasons for poor uptake in low vigour seeds are not known, but an insight into the pattern of respiration changes in normal germinating seeds, indicates possible problem areas for low vigour seeds.

During early germination, both high and low vigour seeds show triphasic patterns of oxygen uptake which have not previously been described for wheat. These three phases can probably be attributed to the following metabolic events:

Phase 1 is characterized by a sharp rise in respiration over the first few hours, from the undetectable rates in the dry seed. It is probably due to the activation and rehydration of mitochondrial enzymes associated with the citric acid cycle and electron transport chain. At this stage mitochondrial structure is reforming (Opik and Simon 1963), but this may be impaired in low vigour seed (Abu-Shakra and Ching 1967) especially under stress. The freezing of membrane lipids including those of the mitochondria has been cited as the cause of chilling injury in imbibing seeds of chill-sensitive plants (Lyons *et al* 1964). Although wheat seeds are not normally chill-sensitive, there may be an increase in membrane viscosity which slows down lesion repair in the cold, and hence we find slow respiration in the low vigour seeds at 10°C.

Low vigour seeds have reduced amounts of certain important respiratory enzymes, such as malic and alcohol dehydrogenases and cytochrome c oxidase (Throneberry and Smith 1955). Levels of cytochrome c reductase are low in dry seeds (Wilson and Bonner 1971) and probably limit

respiration rates, but these levels rise rapidly on imbibition. Mayer (1973) has suggested that the activation of cytochrome c reductase could be a controlling factor of germination and that this activation may be dependent on very early protein synthesis. In low vigour seeds, protein synthesis is impaired during early germination (Ching 1982). Also important during phase 1, is a rise in ATP levels (Pradet et al 1968) which may be needed for protein synthesis and hence the rapid synthesis of new respiratory enzymes. Low ATP levels are reported in low vigour seeds (Ching 1973; Ching 1982) and this may be due to their mitochondria not being able to carry out oxidative phosphorylation as their mitochondria are uncoupled (Abu-Shakra and Ching 1967). However, respiration during the earliest stages of germination does not involve oxidative phosphorylation and is largely anaerobic (Mayer 1977) so that ethanol accumulates as a result. It may be significant that low vigour seeds have been found to be less tolerant to ethanol than those of high vigour (Ellerton and Perry 1983).

Phase 2 is a lag phase in which oxygen uptake hardly increases. Apart from some unpublished results using infra-red gas analysis (Goldsworthy and Dover - personal communication) it has not been reported before for wheat. Many other studies on the respiration of germinating cereals have not shown lag phases (Bewley and Black 1978), but this may be due to the investigators not taking readings at the critical times. If they were using highly vigorous seeds, then the lag phase could be very short in which case it would be easy to overlook. It is considerably longer in low vigour seeds, and accounts almost entirely for the difference in the pattern of respiration at 25°C. between the two types of seed. Bewley and Black (1978) have reviewed the possible causes for the lag phase and conclude that it is a period of temporary anaerobiosis during which new and efficient mitochondria are formed. By efficient, they imply mitochondria that are fully coupled as opposed to the preformed mitochondria which are either partially or completely uncoupled. Abu-Shakra and Ching (1967) found that the uncoupled mitochondria from low vigour soybean seeds were never replaced; this abnormality was passed on to all new

mitochondria. This was not the case with vigorous plants, where all new mitochondria were completely coupled. The explanation for the extended lag phase in low vigour seeds may be that as protein synthesis is slow (Ching 1982), the formation of new mitochondria is correspondingly slow.

The final phase begins when the radicle protrudes through the pericarp and testa and continues with a rapid, linear increase in oxygen uptake. Once the outer, oxygen impermeable, layers are ruptured, the seed can take up oxygen rapidly and begin wholly aerobic respiration. The continued rise in uptake is fuelled by new mitochondria in the dividing cells of the growing embryo. The rate of increase in high and low vigour seeds is the same at 25°C., implying that growth and mitochondrial efficiency are similar. At 10°C. the low vigour rates never match those of the high vigour seeds suggesting that the mitochondria are either fewer in number or less effective. From the discussion above it would seem that both are probable, as the both repair and synthesizing machineries are impaired when the low vigour seeds are stressed.

4.5. SUGAR LEVELS IN GERMINATING SEEDS

During the early phases of germination there is no mobilization of endospermic carbohydrates, so that all respiration must be fuelled from embryonic reserves. The only two sugars which were found to decrease during this period were raffinose and sucrose, presumably they were utilized for respiratory purposes. Dissection of the seeds showed that at this early stage, both sugars were virtually exclusive to the embryo. Analyses of the sugars in dry and germinating seeds of some 81 species have shown that the principal sugars stored in the embryo and used during these early stages are the tetrasaccharide stachyose (galactose-galactose-glucose-fructose), the trisaccharide raffinose (galactose-glucose-fructose) and the disaccharide sucrose (glucose-fructose) (Duperon 1955). All of the species contained sucrose, 70 contained raffinose and 35 contained stachyose; (no stachyose was found in wheat). All three sugars are reported to be important in the early germinative processes of cotton, especially in respiration (Doman et al 1982).

There were no differences in the levels of these sugars between dry high and low vigour seeds. The drying damage did not destroy embryonic sugars in the present work and there was no sugar loss with low vigour caused by excessively long storage of barley seeds (Abdul-Baki and Anderson 1972). This implies that there is the same amount of potentially respirable substrate in both types of seed and hence the reduced respiration rates in the low vigour seeds cannot be due to insufficient substrate. These low rates are therefore due either to ineffective mobilization of the embryonic sugars or to lesions in the respiratory machinery. The results presented in this thesis do not completely resolve this problem, but it is likely that there is an element of each fault involved. The early utilization of both raffinose and sucrose is delayed in low vigour seed, with the high vigour seed utilizing its' stores in half the time. This slow hydrolysis of the sugars may be due to low hydrolytic enzyme activity or to a build up of the end products of hydrolysis (the monosaccharides which would normally be rapidly respired) which in turn prevent further substrate breakdown by product inhibition. If the latter were the case, an increase in the concentrations of these monosaccharides would be detected, so that their levels were greater in low vigour seeds than in the high vigour ones. This was never so, as high vigour seeds always had higher levels of both glucose and fructose, and the fact that galactose was never detected in either type suggests that once the sucrose and raffinose were hydrolysed the products were very rapidly metabolised. This leads one to the conclusion that the sucrose and raffinose hydrolysing enzymes are impaired in low vigour seed, possibly as a result of heating. As these enzymes are required during the very earliest stages of imbibition it would be reasonable to expect them to be present in the quiescent seed in an inactive form, rather than be synthesized *de novo*. Endogenous seed proteins may indeed be affected by hot air drying as has been shown with dehydrogenase enzymes (Wellington and Bradnock 1964). Additionally, any damage to vacuolar membranes could cause the release of digestive enzymes on imbibition thus causing the proteolysis of essential stored enzymes. Further work is required to elucidate whether these enzymes are indeed activated at imbibition, and if so, whether hot air drying

could partly denature them. There may, of course, be some *de novo* synthesis of these enzymes at a slightly later stage of germination. It has already been established that protein synthesis is impaired in low vigour seeds (Ching 1982). Thus the low vigour seeds may be failing for three reasons; by having disrupted respiratory machinery; by partial denaturation of pre-existing sugar hydrolysing enzymes and by being unable to make good the enzymic lesions because of inadequate protein synthesis.

Apart from raffinose, which became exhausted early on as it did in germinating barley (James 1940), the levels of the sugars investigated all increased during later germination and subsequent growth. The initial source of these sugars must have been the endosperm which would explain why their levels did not increase until germination was well advanced; barley seeds show similar sugar increases but fail to do so when the endosperm is separated from the germinating embryo (James 1940). The first sugar to rise in concentration was maltose, which was not surprising, as it is a direct product of starch hydrolysis. Glucose, another product of starch degradation, rose slightly later reflecting the fact that it is the product of maltose hydrolysis. The amount of maltose per grain was usually higher than that of glucose, which probably indicates that the glucose was rapidly taken up by the scutellum and metabolised to other compounds. This is substantiated by the observation that sucrose and fructose levels rose at the same time as those of glucose; the only source of these two sugars is endospermic glucose, which is their precursor, suggesting that glucose was indeed rapidly converted to sucrose and fructose amongst other compounds. Further evidence comes from experiments where embryos were fed with radioactive glucose and amongst the first compounds to become labelled was sucrose. Similarly rising levels of sucrose, glucose, maltose and fructose with increasing germination time have been reported in rice (Palmiano and Juliano 1972; Nomura *et al* 1969), cotton (Doman *et al* 1982) and barley (Abdul-Baki and Anderson 1970; James 1940). It is reassuring that the more sophisticated techniques of HPLC indicate similar results to the studies using simpler colourimetric assays for sugars.

The observation of rising sugar levels applied to both high and low vigour seeds germinated at optimum and sub-optimum temperatures. However, there were substantial quantitative differences between these levels in the two seed types. At 25°C. the major difference was the presence of a lag phase in the low vigour seeds' sugar production which put them about one day behind the high vigour seeds, which is the same length of time noted for their lag in fresh weight accumulation. In fact, all four growth curves (high and low vigour at two temperatures each) were similar in shape to the corresponding graphs of sugar content, suggesting a close relationship between sugar content and growth. At 10°C. this lag phase was much extended in low vigour seeds, and they never accumulated even half of the sugar found in high vigour seeds. From these graphs it is difficult to resolve whether the low sugar levels were the cause of the poor growth or whether they were merely a reflection of the generally inadequate metabolism of the stressed low vigour seeds. However, when glucose *concentrations* were ascertained per unit fresh weight of plant, it was found that they were much lower in low vigour plants, indicating that for a certain size of plant there was less free glucose available for further growth. This suggested that a lack of glucose was limiting the fresh weight increases in low vigour seedlings. The relationship between fresh weight and sugar concentration was not investigated for the other sugars, but as glucose is either the product of or precursor for them, it would be expected that similar results would be obtained. The sucrose content of germinating oat, wheat, barley and rye grains has been shown to correlate with vigour (Crocker and Barton 1957).

4.6. ALLEVIATION OF LOW VIGOUR WITH EXOGENOUS SUGARS

The discussion above leads to the conclusion that the poor growth of low vigour seedlings is in part due to insufficient sugar. By supplying these sugars, it should be possible to alleviate the symptoms. Experiments with isolated embryos showed that, in part, this was the case.

When isolated embryos were incubated in a sugar-free buffer solution their growth was slight, but there was still a significant lag between the increase in fresh weight of the

low vigour embryos and that of the high vigour ones, suggesting that factors other than sugar supply affect vigour. As expected, this lag was accentuated when the embryos were incubated at 10°C. In either case there was little difference between the growth patterns of embryos incubated in glucose or sucrose solutions. As soon as sugar was added to the medium the rate of increase in fresh weight rose dramatically, a phenomenon noted before in isolated embryos (Bray 1979). It is interesting that after the first 24 hours incubation in the presence of glucose or sucrose at 25°C., the increase in fresh weight for high vigour embryos was about twice that without sugar. This implies that during normal germination, i.e. when the embryo is attached to the rest of the grain, the amount of sugar available to the embryo is a rate limiting factor in its growth, as the endosperm carbohydrate reserves are not yet mobilized. It would seem likely then, that sugar level control of growth is not unique to low vigour seeds.

Low vigour embryos did not grow as well as high vigour ones at 10°C., but they did grow much better than they had done when attached to their endosperms. Their maximum rate of fresh weight increase was 79% of the high vigour maximum rate, when incubated with glucose, and this fell slightly to 71% in sucrose solutions. This can be compared with 38% of the high vigour rate in sugar-free media and 48% for whole seeds grown on drip-slides. It seems that by supplementing germinating low vigour embryos with exogenous sugar, some of the symptoms may be alleviated. Siegel (1953) found that by adding sucrose to heat-injured kidney beans he could reduce or overcome their otherwise poor germinative performance. The addition of sugar to the incubation medium of low vigour embryos did not remove the lag phase, but it did reduce its duration. This indicates that the lag is not caused by lack of sugars, which concurs with the evidence from the measurement of the concentrations of endogenous sugars in the freshly imbibed seed, as discussed before. Events during this period, such as protein and nucleic acid synthesis, could be fuelled by the adequate embryonic sugar supplies, but lesions in their mobilizing systems might lead to poor initial growth. The observation that the lag phase is shortened by the addition of exogenous sugar suggests that in the intact seed,

recovery from the poor start may be hindered by acute sugar starvation. The exogenous sugar may help to generate ATP which could be used for protein synthesis and thus allow the embryo to grow properly.

Although this sugar supplementation alleviated the syndrome, it did not cure it, which indicates that either the heating damage has irreparably affected some of the seeds, or that sugar shortage does not constitute all of the problems in low vigour seed. There is probably some truth in both possibilities. Sugar utilization is one of the earliest metabolic events to be impaired in low vigour barley embryos (Abdul-Baki 1969) and was therefore investigated in these low vigour wheat embryos.

4.7. THE CARBOHYDRATE METABOLISM OF THE SCUTELLUM

Several studies of the metabolic activity in low vigour seeds have suggested that one of the first lesions to appear is that of an inability to fully utilize exogenous glucose (Abdul-Baki 1970; Abdul-Baki and Anderson 1970; Anderson 1970; Siegel 1953) as well as endogenous sugars (French 1959). The inability to properly utilize the sugars always preceded any loss in viability and was not related to the amount of sugar taken up, which was the same in high and low vigour seeds. These studies were carried out at non-stress temperatures and so at first sight might seem at variance with the results discussed in the previous section. However, in each case the rate of sugar utilization was measured during the first few hours of imbibition i.e. during the lag phase which features so prominently in low vigour seeds.

The scutellum is the organ most directly concerned with sugar metabolism in the germinating seed, and as such any damage to it could have serious consequences. The scutella of low vigour seeds were indeed lesioned in that they stained less intensely with tetrazolium than did the scutella of vigorous seeds. The same lesioning has been noted in the scutella of low vigour barley seed which, like the seeds used in this study, were induced by high temperature drying damage (Wellington and Bradnock 1964). Preliminary studies, using iodine staining of moist starch

in order to map the presence of water, have shown that most of the water leaving the seed during the drying process goes through the scutellum and hence out of the embryo. As the water evaporates from the surface of the embryo, it would cool the embryo so that this part of the seed may remain cooler than other parts. The scutellum, however, would remain as moist as the embryo but would not benefit from the evaporative cooling, thus staying hot and moist for a longer time which would be likely to induce tissue damage.

Scutellum damage was not only noted by histochemical staining, but was also indicated by measuring the conversion of [^{14}C] glucose to labelled sucrose in scutella of high and low vigour seeds which had been germinated at 10°C. and 25°C. Sucrose was synthesized by the scutella at an early stage after the application of the labelled glucose; this has been found in other studies with wheat embryos (McDonnell 1980). At 25°C. there was a conspicuous lag phase in the production of sucrose by low vigour scutella compared with high vigour ones, but by 100 hours of germination the amount of labelled sucrose was the same in both. The early differences were not due to a lack of glucose uptake by the low vigour scutella, as approximately the same amount of glucose was taken up as was by the high vigour tissues. However, this could have been due to the passive diffusion of glucose into the free spaces between the cells, so that it may not be a reflection of the activity of the energy requiring glucose uptake mechanisms. The situation was quite different at 10°C. with the low vigour scutella again taking up as much glucose as the high vigour ones but now they produced far less sucrose. Their performance had hardly improved by 200 hours of germination. The gross sucrose synthesizing capacity of germinating low vigour seed scutella appears to be severely lesioned, and again, the situation is exacerbated at a stress temperature.

4.7.1. Sucrose Metabolism in the Scutellum

The previous experiments using labelled glucose indicated that the scutellum is likely to be the site of sucrose synthesis during germination. This has been appreciated

for nearly 25 years (Edelman et al 1959) and over this period much effort has been applied to the mechanisms involved. All the enzymes necessary to convert endospermic glucose into sucrose are present in the scutellum of germinating wheat (Keys 1959) and a scheme for this synthesis is shown in Figure 53. There are three sucrose synthesizing enzyme systems known and activities of two of these were found in this study. No activity of the third enzyme, sucrose phosphorylase, could be detected despite numerous attempts, so that it seems it does not exist in wheat scutella. There have been a few unconvincing early reports of the activity of this normally bacterial enzyme in higher plants (see Whittingham et al 1979) but it has not been discovered in careful and comprehensive studies (Hatch et al 1963).

Two pathways which could bring about sucrose synthesis are thus in existence in the scutellum and it is a matter of considerable speculation as to which one is the most important and why there should be two systems at all. Since the early reports of the discovery of sucrose synthetase (Cardini et al 1955) many studies have been made of this enzyme, most of which report a K'_{eq} for sucrose synthesis of between 1 and 8 (Akazawa and Okamoto 1980) dependent on pH (Pontis 1977). The K'_{eq} in this study varied between 1.4 and 1.9. The reaction is clearly reversible, and would favour sucrose synthesis particularly if any sucrose produced was rapidly loaded into the phloem. However, tracer studies suggest that this is not a synthetic enzyme *in vivo*. The reaction requires that free fructose be a precursor of sucrose, but experiments using either photosynthetically assimilated [^{14}C] CO_2 (Whittingham et al 1979) or [^{14}C] glucose in cereal seeds (Edelman et al 1955) show that sucrose becomes labelled before free fructose. Furthermore, the levels of sucrose synthetase are higher in the non-photosynthetic tissues (sucrose utilizing) of mung-bean plants than they are in the actively sucrose synthesizing photosynthetic tissues (Delmer and Albersheim 1970). Perhaps the main function of sucrose synthetase is sucrose degradation. Arguments presented by many other workers also point to a sucrose degrading role for sucrose synthetase. If this is so, then it might seem peculiar that the enzyme has a K'_{eq} that is

slightly in favour of synthesis. It must not be forgotten though, that *in vitro* studies do not necessarily reflect the real situation *in vivo*, where the rapid removal of UDPG could drive the reaction in favour of sucrose breakdown. For example, in potato tubers sucrose synthetase catalyses sucrose cleavage more rapidly than sucrose synthesis at physiological pH (Pressey 1969). If sucrose synthetase is working in the reverse direction in this way, then one of its major functions could be the generation of nucleoside diphosphate sugars and to allow the cell to use the energy of the glycosidic linkage in sucrose for the synthesis of other saccharides. As Pontis (1977) has pointed out, the evidence suggests that there is a strong link between increasing polysaccharide synthesis and increasing sucrose synthetase levels. In germinating cereals, these nucleoside diphosphate sugars are very important for cell wall polysaccharide biosynthesis (Grimes *et al* 1970), and for the early starch synthesis observed in the scutellum (Doman *et al* 1982; French 1959). However, when UDP and the other nucleoside diphosphates are available, sucrose synthetase will preferentially synthesize UDPG rather than any of the other nucleoside diphosphate glucoses, even if ADP, TDP, CDP or GDP are present in amounts greater than the concentration of UDP (Grimes *et al* 1970). So the reverse reaction of sucrose synthetase would not generate the other nucleoside diphosphate glucoses necessary for polysaccharide synthesis.

The energy of the glycosidic linkage of sucrose could still be used for the synthesis of the other nucleoside diphosphate sugars if UDPG were used for the formation of glucose 1-phosphate (G1P) by the following steps:



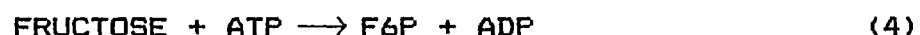
Fructose, released by sucrose degradation, could also be used to generate G1P by the following reactions:



The enzymes for these reactions are (1) sucrose synthetase (2) UDPG pyrophosphorylase (3) fructokinase (4) glucosephosphate isomerase (5) phosphoglucomutase, and all are present in the wheat scutellum (Keys 1959). The G1P would then be converted to various nucleoside diphosphate glucoses by the respective pyrophosphorylases. Versions of this scheme are proposed for starch synthesis (Duffus and Cochrane 1982; Fekete and Vieweg 1979) in the developing grain. There is one major drawback to this theory which is represented by the presence of an active inorganic pyrophosphatase in plant cells, which has been shown to be present in the endosperm during cereal seed development (Baxter and Duffus 1973). If pyrophosphate and pyrophosphatase are not separated by some form of compartmentation then the scheme will not work. There must, therefore, be some inhibition of pyrophosphatase activity, if sucrose synthetase is to generate the other nucleotise sugars in the way described above. Any inhibition of pyrophosphatase will disrupt general phosphorus metabolism and will consequently affect such biochemical events as protein synthesis. Although it is possible for this to happen, it seems a drastic method for the cell to employ in order to bring about interconversions of sugar nucleotides. It is more likely that there is a specific enzyme which effects this interconversion without biochemical disruption, but as yet it is unknown. Any enzyme which could carry out a reversible transfer of glucose from either ADPG or UDPG to a common substrate could, in effect, cause the equilibration of the two nucleoside diphosphate glucoses. The whole field of sugar nucleotide metabolism in germinating seeds warrants a careful investigation.

Studies on the K'_{eq} of sucrose phosphate synthase show that the reaction is fully displaced towards the synthesis of sucrose 6-phosphate ($K'_{eq} = 3,250$) and, unlike sucrose synthetase, it is not freely reversible (Pontis 1977). Additionally, as it is an allosterically controlled enzyme, it would be able to increase its activity as the concentrations of its substrates, UDPG and F6P, increased during periods of active photosynthesis (Preiss and Kosuge 1970). Sucrose synthetase could not do this. These results suggest that sucrose synthesis is mediated via sucrose 6-phosphate (S6P) synthase and that it is not synthesized from free fructose by sucrose synthetase.

This study has shown that both sucrose synthetase and sucrose phosphate synthase/phosphatase are present in the scutellum at the same time, which has also been noted in rice scutella (Nomura et al 1969). In both of these studies the levels of the former enzyme was always less than that of the latter ones and declined during germination, whereas the sucrose phosphate synthase/phosphatase enzymes increased. This was not so in rice scutella where the levels of all three enzymes increased with advancing germination (Hawker 1969). If, both systems were active together at the same location, then a futile cycle would be set up in which the following reactions all took part:



The enzymes involved are (1) sucrose 6-phosphate synthase (2) sucrose 6-phosphate phosphatase (3) sucrose synthetase (4) fructokinase, and the reactions lead only to the hydrolysis of one molecule of ATP to ADP and Pi. Consequently, without some form of compartmentation this futile cycle will run. There are two possibilities which would avoid this situation. The first is that in the

scutellum, sucrose synthetase is involved with sucrose synthesis and not degradation as is proposed in other tissues. As sucrose synthetase does not use ATP in its synthesis of sucrose, whereas sucrose phosphate synthase/phosphatase does, it would make energetic sense for at least some sucrose synthesis to occur in this way. This could be brought about by the regulation of sucrose levels or that of magnesium. High magnesium ion levels inhibit sucrose synthetase in the degradative direction (Whittingham et al 1979), so that high magnesium concentrations in scutellum cells would prevent its operation in the futile cycle. A study of magnesium concentrations in the scutellum would give an indication as to whether this was probable.

The second possibility is one of actual cellular compartmentation, that is sucrose synthesis via S6P might only occur in the cells adjacent to the phloem thus facilitating easy phloem loading. Sucrose synthetase might not be present in these cells, thus avoiding the futile cycle. These possibilities are still a matter of speculation, and the real relationship between the two pathways in the germinating cereal scutellum still remains to be solved.

There are no previous reports of the effects of low vigour on the activities of these enzymes. One early indication that there might be some connection, was the observation by French (1959) that in heat-damaged barley embryos the rapidity of starch accumulation during the first 18 hours of germination was much reduced, compared with unheated embryos. As has been suggested above, sucrose synthetase, amongst other enzymes, could be intimately involved in starch synthesis, and measurement of its activity showed that it was low in stressed low vigour seeds. It is not known whether any sucrose synthetase is synthesized *de novo* at germination or whether it is a remnant from the developing seed. In either case, it might be expected to be of low activity in low vigour seed as it may have been partly denatured by the heating process, and as we know protein synthesis is impaired in low vigour seeds. Sucrose phosphate synthase and sucrose phosphate phosphatase are synthesized *de novo* on germination (Nomura et al 1969) and

so it was not surprising that the activities in low vigour seeds were low at both sampling intervals at 10°C. and were also low after 50 hours of germination at 25°C., but they had caught up with the activities of the high vigour seeds by 100 hours. This again represents a lag period during which protein synthesis is delayed in the low vigour seed. Similar findings were noted for UDPG pyrophosphorylase, which suggests that it may also be synthesized *de novo*, though this has not been investigated. The activity of invertase was extremely low in both high and low vigour seeds, which further indicates the sucrose synthesizing nature of the scutellum. Invertase was also found to be extremely low in germinating rice scutella (Nomura et al 1969).

The measurement of the rate of incorporation of [^{14}C] glucose into [^{14}C] sucrose indicated that sucrose synthesis was poor in low vigour seeds, especially when they were germinated under stress conditions. This can now be explained in part by the observation that the activities of most of the important enzymes involved in sucrose metabolism are low in the same seeds, and again the situation is worsened by germination at 10°C. No single enzyme was responsible for the low sucrose synthesis, indicating that drying damage does not, at least in this case, specifically affect one protein. It is not surprising that the embryos of low vigour seeds have difficulty in growing successfully if they do not receive sufficient carbohydrate, in the form of translocatable sucrose, from the endosperm. A lesioned scutellum would more than likely lead to very slow growth or death from starvation.

4.8. THE PRODUCTION OF *ALPHA*-AMYLASE DURING GERMINATION

Even though the scutellum of low vigour seeds is less able to convert glucose to sucrose than its high vigour counterpart, it may still bring about some conversion. For this to occur a supply of glucose is required, from the endosperm, which is the ultimate carbohydrate source for the growing embryo. The glucose is released by the action of hydrolytic enzymes on the starch granules and, as has been stressed before, *alpha*-amylase is the most important

of these enzymes. Any significant inhibition of the synthesis of *alpha*-amylase would have a serious effect on the carbohydrate supply to the embryo. When low vigour seeds were stressed the increase in activity of *alpha*-amylase per grain was indeed inhibited. This was not merely a reflection of poor growth but an inhibition of the increase of the activity per unit fresh weight of plant. This suggests that the biochemical machinery that brings about *alpha*-amylase synthesis is affected to a greater extent by loss of vigour than other parts of metabolism which bring about fresh weight increase.

A decline in the amylolytic activity of deteriorating seeds has been noted in other investigations (Anderson 1970; Ayers *et al* 1976; Ching 1982; Harrison and Perry 1976; Siegel 1950). Such reductions in the activity of *alpha*-amylase could be due to any of three factors: an actual decrease in the amount of *alpha*-amylase protein; a reduction in the specific activity of the enzyme caused by conformational changes in the protein, or an increase in the concentration of inhibitors. Protein inhibitors of *alpha*-amylase have been reported in several species (Blano-Labra and Iturbe-Chinas 1981; Maeda *et al* 1982) and could be responsible for the decline in *alpha*-amylase activity. The loss of easily available calcium ions has been cited as a cause for depressed amylase levels in aleurone cells (Jones and Jacobsen 1983) and a new group of *alpha*-amylase inhibitors, the purothionins, is known to affect calcium levels *in vivo* (Jones and Meredith 1982). Low calcium levels may be detrimental to *alpha*-amylase synthesis but so are very high calcium levels (Irshad and Sharma 1981). Perhaps a study of the availability of calcium ions in low vigour seeds would yield interesting results.

An investigation into several fundamental properties of *alpha*-amylase showed that there were no differences between the enzyme extracted from high vigour seeds and that obtained from low vigour seeds. The K_m 's, temperature and pH optima, antigenicities and the isoenzyme complements were all identical. Other workers, using starch-gel electrophoresis (Ellis 1979), found no differences in the electrophoretic behaviour of proteins from high and low

vigour seeds (Ellis - personal communication) suggesting that loss of vigour was not connected with the physical properties of the protein molecules. These results strongly suggest that the low activity of *alpha*-amylase is simply due to a reduction in its synthesis during the germination of stressed low vigour seeds.

4.8.1. Some Biochemical Properties of *Alpha*-Amylase

As was mentioned above, several biochemical properties of *alpha*-amylase were investigated in order to compare the enzymes of high and low vigour origins. The *alpha*-amylase K_m for *beta*-limit-dextrin has not been reported before and so cannot be compared with previous findings. However, the pH and temperature optima have been studied in one other investigation (Sargeant 1980a) and the values of pH 5.7 and 58°C., found in this study, agree closely with these.

The most extensively investigated feature of cereal *alpha*-amylase, in recent years, is that of the isoenzyme complement. Analytical isoelectric focussing showed that there were two distinct groups of isoenzymes. Group 1 was designated as those isoenzymes with a pI of 6.0 - 6.5 and group 2 comprised isoenzymes with a pI of 4.5 - 4.8. Various workers have named the groups of isoenzymes in different ways, but to avoid confusion all isoenzymes will be referred to as group 1 or 2, with group 1 having the more basic pI. Several investigations have discovered the existence of two isoenzyme groups in cereals (Daussant *et al* 1983; Jacobsen and Higgins 1982; Kruger 1972, 1976; Olered and Jonsson 1968, 1970) and it is reported that there is a third group with an even more basic pI (Daussant and Renard 1972; Daussant and Hill 1979). There was no evidence for this third group of enzymes in the present study, although attempts to identify it were made.

The two groups of isoenzymes differ in various ways, such as possessing slightly different pH and temperature optima and different thermal stabilities (Jacobsen *et al* 1970; MacGregor 1978a; Marchylo *et al* 1976; Sargeant 1980a). A recent thorough study of the properties of each group has shown that they differ in other ways too, such as charge characteristics, serological typing, peptide mapping and

time course of production (Jacobsen and Higgins 1982). In the present study it was found that group 1 isoenzymes appeared before group 2 ones during germination. This has been discovered in other studies too (Daussant *et al* 1980, 1982; MacGregor 1978b). Evidence from radiolabelling and immunoprecipitation has proven that both groups of isoenzymes are synthesized *de novo* during germination (Daussant and Corvazier 1970; Daussant and MacGregor 1979) and it now seems likely these two groups of isoenzymes are encoded by two groups of multiple genes (Jacobsen and Higgins 1982; Miyata and Akazawa 1982).

The evidence presented above strongly suggests that the two groups of isoenzymes are real, and are not artefacts of extraction processes. This latter point was also made by Manners (1979) who stressed that the greatest care must be taken in attempts to isolate multiple isoenzymes. He points out that isoenzymes are unlikely to be duplicate forms of the same enzyme for precisely the same function. Thus the question arises, what is the function of these isoenzymes in germinating wheat?

Studies on the binding of *alpha*-amylase isoenzymes to intact starch granules have indicated that whilst group 1 isoenzymes can easily adsorb to the granules, group 2 isoenzymes do not (Sargeant 1979; Sargeant and Walker 1978). As adsorption of the *alpha*-amylase is a pre-requisite for the digestion of undamaged starch (Manners 1979), this means that group 2 isoenzymes would be unable to participate in the amylolysis of undamaged granules. Sargeant suggests that the function of the group 2 isoenzymes might therefore be to attack the soluble starch that is released by the action of the group 1 isoenzymes on the intact granules. This would explain why the group 1 isoenzymes are released before group 2 during germination, as some degree of amylolysis by group 1 must occur before group 2 could have a role. However, recent work by Weselake and Hill (1983) has shown that if the isoenzymes are isolated by affinity chromatography, rather than by isoelectric focussing, then both groups bind equally well to intact starch granules. They suggest that this is due to the removal of *beta*-limit-dextrin which may still have been bound to the non-catalytic site of the group 2

isoenzymes: isoelectric focussing does not effect this removal. It is this non-catalytic site, they surmise, which facilitates the adsorption of the protein to the starch granule. If this is so, it indicates that the theory of Sargeant is not quite correct, although the primary function of the group 2 isoenzymes could still be to degrade solubilised starch.

4.8.2. Localisation of *Alpha*-Amylase isoenzymes in Grains

For many years it had been accepted that the scutellum was the source of diastase in germinating grains, but then the secretory role of the aleurone was revealed (Paleg 1960). From this point, until recently, the role of the scutellum in amylase secretion was thought to be subservient to that of the aleurone. Modern techniques of electron microscopy, starch film gels and immunohistology have now shown that the scutellum plays a very significant part in the digestion of endosperm starch.

Using the starch film technique, Okamoto and Akazawa (1979a) found that *alpha*-amylase was first released by the scutellum of germinating rice grains, and only after two days of germination did the aleurone begin to secrete the enzyme. Using the same method, Okamoto et al (1980) found the same pattern of release in barley, wheat, rye, oats and maize, and they concluded that the scutellum was more important than the aleurone in early germination. They also found that the first part of the aleurone to secrete *alpha*-amylase was that part nearest to the embryo, and suggested that this was because it would have been the first to have received the GA signal from the embryo. Electron microscopical studies on germinating sorghum have also shown that endosperm modification begins at the scutellum first (Glennie et al 1983) and have indicated that in this species the scutellum is solely responsible for releasing all of the *alpha*-amylase.

The use of monospecific antibodies has proven that in barley the initial *alpha*-amylase is indeed secreted by the scutellum and that the aleurone only produces the enzyme at a later stage in response to GA (Gibbons 1980, 1981). In the present study, monospecific antibodies were raised

against the two groups of isoenzymes and were used to map the positions of these isoenzymes during germination by immunofluorescence. In dry seeds no isoenzymes were found, and the only fluorescence noted was the autofluorescence of the outer seed layers, as was found in other studies (Okamoto and Akazawa 1979b). At the onset of germination the cells of the scutellum epithelium were seen to fluoresce, when stained for group 1 isoenzymes, and the isoenzymes were observed to be present in the endosperm immediately adjacent to the epithelium. The group 2 isoenzymes did not appear until a day later but were also observed to be confined to the scutellum epithelium and its immediate environs. At this stage, the aleurone was just beginning to fluoresce when stained for group 1, though only the few cells next to the scutellum did so. As germination progressed the areas of fluorescence in the endosperm increased, for both isoenzymes, until the whole endosperm, and the surrounding aleurone cells, were fluorescent. These results confirm those of other workers in that the scutellum epithelium was solely responsible for the secretion of *alpha*-amylase during the early germinative stages, and have shown for the first time that both groups of isoenzymes are released from this tissue *in vivo*.

Other investigations have provided evidence that both groups of isoenzymes are synthesized in the scutellum epithelium. By raising antibodies against the two groups, Daussant *et al* (1983) were able to show that the two antigens were present inside the cells of the epithelium. Other studies have involved extracting m-RNA from the tissue and translating it *in vitro*. The necessary m-RNA for *alpha*-amylase translation has indeed been isolated from the scutellum (Miyata *et al* 1981; Miyata and Akazawa 1982; Okamoto *et al* 1982). There is now little doubt that one of the main functions of the scutellum during germination is to secrete *alpha*-amylase, in order to enable starch degradation to occur before the activation of the aleurone is completed.

Low vigour seeds showed the same pattern of release as the high vigour ones, when germinated at 25°C., but all the events took a day longer to commence. At 10°C. high vigour seeds still gave this pattern, but low vigour seeds did

not. At this temperature, a range of abnormalities was encountered. These included the secretion of no enzyme, in ungerminated seeds; slow secretion of enzyme by all of the aleurone cells; amylase secretion by only some of the aleurone cells, and no secretion of the enzyme by the scutellum epithelium. Of these, the most common observation was that of patches of non-fluorescent aleurone cells interspersed with fluorescent ones, suggesting that parts of the aleurone were not functioning at all. It was shown that the lack of fluorescence was not due to the loss of cell contents during sectioning. It would seem that one important cause of heat induced low vigour could be the loss of function of some aleurone cells, which in turn causes a significant decrease in the amount of *alpha*-amylase. Measurements of the activity of maltase during germination showed that it too was much decreased in stressed low vigour seeds. The loss of function of the aleurone cells does not, therefore, apply only to *alpha*-amylase.

4.8.3. The Degradation of Starch Granules

The degradation of intact starch granules was studied by interference and scanning electron microscopy, and it was noticed that the two types of granule, A and B, were degraded in different ways. The small B-granules showed only surface erosion without any signs of internal decay, a phenomenon noted by others (MacGregor and Ballance 1980; MacGregor and Matsuo 1982). A contrary report by Dronzek *et al* suggested that the interior of the small granules was preferentially degraded, rather than the outside. It could be, though, that the small granules they studied were small A-granules as opposed to B-granules; two types of small granule have been identified in wheat (Meredith 1981).

The A-type granules undoubtedly had greater internal decay than surface erosion, with the equatorial groove being particularly susceptible to attack. Similar findings have been reported by others (Macgregor and Matsuo 1982; Sargeant 1980b). It seems that the first two or three lamellae on the outside of the granule get degraded, and that this allows the enzymes to enter and bring about internal decay. The outer lamellae of the granules seemed resistant to

attack, as after several days of germination a large number of "empty shells" were visible. Why these outer layers should be so much more resistant to *alpha*-amylase action is not clear, but it may be connected with their high phospholipid content (Sargeant 1980b, 1982) or the presence of inhibitory proteins (Fursov et al 1982).

Although extensive studies were made, no difference between the patterns of starch granule degradation in high or low vigour seeds was found. It seems that the ability of the enzyme to bind to and degrade intact granules is not affected by loss of vigour. This would fit in with the previous observations of the parity of physical characteristics between the enzymes extracted from the two types of seed. Low *alpha*-amylase activity is due to the insufficient synthesis of the enzyme and not to a reduction in its specific activity.

4.8.4. *Alpha*-Amylase Synthesis in De-Embryonated Grains

There could be two causes for comparatively little *alpha*-amylase in low vigour seeds, either the cells that secrete the enzyme are lesioned so that they produce less enzyme even in the presence of sufficient elicitor, or there is less elicitor about *in vivo*. It has already been shown that the aleurone cells of low vigour seeds do not function properly but this could be due to the absence of a high enough concentration of the elicitor GA. It is also possible that these cells require a higher concentration of GA to induce enzyme synthesis than is required by uninjured cells. It is known that when embryo-less seeds are incubated with a sufficient quantity of GA, they secrete *alpha*-amylase into the medium (Fadeel et al 1980; Moll and Jones 1982; Jones and Jacobsen 1982; Palmiano and Juliano 1972), but if the seed has been too rapidly dessicated it may not respond to the GA (Nicholls 1979). The scutellum shows similar responses to exogenous GA (Marchylo et al 1980).

Experiments with de-embryonated grains showed that both high and low vigour grains responded in similar ways to increasing concentrations of GA at 25°C., but that the

magnitude of the response was greater in high vigour grains. Similar dose-response curves were reported by Hardie (1975) in barley grains. It seems that concentrations of GA greater than those observed *in vivo* cannot re-invigorate the low vigour grains, a point noted by Hanson (1973) during his studies on low vigour wheat. High vigour grains showed similar responses at 10°C. but low vigour grains performed very much worse. Here too, elevated GA concentrations could not induce the aleurone cells to secrete *alpha*-amylase to anything like the same extent as high vigour ones. Clearly, low vigour aleurone cells are inherently unable to respond in the expected way when subjected to stress. Perhaps this is the same phenomenon that Nicholls noted (1979) when he reported the inability of the aleurone cells in rapidly dessicated grains to respond to exogenous GA.

Low vigour seeds not only suffer from an inability to respond to GA, but they are also subjected to depressed levels of the hormone during germination under stress. Measurements of the concentration of GA equivalents indicated that low vigour seeds germinated at 10°C. were severely depleted in the hormone, presumably because of the inadequate metabolism of the embryo. Thus not only were low vigour aleurone cells less able to respond to GA than high vigour ones, but there was less GA around for them to respond to. It must be borne in mind though, that the measurement of depressed GA levels may really have been a measure of elevated antagonist levels, especially ABA. The possible significance of this will be discussed in the next section.

4.9. THE LAG PHASE - AN INTEGRATED RECOVERY PERIOD?

One of the most noticeable events during the measurement of many biochemical and physiological processes, in this study, was the presence of an extended quiescent period at the onset of germination, in low vigour seeds. When these seeds were germinated at an optimum temperature, this lag period was often the only feature that differentiated the process under observation from that in high vigour seeds. Under stress conditions, the lag was much extended and the seeds usually showed few signs of eventually matching the

rate of the process in the high vigour seeds. The frequency of recurrence of the lag phase suggests that it serves some fundamental purpose which is essential for the survival of the seeds. It does occur in high vigour seeds too, but it is much reduced, and has been assigned to a period in which essential protein synthesis must take place (Brooker et al 1977). The lag phase in low vigour seeds is similar to period of dormancy, where a fully imbibed seed does not germinate under the conditions which are normally favourable for it to do so. This begs the question, is the lag phase merely an inevitable event caused by sluggish biochemistry or is it a physiological state of dormancy enforced by the seed during which essential repair processes occur, so that the seed may subsequently germinate in an orderly way?

Much attention has been paid to the early events of germination, but less work has been done on these processes in low vigour seeds. Most studies of the biochemistry of low vigour seeds have concentrated on protein and nucleic acid metabolism, which are indeed considered amongst the most fundamental events that occur during these stages. However, enzyme activation must take place before gene activation and protein synthesis, as preformed enzymes are needed to drive the reactions of protein synthesis. It is obviously impossible to synthesize new proteins if newly synthesized proteins are themselves required for the reactions. Such enzyme activation could be the controlling factor in germination (Mayer 1973), and may be delayed in low vigour seeds. The short lag phase of about 6 hours in the growth of high vigour wheat embryos is due to the need for protein synthesis before cell elongation can begin (Brooker et al 1977). That protein synthesis is impaired in low vigour seed is well established (Ching 1982; Dell'Aquila et al 1976). The lag phase could therefore be simply due to slow protein synthesis, in low vigour seeds.

There have been several investigations into the causes of the impaired protein synthesis in low vigour or non-viable seeds. When seeds begin imbibition they already have some stored m-RNA with which to start protein synthesis (Dure 1977), but this is probably not sufficient, nor of the right kind, to allow germination to proceed. Even if it

were sufficient and of the right type, it may have lost integrity in deteriorated seeds (Barker and Bray 1972). Soon after imbibition there is an increase in poly(A⁺)-RNA and r-RNA which should stimulate protein synthesis. Poly(A⁺)-RNA is a species of m-RNA which has been post-transcriptionally modified by the addition of a polyadenylic acid tail, at the 3'-terminus. The role of the tail is unclear, but it may be to stabilise the m-RNA molecule (Bray 1983). Wheat embryos of reduced vigour contained lower levels of poly(A⁺)-RNA during early germination, and had poorer rates of protein synthesis (Blowers et al 1980; Peumans and Carlier 1981). In a recent investigation Smith and Bray (1982) found that there was an initial decline in the concentration of poly(A⁺)-RNA in both high and median vigour wheat embryos, followed by a rise. They suggested that the initial decline in the poly(A⁺)-RNA levels could be a prerequisite for germination, and the fact that this decline was slower in median vigour seeds would account for their delayed germination. Both the rates of decline and subsequent increase were much reduced when the median vigour seeds were germinated at 10°C., as was the rate of germination. The function for the initial decline could be to destroy the preformed m-RNA which coded for proteins required during seed development. These proteins could be without function in the germinating seed or at worst actually deleterious to it. The lesser decline in the poly(A⁺)-RNA in the lower vigour seeds may worsen their germination potential, by allowing the consequent translation of useless or harmful proteins.

Poly(A⁺)-RNA is not the only RNA species to be affected by loss of seed quality. In pea axes, loss of viability was associated with the appearance of lesions in the processing of r-RNA precursors as well as a significant loss of overall RNA synthesizing activity (Bray and Dasgupta 1976). Ribosomes were increasingly fragmented in dry rye embryos as viability declined (Roberts et al 1973), as was also the case in imbibing pea axes (Bray and Chow 1976b). Polysomes were unable to form in non-viable pea axes (Bray and Chow 1976b), and t-RNA was less able to bind to the ribosomes in low vigour seeds (Bray 1979). The loss of the elongation

factor EF-1 has been implicated in low viability seeds too (Bray and Chow 1976a; Dell'Aquila *et al* 1976; Osborne 1977). The lag phase in the low vigour seeds could be due to their need to repair these lesions.

Not only is there damage to RNA metabolism but also to that of DNA. As viability and vigour decrease, there is a corresponding increase of deoxyribonuclease (DNAase) activity which leads to lesions in the genome (Bray 1979; Roberts and Osborne 1973). The absence of DNAase activity in viable rye embryos was due to the presence of a heat-labile inhibitor, and during loss of viability the inhibitor was inactivated, thus unmasking the DNAase (Cheah and Osborne 1978). It is interesting to speculate whether the hot air drying damage used in the work reported in this thesis could inactivate such an inhibitor, thus inducing DNA lesions. In a recent review, Osborne (1982) presents evidence that DNA repair occurs within the first two hours of imbibition, which corresponds roughly to the time needed to invigorate low vigour seeds by a brief period of imbibition (Goldsworthy *et al* 1982) and that three times the amount of [Me-³H] thymidine is incorporated into DNA by 52% viable rye embryos than by 96% viable ones. By three hours the position is reversed, the 96% viable embryos incorporated ten times the amount that the 52% viable ones did. This suggests that the low viability seeds attempt to repair their genetic damage but are not wholly successful in doing so. Osborne puts forward the suggestion that the DNA could still be faulty after the initial repair, so that the DNA sequence for DNA ligase is impaired. Hence an imperfect ligase is produced, explaining the slow rate of DNA synthesis in the later stages of germination. This, she surmises, would result in a self-perpetuating lesion in the DNA from which the seeds could never recover. It is also possible that the essential cofactors and metabolites necessary for continued DNA synthesis are lost from the cells, during imbibition, because of leaky membranes. It could also be that subsequent DNA turnover is reliant on a source of ATP and, as has been discussed previously, this may not be forthcoming in low vigour seeds. This observation of very early DNA repair is one of the first reported instances of a biochemical process that is faster in low vigour (viability) than high vigour seeds. It would

seem apparent that the lag phase in low vigour seeds is not due to a slowing down of their entire biochemical machinery, but that there is a rate limiting step. This could well be DNA synthesis, which if slowed down would, by "chain-reactions", slow down most other biochemical events which require nucleic acid and protein synthesis.

4.9.1. The Involvement of Hormones

The observation that low vigour embryos did not secrete GA equivalents to the same extent as their high vigour counterparts has already been discussed, and as was pointed out there, it is impossible to tell whether this was due to a decrease in GA itself or an increase in GA antagonists. Whichever is the case, it does not alter the fact that there is a smaller hormonal signal from the embryo, which will cause the response by the target tissue to be correspondingly less pronounced. As GA controls many of the biochemical processes that have been investigated in this project, it is worth speculating how the hormone levels, metabolism, and survival of low vigour seeds may be interlinked.

As was discussed in the section above, impaired nucleic acid and protein synthesis in the embryo is probably one of the major causes of the lag phase in low vigour seeds. There are no reports of the effects of vigour on the corresponding metabolism of the aleurone cells. It is known that GA promotes an increase in the m-RNA which codes for *alpha*-amylase in isolated barley aleurone layers, and that this is directly related to an increased rate of *alpha*-amylase synthesis. Consequently a decrease in the amount of GA released by the embryo would result in decreased metabolic activity of the aleurone. The reason for the possible decrease in GA levels is not known but it is interesting to note that in two studies, GA synthesis was linked to the degree of sucrose metabolism in the scutellum (Ingle *et al* 1964; Radley 1969). The aleurone would therefore not begin its enzyme synthesis until the signal from the embryo was received, which in turn would not occur until the metabolism of the scutellum was in a sufficiently active state. This is logical since it would be of no advantage, or possibly even dangerous for, the

aleurone to release hydrolytic enzymes if the scutellum could not metabolize the products; the endosperm would be awash with unusable, but osmotically active sugars.

Any speculation on decreased GA levels in low vigour seeds must be accompanied by a consideration of the effects of elevated levels of ABA. Any bioassay of the concentration of "GA equivalents" is really a measure of the relative amounts of promoter and inhibitor; to resolve which is dominant would necessitate a careful chemical investigation which was beyond the scope of this project. However, this would make a valuable contribution to our understanding of the causes of slow development in low vigour seeds.

Mevalonic acid is the common precursor of both ABA and GA, and treatments that induce germinability shift the pathways from ABA to GA synthesis (Addicott and Lyon 1969); conversely, stress shifts the pathway towards ABA synthesis (Khan 1977). An alternative possibility for elevated antagonist levels is that ABA is stored in the cell vacuoles, and when stressed the cells release the hormone (King 1982). There is no doubt that when low vigour seeds are germinated at 10°C. they are under considerable stress, and ABA may be synthesized or released as a result. Of course, if the ABA were left over from seed development and was confined to the cell vacuoles, then any treatment that disrupted the integrity of those vacuoles would be likely to liberate the ABA. As has been mentioned before, cell membranes are indeed disrupted and leaky in low vigour seeds (Ching and Schoolcraft 1968).

If ABA was in excess in low vigour seeds, then it would explain many of the observations that have been discussed above. For example, ABA inhibits DNA synthesis in wheat grains (Chen and Osborne 1970). This could explain the slow rates of DNA synthesis found in the low viability rye embryos discussed before; if ABA had leaked out of ruptured vacuoles it would prevent DNA synthesis. The observed early DNA repair may have occurred before the ABA had begun to take effect. ABA can also decrease the GA enhanced synthesis of poly(A⁺)-RNA as well as that of other RNA species (Ho 1982); it affects *alpha*-amylase synthesis (Walton 1980) and it can inhibit sugar loading into cells (King 1982). ABA also inhibits the GA induced rises in

membrane potentials (Walton 1980) which may explain the low value of the surface potentials in germinating low vigour seeds. It is easy to see that if ABA was at a high concentration in low vigour seeds, the general level of metabolism would be reduced. This would be of value to the damaged seed, as it would delay germination while lesions were being repaired.

4.9.2. The Involvement of Membranes

There is little doubt that membrane integrity is impaired in low vigour seeds (Ching and Schoolcraft 1968; Cuming and Osborne 1978b; Kaloyereas 1958). It is thus essential that membrane repair is undertaken before germination commences, else the seeds will never function correctly. In imbibed, but dormant, wild oat embryos membrane lipid and protein turnover occurs at a sufficient rate to repair any lesions that may have arisen; this dormancy was maintained by ABA and broken by rising GA levels (Cuming and Osborne 1978a,b). If this were the case in the lag phase of low vigour embryos too, it would explain why such a period of enforced dormancy would be so efficacious.

Apart from maintaining dormancy and thus allowing membrane repair to proceed, ABA could have other effects on membranes, such as controlling sugar transport. The mechanism of sugar uptake into plant cells is presumed to be one of sugar-proton cotransport, which is driven by the H^+ gradient across the plasmalemma (Baker 1978; Komor *et al* 1981). Evidence for this includes the observation that on the addition of glucose to the external medium, membrane potentials (that is the transmembrane electric potentials created by the H^+ gradient) rapidly depolarise (Hauer and Hofer 1978; Kennedy and Stewart 1982; Novacky *et al* 1978). The higher this membrane potential, the more sugar could be taken up and the higher was the carbon substrate level inside plant cells (Novacky *et al* 1978). The converse is also true, in that any agent that depresses the membrane potential will inhibit sugar uptake and therefore slow down growth (Hauer and Hofer 1978; Kennedy and Stewart 1982); wounding (Koopowitz *et al* 1975) and ABA (Ho 1982), will do this. It is interesting that the compound fusicochin can raise membrane potentials (Lado *et al* 1976), and has the

additional affects of overcoming ABA induced dormancy and alleviating the effects of temperature stress (Heydecker 1977). Perhaps there is some connection between ABA levels in low vigour seeds and the metabolism of the simple sugars, which is mediated via membrane potentials.

Preliminary experiments reported here have indicated a connection between vigour status and the seed surface potentials. As has been mentioned before, these surface potentials are not a direct measure, but are certainly a reflection, of the seeds' membrane potentials. If all of the membrane potentials in the seed were equal then the surface potential would be nil. This was the case in non-viable seeds. However, because the active cells do not have equal membrane potentials, an overall potential is generated between different parts of the seed, the size of which reflects the differences in the sizes of the membrane potentials of the individual cells. Thus a large surface potential indicates areas of cells with high membrane potentials (and hence metabolically active) which are separated from areas of cells with lower membrane potentials; in the seed this could be the cells of the scutellum versus the cells of the embryo. Because of the complex ion conducting pathways in the seed, it is impossible to locate precisely the origins of the large membrane potentials from surface measurements.

One other investigation has linked seed surface potentials with vigour. Jones *et al* (1950) found that surface potentials increased with advancing germination and that the rate of increase was proportional to the final yield of the plants. They also irradiated maize seeds with various intensities of X-rays and in so doing reduced viability; the larger the dose the greater the loss of viability. As the viability declined so did the magnitude of the surface potentials, indicating that the health of the plants was reflected by the size of their membrane potentials. High yielding maize and barley varieties have higher membrane potentials than lower yielding varieties (Morgutti *et al* 1981). The experiments described in this thesis, confirm the observations that surface potentials increase as germination proceeds and that the lower the vigour of the seeds the lower are the surface potentials. If these low

surface potentials derive from low membrane potentials, it could explain the poor growth rates of low vigour seeds by, amongst other things, sugar co-transport.

SUMMARY

1. Mechanical damage to seeds during harvesting caused a loss of viability and vigour.
2. Post-harvest hot air drying induced low vigour in high moisture content seeds, but did not affect viability.
3. The low vigour seeds had more than 90% germination at 25°C. but this decreased to about 55% at 5°C.
4. A new vigour test based on fresh weight measurements was developed, and was used to show that at 10°C. the maximum slopes of the growth curves for low vigour seeds were half those of their high vigour counterparts but at 25°C. they were identical.
5. Respiration rates were depressed in stressed low vigour seeds, but at 25°C. they were not. However, at the higher temperature a significant lag phase in the onset of increased respiration was still noted.
6. The amount of sugar stored in quiescent low vigour seeds was the same as that in high vigour ones, but the former utilized them at a slower rate during germination.
7. Endospermic glucose supplies were poor in low vigour seeds, as were embryonic glucose and sucrose levels.
8. The supplementation of sugar to germinating low vigour embryos substantially relieved their slow-growth symptoms.
9. The scutella of low vigour seeds did not synthesize sucrose as fast as those of higher vigour apparently due to the decreased activities of several important enzymes.
10. Two enzyme pathways capable of synthesizing sucrose were discovered in the scutellum, and the roles of each are discussed.
11. Stressed low vigour seeds suffered from a shortage of *alpha*-amylase during germination, but the biochemical characteristics of this enzyme were identical to those of

the *alpha*-amylase from high vigour seeds.

12. By raising monospecific antibodies against the two groups of *alpha*-amylase isoenzymes, it was found that the initial source of these isoenzymes in germinating grains of high or low vigour was the scutellum epithelium. The aleurone became important at a later stage.

13. When germinated at 10°C., many low vigour seeds were found to have some non-functional aleurone cells, which explained why the synthesis of *alpha*-amylase was reduced.

14. High concentrations of GA could not induce low vigour aleurone cells to increase their synthesis of *alpha*-amylase.

15. The possible role of hormones in the retarding of the metabolism of low vigour seeds is discussed.

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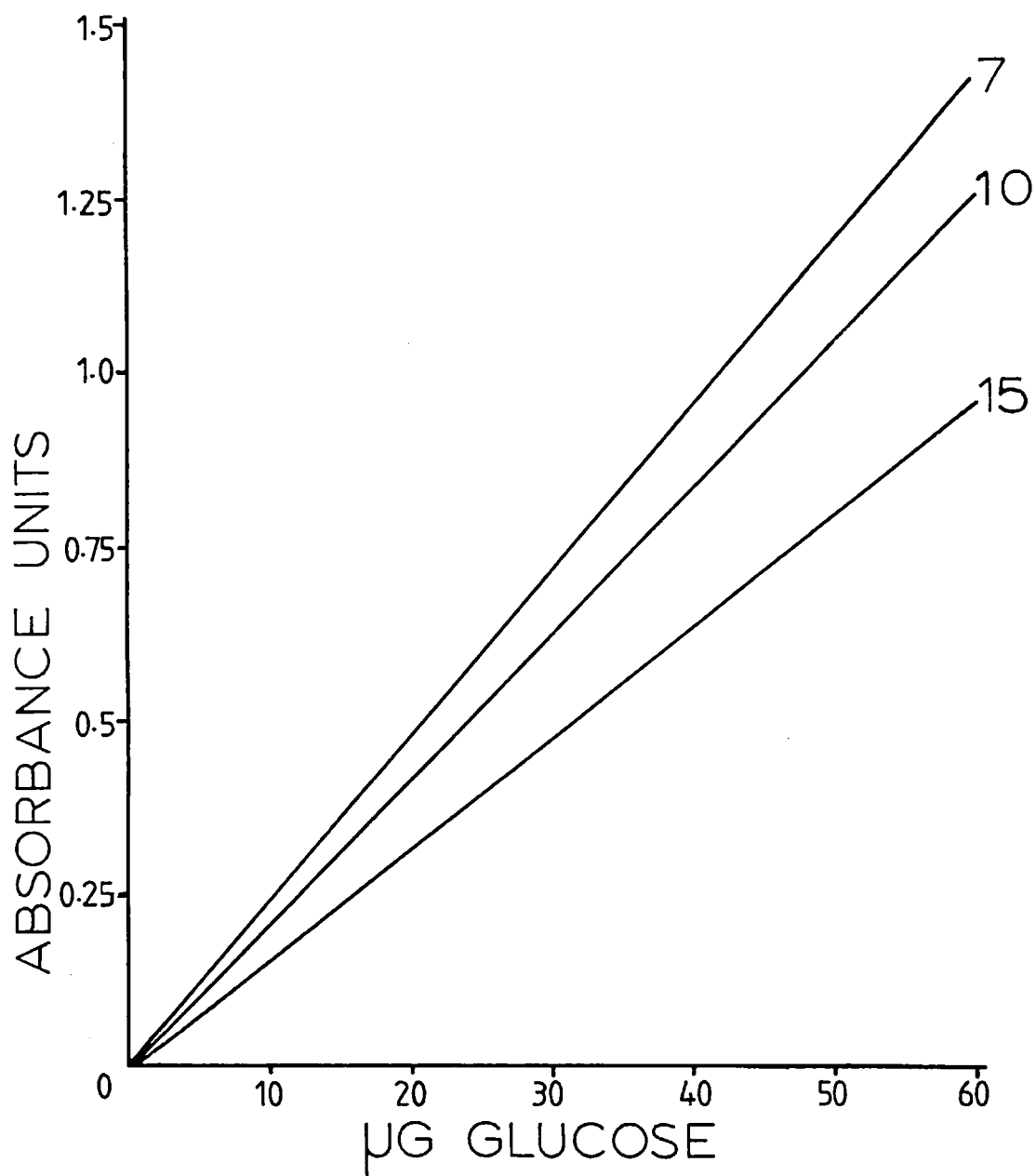
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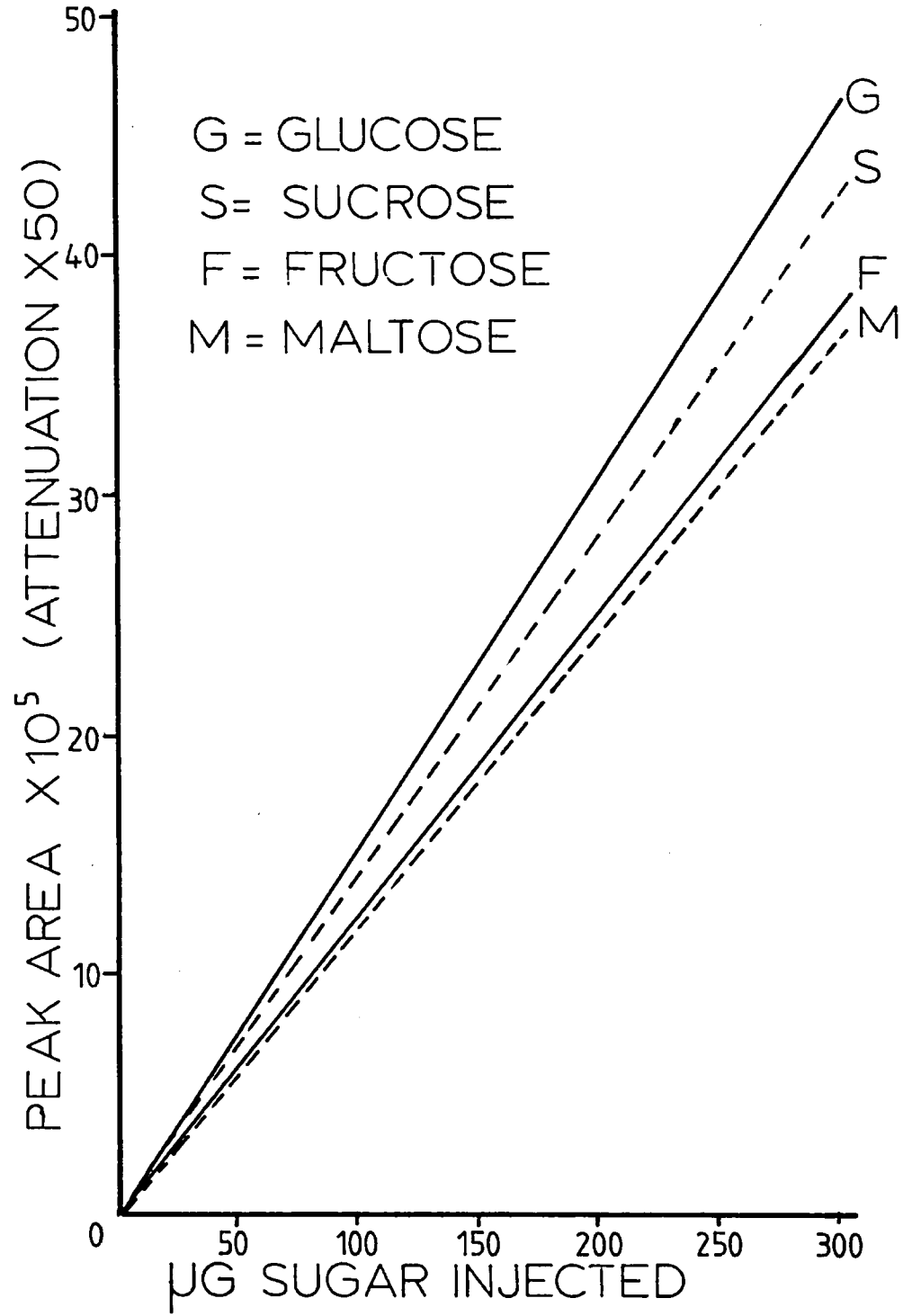
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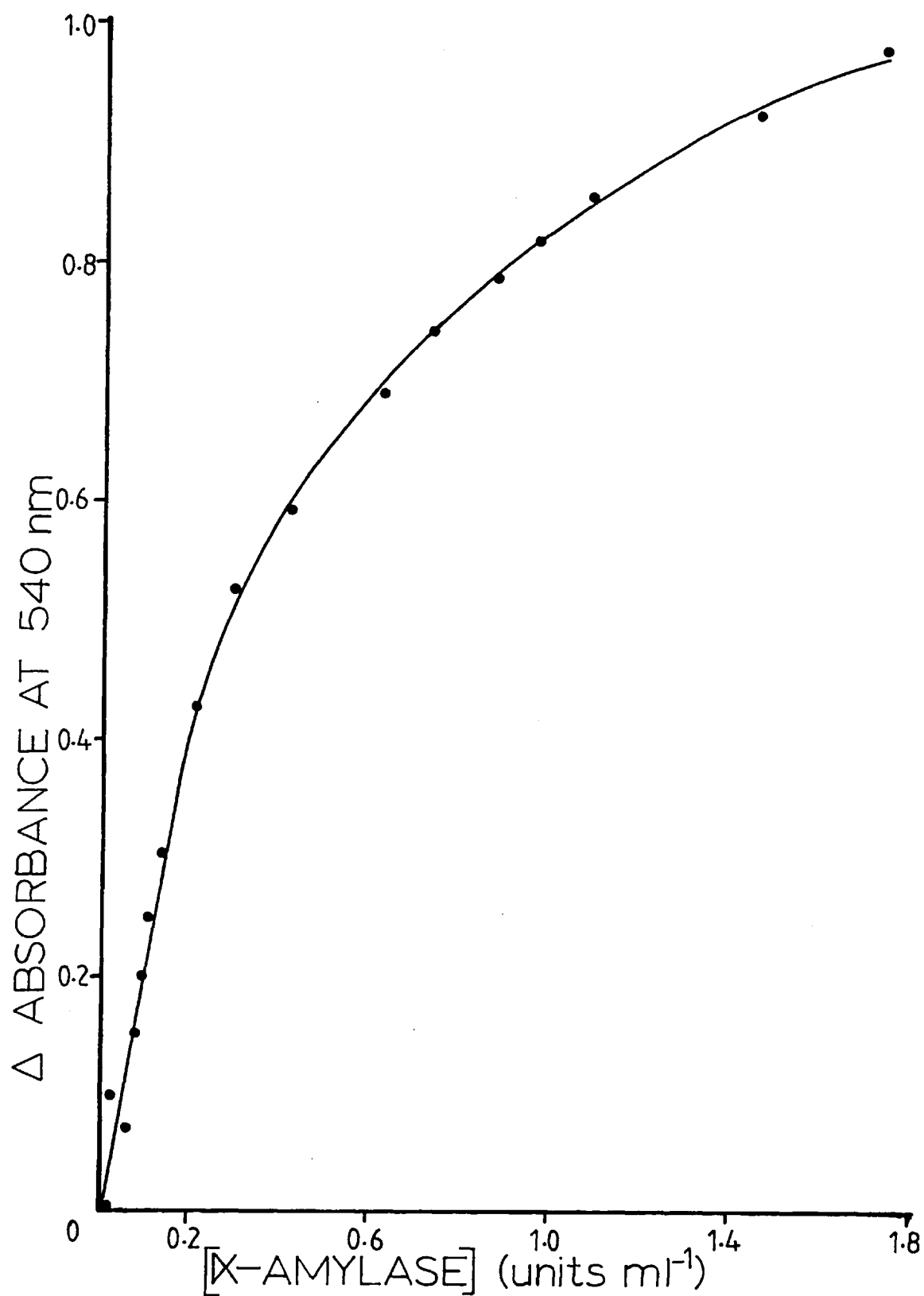
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APPENDIX A. Calibration curves (regression lines of 25 points each) for the enzymic glucose assay, for readings taken after 7, 10, and 15 minutes incubation.



APPENDIX B. Calibration curves for four sugars using HPLC separation, and quantification with a refractive index detector set at X50 attenuation. Each line is the regression of 40 points.



APPENDIX C. Calibration curve of international units of α -amylase activity versus decrease in absorbance using the *beta*-limit-dextrin assay.