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GENERAL INTRODUCTION.

This thesis is concerned with several aspects of the biology of the caryopsis of species of the Gramineaceous genus, Bromus. The bulk of the work is concerned with the carbohydrate metabolism of the grain and in particular, with the metabolism of the hemicellulosic materials known to be present in very large quantities, MacLeod and Sandie(1961). Throughout the thesis an attempt has been made to correlate biochemical findings with structural and ultrastructural investigations. In the past, many interesting discoveries have been made in biochemical and biological fields but only too often a true liaison between them is lacking. The biochemist studies a particular reaction, frequently using in vitro techniques, and often assumes that this reaction definitely takes place in the living plant; this is dangerous because it seems probable that in many cases barriers exist in the plant which are not found in the test-tube. These barriers may be structural in form, separating two compounds which come together only when the cell is ruptured as occurs for instance in the preparation of enzyme extracts. The barrier may simply be a chemical one: eg. synthesis of many carbohydrates can be induced using high substrate concentrations but it is questionable whether these very high concentrations occur in the cell. This problem of correlating the different aspects of a particular problem will be discussed again.

Up to the present day there have been innumerable studies on cereals, mainly like this one, concerned with the grain

itself. Economic influences probably led to barley being the grain most commonly examined and particular interest in the barley grain has come from the brewing and malting industries. The production of malt is dependent, basically, on the first steps in the germination of the barley grain, ie. the mobilisation of the food reserves from the main storage tissue of the grain, the starchy endosperm(Fig. 2). In normal germination, this mobilisation of food reserves is controlled by the embryo and the low molecular-size degradation products are absorbed by the embryo to supply energy giving substrates and building blocks for the growing plant. The maltster, however, wants to utilise the most important of these degradation products, the sugars; therefore he wants a maximal availability of reserves, with a minimal embryo growth rate. The most important of the food reserves present in the grain is starch but it is enclosed in large cells with thick cell walls, Fig. 3 . The degradation of reserves is consequently due to action of two main enzyme groups, those responsible for the breakdown of the cell walls of the endosperm, and the amylases which break down the starch grains.

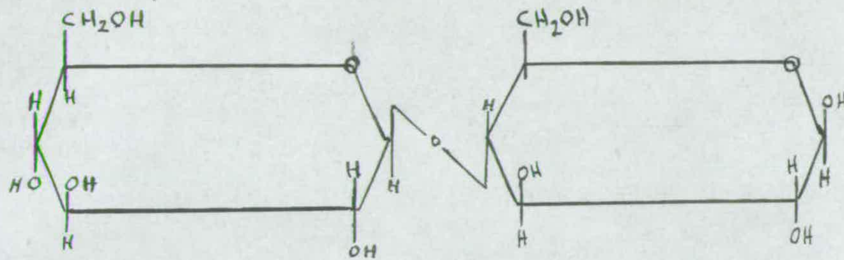
Until recently, MacLeod and Napier(1959), it was believed that the walls of the starchy endosperm cells were largely made of cellulose and consequently the enzymes which broke them down were termed cellulases. In fact, the cereal grain is frequently quoted as being a rich source of cellulase. For barley it is now known that little(less than 0.04%) if any cellulose is present in the wall of the starchy endosperm cell and it is now generally believed that the high percentage of hemicellulose found in the barley grain is the major constituent of this wall.

The reason for using Bromus for these studies is based on several factors, the main one being that the Bromus grain has extremely thick starchy endosperm cell walls and should therefore provide excellent material for the study of hemicellulose metabolism. Earlier studies have revealed that the possession of thick walls in the starchy endosperm is accompanied by a high percentage of hemicellulose and, interestingly enough, by a powerful hemicellulase system. The bulk of this work is therefore concerned with a detailed study of the biological significance of the degradation, synthesis and interconversions of these hemicellulosic materials and their intermediates.

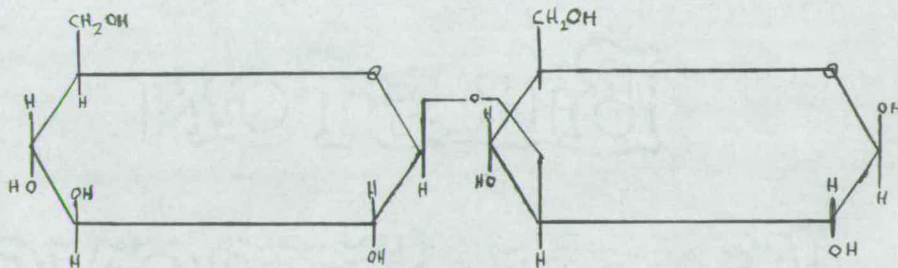
The hemicelluloses found in the cereal grain can be divided into two main groups. Firstly, there are the β-glucans which, like cellulose, are made of glucose units linked together in β-configuration to form extensive polymers. The other group, the pentosans, consists of polymers of pentose sugar units, eg. xylose and arabinose. As already stated, the glucans are similar to cellulose in that they are made up of β-linked glucose units but (in barley and Bromus) they contain not only β1:4 linkages (as in cellulose) but also β1:3 linkages. Thus the two main disaccharide units of β-glucan are cellobiose and laminaribiose, as shown on the next page. In barley, these β1:4 and β1:3 linkages appear in approximately equal numbers but they do not occur in any simple repeating pattern, Preece and Hoggan (1956).

The pentosans of the cereals contain xylose and arabinose in varying ratios depending on the species examined but the usual ratio of xylose to arabinose is 60:40. It appears that

such an araboxylan polymer often consists of a backbone chain of β1:4 linked xylosyl residues with side chains of arabofuranosyl residues linked by β1:3 bonds to the xylose backbone, Perlin(1959).



Cellobiose, 4-O-(-β-D-glucopyranosyl-D-glucopyranose.



Laminaribiose, 3-O-(-β-D-glucopyranosyl-D-glucopyranose.

Extraction techniques used for the preparation of hemicelluloses have resulted in their being classified into three main groups depending on their relative solubilities :-

i) water-soluble(40°C.) hemicelluloses,

ii) autoclave-soluble hemicelluloses,

iii) alkali-soluble(cold 4%NaOH)hemicelluloses.

As yet there in no evidence that these three fractions do in fact appear in distinct structural forms in the cell itself.

Most of the earlier studies have been concerned with the water-soluble hemicelluloses, especially β-glucan. Similarily, studies in the enzymic degradation of hemicelluloses have been

mainly concerned with the enzymes responsible for the breakdown of these water-soluble hemicelluloses.

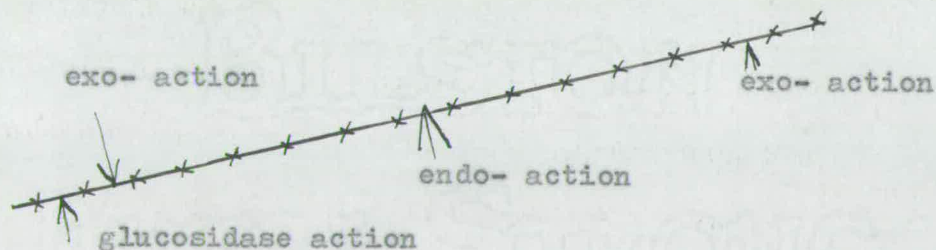
The enzymes responsible for the degradation of β-glucan can be divided into three groups :

i) endo-β-glucanases, which attack the inner linkages of the polymer,

ii) exo-β-glucanases, which attack the ends of chains, producing low molecular-weight glucosides which in turn are degraded by,

iii) the β-glucosidases, cellobiase and laminaribiase which degrade cellobiose and laminaribiose respectively, producing glucose.

It is of exceptional interest that such a high degree of uniformity exists in the patterns of enzymic degradation of water-soluble polysaccharides, for this triple stage degradation pattern is responsible for pentosan and starch degradation.



As with amylase action on starch, it has been found that even continued action of these enzymes will not breakdown a small resistant fraction of the polymer.

During earlier studies on glucanase action in barley, Garg(1958), it was found that the β1:6 linked glucodisaccharide, gentiobiose, appeared. The formation of this uncommon

disaccharide was attributed to the transglycosylation properties of the enzyme preparations. Other studies have shown a similar picture in Bromus and in fact it was shown that if the enzyme extracts were incubated with cellobiose a complete series of gluco-oligosaccharides could be synthesised, MacLeod and Sandie(1961). This powerful transglycosylation activity forms the subject of Part III of this thesis.

Much has been written about the action of the hemicellulases and their chemical action now seems clear, but it is only recently that authentic information has become available as to the origin of these enzymes and the factors which control their production. Even to-day, it is still the general belief that the scutellum is the main source of hydrolytic enzymes but recent studies, including work in this department, MacLeod and Millar(1962), has established that the aleurone layer, the layer of cells round the periphery of the starchy endosperm(Figs. 2 & 3) is the origin of a very high percentage (probably of the order of 80%) of the hydrolytic enzymes responsible for starch and hemicellulose degradation. It has been shown that contact between embryo and aleurone is necessary for the induction of hydrolase release, Schander(1934); and MacLeod and Millar(1962). It has also been demonstrated that if gibberellic acid is added in trace quantities to grain slices containing only aleurone and starchy endosperm a rapid release of hydrolases is obtained,(Yomo,1958;Paleg,1960; and MacLeod & Millar,1962). It has therefore been postulated that the release of hydrolases from the aleurone in an intact germinating grain is in response to the production of a

gibberellin from the embryo, and gibberellins have indeed been detected in the embryo, Radley(1959). The need for the translocation of a substance such as a gibberellin from the embryo to the aleurone would explain the results of Schander(1934) with his 'ringing' experiments with rice grains.

The work covered in this thesis includes a brief examination of the basic properties of the hemicelluloses and hemicellulases in Bromus ; a very extensive examination of transglycosylation powers of enzyme preparations; and an examination of hydrolase production in developing and germinating grains, and in response to the addition of gibberellic acid. At all times attempts were made to correlate findings in Bromus with studies in barley and to interprete all such findings in a biological manner. This involved concurrent structural(incl. ultra-structural) and biochemical approaches to the common problems of carbohydrate metabolism in the Gramineaceous caryopsis.

The Taxonomic Position of the Genus *Bromus*.

The determination of the exact taxonomic position of *Bromus* has always been a problem. As it has usually been found difficult to separate *Bromus* and *Festuca*, the exact limitations of the genus have still not been settled and authors such as Lindman(1926), Stahlin(1935), Hitchcock(1935) and Hubbard(1948) are at variance. Only in 1941 did Cugnac and Belval show good evidence for the correct position of *Festuca gigantea*, which has sometimes been referred to *Bromus*. Morphologically *Bromus* and *Festuca* are superficially similar, and some authors have placed both in the same tribe, the Festuceae.

In 1880-82, Harz classified the bromes close to *Hordeum*, *Elymus* and the other genera of the tribe Hordeae. Harz was generally influenced by the work of Nageli(1858) who found that the starch grains of *Bromus* were simple and those of *Festuca* compound. Nageli also found that the majority of the Hordeae have simple starch grains whereas the majority of the Festuceae have compound ones. Many later workers followed Harz's example and placed *Bromus* and *Brachypodium* in the tribe Hordeae, in the sub-tribe Brachypodiinae. Hayek gave the Brachypodieae tribal rank, the tribe comprising the two genera *Bromus* and *Brachypodium*, but his most startling innovation was the placing of Brachypodieae between the tribes Bambuseae and Triticeae(Hordeae). This implied that the two genera were even more primitive than had been shown by previous workers. His classification thus set *Bromus* farther apart from *Festuca* than any previous classification.

Some indirect evidence has been found for the relationship between brome grasses and members of the Hordeae. Cugnac & Belval(1941) discovered that Bromus contains a laevorotatory glucoside, which differs chemically and physically from that found in many other grasses, including Festuca, but is related to the triticin found in species of Agropyron(Hordeae). Stebbins(1949) has pointed out that there is a similarity in the chromosomes of the two groups. Hubbard(1934) placed Bromus with Festuca, but in 1948 he expressed doubt as to the correct position of Bromus. He pointed out that both Bromus and Brachypodium differ from other genera of the Festuceae in possessing a nucellus with an outermost cell-layer of thick-walled cells whilst the ovary bears an appendage with styles borne laterally on it. The work of Cugnac & Belval(1941) perhaps influenced Hubbard's conclusion considerably. These workers questioned the apparent similarities of Bromus and Festuca and carried out chemical and genetical studies to see if the affinities were natural ones. Besides the distinct glucosidal property of Bromus mentioned above, they also found that Festuca contained a glucoside which is found in Lolium but not in Bromus. Harz had pointed out that species with compound starch grains would not hybridise with those that had simple grains. Cugnac and Belval's findings supported this work as all of their attempts to cross members of the genera Bromus(simple starch grains) and Festuca(compound starch grains) failed.

After considering the recent work of MacLeod & McCorquodale (1958), it appears that there is good biochemical evidence for

the separation of the genus Bromus from Brachypodium. The relationships of these two genera have been under some dispute for many years, and even recently taxonomists like Clapham et al.(1952), link them together whilst Hubbard(1934) splits them into two separate tribes. However, the study of the water-soluble carbohydrates of seeds of Gramineae has established that on a biochemical basis the Bromeae form a very natural tribe, quite distinct from the Brachypodieae, Festuceae and Hordeae, as the Bromeae contain none of the trisaccharides of the raffinose-type found in these other tribes.

In the present work, Bromus inermis,(kindly supplied by Dr.W.O.S.Meredith, of the Grain Research Laboratory,Winnepeg) has been used extensively, as well as various indigenous species of Bromus.

Taxonomic Features of Bromus inermis,Leyss.

This species is divided into several sub-species, some of which are further sub-divided into varieties. Bromus inermis belongs to the Section Bromopsis of the genus Bromus.

Bromus inermis Leyss. subsp. inermis.

A native of Europe, Siberia and China, this grass was introduced into N.America about 1884. It is widely cultivated in that country as a pasture grass for hay, especially in mixtures with alfalfa, and is also used extensively in soil conservation work. Two types, a southern type from Europe, and a northern type from Siberia have been introduced. The extreme hardiness, drought resistance and long-lived perennial

habit of the species, plus the aid of man, has enabled it to become more widespread than either of its native varieties or any one of the other native species. It apparently gets its initial start wherever man has disturbed the soil. The ecomomic interest has aroused considerable attention and it has been the subject of numerous studies. Chromosome complements of 42,56 and 70 have been recorded, but in N.America 56 is the consistent number present. Evidence exists for introgressive hybridisation between var.inermis and var.purpurascens. Intermediates have been seen but are not common. The main difference between var.inermis and native varieties lies with the glabrous nodes. The regular absence of an awn from v. inermis has been disproved after a complete examination of the variety throughout its range. Both native and Eurpoean varieties are octaploid.

Bromus inermis, also known as Hungarian Brome, has been cultivated in Britain. Being drought resistant it persists on sandy and stony soils, and has become naturalised in widely dispersed localities in England. It may be distinguished from B.erectus by its extensively creeping rhizomes, hairless leaves and usually blunt, awnless, hairless lemmas.
The origin of B.inermis is likely to be from a tetraploid cross in the Old World Range, ie. in Eurasia, in areas more southerly than the present range of octaploids.

British Species of *Bromus* Examined.

The most common species of *Bromus* to be found around the Edinburgh area is probably *Bromus mollis* and although this species is obviously morphologically distinct from the Canadian supply of *B.inermis* used, it is very similar anatomically. The same applies to the other British species examined, ie. *B.sterilis*, *B. erectus* and *B.ramosus*. Studies made by MacLeod & McCorquodale(1958) and MacLeod & Sandie(1961) have shown that biochemically all these species are very similar, with only slight quantitative differences in their carbohydrates. As their structure is basically the same, *B.mollis* will be used as an example from the morphological point of view, whilst *B.inermis* will be examined and described anatomically.

PART I.

An Examination of the Structure and Metabolism of the *Bromus* Grain.

Section A - Structural Examination of the *Bromus* Grain.

i) Morphology of *Bromus* Spikelet,

ii) Anatomical Examination of *Bromus* Grain,

iii) Histochemical Examination of Aleurone-Endosperm,

iv) Ultrastructure of Aleurone-Endosperm Tissues.

Section B - Metabolism of Germinating *Bromus*.

i) Free Sugars Released During the Germination of *B. inermis*,

ii) Quantitative Examination of Sugar Formation,

iii) Metabolite Requirements of Excised *Bromus* Embryos.

SECTION A.

A Structural Examination of *Bromus* Grain.

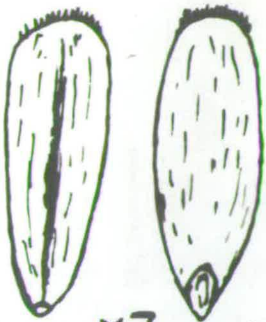
The work reported in this part of the thesis is intended to provide a background to the subsequent biochemical studies. In the general introduction it was stressed that this is a biological study using biochemical methods not as ends in themselves but in relation to the biology of the grains. It is therefore hoped that the work reported in this section can be correlated with the biochemical findings to produce an overall picture of the biology of the grain. The work has been divided into the various sub-sections listed on the previous page.

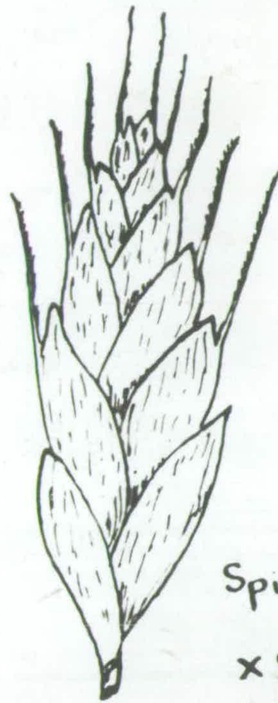
* * * * * *

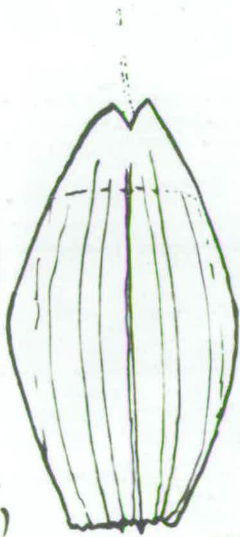
i) Morphology of *Bromus* Spikelet.

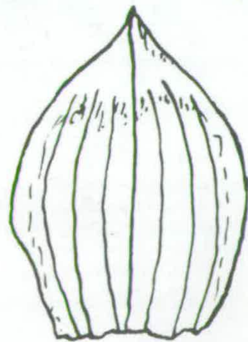
The drawings in Fig. 1 , illustrate the basic floral morphology of *B.mollis*. The essential difference between *B.mollis* and *B.inermis* is that *B.inermis* possesses blunt, awnless lemmas(cf.*B.mollis*). The caryopsis of *B.inermis* is longer(6-10m.m.) than that of *B.mollis*, giving it a more linear-oblanceolate appearance. It is darker in colour, being rich in anthocyanin and often a dark amber, to almost purple-black colour. The caryopsis of *B.inermis* is often closely adherent to the palea and lemma, making the dehusking techniques difficult(see page 41 and appendix I).

FIG. 1 - Floral Morphology of *Bromus mollis*. L.









ii) Anatomical Examination of Bromus Grain.

Although several species of Bromus were used in this study all illustrations refer to B.inermis. After soaking grains in detergent overnight, transverse and longitudinal sections were cut by hand and examined microscopically. This simple study revealed that the internal structure of the grains of the various species of Bromus was very similar but that they had certain distinctive differences from barley. These features are illustrated in Fig. 3 , and are listed on the following page.

The embryo lies at the base of the main storage tissue, the endosperm, separated from it by a distinct layer of cylindrical cells. This layer is the scutellum and has attracted considerable attention in respect of its role as a secretory layer as well as an absorptive layer,(Brown,1890; Lehmann and Aichele,1931; and Engel,1947). This matter will be considered later in Part II of this thesis.

This study, however, is more concerned with the structure and function of the starchy-endosperm and the aleurone layer. The following anatomical features of these regions are based on several transverse sections cut from different species of Bromus. Regularly noted are the following :

i) very large endosperm cells, approx.150μ long, making up the bulk of the grain,

ii) very high starch content of the true'starchy' endosperm tissue, ie.the main food reserves of the grain,/

/ iii) an aleurone made up of one layer of cells, approx. 50μ thick, (cf. barley which has 3-4 layers of cells). The aleurone cells are filled with dense contents.

iv) the relatively small number of endosperm cells per unit volume; only about four cells bridge the gap between opposite sides of the grain. This gives a high value for the ratio - number aleurone cells/ number of endosperm cells, (cf.barley). This also results in a high value for the ratio-volume aleurone/volume starch reserve, (cf.barley).

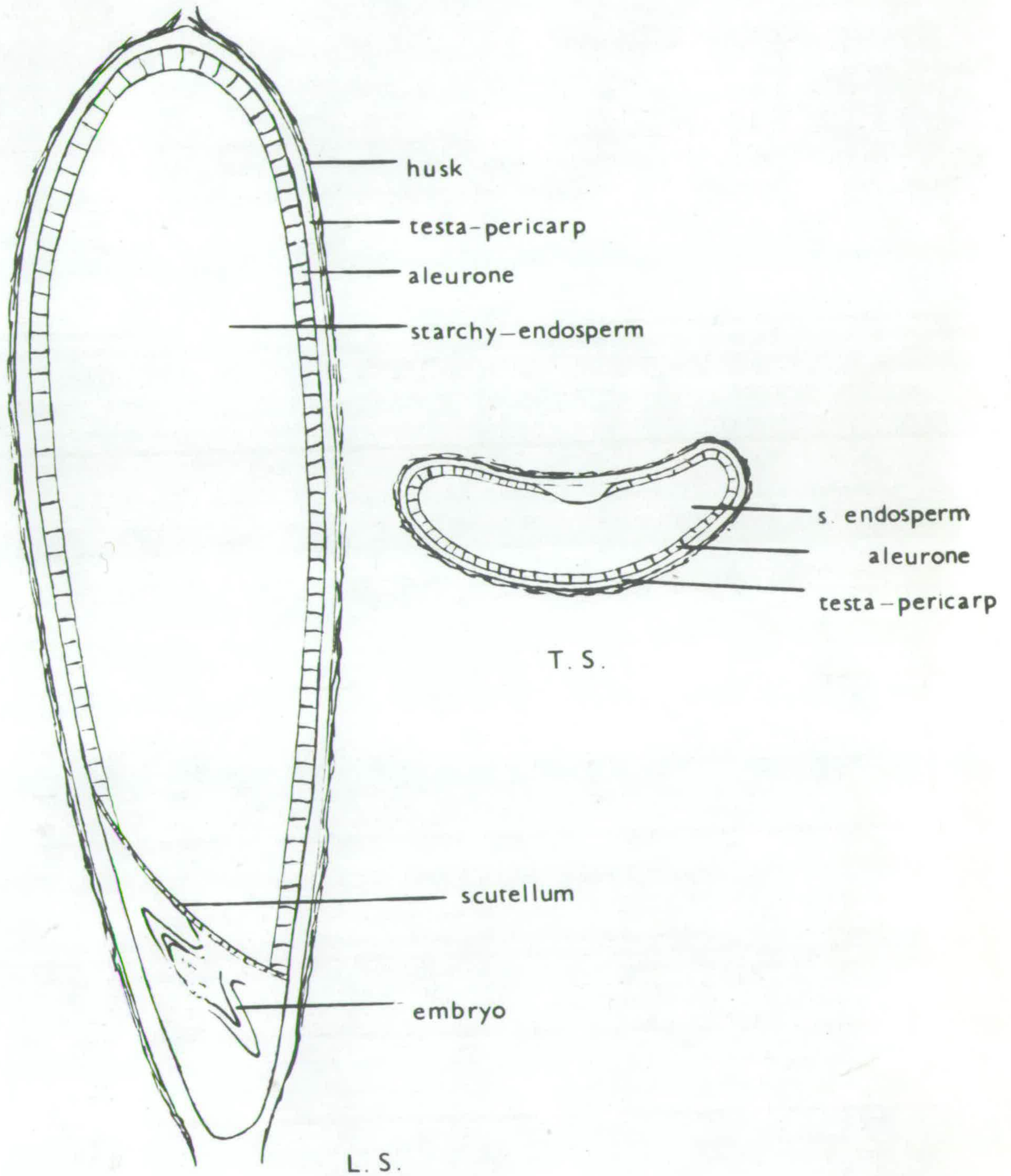
v) the walls of the starchy endosperm cells are very thick indeed; they are much thicker than those of barley, thereby probably explaining the higher percentage of hemicellulosic material in Bromus. This again means that the value - volume hemicellulose/volume starch is much higher in Bromus than in barley; emphasising the unsuitability of Bromus as a grain for the maltster but indicating that it would be an excellent grain for studying hemicellulose metabolism.

vi) outside the aleurone layer there is a very thick band of wall material, probably cellulosic, which is stratified and constitutes the fused testa-pericarp.

This next part of this thesis is devoted to a more detailed examination of the aleurone-endosperm using histochemical and related techniques in an attempt to establish the nature of the various materials present in these tissues. This is followed by a detailed ultrastructural examination, by electron microscopic methods.

FIG. 2.

Sections through Grain of Bromus inermis.



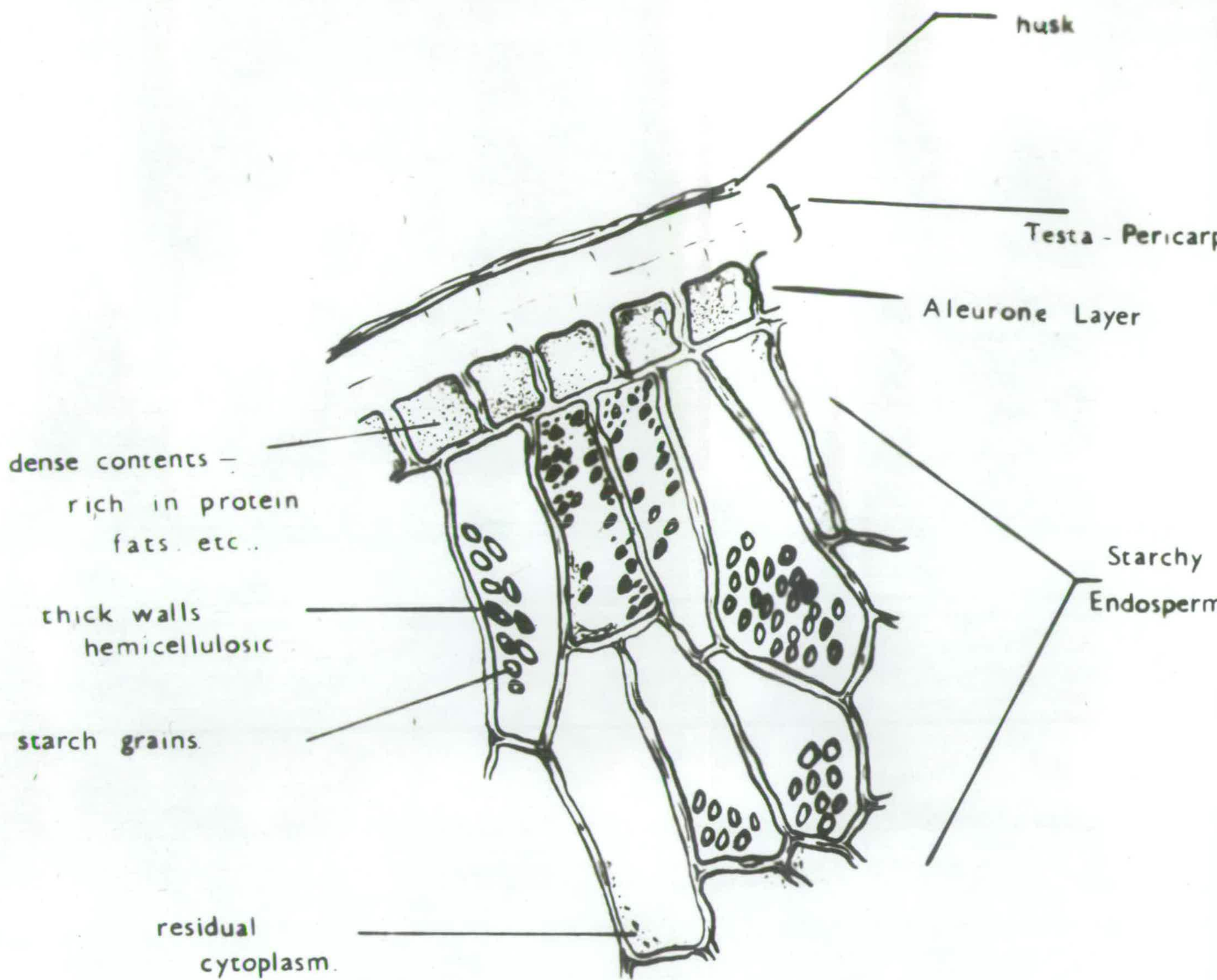


FIG. 3. High Power Section Through Grain of *Bromus inermis*. (diagram)

iii) Histochemical Examination of Aleurone-Endosperm.

A Comparison of the Cell Walls of the Aleurone and Starchy-Endosperm.

Previous studies, MacLeod & Napier(1959), have established that, in barley, the walls of the aleurone cells contain a high percentage of cellulose, whereas those of the central endosperm cells contain very little true cellulose. These findings were based on chemical analyses of successive 'rubbings' from barley grains. This present work is an attempt to correlate such chemical findings with definite observable features. The following staining methods were used :-

<u>Periodic Acid - Schiffs Test for Carbohydrate.</u>

Method. a) Stain sections for 5-30 min. in 0.5% periodic acid,

b) wash in running water, for 10 min.,

c) stain in Schiff's reagent for 10-15 min.,

d) rinse in water,

e) rinse in 2.0% sodium bisulphite for 1-2 min.,

f) wash in running water,

g) dehydrate and mount.

Results. All the cell walls gave a positive reaction with this test. The walls of the husk cells gave a deep red coloration. The endosperm walls stained weakly but distinctly. The 'sclerenchyma' type cells of the furrow were deeply stained. After treatment of sections with cold 4% NaOH all the residual cell wall material gave a positive reaction to

this test. The material removed by 4% NaOH, mainly hemicellulosic in nature, also gave a positive reaction. There was a pink coloration in the cytoplasm of the aleurone cell and there were faint pinkish areas inside the endosperm cells.
Conclusions. This test is only of value in confirming the presence of carbohydrate. It has provided evidence of the presence of carbohydrate in the cytoplasm of the aleurone cells and also traces in the endosperm, in addition to the starch.

<u>Iodine-Pot.Iodide - Sulphuric Acid Test for Cellulose</u>.

Method. a) Soak section in I_2KI(2% KI to which 0.2g. I_2 is added) for 15 min.,

b) add one drop of 65% H_2SO_4 and allow to diffuse under cover-glass.

Result. The walls of both aleurone and endosperm gave a positive reaction. The slowest reaction, with the weakest coloration, was in the starchy endosperm walls. It should also be noted that the treatment with acid caused the aleurone to split away from the testa-pericarp. This stain also gave the usual blue coloration with the starch present in the endosperm, but gave no such coloration in the aleurone.
Conclusions. This stain does not distinguish between true cellulose and hemicelluloses, but merely established the presence of such materials in both types of wall. It proved that starch was absent from the aleurone cells at this stage of grain development.

Aniline Blue Test - This stain is used to indicate the presence of β1:3 linked glucosans, such as callose(Currier,1957).

Method. Direct staining of sections in a 0.005% solution of aniline blue in 50% ethanol for varying periods of time.
Results. There was a strongly positive reaction in the cell walls of the husk but no reaction in the testa-pericarp, aleurone or starchy endosperm.
Conclusion. The finding of a possible β1:3 linked glucosan in the husk is in agreement with Aspinall & Kessler(1957). The chemical findings of other workers and those presented later in this thesis suggest that the hemicellulosic materials of the grain must be in the walls of the endosperm; this negative result therefore suggests that this stain can react only with pure β1:3 glucans and not with the β1:3 linkages of a mixed hemicellulose of the type found in barley and Bromus.

It seems probable that no histochemical test is cellulose specific to the exclusion of hemicelluloses and therefore such methods are at present of no use for establishing the presence or absence of cellulose in the endosperm cell wall.

When sections were washed in 4% sodium hydroxide a considerable portion of the endosperm cell wall was removed but there still remained a layer which gave a positive reaction with all three of the carbohydrate tests. Similarily, treatment of sections with hot 4% NaOH still did not remove all carbohydrate from the endosperm cell walls. The final residual wall material

may be true cellulose or it may be a very resistant hemicellulose. A similar residual material remains for a considerable time during the germination of the grain.

One accepted feature of cellulose in plant cell walls is the organisation of molecular chains into distinct crystalline forms - micelles and higher aggregations, microfibrils, which are detectable by electron microscopy. Crystalline organisation can be detected by using polarised light, when such materials possess birifringent characteristics. An examination of hand-cut sections of the grain was carried out using polarised light microscopy. It was found that the walls of cells in all parts of the grain were anisotropic. Optical anisotropy, as already stated, implies that molecules are in some form of orderly arrangement as in cellulose, and therefore it is reasonable to expect the walls of the husk, testa-pericarp and aleurone to be anisotropic as it has been established that they contain cellulose. On the other hand the anisotropic nature of the endosperm walls introduces an interesting problem. It implies that either they contain cellulose in large amounts unlike barley, or that they contain another component which is anisotropic. After treatment with cold 4% NaOH, these endosperm walls still possessed optically anisotropic characteristics. This possibility of a hemicellulose with an orderly molecular arrangement is in agreement with findings with other hemicellulosic materials such as the β 1:3 glucans - paramylon and callose (Deflandre, 1934; Clarke & Stone, 1960). This problem of molecular organisation in the endosperm cell wall will be considered more fully in the section on ultrastructure.

Previous studies with barley and *Bromus*, MacLeod and McCorquodale(1958), suggested that the cells of the starchy endosperm were not joined by pectic materials as they are believed to be in most plant tissues. This was based on the apparent absence of pectic materials from chemical analyses of the endosperm as well as on a series of simple microscopic observations. It was found that, in *Bromus sterilis*, when pieces of endosperm were squashed in water the individual cells separated very easily. This would imply that either the cells were only held together by the(mechanical) enclosing effect of the surrounding aleurone layer or that any 'cementing' layer was extremely soluble in water. MacLeod & McCorquodale also found that the endosperm cells of *B.mollis* and barley did not separate in pure water but that treatment with papain allowed separation under very slight pressure. This implied the involvement of a proteinaceous material as a binding agent. Up until now this theory has neither been proved nor disproved by any other workers, though similar observations for wheat have been made by other workers . Similar results were obtained during this study, using *B.inermis*, *B.mollis*, *B.sterilis*, *B.erectus*, *B.ramosus* and barley. Sections of *B.inermis* were also tested for the presence of pectic acid using the ruthenium red reaction. A positive reaction was obtained only in the husk, and not in the starchy endosperm *or* the aleurone.

A Brief Histochemical Examination of the Cytoplasmic Contents of the Aleurone - Endosperm.

It is now generally accepted that the endosperm proper is a storage tissue, whereas the aleurone layer is responsible for the secretion of hydrolytic enzymes. A histochemical differentiation of these two tissues was thought desirable. The following tests were performed :-

I. Simple iodine tests established the presence of large numbers of starch grains in the starchy endosperm but none in the aleurone,(see also page 18).

II. Lipid Crimson Test. This reagent is used to establish the presence of lipid materials.

Method. Sections were cut by hand, stained for varying times up to 30 minutes in an aqueous solution of lipid crimson then dehydrated and mounted.

Results. The aleurone layer gave a positive reaction but no reaction was observed in the starchy endosperm. Careful examination of many sections indicated that fatty materials of the aleurone were present in distinct granules about 1-2 μ. in diameter and that these appeared to be evenly distributed throughout the cell.

Conclusion. The aleurone is rich in lipid material, apparently in distinct granules. Later in this thesis this problem will be dealt with in connection with the ultrastructural findings in the aleurone.

III Protein Stains. Histochemically the plant proteins are extremely difficult to study and many so-called protein stains have been found to be far from specific.

A good general protein stain used was a 0.1% aqueous solution of Ponceau de Xylidine. Using this stain, protein was detected in large amounts in the aleurone layer. These cells gave a fairly uniform staining reaction. Deposits of protein were also detected in the starchy endosperm cells.

Another protein stain used was the combined - 2.2-dihydroxy-6.6-dinaphthyl disulphide(D.D.D.) : Fast Blue 2R stain which reacts with -SH containing proteins. A positive reaction was obtained in the aleurone and a very slight reaction in the endosperm.

IV Feulgen Staining for D.N.A.. In contrast to the non-specific protein stains, Feulgen staining is specific for D.N.A..

Method. a) fixation, usually 24 hr. in neutral formalin, 10-20% at room temperature,

b) rinse in water,

c) place in 1.N HCl for 1 minute,

d) hydrolyse in N.HCl at 60°C. for 5 minutes,

e) rinse in cold 1N.HCl for 1 minute,

f) rinse in water,

g) stain in Feulgen for 3-5 hr.,

h) rinse in 50% sulphurous acid for 10 minutes,

i) repeat (h) twice,

j) dehydrate and mount.

Results. Nuclei of aleurone cells stained intensely; all in interphase(metabolic) state. The nucleus of the aleurone cell was relatively large, about 15μ. in diameter. Small

fragments stained up in some of the starchy endosperm cells; these were apparently nuclear debris.

Conclusions. All aleurone cells possess large nuclei in the non-dividing, metabolic state. Endosperm cells sometimes contain nuclear remains but almost certainly in a non-functional form.

V. Tetrazolium Staining. This is reputed to stain viable tissues, undergoing respiration.

Method. Sections were cut and washed and then placed in a filter-tube containing 0.1% aqueous 2-iodophenyl-3-nitrophenyl tetrazolium chloride. These sections were left in this solution under vacuum until a red coloration could be seen in the sections.

Results. The aleurone cells stained intensely and if the routine was controlled carefully it was found that the staining first appeared in distinct granules(less than 1μ.) but rapidly spread throughout the whole cell.

Conclusion. This stain was reputed to indicate mitochondrial action but now appears to indicate the presence of cytochromes whether they are present in mitochondria or not. Other work in this department, Duffus(1964) suggests that tetrazolium staining is not indicative of viable grain, therefore at present no further attention is being paid to this reaction.

iv) Ultrastructure of Endosperm and Aleurone Tissues.

The major part of this work has been included in a publication, MacLeod *et al.*(1964). For this study it was decided that apart from examining the structure of the aleurone-endosperm tissues in *Bromus inermis*, similar tissues from barley should also be investigated. The reason for the addition of this cereal to this part of the study was two-fold. Firstly, it is often easy to introduce artifacts into electron microscopic techniques and therefore an inaccurate impression may be gained regarding cellular structure; but by examining two unrelated species of Gramineae known to be biochemically very similar, the chances of this error are reduced. The second reason is based on the previous history of grain studies. The majority of earlier studies have been made using barley, Preece(1957), and frequent comparisons are made between species during this thesis. At the time of this investigation biochemical changes relating to release of hydrolytic enzymes in both *Bromus* and barley have been found to be almost identical in pattern, MacLeod *et al.*(1964). It was therefore decided that if an attempt was to be made to relate biochemical findings to the ultrastructural level, both species should be examined simultaneously.

The information included in this part of the thesis is based on observations on the ultrastructure of the aleurone-endosperm of the mature grain prior to germination ; a preliminary account of structural changes during germination and in response to the addition of gibberellic acid will be given in Part II, section D of this thesis.

Technique.

The barley which was used was the variety Procter from the 1963 harvest. The grains were dehusked in 50% sulphuric acid, then 2.0 m.m. thick slices of endosperm-aleurone were cut. These slices were cut in half, through the furrow and steeped in water at 25°C. overnight. These endosperm fragments were then ground in a glass homogeniser until all the starchy tissue was suspended in the distilled water used as grinding medium. The aleurone was separated by passing the mixture through a sieve and washing the residue thoroughly : the isolated aleurone was then processed in the manner described on the following page. Preparations were also made from slices containing aleurone and starchy endosperm; these gave electron micrographs which were similar to those from isolated aleurone, and it thus appears that the separated aleurone is essentially unchanged in its ultrastructure. All barley tissue isolation and subsequent embedding was carried out in this laboratory by J.H.Duffus who is investigating biochemical changes in barley in response to gibberellic acid. Duffus' techniques of embedding were based on data obtained by the present writer from studies with Bromus.

For Bromus, caryopses of B.inermis were dehusked, washed and incubated at 25°C. overnight. 1.0m.m. slices were cut transversely through aleurone-endosperm and smaller(approx. 0.5 m.m.) fragments were cut from these slices to include aleurone and starchy endosperm cells. As these slices were prepared from endosperm-aleurone which had been in contact with the embryo for 18 hours, other fragments were examined from dry, unimbibed grain.

With the various samples of barley and Bromus

described, aleurone could be examined after the following treatments :-

i) dry, unimbibed grain of Bromus,

ii) imbibed(18 hr.) aleurone isolated from barley but separated from embryo before the 18 hr. imbibition period.

iii) imbibed(18hr.) aleurone from barley aleurone-endosperm slices, which had had no contact with embryo during imbibition period,

iv) aleurone from intact grain of Bromus after incubation for 18 hr., in contact therefore with the embryo.

The cell wall studies are based almost entirely on Bromus.

Processing of Tissue for Electron-Microscopic Examination.

Fixation. Fragments were fixed in veronal buffered 0.6% potassium permanganate, pH 7.4, at 5°C., over a range of times varying from 30 minutes to 4 hours.

Attempts were made to use osmic acid fixation but no success was obtained.

Prefixation of tissue in neutral formalin plus subsequent fixation-staining in potassium permanganate was frequently used with success.

The exact fixation methods used are given with each electron micrograph.

Dehydration. This was carried out using a series of increasing concentrations of ethanol. If any tissue had to be stored at this stage overnight it was kept in 70% ethanol at 0-5°C..

Embedding. Although it is generally accepted that permanganate fixation followed by methacrylate embedding can produce innumerable artifacts, the technique, used in this study produced excellent results. Distinct membrane preservation was achieved and results were in general similar to those obtained by other workers who used Araldite and Epon resins,(Buttrose,1963; Buttrose,1963; Paleg and Hyde,1964; and Nieuwdorp,1963.)

Dehydrated tissue was embedded in methacrylate monomer mixture (88% butyl methacrylate + 12% methyl methacrylate) by passing it through a series of ethanol-methacrylate, pure methacrylate and methacrylate:catalyst mixtures. The polymerisation of the monomer mixture was completed at 60°C.,overnight, in the presence of 1.0% dichlorobenzoyl peroxide catalyst,

Sectioning & Subsequent Treatment Prior to Examination. After a period of at least 24 hours, the polymerised blocks were trimmed and sections cut from a 1 m.m. square cutting face. A Cambridge ultramicrotome was used for sectioning, using ¼ inch thick plate glass knives. The sections cut were usually of 500Å but much of the high resolution work employed sections of 250-300Å. Cutting was onto a solution of 15% ethanol to soften sections and thereby ease spreading. Any ripples present in the sections were evened out by exposure to xylene vapour and the sections were mounted directly onto uncoated grids(Athene, type 200) and subsequently coated with a thin layer of carbon on the upper surface only. This carbon layer was to stabilise the specimen in the electron beam. The coating unit used was an Edwards Speedivac Coating Unit. The specimens were examined and photographed in an Akashi TRS 50 E1 electron microscope fitted with a specially modified

D.C. filament supply and a 50kv. E.H.T.. The photographs were taken on Ilford N50 line cut film and generally printed on Hard or Extra Hard Glossy paper(Ilford). Development of film and paper employed Ilford Contrast FF Developer at the standard dilution and a development time of 1 minute.

Observations.

Internal Structure of the Aleurone Cell.

The general structure of a typical aleurone cell from Bromus is shown in Plate I. Most of the organelles now accepted as normal in a plant cell are found in the aleurone cell, eg. nucleus with double membrane, mitochondria, plastids, endoplasmic reticulum and plasmalemma. Numerous vacuoles are found which are enclosed in distinct unit membranes, typical of plant vacuoles(Poux,1962; Graham,Jennings et al.,1962). Other unusual inclusions are also found but not all of these organelles have been identified with certainty.

Nucleus (N). The nuclei are from 15-20μ in diameter and on occasions nucleoli have been demonstrated. The nuclear envelope (N.E.) is distinctly a double layered structure, each layer being about 150Å in thickness. The outer membrane passes into the cytoplasm and is apparently continuous with the endoplasmic reticulum(E.R.), Plate I. Holes, about 500-800Å in size, are visible in the inner membrane, as indicated in Plate I.

Mitochondria (M). In Bromus(preparations from intact grain soaked overnight), membrane-enclosed organelles(M) of the mitochondrial type, 0.7 - 1.4μ in diameter, can be seen in the aleurone cells and a close examination shows that they are of the same internal structure as those reported from other plant

cells. No such mitochondria have been found in dry,unimbibed *Bromus* aleurone or in barley aleurone(slices cut from dry grain and steeped overnight). It thus seems that the presence of recognisable mitochondria depends on the developmental stage of the grain. It is possible that there is some connection between the appearance of mitochondria and the small vacuoles(V) which are often present in fairly large numbers,(MacLeod *et al*.,1964; Polakis *et al*.,1964), Plate 12 . Paleg and Hyde(1964) suggested that the apparent absence of mitochondria is due to the masking effect of the dense cytoplasm. This matter will be discussed later.

Plastids (P). Structures resembling proplastids(P) have been detected in some aleurone cells, Plate 1 , but it is possible that these are simply mitochondria of the type reported by Hrsel *et al*.(1961) to be present in the scutellum of wheat.

Vacuoles and Vacuolar Inclusions. In all specimens of *Bromus* and barley aleurone examined, deposits of a medium electron-dense material are found(Pr), Plates 1,6,8 ,12 and 35 . These deposits are irregular and are enclosed in a unit membrane, Plates 1 and 8 . Within these deposits are found regular, electron transparent,ovoid structures(V), again enclosed in a unit membrane, Plates 4,5,8 & 13 . In many of the cells of barley aleurone examined, dense structures(I) of the same shape as the transparent structures(V) are found. In barley this inclusion can vary in density from the transparent type, characteristic of *Bromus*, Plate 1 , through intermediates, Plate 4, to the intensely electron dense type seen in Plates 4 & 5. This variation in density cannot be due to a fixation artifact as the relative positions of the inclusions vary from cell to cell, ie. in one cell dense inclusions can be near the outside when the

transparent inclusions are towards the centre, in another cell the opposite may be the case, whilst in others the two types may be mixed throughout the cell(the last example is the commonest). These organelles can be interpreted in at least two ways. Firstly, there may be a developmental connection, one form changing into another as was postulated by the present writer, MacLeod et al.(1964). This view is based on the observation of a series of intermediate forms, Plate 4 (I,1-3). If this gradation is accepted there is still no evidence to suggest the possible direction of any trend. The second interpretation, Paleg and Hyde(1964) emphasises a distinction between the electron transparent and electron-dense inclusions but these workers have not published electron micrographs of the intermediate forms which have been clearly shown in this study. They support Buttroses earlier work(1963) which claimed that the medium density material was vacuolar in origin and that it and the inclusions present in it constitute the 'aleurone grain' of light microscopists. This theory is probably correct as these deposits are the only organelles comparable in size and possible chemical constitution to the 'aleurone grain'. It seems probable that the medium density material(Pr.) is proteinaceous in nature. The observable amounts of this deposit are in approximate agreement with the protein-N contents of the respective grains examined. If the transparent and dense inclusions are distinct, a problem arises not only in interpreting the intermediates of the types seen in Plate 4 , but also(since one of these membrane-enclosed inclusions is found to contain a central transparent area and a peripheral denser zone) in accounting for another possible grade of intermediate, PLATE 13.

The transparent inclusions can frequently be seen with smaller inclusions either budding off from them or fusing to them, Plates 9&10 . The direction of this mechanism is not definite at present but varying stages can be seen, Plates 9,10,11, and 12 . Small inclusions are frequently found near larger inclusions, Plate 11 or scattered throughout the cytoplasm, Plate 12 . A preliminary comparison can be made between dry,unimbibed Bromus aleurone and aleurone from imbibed intact grains. In the dry grain there appears to be few vacuoles but in many(but not all) of the imbibed aleurone cells there are often large numbers of vacuoles, small and large in size, Plates 1 and 12 . As yet no accurate short interval time course study has been made to establish whether the mechanism involves budding of large vacuoles or fusion of small vacuoles. Paleg and Hyde(1964) favour the fusion theory during gibberellic acid treatment of isolated aleurone but the present work appears to support the view of multiplication of inclusions by budding in the early stages of grain germination. This may well be followed later in development by a fusion, especially in the presence of the powerful influence of unnaturally high concentrations of gibberellic acid and the unbotanical incubation temperatures used by Paleg & Hyde(1964). This problem will be discussed in relation to germination in Part II,section D of this thesis.

Unidentified Bodies(U.B.). These small structures(U.B.) are 0.1 - 0.4μ in diameter and are present in both barley and Bromus. These regular vesicles are found in two distinct areas of the cytoplasm. They surround the vacuolar deposits(Pr) just described, Plates 1,6 and Fig.4 and also tend to be aggregated along the wall, just inside the plasmalemma,

Plates 6 and 7. When examined in the dry grain of Bromus, Plate 3; and in the isolated slices of barley, Plate 4 & 5, these vesicles appear transparent and careful examination is needed to observe a fine single limiting membrane, Plate 6, (see arrow). In this transparent form they are usually 0.3-0.4μ in diameter. Occasionally a trace of electron-dense deposit is visible in these U.B.'s. In the Bromus aleurone which comes from intact grains, after soaking for 18 hours, the U.B.'s appear to have a very dense outer layer and in some cases may almost appear totally electron-dense, Plates 1 & 8. This change from almost completely transparent to electron dense is almost certainly developmental in nature and will be discussed in Part II, section D of this thesis. This change appears to require the presence of the embryo and is not the simple hydration problem suggested by Buttrose(1963), since the U.B's remain electron transparent in barley aleurone which has been soaked overnight at room temperature without influence from the embryo.

Dense Bodies (D.B.). These small inclusions are only visible in barley aleurone cells, Plates 4,5 & 6. It is possible that they are artifacts resulting from $KMnO_4$ fixation but it is unlikely. Their possible significance is unknown but similar structures have been found in cereal embryos, Setterfield et al.(1959) and are seen in the electron micrographs of Paleg and Hyde(1964), who, however give no mention of them in their text. Electron micrographs of gibberellic acid treated slices show dense bodies of this type to be distinctly vesicular.

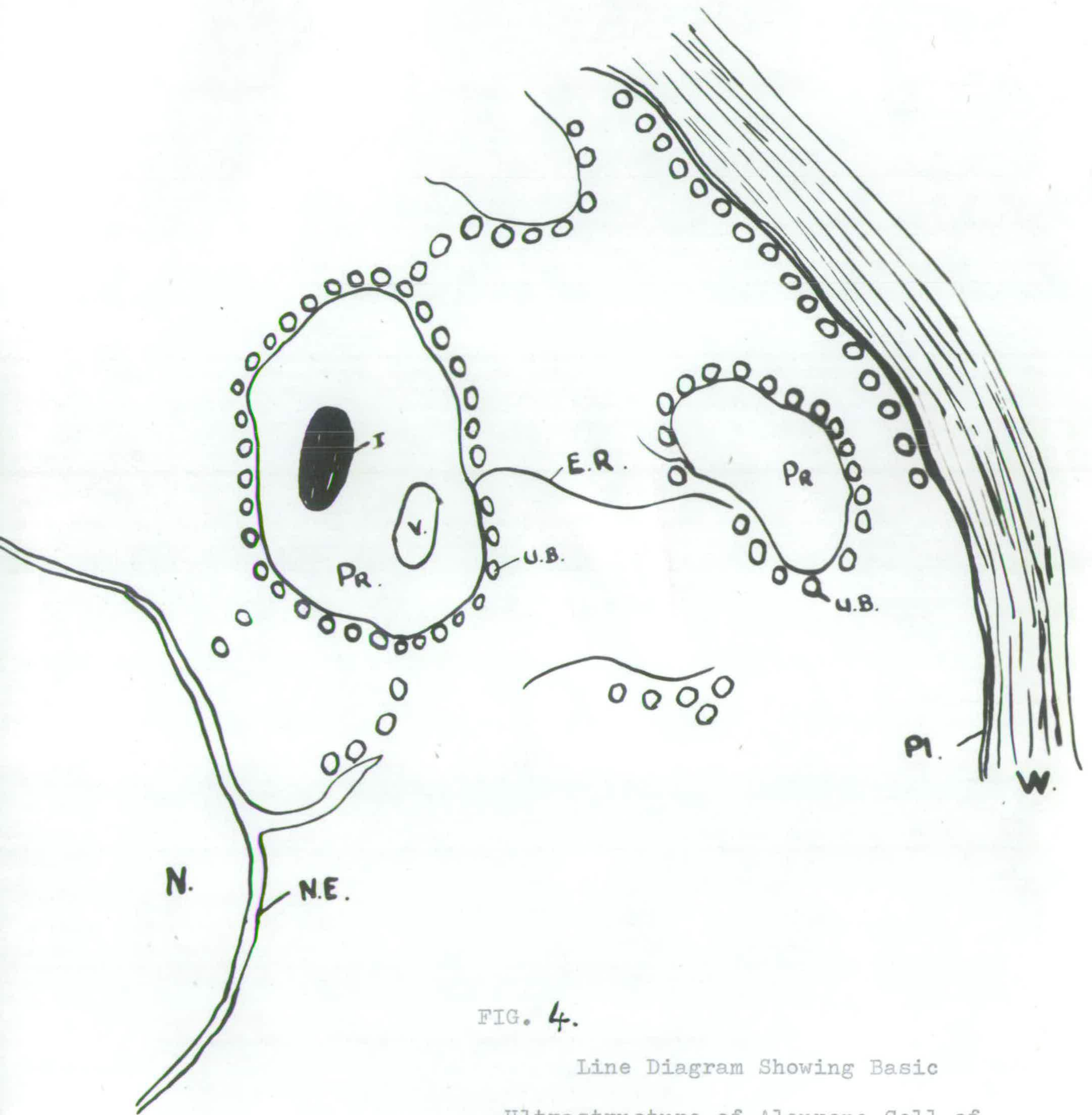


FIG. 4.

Line Diagram Showing Basic Ultrastructure of Aleurone Cell of Ungerminated Grain of Barley. x15000

Internal Structure of the Starchy Endosperm Cell.

This part of the investigation has received only a limited amount of attention but enough evidence is available to give a basic impression of the structural form of the reserves present in these storage cells. In Plate 14 the main storage product, starch, can be seen in the form of starch grains(S) of two basic sizes, the common starch grain of 1.5 - 4.0 µ and the less common but distinct smaller grains of under 1.5 µ. These smaller grains are commoner in endosperm cells adjacent to the aleurone layer. The other large structure present in these starchy endosperm cells is a membrane-enclosed deposit of medium density(PR) which is probably proteinaceous in nature, but which may include phytate deposits. Fine detail of the cell structure is lacking and no organelles resembling mitochondria have been detected. As would be expected from results with optical microscopy, which indicate only occasional remains of degenerating nuclear material, no evidence was found for the presence of a nucleus. Careful examination shows that the original plastid membrane is still present in the proximity of some of the starch grains and on occasions it is still present round the entire grain, Plate 15. If an endosperm cell is burst during embedding the contents are often still recognisable as with the protein deposit, Plate 16, which is directly outside such a broken cell. In Plate 16 the limiting membrane is very clearly seen, with the disrupted contents inside.

Plate 29, shows an endosperm cell of germinating barley. It shows thinner walls than Bromus(early stage of germination when optical microscopy indicates little or no wall dissolution), emphasising the difference between these two grasses.

In Plate 21 there also appears to be two types of electron-dense deposit, possibly one being protein and the other phytate, which is known to be present in fairly large concentrations, J.P. Cook (1964). In the same plate one can also see membranes in the ground cytoplasm, presumably plastid in origin.

Comparison of Cell Wall Structure in Aleurone and Starchy Endosperm.

This section should be considered along with the anatomical and histochemical observations reported earlier in this thesis, and with the chemical findings of Part II, section A.

Techniques. For the examination of sections through the wall, the same basic methods were used as described for the examination of cytoplasmic organelles but special techniques were devised for examining the surfaces of the cell walls. Two main methods were employed. Firstly, the preparation of a carbon replica of the surface which was then shadowed with gold-palladium alloy to bring up the relief detail. This method was used in the early stages of the study but was replaced when another technique was found to give higher resolution of detail. The second method involved the direct examination of cell wall preparations. There were two basic variations in this method:-

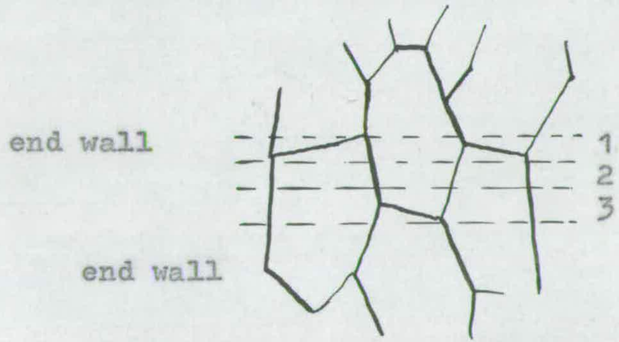
a) examination of wall preparations dried onto electron microscope grids,

b) examination of end walls of cells in hand sections of aleurone-endosperm dried on to grids.

a) A suspension of the cell type to be examined was dried directly onto an uncoated grid. During the drying some of the cells rupture and expose single cell walls which are just thin enough to view with an electron microscope. A very thin carbon film is deposited over the dried wall preparation to stabilise it in the electron beam. The relief detail of the wall s surface was brought up by the standard metal shadowing technique using gold-palladium alloy. The alloy was deposited at an angle of 15°. This preparation was then examined in the electron microscope.

Some diffulties were encountered in the preparation of absolutely pure suspensions of starchy endosperm cells, free from all aleurone cells. The final stages of isolation were, however, carried out under an optical microscope where the aleurone cells could be identified and removed.

b) The most efficient and reliable method of examining cell wall surfaces used, was to cut hand sections from the grain and dry these sections onto a grid. Although the sections themselves were far too thick for examination, any intact end walls(see diagram below) could be examined.



section 1 would have two end walls for examination.
section 2 would have no end walls for examination
section 3 would have one end wall for examination

This technique enables one to locate the different tissues under the lower powers of the electron microscope, Plate 27 , and identify, with certainty, the cell type being examined (ie. aleurone or endosperm cell; and if endosperm, then which part, outer or inner cell). The structural detail of the wall can be seen under magnifications of 5,000 to 30,000 times, Plates 26 and 28 .

Observations.

Aleurone Cell Wall.

The structure of a typical aleurone cell wall, seen in section, is clearly illustrated in Plate 17 . The wall has a fibrillar structure similar to that seen in normal cellulose cell walls. It is reasonable, taking its appearance in conjunction with the results of earlier biochemical studies, MacLeod and Napier(1959), to attribute this fibrillar structure largely to cellulose. This fibrillar structure can be very clearly seen in Plate 19 and 20. Prolonged treatment with potassium permanganate, which causes oxidation of interfibrillar material and also causes a swelling of the wall with subsequent separation of the resistant fibrils, gives a clear demonstration of the fibrillar organisation. A middle lamellar region is clearly demonstrated in Plate 18 . Similarily, a dark staining inter-cellular substance(I.S.) has also been demonstrated in the aleurone, Plates 17 and 19 . The intercellular connections, plasmadesmata, are very distinct in both barley and Bromus aleurone, Plates 30 and 31 , passing between adjacent aleurone cells but have never been observed passing into starchy endosperm cells.

Plate 26 shows the microfibrillar detail of the surface of an aleurone cell wall, supporting the evidence from sections, regarding microfibrillar organisation.

Starchy-Endosperm Cell Wall.

In Plates 22 and 23 it can be seen that the endosperm wall of Bromus is exceedingly thick, though it has a rather less well-defined structure than that of the aleurone. Nevertheless, there is still a considerable degree of organisation, possibly fibrillar, still present. The distribution of cellulose in Bromus is not known but preliminary studies suggest that cellulose is not present in large quantities in the wall of the starchy-endosperm cell. The organised material, which is birefringent(page 20), may therefore be hemicellulosic as hemicelluloses are certainly present in large concentrations in Bromus. Close examination of the micrographs Plates 23&24, suggests that this wall is in fact made of more than one distinct layer : there is a well-defined outer region, ($W.H._1$), within which can be seen one or possibly two more amorphous layers(WH_2 and WH_3), the innermost of which appears to extend into the lumen of the cell as a form of matrix round the peripheral starch grains. It is possible that these three layers may correspond to the hemicellulose fractions obtained by earlier workers, MacLeod and Sandie(1961), but at present no substantial evidence in favour of this possibility is available. Plate 28 shows the surface of an endosperm cell from just under the aleurone layer and it shows a distinct fibrillar structure. As this cell is from just under the aleurone it is possible that some of this organisation, probably the larger microfibrillar structures, is due to the

presence of a limited quantity of cellulose but this could not explain the amount of distinct organisation as seen in sections.

Plate 21 shows the wall between an aleurone cell and a starchy-endosperm cell.

Discussion.

This part of the thesis is intended to supply a structural background to the biochemical studies of the remaining parts of the thesis therefore only a few general comments are needed at this stage. Cereal grains are rather difficult objects as regards preparation for examination for conventional optical techniques, therefore it was not unexpected to find that considerable work was needed before positive results were obtained in the electron microscopic studies. In fact, over a year passed before satisfactory results were obtained. Although the methods used, permanganate fixation followed by embedding in methacrylate, can lead to implosion of vesicles and formation of various types of artifact, these methods are the only ones which have given consistently successful preparations. Membranes have been well preserved and the familiar subcellular organelles present a normal appearance : it is therefore reasonable to assume that the unfamiliar unidentified bodies are also authentic cell components. For preparations from developing wheat caryopses, Buttrose(1963) has additionally used fixation with osmic acid and Araldite embedding, and the fine structure of aleurone from ripening wheat appears to be essentially similar to that now reported for Bromus and barley. Other recent publications, (Paleg and Hyde, 1964; Nieuwdorp, 1963) support the present findings for the structure of the secretory tissue of the

aleurone in the ungerminated grain.

However, it is not yet desirable to speculate extensively on the nature and function of the less familiar organelles detected in the aleurone. A brief report regarding the possible significance of some of these inclusions will be more usefully made after studying tissue from germinating and gibberellin treated material.

SECTION B.

Metabolism of Germinating *Bromus*.

The major portion of this thesis is devoted to the mechanisms of carbohydrate degradation and interconversion in the aleurone-endosperm tissues of the grain. These studies were based mainly on *in vitro* incubations and it was thought desirable to obtain some details of the *in vivo* mechanisms and requirements of the grain. This study was based on two types of experiments.

1. A qualitative and quantitative examination of the alcohol-soluble sugars of the entire grain at varying stages of germination.

2. The nutritional requirements of excised embryos.

A series of simple experiments on the physiological requirements of the germinating grain established that optimum growth was obtained between 25°C and 27°C. ; all work was therefore carried out around 25°C.. Although this is slightly high for a temperate plant; it is convenient for laboratory purposes and is not as high as that used by many other workers who insist on using un-botanical temperatures such as 37°C., the 'animal biochemists temperature'.

Free Sugars Released During the Germination of B.inermis.

Qualitative Study,(chromatographic). A sample of grain was dehusked in 50% sulphuric acid for 4 hours(see appendix) and was then divided into six equal portions(100 g.). The first was ground in a Casella mill, and enzymes deactivated by plunging into boiling 85% ethyl alcohol which also extracted much of the sugars present. This boiling was repeated for a further two occasions, each time under reflux. Between each extraction the grain was separated from the extract by filtering through muslin. The time of each extraction was 30 minutes. The three extracts were bulked and then left in a refrigerator overnight to allow fats to separate out ; they were then filtered off. After a final centrifugation and Celite filtration the alcohol was distilled off under reduced pressure. The aqueous solution remaining was left in a refrigerator overnight then filtered through Celite. The sample was then concentrated and chromatographed on Whatman No.1 paper using a butanol: acetic acid: water(4:1:5) solvent(in descending technique).

The other five samples of grain were placed on moist filter paper in sterile Petri dishes and incubated at 25°C.. After 24 hours one sample was removed ; the others were left for 48 hr.; 72 hr.; 96 hr.; and 144 hr. respectively. After removal from incubation the grains were killed by plunging into boiling alcohol. Sugar extraction was by the method already described.

In each case, three chromatograms were run and different sugar detection methods were used.

1. Aniline hydrogen oxalate, which detects sugars whether hexose or pentose, whether reducing or non-reducing.

2. α-naphthol-phosphoric acid, which detects only ketose sugars, incl. sucrose.

3. Pretreatment of extract (alcohol-free) with crude α-amylase preparations, which remove α-linked oligosaccharides; followed by spraying with aniline hydrogen oxalate.

In all samples examined, the following sugars were detected:- glucose, fructose, sucrose, maltose (trace), maltotriose (trace) and a series of fructosans. Maltose and maltotriose were removed by the crude α-amylase treatment which contained maltase. The fructosan series was tentatively identified by means of the relationship log 1-Rf/Rf: saccharide level.

Extracts from grains grown for 96 hours and 144 hours contained free pentose sugars. These never occur in the normal malting of barley, Preece (1957) nor are they likely to occur during the normal low-temperature germination of Bromus where all pentoses released will be rapidly utilised in synthesis. This experiment was, however, at the relatively high temperature of 25°C which would favour hydrolysis, resulting in a build-up of low molecular-weight carbohydrates; a similar effect has been recorded in barley, Preece (1957).

Quantitative Examination of Sugar Formation. Grain samples were incubated for periods ranging from 0-72 hours. The sugars were extracted in the same manner as was used for the chromatographic study. After filtration the alcohol was boiled off and the concentrated sugar extract remaining was diluted with distilled water, to a volume of 100 ml.. The reducing sugars present were determined quantitatively using the standard Somogyi method, Somogyi (1945). The results of these determinations are given in Table 1 . These values represent

reducing sugars present at the various stages of germination but do not give a measure of sucrose and fructosan levels. These were obtained from the values for total sugar content, after hydrolysis of extracts by boiling in N.H_2SO_4 under reflux, followed by neutralisation.

From Tables 1 and 2 it can be seen that there is a nett production of low molecular carbohydrates during germination, caused presumably by the breakdown of high-molecular storage products. The pattern of this release is given below :-

i) over the first 24 hours the reducing sugar level falls, presumably as a result of uptake by the growing embryo,

ii) over the period 24-48 hours the level of non-reducing sugars(sucrose and fructosans) rises slightly, probably as a result of the hydrolysis of insoluble high-,molecular compounds, (eg. cell wall materials and starch) and the transformation of the products of hydrolysis,

iii) this production of non-reducing sugars continues and rises rapidly after 48 hours, with a concurrent increase in reducing sugars,

iv) by 72 hours the content of non-reducing sugar is very high, presumably supplying directly,or indirectly respiratory substrate, etc. for the now rapidly growing embryo. It is also at this time that hydrolytic enzyme activity in the endosperm is very high indeed and the supply of breakdown products will be readily available. This will be discussed in Part II of the thesis.

It seems probable from the results of this study that sucrose and the fructosans act as an intermediate, transient reserve, resulting eventually from the hydrolytic breakdown of larger molecular reserves, starch and hemicelluloses.

TABLE 1. - Reducing Sugar Production During Germination.

Incubation Time (hr.)	0.0	24.0	48.0	72.0
Sugar Content, mg. per 100g. grain.	324	183	299	377

TABLE 2. - Total Sugar Release During Germination.

Incubation Time (hr.)	0.0	24.0	48.0	72.0
Sugar Content, mg. per 100g. grain	864	1328	1375	2243

The upper table represents reducing sugars such as glucose, fructose, etc.; the lower table (after hydrolysis of extract) represents reducing sugars plus non-reducing sugars such as sucrose and the fructosan series.

Metabolite Requirements of Excised *Bromus* Embryos.

A considerable portion of this thesis is devoted to sugar production (and interconversion) from the reserves of the endosperm. If any final opinions are to be formed regarding the possible role of these sugars in germination, it is obviously important that one should have some idea of the nature of carbohydrate metabolites suitable for embryo growth. This study was performed to record the growth rates of excised embryos supplied with sugars which have been examined in other *in vitro* biochemical studies,(Parts II and III). Work of a similar kind, using barley, was carried out 75 years ago by Brown and Morris(1890) and reported in a paper which contains much interesting information.

Techniques.

Embryo Excision. The size of the *Bromus* grain made it virtually impossible to remove the intact embryo from the dry(hard) grain without either including some endosperm or damaging the embryo. Embryos were, therefore, removed from imbibed grains. The grains were dehusked, washed in sterile water and kept at 25°C overnight; after 18 hours the small embryos were removed. During the first 24 hours it is likely that(as in barley,James,1940) the embryo is utilising free sugars present before obtaining new supplies from enzymolysis in the endosperm.

The dehusking process sterilises the grain, although if imperfectly dehusked, fragments of husk could carry over fungal spores,etc.. The sterile grain was placed on sterile filter paper in presterilised Petri dishes. These were left for 18 hours and then the embryos were removed from the grains by dissecting needles, under a lens. They were then washed in

sterile water in a Petri dish, where they were stored until required, later the same day. This period of excision, followed by plating out was the most probable time for contamination, but to reduce this to a minimum all instruments were sterilised. The plating out was carried out in an innoculation cabinet which had been pre-sterilised by U.V. radiation. The embryos were placed in small(2 inch diameter) sterile Petri dishes on sterile filter paper pads. The culture solutions were all sterile and were kept in the cabinet. These extreme precautions were taken to avoid contamination following the results of preliminary attempts at embryo culture when it was found that occasional contaminants present only in trace numbers caused considerable changes in the culture solutions(particularily sugar interconversions).

Embryo Culture. Excised embryos were plated out in batches of 10 embryos per dish. 2 ml. of culture fluid was added. Incubation was at 25°C for 3 days(72 hours). After this period measurements of total seedling length, root length, and shoot length were made and mean values obtained. The results of this study are listed in Table 3 . The mineral solution used in some incubations was Knops essential element solution.

* * *

From Table 3 several interesting facts can be seen.

1. Sucrose, glucose and fructose all produce considerable embryo growth.

2. The addition of mineral salts to the nutrient supply, greatly enhances seedling growth, especially root growth(which is often two-fold).

3. Cellobiose is readily utilised as a substrate. This

disaccharide is a breakdown product of cell walls in the endosperm yet is seldom detected free in the grain.

4. Raffinose allows a slow embryo growth rate, an interesting finding when examined in conjunction with the findings of MacLeod and McCorquodale(1958), who were unable to find raffinose in Bromus grains.

5. Galactose could not be utilised by the embryo.

6. Embryo growth on 1% starch is almost negligable at the start but if left for longer than 3 days the growth rate increases slightly, probably due to a release of amylase followed by an uptake of the degradation products. The presence of hydrolytic enzymes in culture fluids was verified; presumably secreted from the scutellum but the possibly of a trace of aleurone remaining cannot be overlooked. Such enzymes included α amylase and endo-β-glucanase. A wide range of sugars, eg. glucose, cellobiose and occasionally laminaribiose, was found in the culture fluids. These sugars were presumably formed by enzymes capable of transosylase action(Part III).

* * * *

At this point, no attempt will be made to discuss the general findings of Part I of the thesis. Points covered in this first part of the thesis will be referred to in the rest of the thesis.

TABLE 3. - Excised Embryo Growth on Varying Sugar/Mineral Salt Substrates.

Substrate	Mean Lengths (mm.)		
	Total Seedling	Shoot	Root
riginal Excised Embryo	2.00	1.00	1.00
Water Control	3.00	1.75	1.25
ineral Salt Solution	2.75	1.70	1.05
1.0% Sucrose	9.40	6.00	3.40
.0% Sucrose + min.salts	10.30	5.90	4.40
5.0% Sucrose	6.45	3.25	3.20
.0% Sucrose + min.salts	13.60	5.20	8.40
1.0% Glucose	8,20	4.30	3.90
.0% Glucose + min.salts	12.80	6.20	6.60
5.0% Glucose	8.50	4.00	4.50
.0% Glucose + min.salts	17.70	6.90	10.80
1.0% Fructose	11.25	6.75	4.50
.0% Fructose + min.salts	16.70	7.10	9.60
1.0% Galactose	2.00	1.00	1.00
.0% Galactose + min.salts	2.00	1.00	1.00
1.0% Cellobiose	9.40	5.80	3.60
.0% Cellobiose + min.salts	15.35	10.95	4.40
1.0% Raffinose	7.65	5.95	1.70
.0% Raffinose + min.salts.	7.30	5.50	1.80
1.0% Starch	2.40	1.40	1.00
.0% Starch + min.salts	2.75	1.75	1.00 **

* the starch culture began to grow slowly after a few days, possibly due to release of amylases from embryo(?).

PART II.

Hemicelluloses and Hydrolases of Bromus sp.

Section A. Hemicelluloses and Hemicellulases present in the Ungerminated Grain of Bromus spp..

Section B. Changes in Hemicellulase and Transferase Activities during the Development of the Grain of Bromus ramosus, Huds..

Section C. Changes in Activity of Hydrolases during Germination and Seedling Growth.

Section D. Ultrastructural Changes in the Germinating and Gibberellic Acid Treated Grain.

Discussion.

SECTION A.

Hemicelluloses and Hemicellulases present in the Grain of Bromus spp..

Hemicelluloses.

The anatomical observations outlined in Part I of this thesis indicated that Bromus spp. contained a very high percentage of cell-wall material. A full chemical examination of these cell walls was made by MacLeod & Sandie(1961) and was briefly examined in this work. The figures obtained in these studies were compared with those of determinations in other grasses(cereals), Preece(1957) and have been summarised in Table 4 . The methods used for the extraction of the different fractions were based on those of Preece and Mackenzie (1955). An outline of the techniques used in hemicellulose extraction is presented in the form of a flow sheet, fig. 5 . The larger part of this study on hemicelluloses in Bromus was devoted to the water-soluble ($40^{\circ}C$) β-glucan. As the majority of the studies with barley had concentrated on that fraction it was hoped that some interesting comparisons could be made with barley. Also, this fraction could be prepared in a relatively pure form.

Bromus water-soluble β-glucan was found to resemble its counterpart in barley in general characteristics. It is an extremely viscous material in aqueous solution, having a viscosity varying from η sp = 3 - 7 ; possessing a specific rotation of $[\alpha]_D = -10^{\circ}$ (0.5% soln.), cf. $[\alpha]_D = -12^{\circ}$ in barley. On hydrolysis, only glucose was released indicating

absence of contaminants (eg. pentosan, etc.). The glucan samples also gave no blue-black colouration with iodine reagent indicating absence of starch. Some preparations did, however, give a greenish colouration with iodine suggesting that some complexing with iodine occurred (Appendix 2).

Water-soluble pentosans from Bromus were shown to be similar to those found in other grasses. They were found to contain both xylose and arabinose residues as in rye araboxylan. Acid hydrolysis indicated a slight contamination with glucose. The specific viscosity of a 0.5% aqueous solution was usually in the range 5 - 8 , and possessed an $[\alpha]D$ of about -135°.

Hemicellulases.

In this study, attention was devoted to the hydrolytic enzymes responsible for the degradation of the water soluble hemicelluloses. In the preliminary investigations both glucanase and pentosanase systems were examined, but the bulk of later work was concerned with the glucanases alone and in particular with endo- β -glucanase. This section examines the enzymic activities of the mature ungerminated grain and is based on the use of acetone-precipitated dried enzyme preparations derived from sodium chloride extracts of ground grain

Enzyme Preparation. A sample of the grain used was finely ground in a coffee mill, care being taken to keep the temperature as low as possible. The ground grain was then mixed with 0.6% sodium chloride to extract the necessary

enzymes, usually in the ratio 30 gm. grain to 200 ml. solution. The technique was a modified version of that used by Preece & Hoggan (1956). The extraction period used was 90 minutes at room temperature. After extraction, the solution was filtered through muslin and centrifuged for 20 minutes at 2,500 r.p.m.. The filtered extract was left to autolyse overnight, centrifuged to remove precipitate and then dialysed for two days against running water. The extract was recentrifuged and the enzyme preparation was precipitated by adding 4 volumes of acetone and then dried with further acetone.

The autolysis allowed breakdown of polysaccharide impurities and dialysis removed free sugars and other low-molecular compounds. The long purification technique, although it produced a polysaccharide-free preparation, it also allowed considerable autolysis of the enzymic protein. For these preliminary studies, however, it was found advantageous to use a precipitated enzyme preparation. Attempts to purify the enzyme by Celite filtration of extracts resulted in a considerable de-activation of the enzyme. Dialysis against 0.6% sodium chloride instead of water slightly reduced the rate of inactivation of enzymic material.

All the results given in this section of the thesis refer to enzymes prepared by the basic technique just outlined with none of the mentioned adaptations.

Substrates. 1. β-glucan - as it was more practical to prepare large quantities of β-glucan from barley (var.Ymer)

which, unlike Bromus, was available in unlimited quantities it was decided that barley β-glucan would be used as substrate for all investigations with Bromus enzyme preparations. Following some detailed studies at the start of this work it was found that several facters affected the yield and viscosity of barley β-glucan, eg. grain sample, temperature of extraction mixture, volume of extraction water, grinding method, etc., and these are described in Appendix 3,-but there was no chemical variation if the basic preparatory scheme was followed.

2. Pentosan - as with the glucan, the araboxylan used was not obtained from Bromus itself. It was prepared from rye by fractionating the water-soluble hemicellulose mixture with ammonium sulphate and using only the fraction precipitating out between 40 and 50% saturation. This preparation had a reducing power of 2.5 g. per mg.; η_{sp}(0.5% soln.) = 5 - 8; $[\alpha]_D = -135^0$. As with Bromus pentosan it was found that there was a slight glucan contamination, about 3 - 5%.

3. Cellobiose - analytical grade of cellobiose from Messrs. Lights & Co. Ltd. was used as substrate for cellobiase determinations.

Measurement of Enzymic Activity. Transformation of soluble β-glucan was measured as endo-β-glucanase, exo-glucanase, and cellobiase action. Endo-β-glucanase activity was measured in terms of an increase in reciprocal specific viscosity, per mg. enzyme per hour, the increase being directly proportional to activity, (Preece & Hoggan, 1956). The

The reaction mixture used was :-

8 ml. 0.6875% β-glucan

1 ml. acetate buffer, pH = 5.0

2 ml. enzyme solution containing 1.1 mg. / ml. enzyme.

Exo-glucanase activity was determined using the same reaction mixtures as was used for endo-glucanase determinations and was measured as an increase in reducing power, using Somogyi (1945) titrations, expressed as increase in mg. glucose equivalent per mg. enzyme, per hour. Incubations were at 25°C. and 5ml. aliquots were used for each determination. Correction was made for the increase in reducing power resulting from endo-glucanase action (MacLeod & Sandie, 1961).

Cellobiase was also expressed as an increase in reducing power. As with exo-glucanase determinations the technique of Somogyi(1945) was employed for determination of the reducing power. When preliminary determinations of cellobiase activity were made by MacLeod & Sandie(1961) and again in this present study, it was found that conditions suitable for equivalent cereal enzymes produced results which suggested that cellobiase was absent from Bromus preparations since no increase in reducing power was apparent over periods of many hours. With some of the enzyme preparations studied a decrease in reducing power was obtained. Chromatographic studies revealed that in fact extensive hydrolytic action had taken place within the first hour but it had been accompanied by concomitant transglycosylation. A study of this trans-glycosylation activity formed a major portion of this work and

further details will be considered in Part III of this thesis. An approximate estimate of true cellobiase action was determined using very low substrate concentrations, 0.05 % cellobiose, where there appeared to be no interference from synthesising mechanisms.

The results of a typical series of determinations of hemicellulase action in Bromus spp. are given in Figs. 6 and 7 .

In previous studies on Bromus (MacLeod & Sandie, 1961), and later in Part I of this thesis, it was recorded that the endosperm cell walls were very thick indeed compared to many other grasses, including barley, and this was supported in quantitative chemical analyses of these walls(Table 4). As previously stated these walls undergo very sudden dissolution during germination and consequently it was reasonable to assume that a very active hydrolytic enzyme system must be present. The results given in Table 5 support this supposition. These results are based on studies for this thesis, studies of MacLeod and Sandie(1961) and of Preece et al.(1956,1957,1958 and 1961). Although these activities were found to be relatively high compared to other cereals, often 20 times as high, they represent only the basal activity of the ungerminated grain. This activity increases tremendously during germination, as can be seen from the figures given in section C of this part of the thesis, thereby explaining the extremely rapid dissolution of the very thick endosperm cell walls.

A more detailed study of endo-β-glucanase activity revealed that this enzyme system was very stable. In solution

it remained active for over a week (lost 10% of activity) at room temperature; several months at $4^{\circ}C$. and as an acetone precipitated powder it remained active over very long periods indeed (over 12-18 months if stored at $4^{\circ}C$.).

Figure 8 indicates that endo-β-glucanase possesses a pH optimum of 4-6, a feature common to many other hemicellulases, (Clark and Stone, 1963).

Location of Enzymic Action. As was mentioned in the general introduction there has been considerable confusion in past years as to the exact origin in the cereal grain of the hydrolytic enzymes, such as the hemicellulases and the amylases. Recent studies have indicated that these enzymes are released mainly from the aleurone and not only from the embryo via the scutellum although there is evidence that some 20% of the total α-amylase of the germinating grain is released from the scutellum, (Briggs, 1964). To establish that endo-β-glucanase was released from the single-layered aleurone in Bromus, a series of simple tests was made. First, grains of Bromus inermis were halved and then enzyme preparations were made from the two sets. Thus one preparation contained extracts from the embryo, aleurone and endosperm(starchy), whilst the other had extracts of the aleurone and starchy endosperm only. Both sets gave preparations active in endo-β-glucanase, therefore there was evidence of activity in the aleurone and/or starchy endosperm. To establish if there was any glucanase in the true starchy endosperm, an extract was made from a pure preparation of starch cells, dissected from several grains. No endo-activity could be detected in the preparation. By

elimination, it can be assumed that Bromus aleurone can release endo-β-glucanase.

This problem of the location of enzymic release is dealt with in greater detail in the section dealing with changes in enzymic activity during germination and the accompanying section on changes in ultrastructure during germination.

* *

Although it would have been desirable to examine the activities of the hemicellulases of Bromus using substrates from Bromus itself, this was impracticable owing to the large amount of grain required for substrate preparation. However, the general characters of β-glucan and pentosan from Bromus resemble those of similar products from barley and rye. Substitution of hemicellulose substrates of "foreign" origin thus seemed to be justified.

From the results presented , it can be seen that Bromus grains possess very large quantities of β-linked pentosans and hexosans. Similarily, they contain an extremely powerful system of β-glycosidases responsible for catalysing the degradation of at least the soluble fractions of these polymers. Previous work, (MacLeod & Sandie, 1961), has shown that Bromus also possesses enzymes capable of partially degrading the insoluble hemicelluloses and this has been verified by some of the histological studies outlined in Part II, section D, of this thesis. In most cases, the aspects of polysaccharide degradation studied in Bromus have been found to resemble events already observed in barley(Preece, 1957), the essential differences

being the very high percentages of hemicellulosic materials present in Bromus grains and the correspondingly high degree of enzymic activity. This degradative power is of the order of over 20 times that of barley,(Table 5).

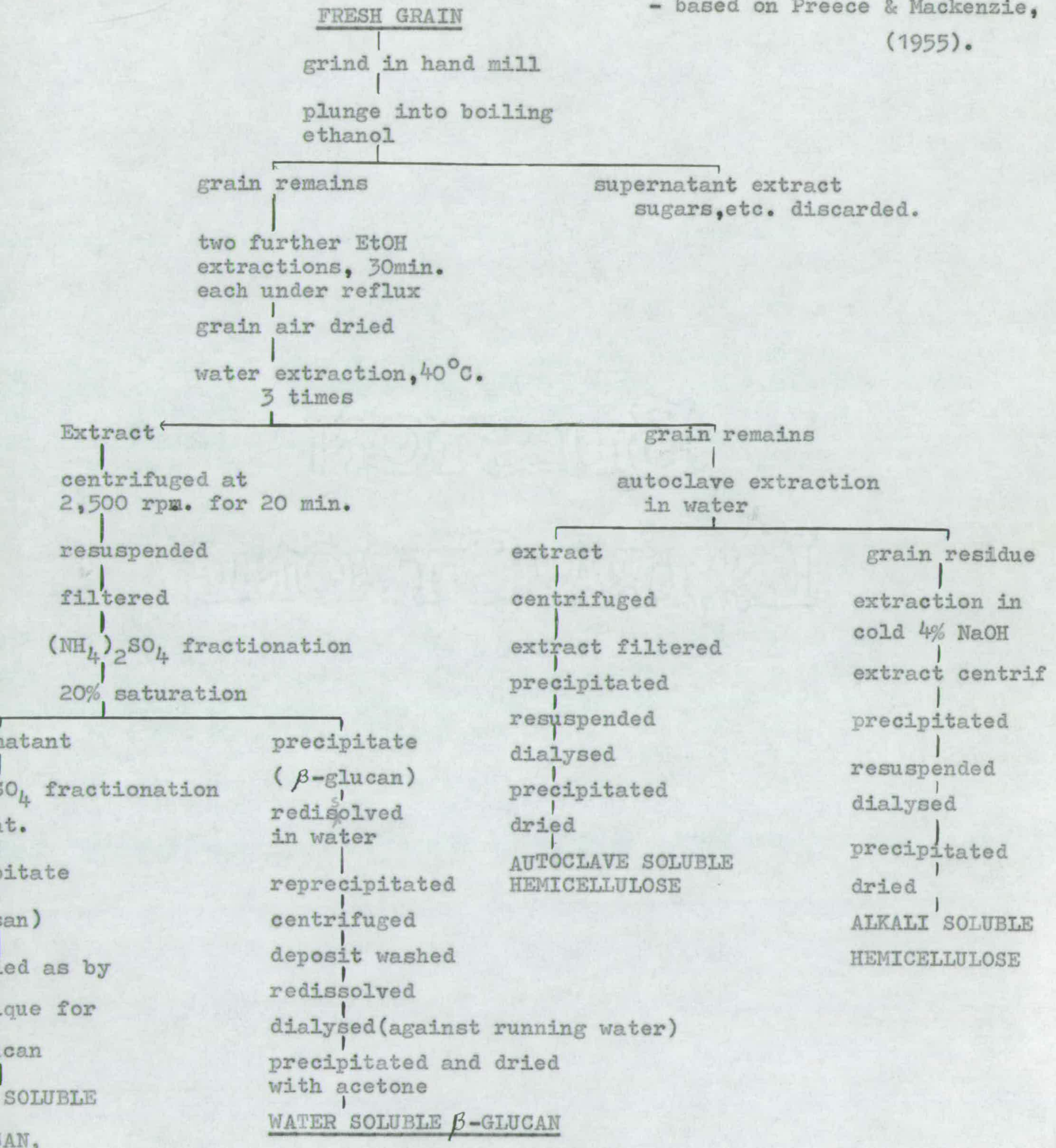
It must be remembered that all the determinations made in this section of the work and subsequent comparisons were based on hemicellulases at their lowest activities. In fact other studies in this respect, MacLeod & Duffus(unpublished data), and section C of this thesis, have suggested that the enzymic activity measured in the ungerminated grain is quite insignificant in comparison to the induced release during germination.

Although this section of the thesis was devoted principally to the study of the enzymic mechanisms responsible for the degradation of endosperm cell-wall hemicellulosic polymers in the Bromus caryopsis, the examination of the cellobiase activity of the enzymic preparations revealed the presence of an extremely powerful transferase system capable of synthesising β-linked oligosaccharides. This again verified the preliminary finding of MacLeod & Sandie(1961) and a detailed study of the mechanisms involved in this transferase action has been formed a major part of the work for this thesis, see Part III.

It can be easily seen that although Bromus, with its extremely thick endosperm cell walls, is useless for the making of malt, it is an extremely suitable grain for a

detailed study of the metabolism of these β-linked cell-wall hemicellulases which play such an important part in the barley grain during malting.

FIG. 5 - FLOW SHEET of Hemicellulose Extraction and Fractionation.



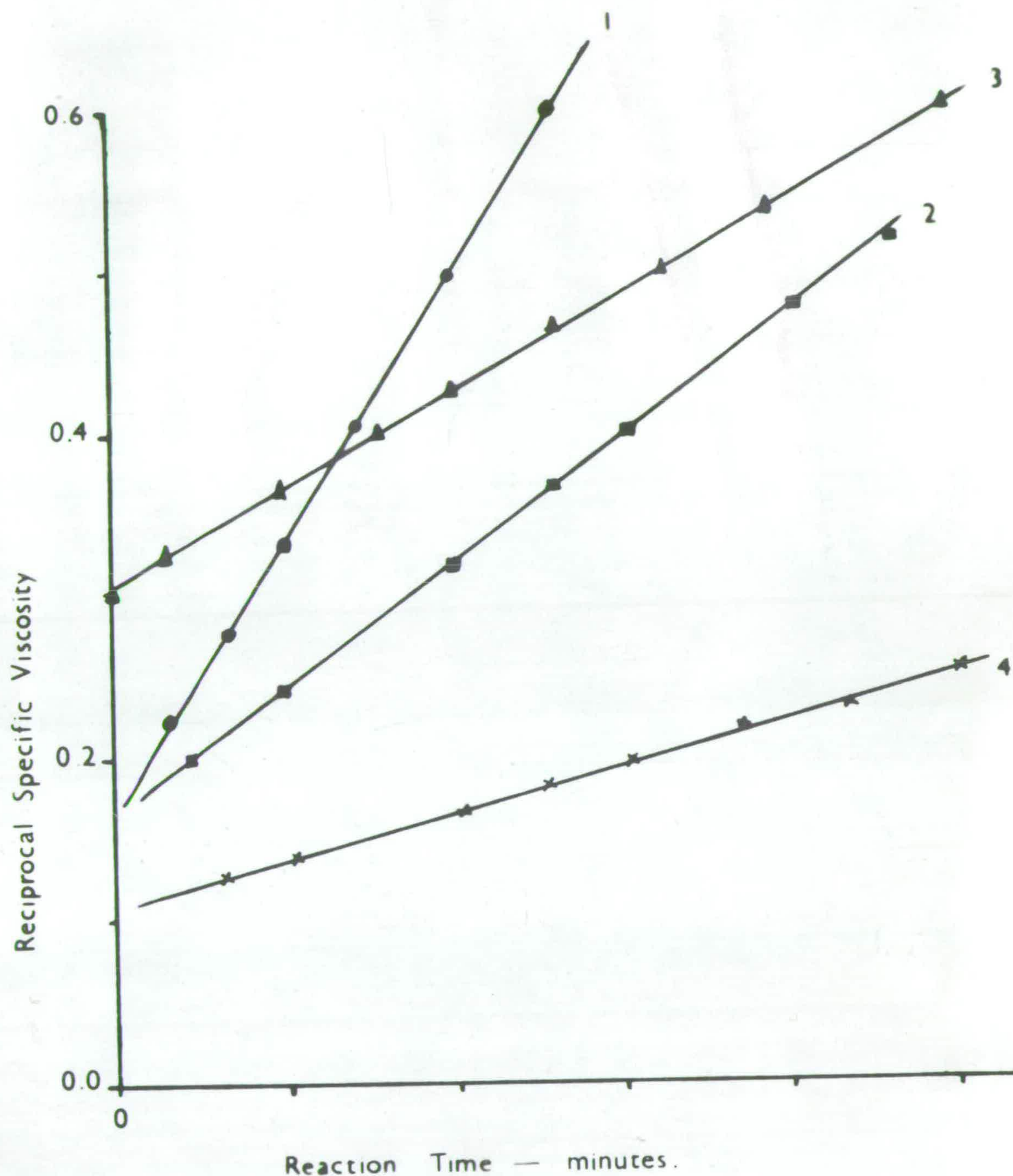


FIG. 6. Typical Results of Determinations of Endoglycosidase Activity. 1, 2, and 3 were glucan substrates with enzyme preparations from different samples of B.inermis; 4 was a pentosan substrate.

* Endo-glucanase Activities - 1 = 0.33
2 = 0.155
3 = 0.115

Pentosanase Activity -(prepn.4) = 0.055

* - activities expressed in same units as used in text.

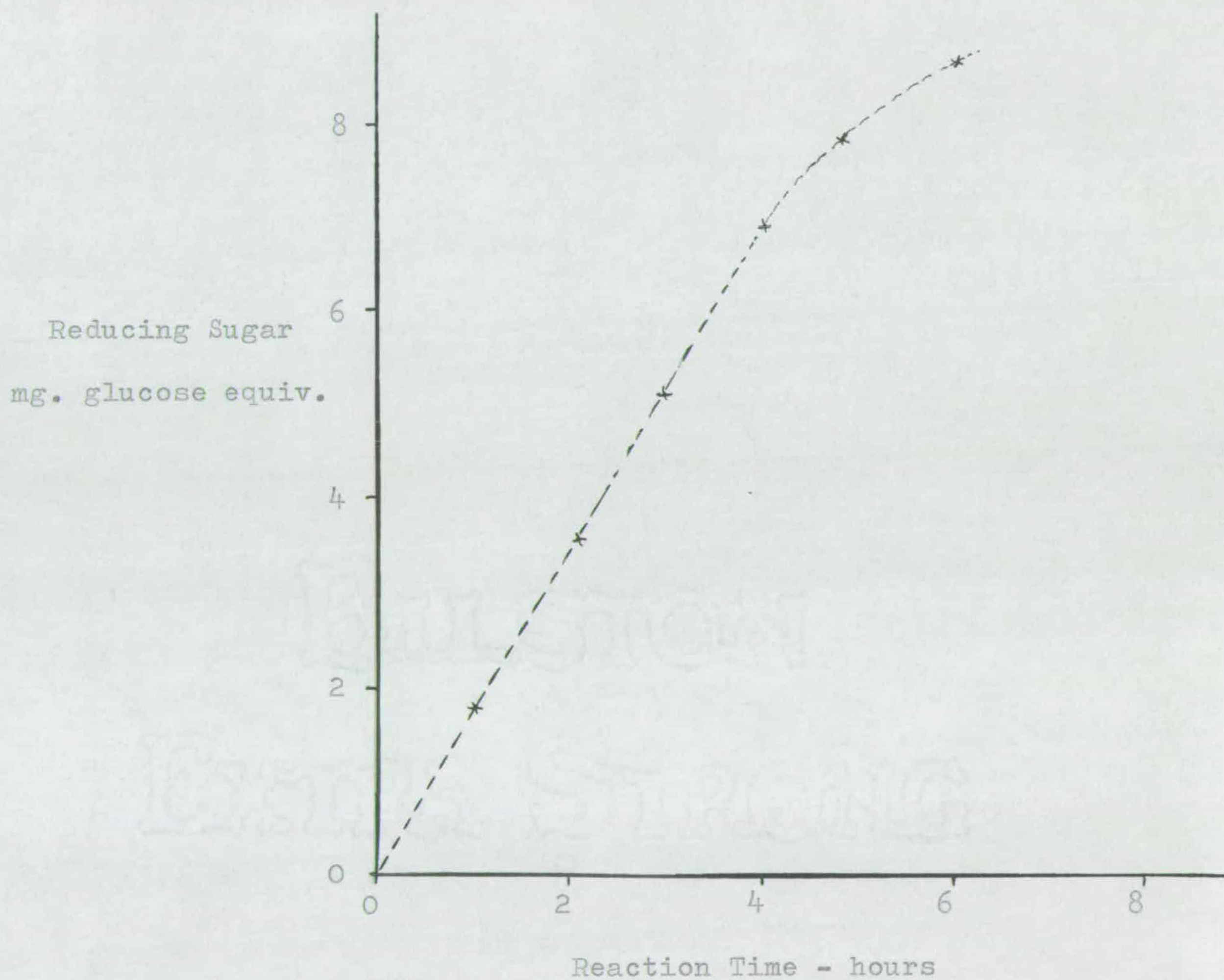


FIG. 7. A Typical Result of a Determination of Exoglucanase and Cellobiase Activity, measured as an increase in reducing power of a glucan substrate.

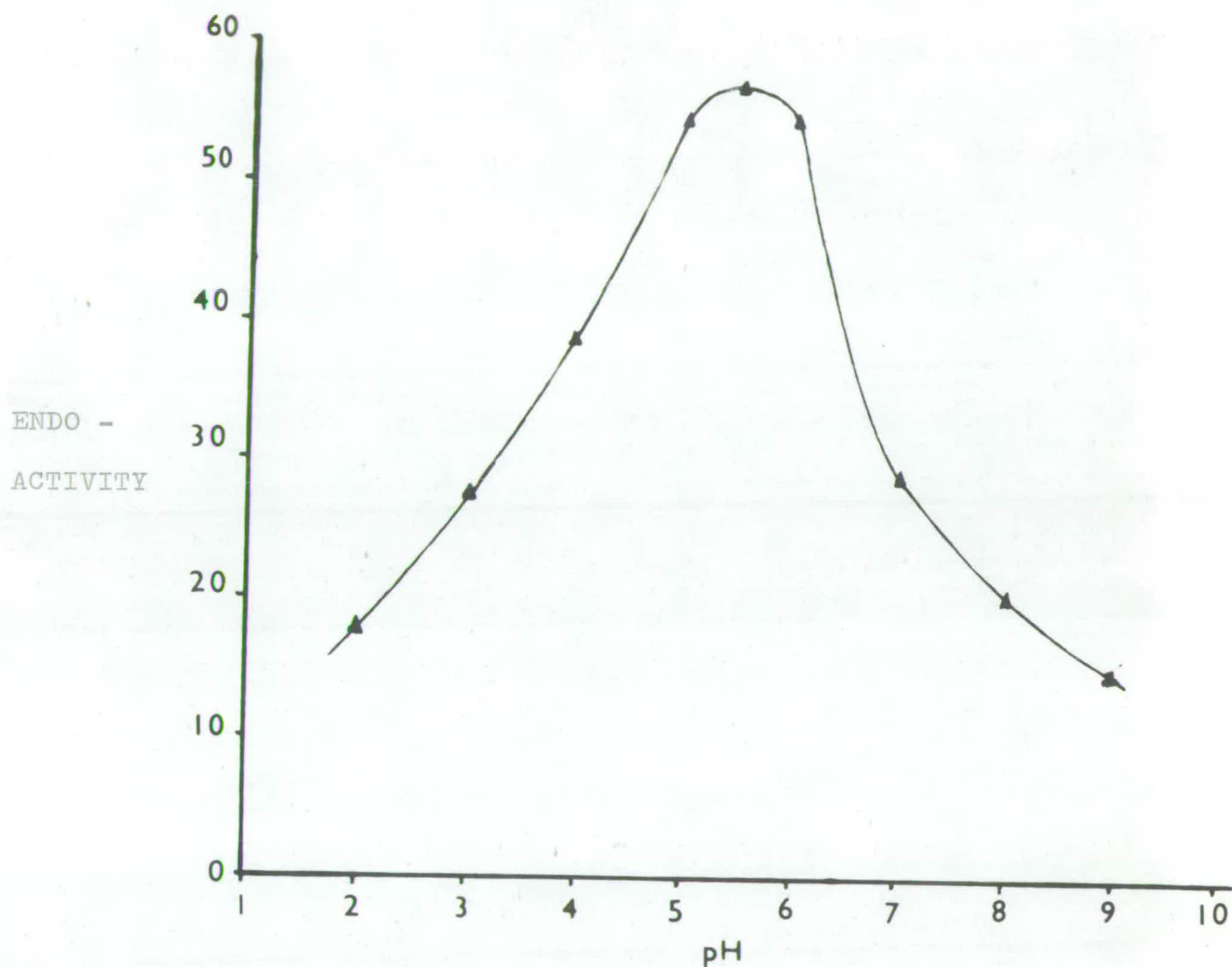


FIG. 8. Effect of pH on Endo-β-Glucanase Activity.

Endo- activity is expressed as increase in reciprocal specific viscosity/ mg. enzyme/ hour X 100.

A Comparison of the Hemicellulose Contents of Various Grasses.

	Hemicellulose Content *			
GRASS	Water Soluble 40° C.	Autoclave Soluble	Alkali Soluble	Total
Bromus spp.	1.3 - 2.3	4.0 - 7.3	5.6 - 9.2	13.4 - 17.3
Barley	1.1	1.0 - 2.0	1.5 - 3.0	3.8 - 6.1
Oats	0.56	-	-	-
Wheat	0.41	-	-	-
Rye	0.83	-	-	-

* all values expressed as percentage of grain dry weight.

\- data not available; the important comparison is between <u>Bromus</u> and barley.

Data in this table is based on figures from several workers, Preece (1957); MacLeod & Sandie (1961), as well as work for this thesis.

	Endo-B-glucanase		Exo-B-glucanase		Cellobiase		Pentosanase	
	Activity	Activity x Yield	Activity	Activity x Yield	Activity	Activity x Yield	Activity	Activity x Yield
Bromus spp.	0.13-0.18	130-240	0.103-0.267	150-250	0.70-0.94	656-2,000	0.032	32
Barley	0.011	8	0.002	2	0.80	572	0.013	4
Oats	0.023	14	0.022	15	-	208	0.027	10

- increase in reciprocal specific viscosity per mg. enzyme per hour.
- increase in reducing power, as glucose, corrected for endo-action, per mg. enzyme per hour.
- mg. glucose produced from cellobiose per mg. enzyme per hour.
- yield of enzyme in mg. per 100 g. grain.

TABLE 6 .

The Effect of pH on Endo-β-Glucanase Activity, in Extracts from *Bromus inermis*.

pH	Endo-β-Glucanase (arbitrary units)†
2.0	17.7
3.0	27.1
4.0	38.5
5.0	54.2
5.5	56.8
6.0	54.5
7.0	29.0
8.0	20.4
9.0	15.0

† - expressed as increase in reciprocal specific viscosity, per hour, per mg. enzyme preparation, x 1,000.

SECTION B.

Changes in Hemicellulase and Transferase Activities during the Development of the Grain of *Bromus ramosus* Huds.

Samples of *B. ramosus* at various stages of maturity were collected from woodland near Edinburgh. The material used for the enzyme extracts included the caryopsis itself plus the lemma and palea, which form the husk in the mature grain. Samples of known grain number were ground in 0.5% sodium chloride solution at room temperature, either by micro-wet grinder or by mortar and pestle; with the techniques employed the extent of extraction was the same whether grinder or by mortar and pestle was used. After grinding, an extraction period of 90 minutes at room temperature was allowed, followed by filtration through muslin and centrifugation at 2,500 r.p.m. for 10 minutes to remove residual plant debris. The supernatant extract was used immediately for the determination of enzymic activity.

At the same time as preparing enzyme extracts from the different grain samples, fresh weight and dry weight determinations were made for 10-corn samples and further samples were stored in deep-freeze (-15°C.) or fixed in 70% ethanol for microscopic examination at a later date.

Endo-β-glucanase activity was measured by the standard viscometric technique of Preece & Hoggan(1956), activity being expressed as increase in the reciprocal specific

viscosity per hour per 10 corns. The incubation mixture consisted of :-

1.6 ml. 0.6875% β-glucan

0.2 ml. 0.1-M acetate buffer, pH=5.0

0.4 ml. of the 2 ml. fraction of enzyme extract from 10 corns,

After mixing the components, 2 ml. of the mixture was quickly pipetted into a viscometer, and viscosity changes were noted over 90 minutes at 25°C..

Results of determinations of endo - β-glucanase activities are given in Fig. 9 . It is interesting to note that results similar to those shown in Fig. 9 have been found for endo-β-glucanase development in the grains of Bromus mollis L., B. sterilis L., Festuca gigantea (L.) Vill., Brachypodium sylvaticum (Huds.) Beauv.. This suggests that this general pattern of fluctuation in this cytolytic enzyme during grain ripening is common to several grasses.

As the samples used for the preparation of enzyme extracts consisted, in these preliminary determinations, of lemma, palea and caryopsis it was decided to fractionate the grain into these components and make extracts from each. This fractionation was felt to be desirable because of the different degree of maturity of these regions of the grain. Thus, the lemma and palea are mature organs at stage 1 in Fig. 9 , they remain actively photosynthetic throughout stage 2 and they become senescent as the grains ripen. The caryopsis, on the other hand, is increasing in dry weight throughout all the stages examined, though it must be remembered that complex developmental changes are taking place within the caryopsis while it

is maturing. Since the stigma is inevitably included in the whole grain analysis, assays were made of endo-β-glucanase in this organ ; the reason for undertaking these determinations is that pollen tube growth is occurring within the stigmatic tissues and a possible contribution of cytolytic enzymes from pollen tube activities could not be excluded, especially during the earlier developmental changes,(stage 1 in Fig. 9). The results of these determinations are given in Table 9 .

It is clear from the results in Table 9 , that the stigma makes no contribution to the total endo-β-glucanase of the caryopsis ; on the other hand, the lemma and palea contribute more than half of the total activity of enzyme at all stages of ripening, the remainder coming from the caryopsis itself.

Pentosanase activity(endo- action) was measured in the same manner as was used for glucanase except that 1.6 ml. of 0.6875% araboxylan from rye replaced the glucan. The results of the determinations of pentosanase activity at three stages of grain development are given in Table 8 . These results suggest that, like glucanase, pentosanase activity rises to a peak during the development of the grain, and declines considerably by the time the grain is approaching ripeness.

In view of the fact that both endo- β -glucanase and pentosanase were found to show a generally similar fluctuating pattern of activity during grain development, it was thought

desirable to study changes in amylase activity over the same period, so affording a comparison with a rather different type of hydrolytic enzyme. A general survey, using the same sodium chloride extracts as used for the determination of endo-β-glucanase, and determining α-amylase in the presence of added excess β-amylase by a modified version of the technique used by Briggs (1961) showed that α-amylase was completely absent from all stages of the developing grain of Bromus ramosus. The presence of β-amylase was verified, but activity was low and it appeared to show no significant change during grain development.

Transferase activity was estimated by determining the change in reducing power of a 0.5% cellobiose substrate solution. Cellobiase action results in the increase in reducing power, whereas the presence of transferase either reduces the extent of this increase or, if it is very powerful, it results in a decrease in reducing power. Incubation mixtures consisted of :-

16 ml. 0.6875% cellobiose
4 ml. enzyme extract from total of 20 corns
2 ml. 0.1-M acetate buffer, pH=5.0.

Incubation was at 25°C. and samples were withdrawn after 30 minutes and reducing power measured by the Somogyi (1945)method. After correction for substrate and enzyme the changes in reducing power were noted. The results of these determinations are given in Table 10 . It appears from the results given in Table 10 that transferase action becomes unequivocal only in the later phases of development of the grain. However,

chromatographic analysis of all the reaction mixtures reported on in Table 10 showed that transferase action gave the same pattern of oligosaccharide synthesis as has previously been detected in the ripe ungerminated grain. In spite of the increase in reducing power at the two earlier stages, - suggesting predominantly hydrolytic action - oligosaccharide synthesis was detected chromatographically in all phases; at the last stage examined a very large amount of trisaccharide had been synthesised.

Uridine-diphosphate-glucose Transferase

Rigorous tests were made for the occurrence of glucosyl transfer from U.D.P.G.. Incubations were carried out with substrates of concentrations of the type earlier used in studying this system (p130), in presence and absence of added Mg^{++}, cellobiose, and E.D.T.A., but no synthesis whatsoever could be detected, even in preparations made from immature grain selected at the stage where the endosperm walls are rapidly increasing in thickness.

In assessing the significance of the figures presented it must be borne in mind that they represent results from random samples collected from a woodland species of Bromus, (B.ramosus). Considerable variation in absolute figures is an inevitable reflection of the rather extensive range of environmental conditions under which the different samples were growing.

However, from the results given, Table 7, it would appear that the activity of the hemicellulases extractable in 0.6% sodium chloride followed two basic trends during the

development of the grain after fertilisation. First there was a rapid increase in activity, presumably from zero, over the phase when the grain was forming - ie. from the ovary at anthesis up to the full-sized (but still soft and green) grain. This was rapidly followed by a decrease in activity as the grain began to harden and turn yellow. At the same time, it is important to note that the peak in activity corresponded to the time at which the lemma and palea had reached full size before beginning to wither. This two-phase trend was observed not only for the whole grain, but also for its separate components - lemma, palea and caryopsis.

The function of a powerful degrading enzyme system, which is apparently active during a phase of growth which must be reliant on synthesis, is rather obscure. In the lemma and palea the increase in hydrolytic activity may bring about the digestion of parts of these structures as they wither, for although analysis of husk from mature barley grain shows that there is still a substantial amount of hemicellulose present, virtually all of the final husk hemicellulose is of the 'resistant' type (Preece , 1957), and material which was susceptible to cytolytic action may have been eliminated enzymically during grain ripening. In both barley and Bromus no apparent hemicellulase activity remains in the withered husk therefore it can be assumed that the cytolytic activity observed in the developing lemma and palea falls steadily during withering, finally reaching zero as the husk dries out completely.

The greatest difficulty in interpreting the observed results rests in assessing the possible function of the powerful hemicellulase within the caryopsis itself. At the time when endo-β-glucanase shows its maximal activity, (ie. when the grain is half its final weight), the enzymes bringing about wall formation in the endosperm must also be at their peak activity, since it is at this stage that the walls thicken - a feature which is reflected in the very high content of hemicellulose present in Bromus endosperm(MacLeod & Sandie, 1961). It is possible that a hydrolytic system is active during the formation of these walls, breaking up some of the hemicellulose chains and so permitting interchange of material with newly-formed walls. The decrease in extractable endo-β-glucanase activity during the drying and ripening period of a grain may be a desirable event for a seed entering a phase of dormancy but the mechanism which brings about this decrease is far from clear. The following possiblities may be considered :-

(1) Glucanase is synthesised during the early development of the grain but this synthesising process is rather suddenly arrested, leaving the enzyme in the soluble form, exposed to proteolytic action.

(2) Glucanase present in the grain becomes inactivated, and deposited as an inert insoluble protein.

(3) Glucanase(and other hydrolytic enzymes) become incorporated into some cellular organelle which protects the enzyme from extraction by saline : such an organelle has been tentatively identified in animal tissue as a lysosome of the type suggested by de Duve (1955).

Present evidence from this work with Bromus indicates that (3) is most unlikely. Thus, although refrigerated storage of grain samples allowed increased extraction of active endo-β-glucanase from immature grain, there is little response to repeated freezing and thawing at the later stages of grain development - a treatment recommended by de Duve for releasing hydrolytic enzymes from lysosomes. Also, fractionation of endosperm-aleurone macerates in the refrigerated centrifuge, using de Duves methods, has failed to give any evidence for lysosomes in grass seeds (Duffus, 1963).

As far as possibilities (1) and (2) are concerned, there is not sufficient evidence available to allow any decision to be taken about the correctness of either. The electron microcopic evidence of Buttrose (1963) suggests that large amounts of apparently insoluble deposits of protein are formed during endosperm development in wheat and present studies on the ultrastructure of Bromus aleurone-endosperm appear to suggest that similar deposits are formed in Bromus, see Parts I and II(d) of this thesis. Whether such a deposition of protein is in any way connected with the sudden change in enzyme activity is quite another matter.

As the endosperm of a grass seed becomes filled with starch granules, the central cells and, later, the peripheral cells, show a marked disorganisation in their nuclei. With the progressive failure of nuclear activity, a diminution in the control of synthesis may be expected and the decline in cytolytic enzymes observed during the last phase of grain ripening may reflect this changeover from a state in which

protein synthesis is active (ie. in young endosperm cells) to one in which the synthesised protein is exposed to proteolysis, without any balanced synthesis to counteract breakdown.

The apparent increase in transferase activity from cellobiose is likely to be a reflection of cellobiase activity in general, in presence of a substrate- enzyme ratio which favours synthesis. It is however very interesting to note that this actively synthesising system is again present in the grain when the wall construction must be a major activity in the developing endosperm. The apparent absence of a U.D.P.G. transferase suggests that the pathway of synthesis of hemicellulose in the endosperm wall may be different from that which has been claimed to operate in the formation of the β1:3 linked glucan (callose) of phloem sieve plates in mung bean seedlings (Feingold et al.,1958) and for hemicellulose synthesis in Bromus seedlings (Part III of this thesis). The failure to demonstrate U.D.P.G. transferase could admittedly be due to some fault in technique but, since the methods used were successful in detecting U.D.P.G. transferase in germinating seedlings, this seems unlikely. It seems more probable that the synthesis of endosperm cell wall hemicellulosic components (or some of them) may involve a transfer of single glucose groups directly on to the wall itself, ie. requiring a high molecular primer, according to the theories of Roelofsen (1959),Colvin(1959) and Colvin & Beer(1962) with their work on cellulose synthesis. It is equally possible that the glucosyl donor may not be U.D.P.G. but some other sugar nucleotide compound as has been recently found to be involved in cellulose synthesis in mung bean seedlings, Hassid et al., (1964).

TABLE 7 .

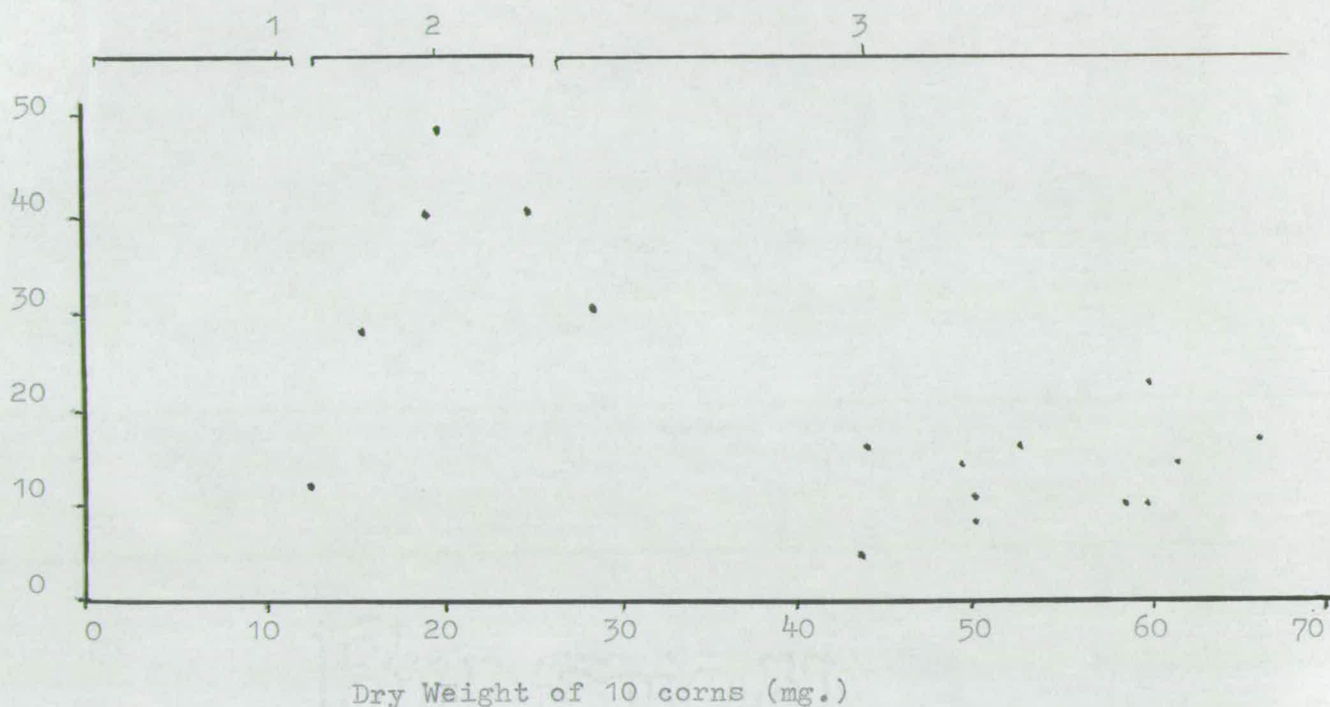
Endo-β-Glucanase Activity of Developing Grains of *B.ramosus*.

Fresh Weight of of 10 corns (mg.)	Dry Weight of of 10 corns (mg.)	Endo-β-Glucanase † (arbitrary units)
27.1	12.5	11.8
30.8	15.0	28.0
54.7	19.1	39.7
61.0	19.8	48.4
68.7	24.8	39.3
80.6	28.6	30.0
128.3	43.6	5.0
137.3	43.8	16.6
131.0	49.3	15.4
149.3	50.2	11.5
156.7	50.3	8.7
149.0	52.6	16.7
152.2	58.4	10.5
147.8	58.8	23.6
144.3	60.2	10.4
240.2	61.0	14.3
135.7	66.2	16.9
169.9	69.7	14.3

† Change in reciprocal specific viscosity/hour/10 corns/ x 100.

FIG. 9. Endo-β-Glucanase Activity of Developing Grain of Bromus ramosus.

ENDO ACTIVITY



The units used in the above figure are the same as those used in Table 7. The three stages of development marked above the graph denote three distinct morphological stages -

Stage 1. The flower is either unfertilised or recently fertilised; anthers are still fresh, stigma fully extended and spikelets open to pollination.

Stage 2. The grain is developing in size during this phase but is still green. The inflorescence is drooping but the spikelets are compact. The anthers are withered and brown or absent, having fallen.

Stage 3. During this phase the grain is hardening and the lemma and palea are yellowing and withering.

TABLE 8 .

Pentosanase Activity of Developing Grains of Bromus ramosus.

Stage of Development (from Fig.9)	mid 2	mid 3	late 3
Fresh Wt. of 10 corns (mg.)	27.8	94.8	132.6
Dry Wt. of 10 corns (mg.)	17.2	33.9	46.7
Pentosanase Activity ‡	0.59	2.75	1.27

‡ Increase in reciprocal specific viscosity, per 10 corns, per hour, x 100.

Substrate used was rye araboxylan.

TABLE 9 .

Location of Endo-β-Glucanase Within the Grain of B.ramosus.

Stage of Development (from Fig. 9)	mid 2	early 3	late 3
Fresh Wt. of 10 corns (mg.)	61.0	80.6	150.6
Dry Wt. of 10 corns (mg.)	19.8	28.6	47.4
Endo-β-Glucanase Activity ‡			
Lemma	32	42	38
Palea	27	29	34
Caryopsis	41	29	26
Stigma	0	0	0

‡ Endo-β-Glucanase activity is expressed as % of total grain activity.

TABLE 10 .

Cellobiase/Transferase Activity of Developing Grains of B.ramosus.

Stage of Development (from Fig. 9)	Dry Weight of 10 corns (mg.)	Cellobiase/Transferase (expressed as change in reducing power of the 'substrate') *
mid 2	17.2	+ 2.1
mid 3	33.9	+ 2.9
late 3	46.7	- 2.2

* Change in reducing power is expressed as mg. reducing sugar, as glucose, corrected for (i) enzyme, (ii) initial substrate.

SECTION C.

Changes in Activity of Hydrolases during Germination and Seedling Growth.

A wealth of information has been provided by biochemists regarding the mechanisms involved in the degradation of cell wall polysaccharides in the grains of the Gramineae. In recent years, a limited amount of research has provided some information on the changes in the balance of the different cytolytic enzymes during germination and subsequent seedling growth, Preece (1957) and MacLeod (1961), but as yet little attention has been paid to the need to relate these biochemical changes to observed structural changes taking place in the grain. The information given in this and the following section of this thesis is the result of a preliminary attempt to integrate the biochemical and structural changes which occur in the germination of the caryopsis of *Bromus*. As this problem of enzyme development is of major importance in the understanding of the process of malting, frequent comparisons have been made between this genus *Bromus* and barley.

Hydrolytic Changes during Natural Germination and Seedling Development. Samples of *Bromus inermis* were dehusked in 50% sulphuric acid (see Appendix I), (which also helped to sterilise the grain). Several samples of 10 grains were then grown on moist filter paper in Petri dishes at 25°C. for the varying periods required. After the required growth period each sample of germinated grains was collected, the embryos were removed and then the remaining portion (aleurone + endosperm) was cut into appoximately 1 m.m. thick slices.

These slices were quickly ground in 0.6% sodium chloride soln. (2 ml. per 10 corn sample) in a micro-wet grinder. After a 90 minute extraction period at room temperature, the debris was removed by centrifugation and the extract used immediately for the determination of enzymic activity. The methods of enzymic assay were exactly the same as those used in section B of this thesis.

The results of the determinations of endo-glucanase and α-amylase in the various seedling stages are given in Table 11 and Fig. 10 . These results are based on triplicate assays for each growth stage. From these figures it can be seen that endo-β-glucanase increases steadily after about 21 - 24 hours until a peak value is reached after approximately 4 days. It appears that α-amylase release starts about 3 - 6 hours after the glucanase and then increases steadily over the 5 days of the study period, ie. its peak activity is later than that of the glucanase. These values are in agreement with studies of other aspects of Bromus germination. For instance, MacLeod & Sandie (1961) observed that the percentage of total β-glucan in B. mollis falls rapidly after 4 days growth at 25°C. and also light microscopy of the germinating grain has shown a rapid dissolution of endosperm cell walls between 4-5 days; these structural changes will be considered more fully in the discussion after section D of this thesis.

The Importance of the Embryo in Release of Hydrolytic Enzymes.

The previous set of determinations was based on grains which had been grown intact and then had the embryos removed before extraction of enzyme material. In this next determination two sets of grain samples were examined, one with intact grains (i.e. plus embryo) and the other with the embryos carefully removed. Both sets were incubated in moist Petri dishes over periods of 24 and 48 hours and the endo-glucanase activities of the different samples were determined. The results of these determinations are listed in Table 12 .

It can be seen that grains grown intact produce a considerable increase in glucanase activity yet the grain maintained with embryos removed (ie. only endosperm - aleurone) show only a small increase over the first 24 hours which then drops slightly over the next 24 hours. From these results it can be implied that the embryo is apparently involved in the release of hydrolytic enzymes. Many similar determinations with barley resulted in the formation of the hypothesis that the embryo secretes hydrolytic enzymes during germination.

However, it was found that if gibberellic acid was added in low concentrations to endosperm - aleurone slices from barley, it induced a very extensive release of hydrolytic activity, Yomo (1958), Paleg (1960), and MacLeod & Millar (1962). It thus seems that the embryo may be indirectly involved in the production of hydrolytic enzymes by producing some substance

/ with an action similar to gibberellic acid which induces the formation and/or release of the hydrolytic enzymes from the cells of the aleurone. It seems that this substance may in fact be a natural gibberellin of the type already extracted from barley grains, Radley (1959) and Lazar (1961). The evidence of the actual transport of some substance from the embryo along the aleurone layer is based on the 'ringing' experiments originally carried out by Schander (1934) with rice and subsequently repeated by MacLeod & Millar (1962) with barley, when such treatment was found to prevent the release of hydrolytic enzymes causing the solubilisation of the endosperm and the consequent germination of the grain.

The Effect of Gibberellic Acid on the Release of Hydrolytic Enzymes from Endosperm - Aleurone Slices of *Bromus*.

A brief investigation of the gibberellic acid - endosperm interaction previously observed in barley was made using similar slices from *Bromus*.

Embryos were removed from 10 corn samples of dehusked *B. inermis* and each of the remaining grains was cut into 1 m.m. thick slices. Slices from 10 corns were incubated in 2 ml. of culture fluid in small (2") Petri dishes at 25°C.. The culture fluid was either distilled water or a solution of gibberellic acid in distilled water (5 p.p.m.). After the incubation period, enzyme extracts were prepared by grinding the slices in the culture fluid and washing out the Petri dish and grinder with 2 ml. of 1.2% sodium chloride, debris was removed by centrifugation and enzyme activity was determined

/ by the techniques already described.

The results of these determinations are given in Table 13. It can be seen that they are in general agreement with those of other workers with barley, eg. MacLeod and Millar (1962).

It was considered that a detailed study of the time-course of this gibberellic acid - induced enzyme release should be made and accordingly endosperm-aleurone slices of *Bromus inermis* were incubated with gibberellic acid (5p.p.m.) for a series of incubation periods ranging from 12 - 48 hours at 25°C.. The results of these assays are given in Table 14 and Fig. 10 . It can be seen that the maximum glucanase activity is reached in about 36 - 42 hours, compared with 96 hours in the intact but untreated grain.

A close examination of the early stages of enzyme release in the intact grain and gibberellic acid-treated slices provides some interesting facts :-

1. Glucanase is released about 3-6 hours before amylase in both sets, (at least a 6 hr. difference in the gibberellic acid treated slices). The following questions suggest themselves - is the release of glucanase necessary before amylase can be released ? Does gibberellic acid cause the release of some other substance which is necessary for the release of amylase ? It is not unreasonable to suppose that a cell wall-degrading enzyme be released before the enzymes responsible for degrading the materials held within these walls, but how such a time lag is controlled is a difficult problem indeed.

2. Glucanase is not released until after 21-24 hrs. in the intact grain yet it is released after only 12-15 hours in the gibberellic acid -treated slices. This 12 hour difference may be due to several factors -

(i) penetration delay of water into intact grain,

(ii) a higher concentration of the stimulating substance, Duffus(1964),

(iii) the time for the embryo to produce endogenous gibberellin (or similarily acting substance),

(iv) the time for this substance to reach the aleurone and thence the aleurone to produce and release enzymes.

3. Even with slices treated with gibberellic acid, no significant release of hydrolytic activity is observed until after 12 hours incubation, then there is an almost explosive release. This delay could be due to :-

(i) the time taken for the hydration of the cells in the slices,

(ii) the penetration of gibberellic acid into aleurone cells,

(iii) the time required before the aleurone produces the enzymes.

These various suggestions as to the mechanisms responsible for the release of hydrolytic enzymes will be considered in full in the main discussion on germination at the end of this Part of the thesis, ie. after section D ; when they will considered along with observed ultrastructural changes.

TABLE 11.

Changes in α-Amylase and Endo-β-Glucanase Release During the Germination of *B. inermis*.

Germination Time (hr.)	α- Amylase *	Endo-β-Glucanase †
12	1.48	49.0
15	2.03	41.0
18	3.33	53.0
21	?	25.0
24	6.40	31.0
38	11.33	320.0
50	22.00	413.0
73	32.9	731.0
96	77.9	1247.0
120	95.6	572.0

* - I.D.C. units, per 10 corns, per hour, (Briggs, 1961).

† - increase in reciprocal specific viscosity, per 10 corns, per hour, x 1000.

TABLE 12.

Effect of Embryo Presence on Endo -β- Glucanase Release.

Incubation Time (hr.)	Endo -β- glucanase †	
	- embryo	+ embryo
24	42	70
48	30	370

† - increase in reciprocal specific viscosity per 10 corns, per hour, x 1000.

TABLE 13.

Effect of Gibberellic Acid on Endo-β-Glucanase Release from Aleurone-Endosperm Slices.

Incubation Time (hr.)	Endo-β-Glucanase †	
	+ 5p.p.m.G.A.*	Water Control no G.A.
24	410	42
48	1,000	30

† - increase in reciprocal specific viscosity per 10 corns(equivalent number of slices), per hour, x 1000.

* - G.A. represents Gibberellic Acid.

TABLE 14.

Effect of Gibberellic Acid on Release of α-Amylase and Endo-β-Glucanase from Endosperm-Aleurone Slices from *Bromus inermis*.

Incubation Time (hr.)	α - Amylase *	Endo-β-glucanase †
12	0.00	26
15	0.00	145
18	0.00	336
21	9.50	423
24	22.10	750
38	50.00	1471

† - increase in reciprocal specific viscosity, per 10 corns, per hour, x 1000.

* - I.D.C. units, per 10 corns, per hour, Briggs(196).

FIG. 10.

ENDO β GLUCANASE.

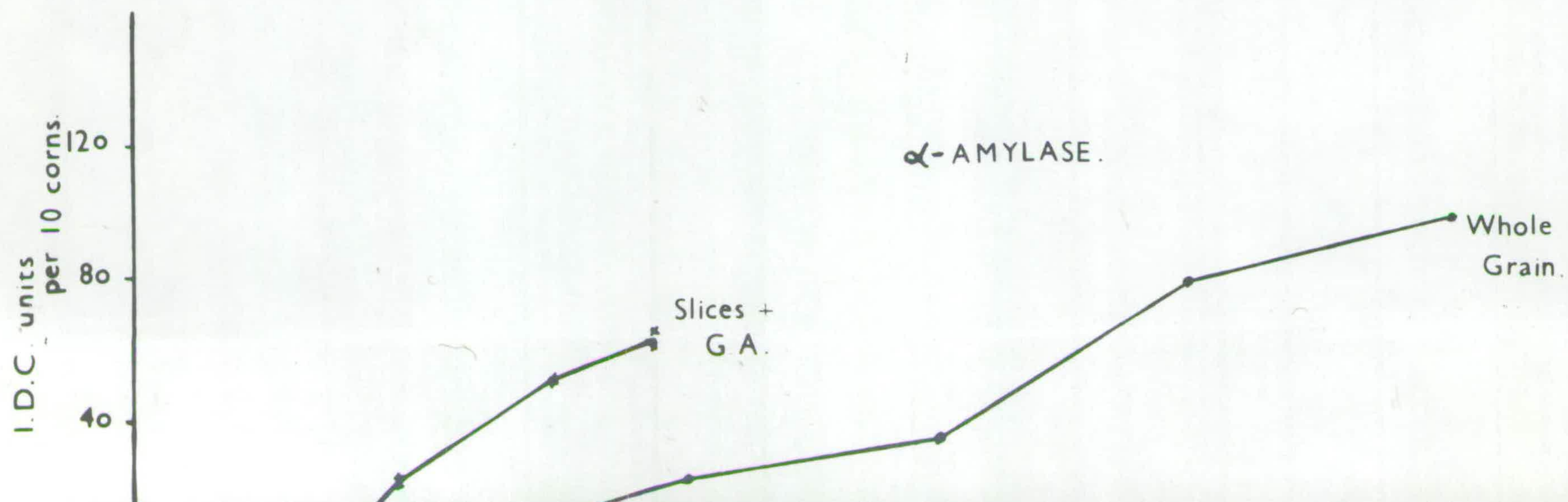
Increase in 1/μ.sp per 10 corns.

1600
1200
800
400
0

0
24
48
72
96
120

Slices + G.A.

Whole Grain.



SECTION D.

Ultrastructural Changes in the Germinating and Gibberellic Acid Treated Grain.

The biochemical studies have focussed attention on the aleurone as a source of the hydrolytic enzymes responsible for the mobilisation of the food reserves of the starchy endosperm. The preliminary ultrastructural studies outlined in Part I of this thesis have shown that the aleurone cell is unlike the normal plant cell in many ways, particularily in that it possesses several unusual inclusions. Unfortunately these studies of the ungerminated grain were interupted by a series of technical problems associated with fixation and embedding of the tissue and this resulted in little time being left to make this study with the germinating grain. A few electron micrographs have been obtained but their quality is very poor. However, electron micrographs obtained from gibberellic acid (G.A.) treated endosperm slices and isolated aleurone preparations from barley which have proved easier to embed than those from Bromus.

Some of these electron micrographs are given in Plates 35-41. It can be observed that the aleurone cells from slices of aleurone-endosperm treated with G.A. for 30 hours at 25°C. show the presence of essentially the same organelles as the ungerminated grain. The medium density deposits(Pr), which are believed to be proteinaceous, are still present and in fact are probably slightly more extensive. They still contain electron-dense inclusions(I) which appear, in some cases, to have a lamellar structure. Fixation of these inclusions has caused many to harden tremendously, with the result that they

fracture when cut, Plate 37. The other unusual organelle found in the ungerminated grain, the small vesicular structure (U.B.) is still present and appears to have increased in numbers, Plate 35. Many electron micrographs suggest that these inclusions may increase in number in the vicinity of the dense inclusions(I.), Plate 39. The association of these organelles(U.B.) with the cell wall and the periphery of the vacuolar deposits(Pr) observed in the ungerminated grain appears to hold true in the G.A.-treated grain. It is also interesting to note that in aleurone from treated endosperm slices the U.B.'s are still apparently electron-transparent at the 30 hour stage examined. This is unusual in that in the isolated aleurone the outer layer of the U.B. becomes electron-dense, Plate 40-41 and Paleg and Hyde(1964). This change from electron transparent to electron-dense was also observed in wheat, Buttrose(1963) and is seen in Bromus if the aleurone is examined after incubation of an intact grain for 18 hours. This change, therefore, seems to require the presence of the embryo or added G.A. and also requires the presence of the starchy endosperm. This problem is at present being investigated further. It seems that this U.B. may well play a very important role in the germinating grain.

In the isolated aleurone it was also found that large transparent inclusions appear in the cytoplasm but their origin is uncertain. They could arise by increase in size of the U.B.'s or they could arise by fusion of the transparent inclusion(V) in the vacuolar deposits, Paleg and Hyde(1964). It has been observed that in the isolated aleurone which has been treated with G.A., the protein deposits(Pr) increase greatly in size. This increase is much greater than that observed in the G.A. treated slices.

Structures of a mitochondrial nature(M) appear in fairly large numbers in the aleurone cell, Plates 37&38 but again considerable doubt exists as to their origin. The small dense inclusions(D.B.) of the ungerminated grain now appear to be distinctly vesicular when examined carefully. It is also possible that their origin lies in the Golgi apparatus seen by Paleg and Hyde(1964).

These techniques have also revealed intense staining in localised portions of the nucleus, Plate 37 . This staining includes the nucleolus(NL). Similar staining has been seen in the ungerminated grain but it appears to be more extensive and intense in the G.A. treated aleurone. It thus seems possible that there is considerable activity(reducing action) in the nucleus but the significance of this is unknown.

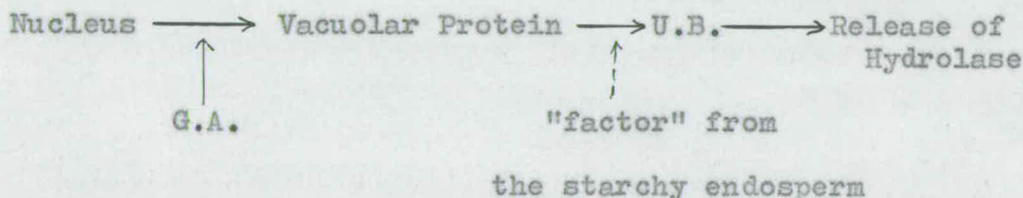
The possible roles of the main cytoplasmic inclusions, the vacuolar deposits(Pr) and the unidentified body(U.B.) are unknown but it is interesting, although dangerous, to speculate a little at this point.

The only structure which appears to parallel the distribution of protein in the aleurone is the vacuolar deposit(Pr). Its medium-density nature with $KMnO_4$ fixation would agree with the supposition that this deposit is in fact proteinaceous. It thus seems possible that this deposit is the source of enzymic protein but whether the deposit observed is already in an active enzymic form or is inert is as yet unknown. This theory agrees with the observed differences between aleurone from slices and in the isolated form. This deposit(Pr) is very extensive in isolated aleurone which has been shown not to secrete hydrolases; but is not so extensive in aleurone from slices which are secreting large amounts of hydrolytic enzymes, MacLeod _et al._(1964).

It is interesting to note that if the non-secreting cells of the isolated aleurone are ruptured, hydrolase release occurs, presumably from a cytoplasmic reserve, possibly the deposit(Pr) described. It is also significant that the U.B.'s are present in large numbers and apparently increasing in number in the secreting cells of the slices. They are present in smaller numbers in the non-secreting isolated aleurone cells. This obviously implies that the U.B. is directly involved in either hydrolase production or release(more probably the second), especially when its association with the cell wall is considered. Further evidence for the involvement of the U.B. in hydrolase release comes indirectly from the work of Buttrose(1963) who established the presence of these structures in the developing aleurone cell, which in Bromus at least has been shown to possess a fairly powerful endo-β-glucanase activity.

The action of G.A. and other factors in the release of hydrolases is considered below.

Suggested Mechanism for Production of Hydrolases.



The action of gibberellic acid would have to be early in the above pathway to explain the differences in ultrastructural changes in isolated aleurone and aleurone from slices; the actual release side of the pathway requires the presence of the endosperm. These suggestions would be in complete agreement with the biochemical findings of MacLeod et al.(1964).

Another interesting series of observations on the structure of the cell wall of germinating and G.A.treated aleurone has indicated the presence of very large numbers of plasmadesmata, Plates 30,31 and 34 . Although insufficient evidence is available at present it seems possible that there are more of the cytoplasmic interconnections in the aleurone of the germinating grain(or G.A. treated) than in that of the ungerminated grain. This may well be due to sampling error but it seems unlikely as all specimens of germinated and G.A. treated grain show aleurone walls with large numbers of plasmadesmata whilst only a few were observed in hundreds of specimens from ungerminated grain. This presence of large numbers of plasmadesmata would obviously simplify enzyme transport in the grain, especially from the outer layers of the aleurone in barley. If this increase is genuine it would create an interesting problem as to how the new pores are formed but could fit in nicely with the early formation of endo-enzymes(endo-β-glucanase) which are capable of cleaving β1:4 linkages, the predominant linkage in plant cell walls.

DISCUSSION.

The possible significance of the presence of hydrolases in the developing grain has been dealt with already, therefore this discussion will be devoted to the pattern of gross chemical, enzymic and structural changes in the germinating and gibberellic acid-treated grain.

Examination of Fig.10 shows that β-glucanase activity starts increasing after about 24 hours and reaches a peak after 4 days(96 hours) at 25°C. Such a finding would suggest that after 4 days the β-glucan content of the grain would be expected to fall rather rapidly. This is exactly what happens, as can be seen if suitable figures are extracted from the data published by MacLeod and Sandie(1961). They showed that over the first three days of growth there is a slight rise in extractable hemicelluloses, presumably due to mobilisation of some of the 'insoluble fraction'. After 3-4 days there is a rapid fall in glucan content, especially from 4 - 6 days , from 8.0% to 2.0%. This drop is also very neatly demonstrated in optical studies which revealed that there was a rapid dissolution of endosperm cell walls after 4-5 days.

Amylase activity rose steadily over the first three days but it continued to rise after the β-glucanase had begun to fall. After dissolution of most of the endosperm cell wall, the mobilisation of starch degradation products would be greatly simplified and the presence of powerful amylase at this stage is not unreasonable.

Careful examination of changes in reducing sugars and fructosans(section B of Part I) shows that over the first 24 hours there is a decrease in reducing sugars which are presumably

being utilised by the embryo, James(1940). Over this period the fructosan level stays constant ; then it increases steadily presumably at the expense of the degradation products of starch, etc. which appear at this point, following the release of α-amylase. As α-amylase activity and, presumably, starch degradation increases, the level of fructosan also rises. The subsequent breakdown of these fructosans results in a rise in reducing sugars. Following this there is an increase in the growth rate of the embryo. Here, the fructosans play the role of a transient reserve between the high-polymer storage products and the free sugars required for growth.

Another important finding regarding the time course of events is that β-glucanase is released about 3-6 hours prior to α-amylase. The exact significance of this is very doubtful but it does help to emphasise one point, namely that these enzymes cannot be enclosed in an active form in a bag-like lysosome structure as suggested by MacLeod and Millar(1962), or they would be released together. The release of a wall polysaccharide degrading enzyme prior to the release of an enzyme resposible for the degradation of materials(eg. starch) within the walls of a cell makes a very interesting picture as it would presumably increase the penetration of enzymes, such as α-amylase, through the walls. Careful examination of the time courses, however, reveals that there is little modification of endosperm cell walls prior to α-amylase release. Therefore any change in wall structure which is going to facilitate α-amylase transport must be at a relatively fine level and must be carefully controlled. Such a mechanism might

involve the removal of some of the hemicellulosic materials from the intermicrofibrillar spaces in the aleurone walls or it might involve the formation of new or unblocking of old plasmadesmata.

PART III.

Transglycosylation in *Bromus*.

Introduction ....

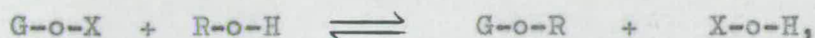
Section A - Glycosidases-Transosylases......

Section B - Nucleotide-Transosylases ....

Discussion ....

INTRODUCTION.

Most carbohydrases have been found to be essentially transosylases which catalyse the reaction -



G-o-X + R-o-H ⇌ G-o-R + X-o-H,

in which G is a glucosyl radical transferred from the donor molecule G-o-X to an acceptor R-o-H, and X is the displaced group which may be glycone or aglycone. Straight hydrolysis occurs if the acceptor is water -

G-o-X + H-o-H ⇌ G-o-H + X-o-H.

Transosylation was first shown by Rabate in 1935, when he presented good evidence for the transfer of 'active' glucose from aryl-β-glucosides to aliphatic alcohols in the presence of β-glucosidase. Since then, many workers throughout the world have provided evidence of transosylase activity in several forms of plant and animal tissue. A survey of the literature indicates that these transosylases can be divided into distinct categories depending on the chemical nature of the donor molecule. The two groups which are of interest in this work are :

(i) transosylase - glycosidases, where the donor is a glycoside but the dominant action of the enzyme is hydrolytic ; and

(ii) nucleotide - transosylases, where the donor is a nucleotide-ose, eg. uridine diphosphate glucose.

During recent research into the possible pathways of polysaccharide biosynthesis, the role of transosylase enzymes

has received increased attention. The enzyme amylomaltase, (maltose-amylodextrin trans-α-glucosidase), found in *E.coli*, (Monod & Torriani, 1948), forms an amylose type of polysaccharide from maltose. Many attempts have been made to find and isolate an enzyme capable of synthesising the β-linked glucosan, cellulose and when this work was in its final stages no definite isolation had yet been made, although a recent paper by Hassid *et al.*,(1964) appears to present positive results. Current research suggests that enzymes of the nucleotide-transosylase type are involved in cellulose synthesis, with either U.D.P.G.,(see - Glaser,1958; Feingold *et al.*, 1958; Brummond, 1960; Peaud-Lenoel,1960; Klungsoyr, 1960; and others) and/or other nucleotide-oses, eg. G.D.P.G., Hassid *et al.*,(1964). Although attempts to synthesise cellulose from U.D.P.G. *in vitro* from plant preparations have failed, animal biochemists have succeeded in synthesising glycogen from U.D.P.G. using particulate preparations from liver, Leloir & Cardini,(1957), whilst workers using the fungal species *Neurospora crassa* have prepared extracts capable of synthesising chitin from U.D.P.acetylglucosamine, Glaser & Brown,(1957). Perhaps the closest the plant biochemist has approached to the *in vitro* synthesis of cellulose is shown by the researches of Feingold *et al.*(1958). These workers have isolated an enzyme from mung bean(*Phaseolus aureus*) seedlings capable of synthesising a β1:3 linked glucan from U.D.P.G., although workers in that same Research School have suggested that U.D.P.G. may not be the glucosyl donor in cellulose synthesis, Hassid *et al.*,(1964) but their work is still in its early stages.

SECTION A.

Glycosidases - Transosylases

1. Trans - β - glucosylation.

The researches of Preece et al. (1957),(1958), into the degradation of β-glucan, a hemicellulosic polysaccharide consisting of glucose units linked by β1:3 and β1:4 glycosidic bonds,(Preece et al.,1960), indicated that enzyme preparations from barley possessed a component capable of synthesising the β-1:6 linked disaccharide, gentiobiose. Later studies, in particular those of MacLeod & Sandie(1961), established the presence, in Bromus spp., of a trans-β-glucosylase capable of synthesising several oligosaccharides from the β1:4 linked disaccharide, cellobiose. Their findings were in agreement with those of Anderson & Manners(1959), who had verified the presence of a similar transferase action with barley β-glycosidase. Normally, however, transferase activity is only detectable using high concentrations of substrate, eg. 20% cellobiose, as used by Anderson and Manners, (1959), but the interesting feature of the following study is that synthesis can be detected using very low concentrations of substrate. It was found that several preparations from Bromus spp. which might have been expected to show considerable cellobiase activity accomplished hydrolysis to only a minor degree. It was thought that this might have been due to competitive synthesising activity. As the determination of cellobiase involves a measurement of the increase in reducing power, synthesis would prevent such an increase and in fact could cause a decrease if it was extensive enough.

Preliminary chromatographic studies revealed the synthesis of up to six new oligosaccharides with Rf values ranging from 0.24 - 0.002 after 5 hours enzymolysis,(MacLeod & Sandie, 1961). The present work has verified the preliminary findings of MacLeod & Sandie,(1961) and also has provided much information into the mechanism of action of these powerful transosylases in Bromus.

Experimental. Preparations of precipitated enzyme,(see the next section on enzyme preparation) from mature ungerminated Bromus inermis grain were incubated with three concentrations of cellobiose at 25°C. to establish the effect of transferase on the determination of cellobiase activity.

After 2 hours incubation :-

Cellobiose Concentration	1.0%	0.5%	0.1%
Change in Reducing Power*	-9.65	-4.37	-0.80

* Reducing power was determined as mg. glucose equivalent, per hour, per mg. Enzyme preparation ; corrected for reducing action of enzyme and substrate blanks.

The decrease in reducing power obtained with 0.5% and 1.0% cellobiose substrates indicates the presence of a powerful synthesising system.

The majority of the results given in this section of the thesis are qualitative in nature, based on chromatographic studies but a rough indication of the quantitative aspects of

/ this transferase activity can be obtained from the symbols used in the tables denoting the approximate amount of each oligosaccharide detected on chromatograms.

<u>Enzyme Preparation</u>. The same 0.6% sodium chloride extraction technique was employed as was used for glucanase determination. After 90 minutes, the extract was filtered through muslin, centrifuged and then allowed to autolyse overnight. This was followed by dialysis against running water for two days at room temperature. After further centrifugation, the enzyme preparation was precipitated out of the supernatant with 4 volumes of acetone. The dried precipitate was used for activity determinations.

Material used for these studies was the mature, ungerminated grain of <u>Bromus inermis</u>, Leyss., obtained from Canada, unless otherwise stated.

<u>Substrates Used</u>. Cellobiose and gentiobiose were obtained from Messrs. Light & Co.Ltd.. It was found that both these products contained traces of contaminating sugars. Cellobiose contained traces of cellotriose and glucose, whilst gentiobiose contained traces (minute) of gentianose. Samples of these sugars were purified by column chromatography and the pure substrates produced the same results as obtained with the original Light's samples when incubated with <u>Bromus</u> enzyme preparations. Initial studies with gentiobiose were carried out using samples of chromatographically pure gentiobiose obtained by alcoholic extraction from roots of <u>Gentiana</u> sp..

Laminaribiose was obtained in a chromatographically pure form from Dr.W.P.Black of the Arthur D. Little Institute, Inveresk. Glucose, xylose, arabinose and fructose used in some of the studies were B.D.H. 'Analar' products. Glucose-1-phosphate was a Light's product.

Other Reagents Used. Buffers used were at a molarity of 0.1-M and were made from Analytical Grade chemicals,

Sodium acetate:HCl buffer for - pH= 2 - 5.4

Citrate buffer for pH = 6.0

Tris buffer for pH = 6.0 - 9.0, and were

all stored at 4°C before use.

Glucose oxidase and catalase were both Light's products.

Determination of Enzyme Activity. Unless otherwise stated, the reaction mixture contained components in the ratio :-

8 ml. substrate,

2 ml. enzyme solution, containing 1.1 mg. precipitated enzyme per 1 ml. distilled water,

1 ml. 0.1-M acetate buffer, pH = 5.0.

Incubation was at 25°C. and enzymic action was stopped by boiling at 100°C. for 5 minutes. After cooling, the reaction mixture was filtered to remove precipitated protein material, etc.. The supernatant was concentrated down to a few drops and tentative identification of oligosaccharides produced was made by means of paper chromatography and related techniques.

Oligosaccharide Characterisation.

Chromatography. The chromatographic technique employed was that of descending paper chromatography on Whatman No.1 paper, using a butanol:acetic acid:water (4:1:5) solvent at 25°C.. Detection of oligosaccharides on the dried chromatograms was by spraying with aniline hydrogen oxalate and heating for 5 - 7 minutes at 100°C.. The identities of the oligosaccharides were determined by a series of co-ordinated techniques,

(i) running of control (known) oligosaccharides on the same chromatograms as the unknowns and comparing rates of movement,

(ii) calculation of R_f and Log $1\text{-}R_f/R_f$ values for all the unknown oligosaccharides produced and basing their tentative identities on the graphic relationship between Log 1-Rf/Rf and saccharide level. This method (French & Wild, 1953), enables the possible saccharide level and linkage to be established.

If a more definite identification was to be attempted, the concentrated reaction mixture was run as a streak across a paper chromatogram and after separation the unknown components were eluted by 30% ethyl alcohol. After elution, the oligosaccharide solution was concentrated, then -

(i) unknown was re-run, with controls, and the Rf and log 1-Rf/Rf were accurately determined,

(ii) the eluted oligosaccharide was co-chromatographed with a true sample of oligosaccharide of the suspected structure of the unknown. If the spots did not separate after prolonged running it was taken as possible that the two

/ oligosaccharides were actually identical,

(iii) electrophoresis of unknown was performed in borate buffer(to determine linkage type at reducing end of molecule) and in bisulphite buffer(to determine molecular complexity),

(iv) on certain occasions it was necessary to hydrolyse unknown oligosaccharides by boiling in 2N.sulphuric acid, followed by identification of the products of hydrolysis.

Electrophoresis.

Determination of Molecular Complexity. This was established by separation in 0.4-M. sodium bisulphite buffer for 6 hours at 50 milliamps and 500 volts. Glucose and cellobiose were used as reference spots for the calculation of $1/M_G$ values. Whatman No.1 paper was used.

Determination of Linkage Type. The mobility of oligosaccharides in borate buffer depends on the linkage type nearest to the reducing end of the molecule; 0.2-M.borate buffer was used and separation was for 3 hours at 1000 volts.

It must be realised that even after the use of the above series of identification techniques, the identity of the oligosaccharide can only be given as tentative ; further chemical methods, such as methylation, would be needed for complete characterisation.

TRANSFORMATION OF CELLOBIOSE.

In this study of the synthesis products from cellobiose substrate, the following aspects were examined :-

i) Preliminary identification of the oligosaccharides produced from cellobiose substrate,

ii) effect of substrate concentration on synthesis,

iii) effect of enzyme concentration on synthesis,

iv) effect of pH on synthesis,

v) effect of incubation time on synthesis -
   a) rate of appearance of oligosaccharides,
   b) stability of enzymes during prolonged incubation.

*   *   *

Spot :-	1	2	3	4	5a - c	5d	6	
Rf	0.2043	0.1210	0.090	0.0729	0.0506	0.0407	0.0312	
Log $\frac{1-Rf}{Rf}$	0.5906	0.8613	1.004	1.1043	1.2733	1.3726	1.4929	
Linkages	0 0 0	1 0 0	0 1 0	0 2 0 0 1 0	0 1 3 2 0 0 0 1 0	0 1 1	1 2 0	β1 : 3 β1 : 4 β1 : 6
Mobility *	+	+	-	+	-/+	-	-/+	
Complexity †	mono	di	di	di/tri	tri/tet	tri	tetra	
Possible Identity ‡	Glucose	G3G	G4G	G6G or G3G3G	G4G4G, G3G6G, G6G3G or G3G3G3G	G6G4G	G4G4G3G	

* - mobility, determined electrophoretically in borate buffer (see method).

† - complexity, based on $1/M_G$ value after bisulphite electrophoresis.

‡ - Possible identity is based on several factors, including extrapolation from graphic relationship between log 1-Rf/Rf (see figs.).

The R_f values given in Table 15, on the previous page, are based on the data from the chromatographic analysis of over fifty incubations of cellobiose substrate with Bromus enzymes from varying preparative methods(see page 50)using several species of Bromus , with particular attention being paid to Bromus inermis. Careful studies of the tri- and tetrasaccharides produced have shown that several different oligosaccharides were formed with similar R_f values. After detailed examination, 'single spots' on first chromatograms have often been shown by prolonged running, drying and re-running, followed by electrophoresis, to be mixtures of two or more oligosaccharides. For example, spot 4 in Table 15 (R_f = 0.0729) has been shown to be a mixture of gentiobiose and laminaritriose, with gentiobiose usually present in much higher concentrations than laminaritriose.

An extensive analysis of all oligosaccharides detected during this study on transosylase has been summarised in Tables 16 and 17 . The figures for mean values of Log $1-R_f/ R_f$ from these data have been used in extrapolating possible identities of synthesised oligosaccharides. The results of these summaries indicate that oligosaccharides produced by transosylase action are of similar chemical identities, independent of whether a glycosidase- transosylase or a nucleotide-transosylase enzyme is involved. It is interesting to note that no evidence has been found for the formation of sophorose, the β1:2-linked glucodisaccharide, which Manners(1960) detected as a product of trans-β-glucosylation. This fact is however in agreement with the findings of Anderson and Manners(1959) using barley /

β-glycosidase. Other sugars sometimes detected in glycosidase-transosylase activity include two phosphate containing compounds, but these have since been found to be regularily present in the enzyme extracts themselves. After elution and hydrolysis they yield only glucose and inorganic phosphate. They have R_G values of around 0.65 and 1.3.

No evidence has been obtained for the synthesis of any α-linked glucosides.

FIG. 11 The Graphic Relationship Between Log 1 - Rf/Rf and Saccharide Level.

a) for oligosaccharides with β1:4 and/or β1:6 linkages, containing only glucosyl units.

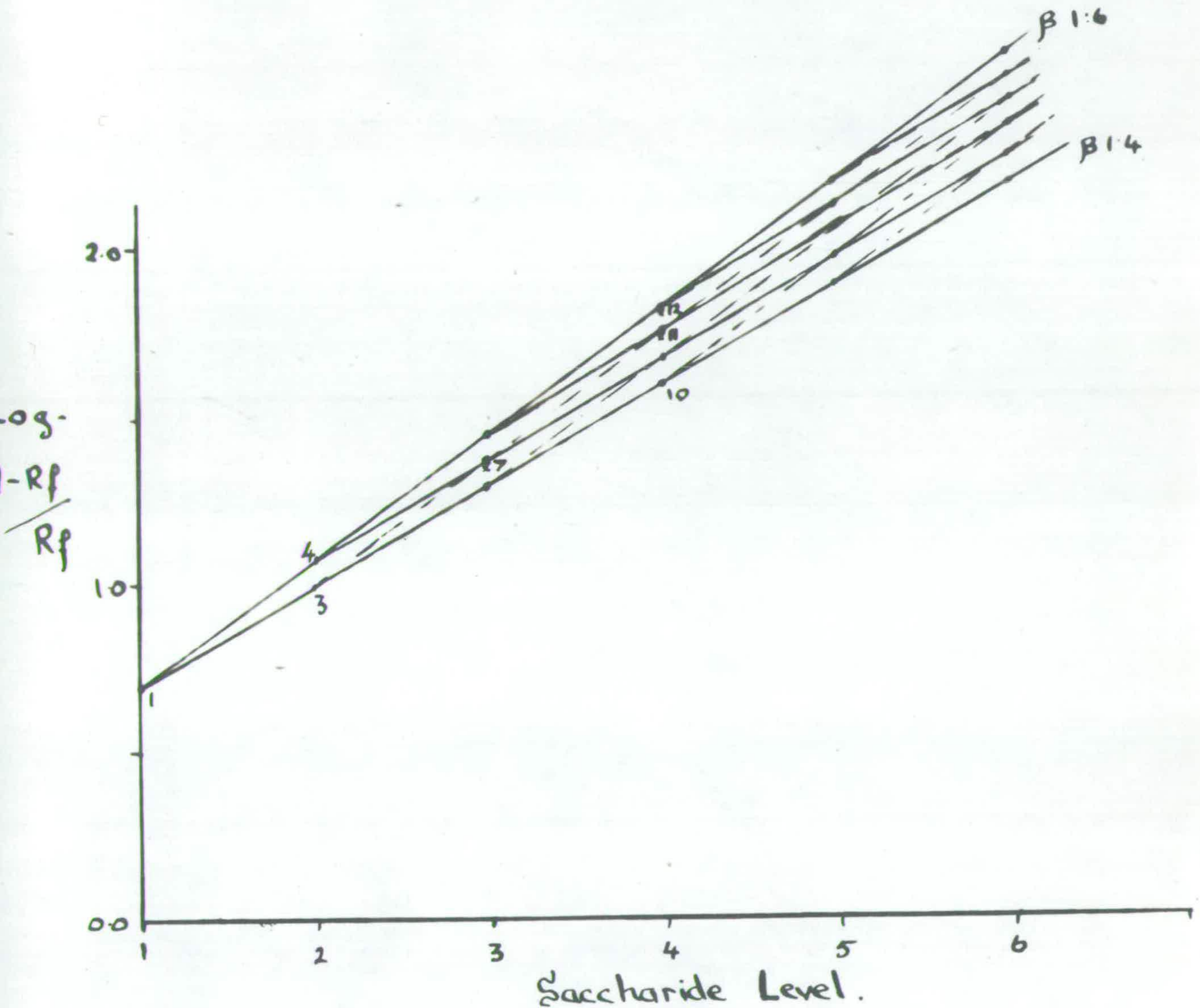


FIG. 11 The Graphic Relationship Between Log 1 - Rf/Rf and Saccharide Level.

b) for oligosaccharides with β 1:3 and/or β 1:6 linkages, containing only glucoayl units.

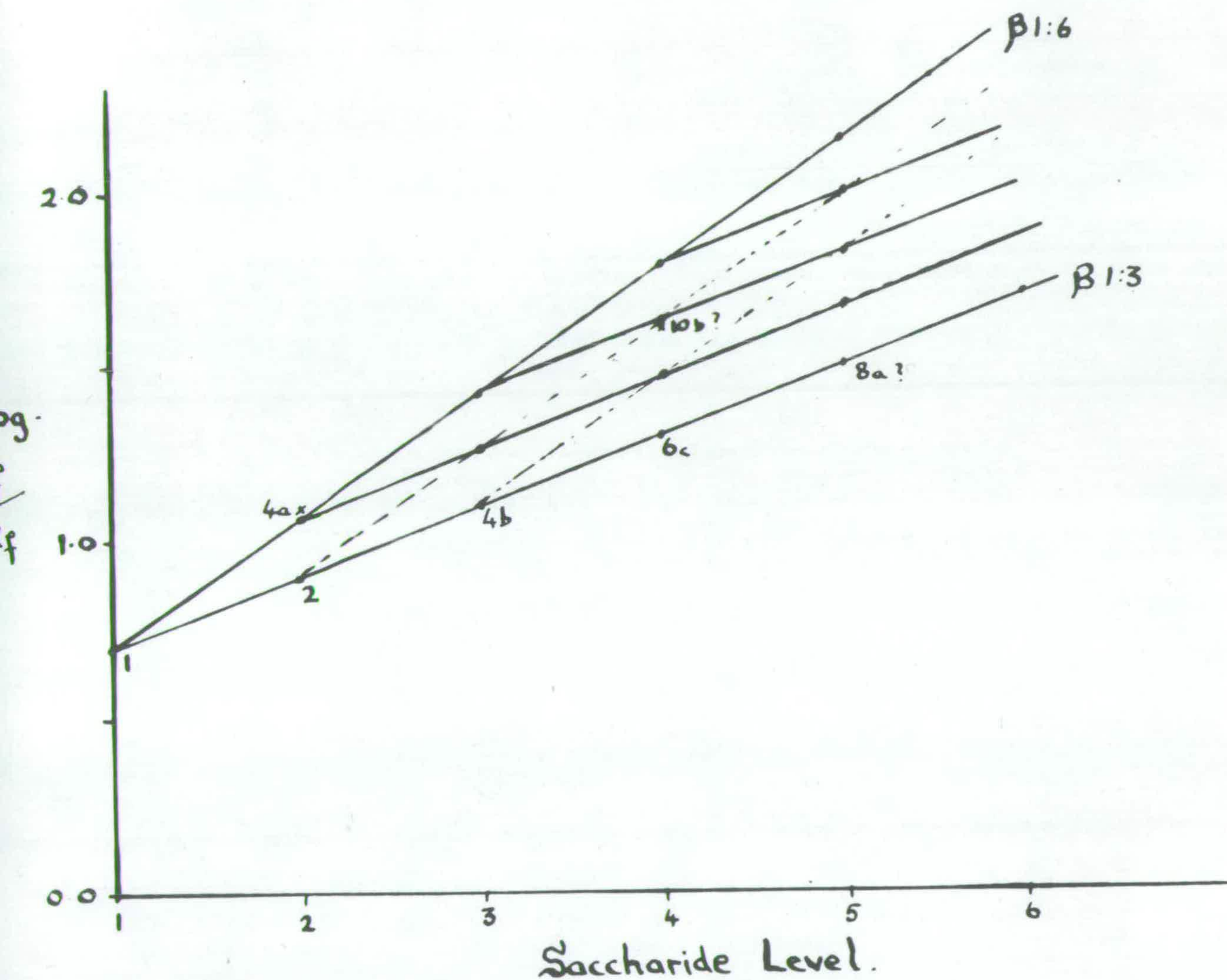
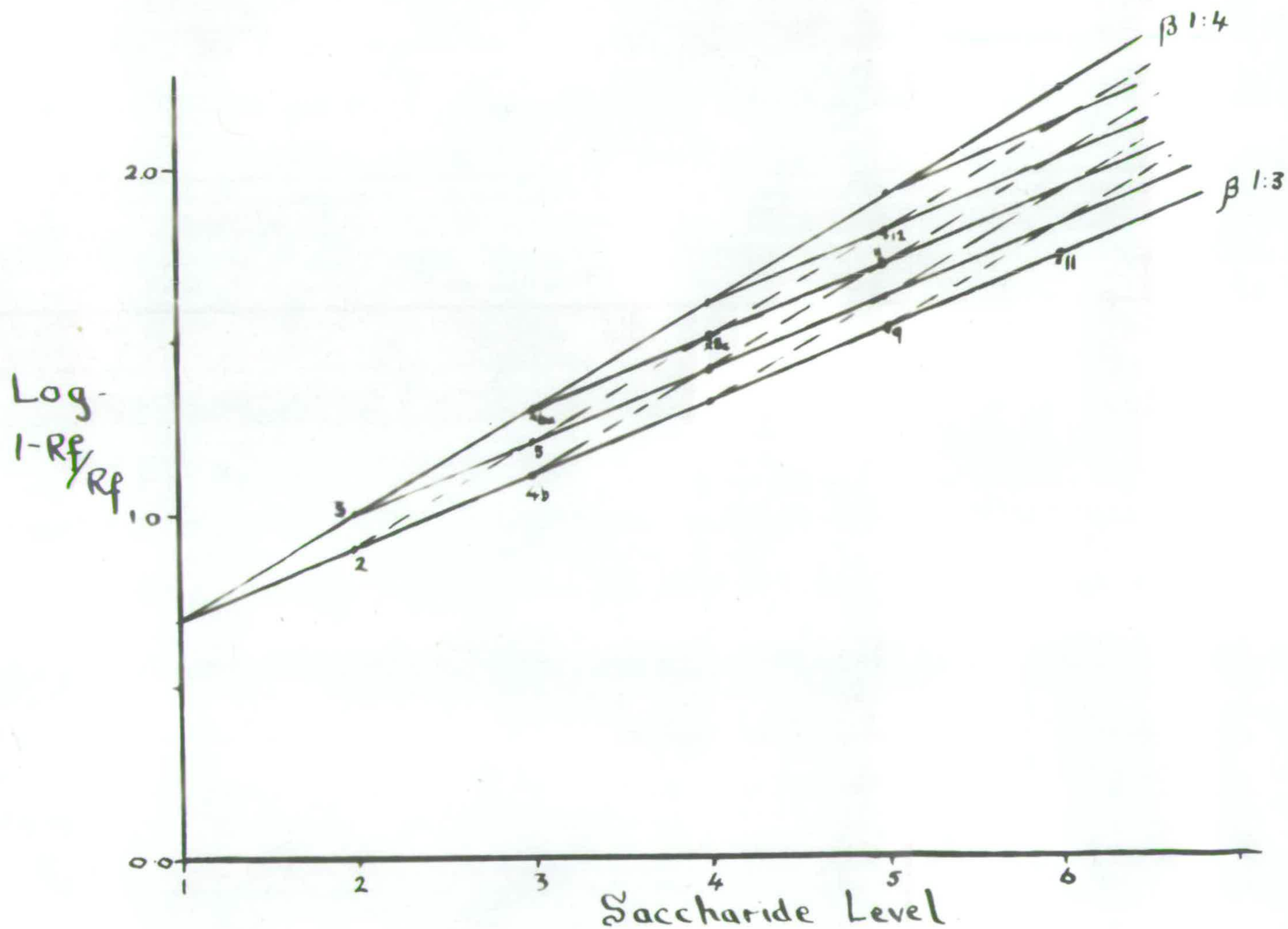


FIG. 11 The Graphic Relationship Between Log 1-Rf/Rf and Saccharide Level.

c) for oligosaccharides with β1:3 and/or β1:4 linkages, containing only glucosyl units.



Explanation to Table 16 , opposite.

This table is not complete ; final steps in characterisation were only made with oligosaccharides regularily appearing in incubations.

? - where a question-mark appears in the table it denotes that no data is available : this only occurs with respect to higher oligosaccharides, in particular those from U.D.P.G. incubations where only trace quantities were detected in relatively few chromatogram

+ - the numbers given to oligosaccharides are only relevant to this table, Table 16 and the associated text, they do not refer to chromatographic spot numbers.

** - Observed Log $1\text{-}R_f / R_f$ are mean values from all chromatographic studies made.

* - Calc. Log $1\text{-}R_f/R_f$ is based on extrapolation of values from the graphic relationship between saccharide level and Log $1\text{-}R_f/R_f$, see Figs. 11 a - c .

† - Mobility is a measure of ionic mobility in borate buffe

‡ - Complexity is a measure of mobility of compound in bisulphite buffer, which is dependant on molecular size

‡ - Linkages - based on extrapolation from Figs. 11 a - c ; o β 1:3 linkage as in laminaribiose is represented by 3^1.

TABLE 16 - Tentative Characterisation of Oligosaccharides Formed by Transosylation.

Oligo-saccharide +		Observed Log $\frac{1-R_f}{R_f}$ **	Calculated Log $\frac{1-R_f}{R_f}$ *	Mobility †	Complexity ‡	Possible Linkages ‡	Possible Structure
1		0.6700	0.700	+	mono-	-	G
2		0.8837	0.900	+	di-	3^1	G3G
3		1.004	1.004	-	di-	4^1	G4G
4	a	1.1083	1.066	+	di-	6^1	G6G
	b		1.087	+	tri-	3^2	G3G3G
5		1.2079	1.200	+/-	tri-	$3^1 4^1$	G3G4G or G4G3G
6	a	1.2864	1.28	-	tri-	4^2	G4G4G
	b		1.29	+	tri-	$6^1 3^1$	G6G3G or G3G6G
	c		1.30	+	tetra-	3^3	G3G3G3G
7		1.3680	1.36	-	tri-	$6^1 4^1$	G6G4G
8	a	1.4828	1.50	+	tetra	$4^2 3^1$	G4G4G3G
9	a	1.5295	1.49	?	?	$6^2 3^1$	?
	b		1.51	?	?	3^4	?
10	a	1.5875	1.59	?	?	4^3	?
			1.61	?	?	$4^1 3^3$	?
11	a	1.7220	1.740	?	?	$6^2 4^1$	?
	b		1.74	?	?	3^5	?
12	a	1.7916	1.78	?	?	$4^3 3^1$	?
	b		1.78	?	?	6^3	?

Explanation to terms used in above Table is given on the page opposite.

TABLE 17 - Full Chemical Identity of Oligosaccharides Characterised in Table 16.

Oligosaccharide	Log $\frac{1-R_f}{R_f}$	Structure	Correct Identity
1	0.6700	G	Glucose
2	0.8837	G3G	Laminaribiose or 3-O-β-glucosyl-glucose
3	1.004	G4G	Cellobiose or 4-O-β-glucosyl-glucose
4 a	1.1083	G6G	Gentiobiose or 6-O-β-glucosyl-glucose
b		G3G3G	Laminaritriose or 3-O-β-glucosyl-laminaribios
5	1.2079	G3G4G or G4G3G	4-O-β-glucosyl-laminaribios or 3-O-β-glucosyl-cellobiose
6 a	1.2864	G4G4G	Cellotriose or 4-O-β-glucosyl-cellobiose
b		G6G3G or G3G6G	3-O-β-glucosyl-gentiobiose or 6-O-β-glucosyl-laminaribios
c		G3G3G3G	laminaritetraose or 3-O-β-glucosyl-laminaritrio
7	1.3680	G6G4G	4-O-β-glucosyl-gentiobiose
8	1.4828	G4G4G3G	3-O-β-glucosyl-cellotriose

Oligosaccharide numbers and values for Log $1-R_f/R_f$ are based on Table 16.

No.1.

CELLOBIOSE SUBSTRATE.

C. 1 2 3 C.

EXPLANATION OF CHROMATOGRAM 1 .

Typical Oligosaccharide Production from a Cellobiose Substrate, using enzyme preparations from Bromus inermis,(see also Table 15 and text on page 92).

Mixture Identities :-

C - Control - of glucose, laminaribiose, cellobiose, gentiobiose and raffinose.

1 -
2 - 3 similar, but separate, preparations
3 - of enzyme from B.inermis.

Oligosaccharide Characterisation(tentative- based on the factors listed on page 89) :-

1 - Glucose

2 - Laminaribiose

3 - Cellobiose

4 - Gentiobiose

5a-c Trisaccharides (see Table 15).

(ii) Effect of Substrate Concentration on Synthesis.

A series of cellobiose substrate concentrations were incubated with B.inermis enzyme for 24 hours at 25°C. and the results of these incubations are given in Table 18 on the following page.

The method of recording the approximate quantity of each oligosaccharide formed was based on a visual estimate of the coloration produced with aniline hydrogen oxalate under controlled spraying techniques. A rough scale has been established where a single + sign represents a sugar which is just on the margin between being visible with the naked eye and being only detectable under ultra-violet irradiation. Thus,

t = trace amount, just detectable under U.V.,

+ = very distinct spot under U.V.,

++ = clearly visible by naked eye but still a relatively faint spot,

+++ = light brown-yellow spot, usually over ½ inch in diameter,

++++ = fairly dark brown spot, often but not always oval in shape,

+++++ = on occasions this sign is given, it denotes a spot which is so dense that it shows 'tailing'.

These signs will be used in many of the later tables in this section of the thesis. They are only intended as a rough guide to the ratio of the different oligosaccharides present and are at their most accurate level when estimating the

/ changes in the amount of one oligosaccharide under varying co conditions, as a degree of coloration is not an accurate method of comparing oligosaccharides of different saccharide levels.

TABLE 18 - Effect of Substrate Concentration on Synthesis.

Cellobiose Concn.	Glucose	Laminar -ibiose	Cellobiose	Gentiobiose	Higher Olig.
20.0	++	+	+++	+	+++
10.0	+++	++	++++	++	+++
5.0	++++	++	++++	++	+++
1.0	++++	+	++	+++	++
0.5	++++	-	+	++	+
0.1	+++	-	-	+	-
0.05	++	-	-	-	-
0.01	+	-	-	-	-

In the above table the cellobiose concentration is expressed as a percentage.

The above table shows that the extent of synthesis is directly related to substrate concentration, the higher concentrations favouring synthesis. Synthesis can be detected with substrate concentrations as low as 0.1% cellobiose. It is interesting to note that low concentrations, 0.1 - 1.0%, favour the synthesis of the β1:6 bond (gentiobiose) and higher concentrations, 5.0 - 10.0%, favour the synthesis of the β1:3 bond (laminaribiose). The possible reasons for these observations will be considered later.

(iii) Effect of Enzyme Concentration on Synthesis.

The enzyme concentration in the standard reaction mixture was 1 mg. enzyme preparation per 5 ml. mixture. Using a substrate concentration of 0.5% cellobiose, pH = 5, a 24 hour incubation at 25°C. was carried out using a range of enzyme concentrations.

TABLE 19 - Effect of Enzyme Concentration on Synthesis.

Enzyme Concentration	Sugar Identity				
	Glucose	Laminaribiose	Cellobiose	Gentiobiose	Trisacc.
50 mg./10 ml.	+++++	-	-	+	-
25.0	+++++	-	-	+	-
10.0	++++	t	t	++	t
5.0	++++	+	+	+	+
2.0	+++	++	++	+	++
1.0	+++	+	+++	+	t
0.5	++	t	+++	t	t
0.1	+	-	+++	-	-
Enzyme Blank -					
50.0	t	-	-	-	-
10.0	-	-	-	-	-

The above table is based on chromatograms (available but not included in thesis). The optimum enzyme concentration with the 0.5% substrate was therefore found to be 2mg. enzyme per 10 ml. reaction mixture, ie. that used in the other experiments. It is also interesting to note that very low enzyme concentrations favour hydrolysis.

(iv) Effect of pH on Synthesis.

The pH generally used for these experiments was pH = 5.0, ie. that used for hydrolytic studies with hemicellulases. A series of 24 hour incubations over a wide pH range was run to note the optimum pH for this transferase activity, using the same buffers in this work as were used for studying the effect of pH on endo- β -glucanase activity.

TABLE 20 - Effect of pH on Synthesis.

pH	Sugar Identity					
	Glucose	Laminari-biose	Cellobiose	Gentiobiose	Tri-sacch.	Higher Oligo.
2.05	++	-	++++	-	t	-
3.05	++	-	+++	?	+	-
4.15	+++	+	+++	+	+	t
4.86	+++	+	+++	+++	+++	t
6.00	+++	++	++++	++	++	-
7.15	++	-	++++	+	++	-
8.15	+	-	++++	?	t	-
9.00	+	-	++++	?	t	-
7.30 **	++	-	+++	-	+++	-

The optimum pH appears to be around 5.0, with a fairly broad peak between pH 4 - 6. The extract itself had a pH of 7.3 which did not alter during a 24 hour incubation. As this was the pH of the salt extract it does not mean that it bears any relation to the pH of the part of the cell where the *in vivo* reaction takes place, if it takes place at all.

EXPLANATION OF CHROMATOGRAMS 2 & 3.

Effect of pH on Oligosaccharide Synthesis from a Cellobiose Substrate.

Mixture Identities :-

C - Control - as in previous chromatograms.

1 - pH = 2.05

2 - 3.05

3 - 4.15

4 - 4.86

5 - 6.00

6 - 7.15

7 - 8.15

8 - 9.00

9 - 7.30 natural pH of enzyme extract, no added buffer used.

Oligosaccharide Spot Characterisation(tentative) :-

1 - Glucose

2 - Laminaribiose

3 - Cellobiose

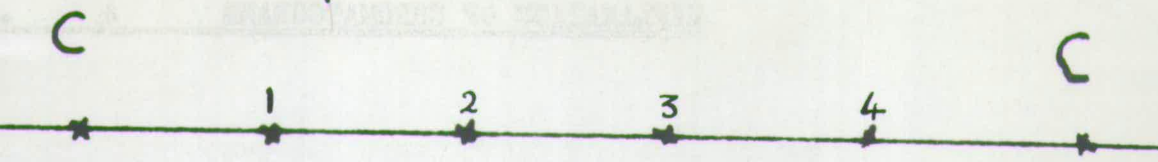
4 - Gentiobiose

5 - Trisaccharide(s)

6 - Tetrasaccharide(s).

No.2.

pH Effect - I



6

5

AFF.

4

ENT.

3

ELL

2

AM.

1

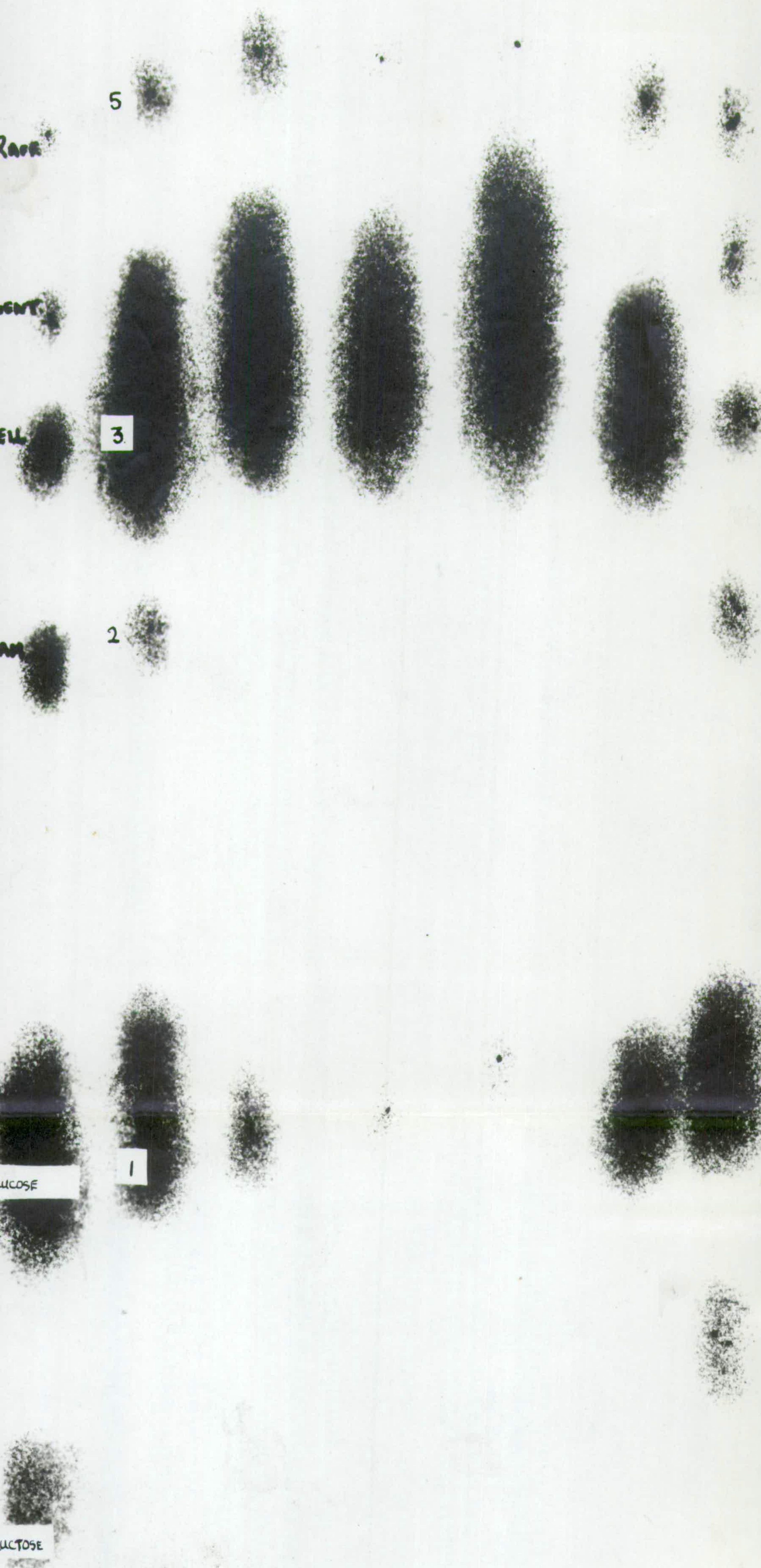
LUC

RUCT.

No.3.

pH EFFECT - II

C 5 6 7 8 9 C



(v) Effect of Incubation Time on Synthesis.

a) Rate of Appearance of Oligosaccharides (during the first stages of the reaction).

It was found in earlier incubations that traces of oligosaccharides other than cellobiose were produced within one hour. If concentrations of cellobiose above 0.5% were used, traces of trisaccharide as well as disaccharide were formed in the first hour. As there was a definite trend towards hydrolysis in solutions of 0.5% and lower, it was decided to make a detailed study of the early stages of transferase using a cellobiose concentration of 1.0%, with incubations at 25°C. The results of that study are given in Table 21 on the next page.

From the table on the following page it can be seen that hydrolysis was so rapid that glucose was detectable after only one minute as a minute trace, rapidly increasing over the first hour. The first oligosaccharide to appear was the β-linked gluco-disaccharide, laminaribiose, which was able to be detected after only 5 minutes incubation. It was not till after 40 minutes, however, that any other higher oligosaccharides appeared; at 45 minutes one was detected about the trisaccharide level, but no further work was carried out to establish if that spot was actually more than one related trisaccharide, as has been established in many of the other incubations. It was interesting to note that gentiobiose did not appear until after 5-6 hours incubation. It has also been established that higher concentrations of cellobiose/

/ substrate actually slow down the appearance of gentiobiose :-

0.1 % cellobiose - gentiobiose appeared after 1 hour,

0.5 % cellobiose - gentiobiose appeared after 5-6 hours,

1.0 % cellobiose - gentiobiose appeared after 6 hours.

This suggests that gentiobiose is, in fact, probably a degradation product of a higher oligosaccharide (eg. a trisaccharide) and is only formed when the overall equilibria are against synthesis and consequently in favour of hydrolysis (as in dilute substrate solutions).

TABLE 21 - Rate of Appearance of Oligosaccharides from 1% Cellobiose.

Incubation Time (mins.)	Oligosaccharide Identity				
	Glucose	Laminaribiose	Cellobiose	Gentiobiose	Trisacch.
0	-	-	++++	-	-
1.0	?	-	++++	-	-
5.0	t	?	++++	-	-
10.0	+	t	++++	-	-
15	+	t	++++	-	-
20	+	t	++++	-	-
25	+	t	++++	-	-
30	++	t	++++	-	-
35	++	t	++++	-	-
40	++	+	++++	-	-
45	+++	+	+++	-	?
50	+++	+	+++	-	t
65	+++	+	+++	-	t
90	+++	+	+++	-	t
5 hrs.	+++	+	+++	t	+
6 hrs.	+++	+	+++	+	+
24 hrs.	++++	+	++	+++	++

b) Stability of Enzymes Involved in the Different Stages of Transferase.

After extended periods of incubation, 36 hours to 14 days, it was found that only particular oligosaccharides persisted. A short study was made to determine whether this persistence of some oligosaccharides was based on the linkages involved.

TABLE 22 - Effect of Prolonged Incubation on Pattern of Oligosaccharide Production.

Cellobiose Concn.	Incubation Period	Glucose	Laminaribiose	Cellobiose	Gentiobiose	Higher Olig.
1.0%	36 hrs.	+++++	+	++	++	+
0.5	36 hr.	++++	-	+	+	t
0.1	36 hr.	+++	-	-	t	-
1.0%	5 days	+++++	t	+	++	+
0.5	5 days	++++	-	t	+	t
0.1	5 days	+++	-	-	t	-
1.0%	14 days	++++	-	t	++	t
0.5	14 days	++++	-	t	++	-
0.1	14 days	+++	-	-	t	-

The above table is based on a series of chromatograms which are available but because of their size have not been included in the thesis.

Prolonged incubation tended to result in the degradation of all oligosaccharides produced after short incubation, except gentiobiose which was degraded very slowly, still being present after 14 days incubation of 0.1% substrate.

Having established that enzymes from Bromus inermis and related species possess transosylase powers capable of synthesising laminaribiose and gentiobiose from cellobiose substrates, it was thought necessary to investigate the possibility of employing these other disaccharides (laminaribiose and gentiobiose) as sources of glucosyl groups in oligosaccharide synthesis.

TRANSFORMATION OF LAMINARIBIOSE.

TABLE 23 - Oligosaccharide Production from Varying Concentrations of Laminaribiose Substrate after 24 hours incubation at 25°C, pH = 5.0.

Laminaribiose Concn.	Oligosaccharide Identity					
	Glucose	Laminaribiose	Cellobiose	Gentiobiose	Trisacch.	Higher Olig.
1.0%	++++	++	+	+	+	+
0.5	+++	+	t	+	t	t
0.1	++	-	-	t	t	-
0.05	+	-	-	t	-	-
Enz.Blank	-	-	-	-	-	-

The above table is based on chromatogram 4 .

Examination of Rf values of the synthesised oligosaccharide at the trisaccharide level, detected on chromatograms after prolonged running, reveals that as with cellobiose, this one spot separates out into 2-3 and that at least one of these spots has a higher Rf than any of the trisaccharides produced from cellobiose. This fact will be considered in the discussion. It is interesting to note that gentiobiose is the only new oligosaccharide synthesised at low laminaribiose concentrations. This synthesis was even detectable with laminaribiose concentrations as low as 0.05%.

Examination of chromatograms from reaction mixtures after only two hours incubation of laminaribiose substrate shows a high degree of synthesis, cf. cellobiose.

TRANSFORMATION OF GENTIOBIOSE.

TABLE 24 - Oligosaccharide Production from Varying Concentrations of Gentiobiose Substrate, after 24 hours incubation at 25°C, pH=5.0.

Gentiobiose Concn.	Oligosaccharide Identity					
	Glucose	Laminaribiose	Cellobiose	Gentiobiose	Tri-	Higher Oligo.
1.0%	+	-	+	+++	t +	-
0.5	++	-	+	+++	t +	t
0.1	++	-	+	++	- t	-
0.05	++	-	t	t	- -	-
Gent.Control	t	-	-	+++	- -	-

The above table is based on chromatogram 5 .

Examination of the Rf values of the synthesised trisaccharides reveals that at least one of these trisaccharides has a lower Rf than any of the trisaccharides produced from cellobiose or laminaribiose. This fact will be considered in the discussion.

Incubation of a pure sample of gentiobiose, ie. after chromatographic removal of the trace of glucose contamination, still yielded the same enzymic products as shown in the above table. Similar results were obtained with a sample of pure gentiobiose prepared from <u>Gentiana</u> sp. roots,(purified on a Charcoal - Celite Column).

EXPLANATION OF CHROMATOGRAM 4 .

Oligosaccharide Production from Laminaribiose Substrate.

Mixture Identities :-

C - Control, as in previous chromatograms.

1 - 1.0% laminaribiose

2 - 0.5% laminaribiose

3 - 0.1% laminaribiose

4 - 0.05% laminaribiose

5 - Enzyme Blank.

Oligosaccharide Spot Characterisation(tentative) :-

1 - Glucose

2 - Laminaribiose

3 - Cellobiose

4 - Gentiobiose + laminaritriose

5 - other trisaccharides

6 - Tetrasaccharide(s).

LAMINARIBIOSE SUBSTRATES [24 HRS.]

No.4

C 1 2 3 4 5 C

EXPLANATION OF CHROMATOGRAM 5 .

Oligosaccharide Production from Gentiobiose Substrate.

Mixture Identities :-

C - Control

1 - 1.0% gentiobiose

2 - 0.5% gentiobiose

3 - 0.1% gentiobiose

4 - 0.05% gentiobiose

5 - Gentiobiose Control(purity check).

Oligosaccharide Spot Characterisation(tentative) :-

1 - Glucose

3 - Cellobiose

4 - Gentiobiose

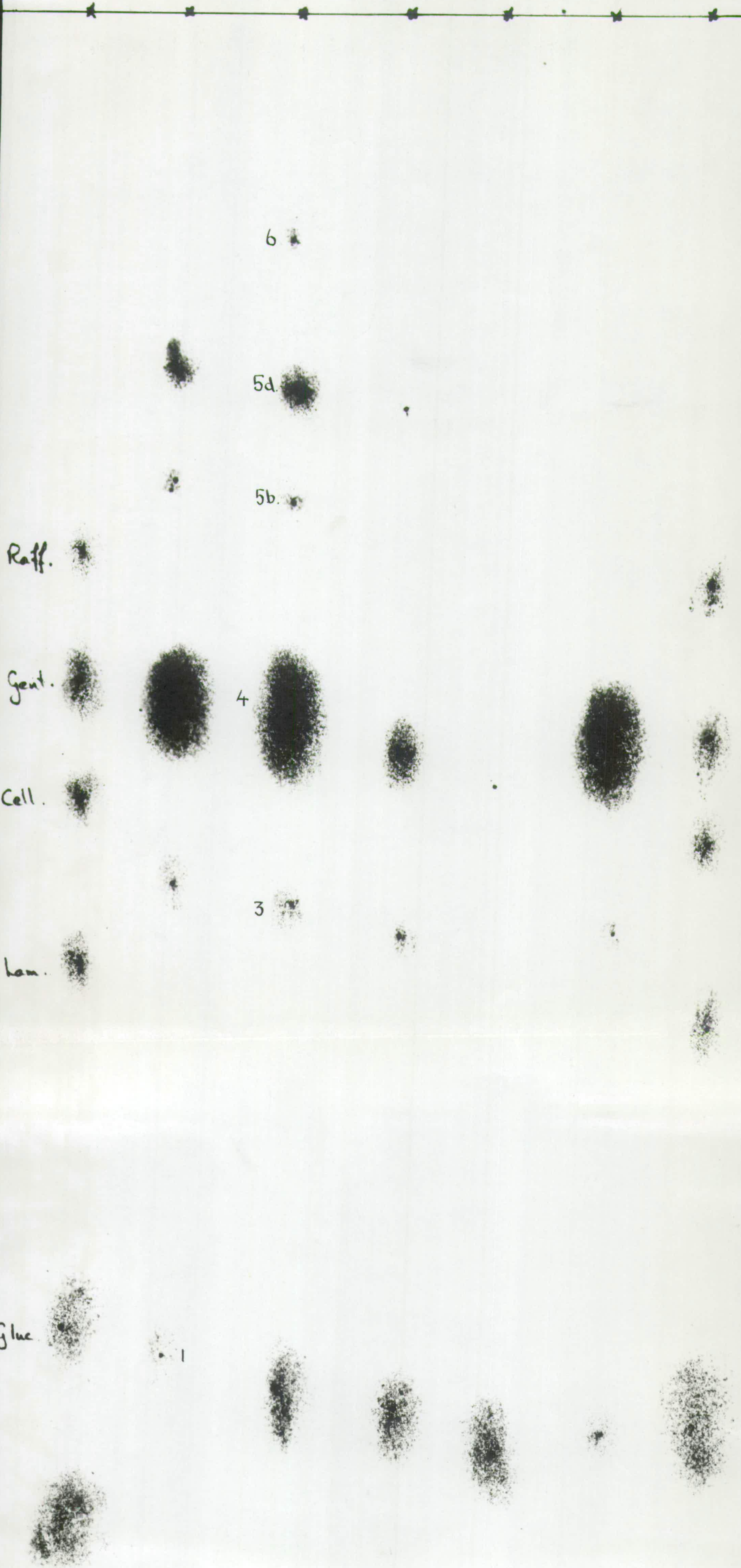
5b - trisaccharide(s)

5d - other trisaccharide(slow moving)

6 - Tetrasaccharide(s).

No.5

GENTIOBIOSE SUBSTRATE [24 HRS.]



The preceding part of this thesis has been concerned with β-linked gluco-disaccharides as sole substrates for transosylase enzymes. Under these circumstances, synthesis involves one compound acting as both glucosyl donor and acceptor. The next part of the thesis is devoted to an investigation made to establish the possibility of other sugars acting as either donors or acceptors of glucosyl groups.

GLUCOSE. No experimental evidence has been obtained during these studies in favour of glucose acting as either glucosyl donor or acceptor in transosylase. Techniques available, however, limited this study in respect that labelled glucose (using C^{14} at carbon atom 6) would have to be employed to establish the involvement of glucose in any reaction also involving cellobiose. Anderson & Manners(1959) failed to obtain any synthesis from glucose substrates during their studies on the transosylase powers of barley β-glycosidase.

GLUCOSE PHOSPHATES. Neither G-1-P nor G-6-P were ever found to be utilised as glucosyl donor or acceptor in the presence of cellobiose or alone. Similarily U.D.P.G. did not in any way alter the transosylase pattern when added to cellobiose substrates. It is likely that enzymes responsible for utilising these sugar esters and nucleotides are very labile (see below) and have therefore become inactivated during the enzyme extraction technique employed. The second half of this section of the thesis is therefore specifically devoted to the enzymology of the nucleotide-transosylases.

PENTOSES. Anderson & Manners(1959), working with barley β-glycosidase considered the possibility of pentose monosaccharides as glucosyl acceptors in transferase action. They were able to demonstrate the synthesis of a mixed disaccharide 3-O-β-D-glucopyranosyl-D-xylose (R_G = 0.86) from a reaction mixture of cellobiose plus a trace of xylose. The following results were based on a single series of experiments with Bromus inermis enzyme preparations but they certainly appear to suggest the presence of a significantly active transferase capable of utilising pentose sugars as glucosyl acceptors. Incubations were run at 37°C. for 36 hours with varying ratios of pentose - hexose substrates and the ratios given are in volumes of the different solutions which are all at 1.0 % concentration.

The actual reaction mixtures employed the following substrate combinations :-

Tube No.	Substrates Used
1	Enzyme Blank - control
2	7.8 ml. cellobiose + 0.2 ml. xylose
3	7.5 ml. cellobiose + 0.5 ml. xylose
4	6.0 ml. cellobiose + 2.0 ml. xylose
5	7.5 ml. cellobiose + 0.5 ml. arabinose
6	6.0 ml. cellobiose + 2.0 ml. arabinose
7	6.0 ml. cellobiose + 1.0 ml. xylose + 1.0 ml. arabinose
8	8.0 ml. xylose.

The results of these incubations are given on the next page and in chromatograms 6&7 .

The products from incubations involving cellobiose and pentose sugars include the usual synthesis products from a cellobiose substrate(eg. laminaribiose and gentiobiose). But these mixed substrate incubations also result in the synthesis of oligosaccharides of the mixed type.

From chromatograms 6 and 7 it can be seen that the synthesis products from mixtures 2,3,4 and 7 included di- and trisaccharides with both xylosyl and glucosyl residues, whilst those from mixtures 5,6 and 7 included oligosaccharides with both arabinosyl and glucosyl residues. No full characterisations of these mixed oligosaccharides were made but they give a pink coloration with aniline hydrogen oxalate.

The most outstanding finding from this study was the ability of this enzyme preparation to catalyse the synthesis of a disaccharide containing only pentose units. This was shown with mixture 8 which had only one synthesis product, a pentose disaccharide, probably xylobiose. This disaccharide must have been synthesised from the pure xylose substrate. This observed synthesis of a disaccharide from a pentose monosaccharide was very interesting indeed, as no parallel could ever be obtained using the hexose monosaccharide, glucose. The pentose disaccharide was only synthesised in trace quantities but the synthesis was apparantly valid as no evidence of contamination was ever obtained.

EXPLANATION OF CHROMATOGRAMS 6 ,and 7 .

Oligosaccharides Produced from Substrates Involving Cellobiose and/or Pentose Monosaccharides.

Mixture Identities :-

C - control - of glucose,xylose,arabinose, laminaribiose,cellobiose, gentiobiose and raffinose.

1-8 - as in Table – on page 104 .

Oligosaccharide Spot Characterisation(tentative) :-

1 - glucose

2 - laminaribiose

3 - cellobiose

P_1 - mixed oligosaccharide

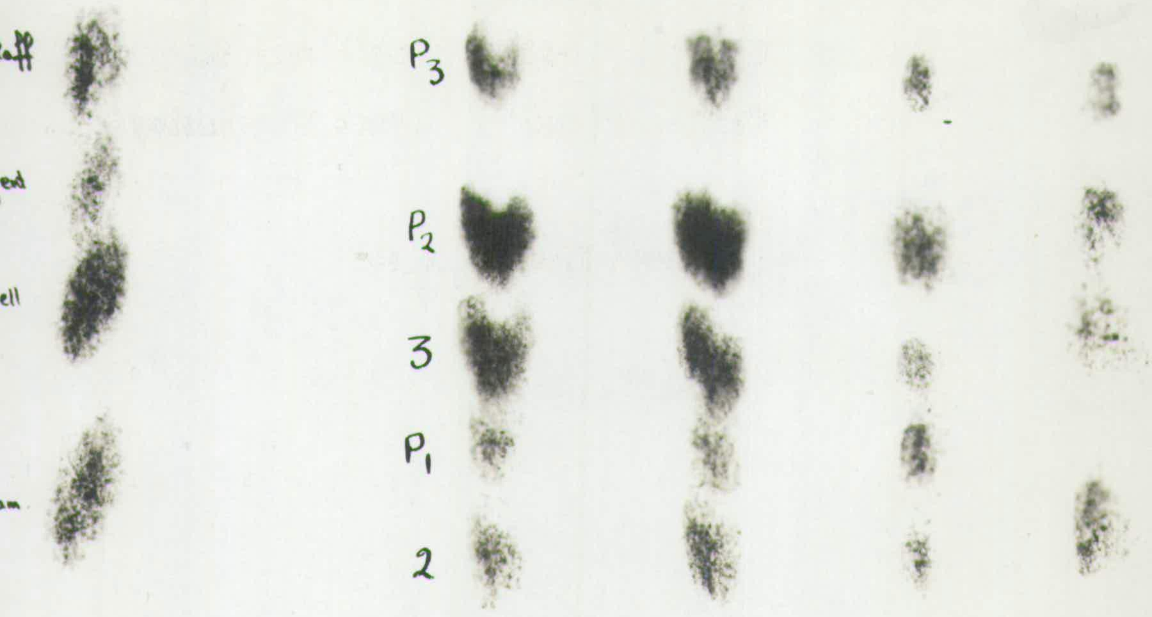
P_2 - mixed oligosaccharide + gentiobiose (?)

P_3 - mixed oligosaccharide + hexose trisaccharide (

Spots P_2 and P_3 contain a pink coloration and on many occasions a yellow-brown coloration.

PENTOSE ACCEPTORS - I. No. 6.

Control 1 2 3 4 Control



Glucose

1

Arabinose

Xylose

No. 7.

PENTOSE ACCEPTORS – II.

Control 5 6 7 8 Control

aff.

ent.

ell.

am.

P_3

P_2

3

P_1

2

pentose disaccharide

lucose

1

arabinose

8

8

xylose

7

Mechanism of Enzyme Action.

A series of experiments was carried out to determine the effects of several established metabolic inhibitors on the products of transglucosylation from cellobiose substrates, (1% cellobiose).

No obvious effect was observed by the addition of :-

4×10^{-4}M. Dinitrophenol, (D.N.P.),

5×10^{-4}M. Sodium azide,

5×10^{-4}M. Sodium salicylate,

10^{-2}M. E.D.T.A.

10^{-4}M. iodoacetate (Na salt) showed little effect on either hydrolysis or synthesis but it did appear to alter the pattern of transferase slightly, resulting in an increase in the quantity of laminaribiose produced. The concentration used was rather low but it seems possible that -SH enzymes may be involved somewhere in this transferase system.
The negative results with E.D.T.A. were also highly significant as they imply that metal ions(eg. Ca,Mg) are not involved in this reaction, a fact which is in agreement with results obtained by many other workers in this field of transosylation.
Further support for the non-requirement of metal ions comes from the fact that repeated dialysis of enzyme preparations does not reduce synthesising powers.
The possible involvement of phosphate in this system is still in considerable doubt. The addition of 10^{-2}M arsenate did not appear to inhibit synthesis or hydrolysis but an interesting fact was that a concentration of 2×10^{-2}M. arsenate caused an increase in the formation of a compound, $R_G = 1.3$, /

/ corresponding to the phosphate previously found in some incubations, see page 93 . This arsenate effect was completely removed by the addition of 2 x 10^{-2}M. Glucose-1-phosphate. Apart from supplying phosphate, thereby removing arsenate effect, the addition of G-1-P in the presence of arsenate produced a significant increase in the amount of gentiobiose formed. The full significance of these results cannot be ascertained from these preliminary findimgs but would certainly warrant further researches, which are however beyond the scope of this *Bromus* study.

Previous workers on synthesising systems of the transosylase nature have reported that glucose resulting from the degradation of substrate can build up to concentrations which are high enough to inhibit further synthesis,(Stettan, 1959). If this is true in this case, the addition of glucose oxidase to remove excess glucose from the reaction mixture might be expected to increase synthesis : this possibility was examined and the results are given in the table on the next page.

TABLE 25 - Effect of Glucose Oxidase on Transferase, using 1% cellobiose substrate.

Incub. No.	Enzymes Present	Oligosaccharide Identity					
		Glucose	Laminari-biose	Cellobiose	Gentiobiose	Tri-sacc.	Tetra-
1	Bromus enz. + notatin	+++	++	+++	++	++	t
2	as in 1 + catalase	+++	++	+++	++	++	t
3	only Bromus enzyme	++++	+	++++	t	t	-

The addition of notatin clearly increased the extent of synthesis. The cellobiose degradation rate was greatly increased and this was accompanied by a tremendous increase in trisaccharide formation, almost as strong a reaction was given by trisaccharides with aniline hydrogen oxalate spray as by cellobiose itself. The net glucose formation has obviously increased, suggesting that the enzyme(s) responsible for degradation/synthesis from cellobiose were capable of maintaining a rate of glucose formation almost equalling the rate of glucose oxidase action. The rate of removal of glucose by notatin will also be affected by the build-up of gluconic acid. The production of hydrogen peroxide as in incubation 1 appears to have no effect on the transferase reaction. This is probably due to a simultaneous breakdown of the peroxide by a catalase enzyme in the Bromus preparation. Simple tests with the Bromus preparation suggest the presence of such an enzyme.

Location of Cellobiase/Transferase in Bromus inermis Grain.

As studies on the mature ungerminated grains of B.inermis, B.mollis, B.sterilis and B.ramosus all indicated the presence of powerful transferase activity it was decided to attempt to locate the position of this synthesising system in the grain.

i) Grains of Bromus inermis were transversely cut in half and extracts were made from the different halves. From this method both extracts would include enzymes from the aleurone and endosperm but only one preparation would have enzymes from the embryo.

It was found that both halves possessed active transferase abilities. This suggests that transferase is present in the aleurone-endosperm, but does not establish the presence or abence in the embryo. It was also found that extracts from small fractions of pure endosperm cells could be prepared by dissecting out the endosperm cells free from aleurone. Attempts were made to use weevils to eat out the endosperm, leaving the aleurone(as can be done with barley), but this was not successful. No extract prepared from pure endosperm fractions could ever be demonstrated to possess transferase activity. This suggests that transferase action is restricted to the aleurone and absent from the starchy endosperm. This observation would be in agreement with the findings of MacLeod & Millar(1962) with regards to hemicellulase location.

ii) Transferase activity was also located in the growing

/ embryo, using preparations from excised embryos. The region of greatest activity appeared to be in the growing root. From the results of embryo culture studies, (see pages 45-47), it appears as if cellobiase/transferase action is also present in the scutellum and enzymes were detected in cellobiose culture media which were capable of transferring glucosyl groups from cellobiose to other cellobiose acceptor molecules. A more detailed report on transferring mechanisms in the growing seedling is given in the section on enzyme development prior to germination, pages 67- .

2. Trans - β - fructosylation.

Studies on the hydrolysis of sucrose by yeast invertase (Bacon & Edelman, 1950 ; Blanchard & Albon, 1950) have demonstrated the synthesis of oligosaccharides from sucrose; these are however subsequently degraded by the enzyme to give glucose and fructose as the only terminal products. Transfer of fructosyl to sucrose gives a series of compounds which are generally termed fructosans. The above workers found that sucrose may be replaced as a fructosyl donor by raffinose or by o-methyl-β-fructofuranoside. Pazur (1952, 1953) obtained similar results with *Aspergillus oryzae* as did Bealing (1953) with *Penicillium spinulosum*. Invertases of higher plants which are β-fructofuranosylases have been shown to have transferring action and to give 1^F- and 6^F-fructosyl-sucrose from sucrose (Allen & Bacon, 1956; Pridham, 1960).

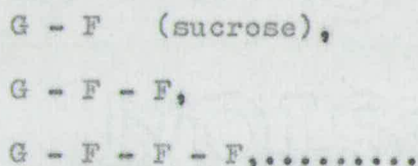
As previous studies, (MacLeod & McCorquodale, 1958), have shown, *Bromus* grains are very rich in fructosans (over 2g. per 100g. dry weight grain) and also in glucose and fructose; raffinose seems to be completely absent from this genus though it is present in 20 other genera of British grasses.

In this study of *Bromus* enzymes it was decided to investigate possible pathways of synthesis of these fructosans.

Experimental. The same techniques were employed as in the study of glucosyl transfer. The acetone precipitated enzyme preparations used in the previous glucosyl transfer studies were also used in this work.

SUCROSE TRANSFORMATION.

Sucrose was incubated with the precipitated enzyme preparations at 37°C. for 24 hours. Sucrose concentrations from 0.5% to 20% were used and in all cases a powerful invertase activity was found, especially below 5.0%, yielding glucose and fructose. Higher oligosaccharides were detected in the reaction mixtures with sucrose concentrations above 5.0%. They were later found to be non-reducing in nature, the result of the presence of fructose residues linked to the reducing end of the glucose moiety. These synthesised oligosaccharides belong to the fructosan series :-



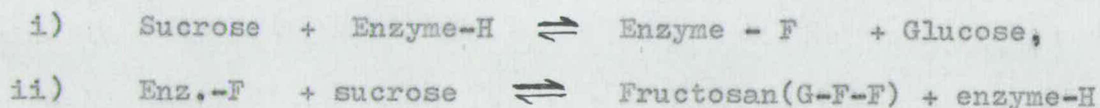
G - F (sucrose),

G - F - F,

G - F - F - F,.........

The trisaccharide under this reasoning will probably be 1^F - fructosyl - sucrose.

Probable mechanism :-



i) Sucrose + Enzyme-H ⇌ Enzyme - F + Glucose,

ii) Enz.-F + sucrose ⇌ Fructosan(G-F-F) + enzyme-H

RAFFINOSE TRANSFORMATION.

This sugar, raffinose, yielded only hydrolysis products when incubated with <u>Bromus</u> enzymes. At pH = 5.0, 37°C. with 24 hr. incubations, with varying concentrations of raffinose(0.5% to 10.0%) the hydrolysis products, glucose,fructose,melibiose and galactose were obtained. The formation of these sugars must/

/ be the result of a series of enzymic reactions :-

i) cleavage of 1:6 linkage between galactosyl and glucose groups, giving galactose and sucrose,

ii) cleavage of linkage between glucosyl and fructose groups, giving melibiose and fructose,

iii) subsequent cleavage of 1:1 linkage in the sucrose formed by reaction i), to yield glucose and fructose,

iv) subsequent cleavage of 1:6 linkage in the melibiose formed by reaction ii), to yield glucose and galactose.

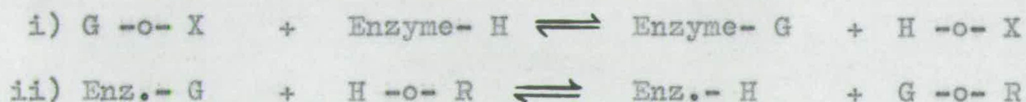
No evidence has been found for the synthesis of any higher oligosaccharides from raffinose substrates. This finding is highly significant when it is remembered that no raffinose has been detected in Bromus spp.(MacLeod & McCorquodale,1958). It is also interesting to note that in the embryo culture studies it was found that Bromus embryos cannot utilise galactose and can only slowly metabolise raffinose, and even then seedling growth is very poor indeed, (see section B(ii), (PART I.) page 47). When embryos were cultured on raffinose, galactose was found remaining in the culture fluid, even after prolonged culture.

Theoretical Considerations of the Observed Glycosidation-Transosylase Activity.

The mass of data supplied in the previous pages on transglycosylation in *Bromus* is based on incubations with enzyme preparations of varying degrees of purification. Preparations from *Bromus inermis*, *B. mollis* and *B. sterilis* all yield the same oligosaccharide synthesis pattern. Preparations taken from 18 and 72 hour germination also yield the same oligosaccharides, as do preparations from immature grains of *B. mollis*, *B. sterilis*, and *B. ramosus*. It seems, therefore, that the enzymes responsible for these transferase actions exist in considerable quantity throughout the lifetime of the grain. The preparations are all very stable, after varied methods of preparation (see enzyme preparation).

The greater part of this work on transglycosylation is actually concerned with transglucosylation, ie. the transfer of glucosyl groups from a donor compound to an acceptor. Anderson & Manners(1959), refer to the enzyme systems which catalyse reactions with cellobiose as glucosyl donor as cellobiase, although purification of their preparations was not attempted. They assumed that the hydrolytic and transferase activity of a carbohydrase represent two examples of the same reaction, enzyme showing dual specificity towards the glucosyl donor and the acceptor.

Basic Equations for the Reactions :-



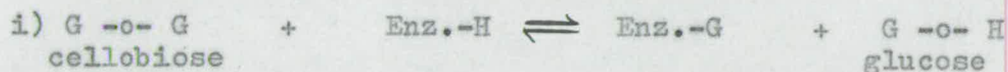
i) G -o- X + Enzyme- H ⇌ Enzyme- G + H -o- X

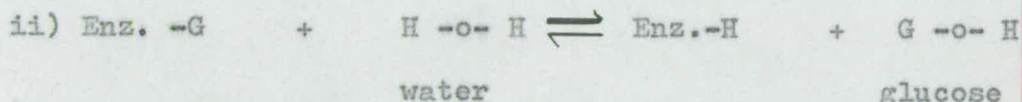
ii) Enz.- G + H -o- R ⇌ Enz.- H + G -o- R

where R = hydrogen atom, G-o-X repres. substrate(glucosyl donor) and H-o-R repres. the glucosyl acceptor.

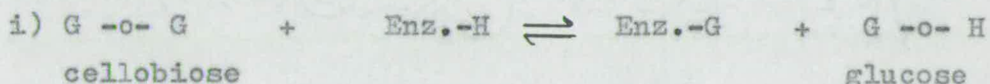
Compounds acting as acceptors may be compounds with hydroxyl groups, eg. carbohydrates or alcohols. Therefore with cellobiose as donor the following reactions may take place :

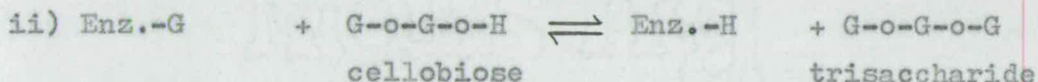
a) at low concentrations the acceptor may be water, H -o- H, yielding as a result only glucose as a product :-





b) if the acceptor is cellobiose,i.e. at high substrate concentration, a trisaccharide will be formed as synthesis is favoured in place of hydrolysis :-





This trisaccharide will possess a β 1:4 linkage from the original cellobiose acceptor but the other linkage to the added glucosyl group will be determined by the enzyme action itself. On this basis, the trisaccharide formed may be of several possible structures. If the new glucosyl group can only be added at the reducing end, the following trisaccharides are possible -

i) G -1:4 - G - 1:4 - G (cellotriose)

ii) G -1:4 - G - 1:3 - G

iii) G -1:4 - G - 1:6 - G ....all with β-linkages.

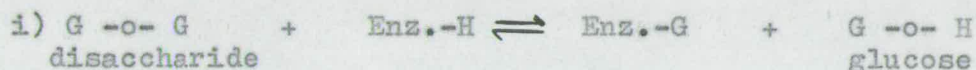
The β1:2 bond has never been detected (sophorose).

If the new glucosyl group can add on to the non-reducing end the other possible trisaccharides are -

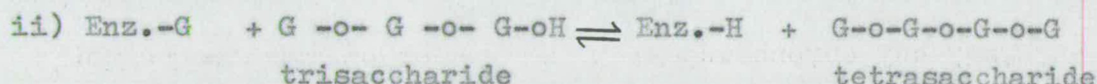
iv) G - 1:3 - G - 1:4 - G

v) G - 1:6 - G - 1:4 - G.

All these trisaccharides are based on cellobiose (G-1:4-G), as glucosyl donor and acceptor. If these trisaccharides then act as glucosyl acceptors the result can again be synthesis if the concentration is favourable -

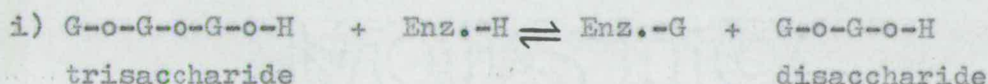


i) G -o- G (disaccharide) + Enz.-H ⇌ Enz.-G + G -o- H (glucose)

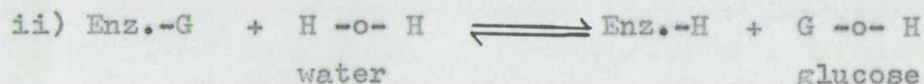


ii) Enz.-G + G -o- G -o- G-oH (trisaccharide) ⇌ Enz.-H + G-o-G-o-G-o-G (tetrasaccharide)

If the trisaccharide concentration is low the mechanism will favour hydrolysis -



i) G-o-G-o-G-o-H (trisaccharide) + Enz.-H ⇌ Enz.-G + G-o-G-o-H (disaccharide)



ii) Enz.-G + H -o- H (water) ⇌ Enz.-H + G -o- H (glucose)

This mechanism could also result in synthesis of new trisaccharide if a disaccharide replaced the water acceptor. As with the formation of the trisaccharide, the added linkage in the tetrasaccharide can be one of three, β-1:3, β1:4 or β1:6, giving therefore several possible structures to the tetrasaccharides. It should be noted that if the trisaccharide acts as a donor in hydrolysis, three possible disaccharides can be formed, cellobiose(G-1:4-G), laminaribiose(G-1:3-G), and gentiobiose(G-1:6-G), depending on the nature of the trisaccharide and the glycosidic bond which is cleaved. When the new disaccharides act as acceptors, the number of possible

/ trisaccharides is increased from five to nine, other possibilities being :-

vi) G -1:3- G -1:3- G (laminaritriose)

vii) G -1:3- G -1:6- G

viii) G -1:6- G -1:6- G

and ix) G -1:6- G -1:3- G.

There is also a similar increase in the number of possible tetrasaccharides. The role of laminaribiose and gentiobiose as glucosyl acceptors has been proved as has their formation from a cellobiose acceptor. The theory of this transferase action is based on the verified formation from cellobiose of:-

i) the disaccharides gentiobiose and laminaribiose from cellobiose and cellobiose from gentiobiose and laminaribiose,

ii) the trisaccharides -

G-1:3-G-1:3-G, laminaritriose,

G-1:3-G-1:4-G, 4-O-β-glucosyl-laminaribiose,

G-1:3-G-1:6-G, 6-O-β-glucosyl-laminaribiose,

G-1:6-G-1:3-G, 3-O-β-glucosyl-gentiobiose,

G-1:4-G-1:4-G, 4-O-β-glucosyl-cellobiose or cellotriose,

G-1:6-G-1:4-G, 4-O-β-glucosyl-gentiobiose,

iii) the tetrasaccharides,

G-1:3-G-1:3-G-1:3-G, laminaritetraose,

G-1:4-G-1:4-G-1:3-G, 3-O-β-glucpsyl-cellotriose.

Evidence from other workers on oligosaccharide synthesis by transferase action supports this step-by-step addition of one glucosyl unit from a donor to an acceptor. Nigram & Cori(1960), working with germinating <u>Phaseolus radiatus</u>, found an enzyme/

/ system which gave rise to oligosaccharides(α-linked) up to the tetrasaccharide level from the original maltose substrate. A similar system was also recorded in rat liver preparations, (Stettan,1959).

It should be observed that glucose itself will not act as glucosyl acceptor or donor. The expected reaction would be :-

i) G -o- G (cellobiose) + Enz.-H $\rightleftharpoons$ Enz.-G + G -o-H, (glucose)

ii) Enz.-G + G -o- H (glucose) $\rightleftharpoons$ Enz.-H + G -o- G -oH (disaccharide)

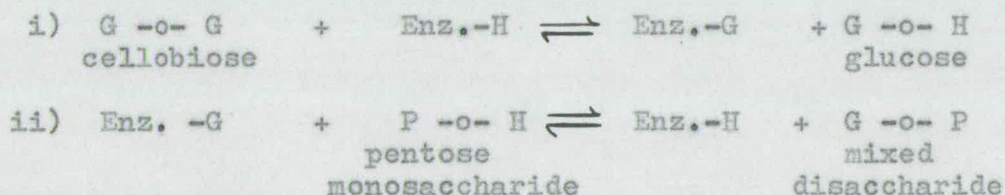
but this does not occur, even if the glucose is supplied as G-1-P or U.D.P.G..

This finding that glucose will not act as substrate is in agreement with Anderson & Manners(1959). It is also supported by Stettan(1959) who established that if water acts as an acceptor of glucosyl groups, ie. hydrolysis, giving rise to glucose only, the reaction is completely irreversible. Thus glucose formed from enzyme action cannot be utilised to resynthesise disaccharide, even in the presence of remaining cellobiose. Stettan (1959) also observed that the amylomaltase from rat liver could not utilise G-1-P in the synthesis of the maltose series of oligosaccharides ; this suggests that her work with the α-linked oligosaccharides is in agreement with our work on the β-linked oligosaccharides in Bromus. Most work on β-glycosidases has failed to indicate synthesis from glucose but occasionally prolonged action of a hydrolytic enzyme results in reversion of the reaction. Such an action has been demonstrated with β-glycosidase from emulsin when it was allowed to act on a 60% glucose substrate for several weeks, Peat et al.(1952). Synthesis products included the

full range of β-diglucosides, trehalose(1:1), sophorose(1:2), laminaribiose(1:3), cellobiose(1:4), gentiobiose (1:6), and higher oligosaccharides, but no evidence of a similar reversion could be found with Bromus preparations. From the chemical point of view it is interesting to note that it has been found that these diglucosides can be synthesised chemically by heating glucose with an ion-exchange resin.

When comparing the results obtained with U.D.P.G. and Bromus preparations with those of other workers using this sugar nucleotide it appears as if the Bromus preparation contains no enzyme capable of utilising U.D.P.G.. This fact is probably the result of the inactivation of such enzymes during the rather crude extraction procedure which is more suited to extracting essentially-hydrolytic enzymes. This supposition is borne out in the next part of this thesis, when it is seen that U.D.P.G. utilising enzymes are extremely labile indeed.

Although glucose failed to act as a glucosyl donor, evidence from this study and that of Anderson & Manners(1959) suggests that pentose monosaccharides can act as glucosyl acceptors :-



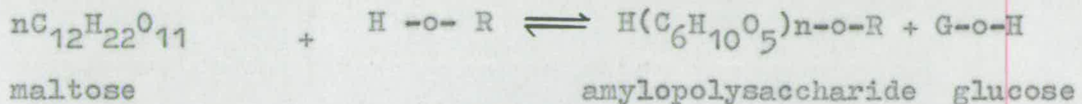
(where P represents a pentose monosaccharide group, eg. xylosyl or arabinosyl).

This results in the synthesis of a mixed disaccharide, 3-O-β-D-glucopyranosyl-D-xylose if the pentose acceptor is xylose. There is even evidence in Bromus for the synthesis of a pentose disaccharide from a pure xylose substrate. This

/ is a very unusual finding, when glucose will not act as a substrate. The full significance of this phenomenon is very questionable indeed.

The suggestion that this transferase action is dependent on the presence of phosphate has still not been proved or disproved, except in that the preparations are still extremely active after extensive dialysis. The amylomaltase activity in rat liver, Stettan(1959), and the transfructosidase of Agave vera-cruz, Bhatia, Satyanarayana and Srinvasan(1955), required no dialysable co-factors such as phosphate. This has also been proved to apply to several other transferase enzyme systems. This suggests that it is highly probable that this transferase system requires no such co-factor, hence explaining the absence of effect of E.D.T.A. and the active transferase action of dialysed preparations. The exact effect of arsenate inhibitor addition is not understood and requires further more detailed chemical study, which is however outwith this study. It is possible that the only effect of arsenate is to inhibit the breakdown of the phosphate compound ($R_G = 1.3$) in the enzyme extract by a phosphorolytic enzyme completely distinct from the transferase pathway.

The effect of treatment with glucose oxidase, recorded in this study with Bromus has also been established in other organisms, Monod & Torriani(1950), working with amylomaltase from E.coli noted that α-linked oligosaccharides large enough to give a blue coloration with iodine could be formed from maltose if the glucose formed was continually removed with glucose oxidase.

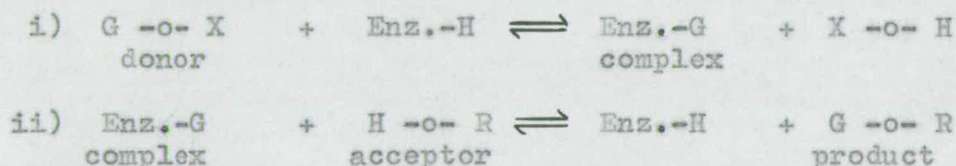


$$nC_{12}H_{22}O_{11} + H\text{-o-}R \rightleftharpoons H(C_6H_{10}O_5)n\text{-o-}R + G\text{-o-}H$$

maltose — amylopolysaccharide — glucose

where H -o- R, the acceptor may be maltose.

Similarily, with amylomaltase from rat liver preparations, Stettan(1959), it was found that oligosaccharide synthesis increased on the addition of glucose oxidase. From the Law of Mass Action, it must be expected that an increase in the concentration of the substrate would favour synthesis,and this has been established with this work in <u>Bromus</u>, using cellobiose. It might be expected that a build-up of glucose from cellobiose hydrolysis would tend to stop further hydrolysis and favour synthesis. In actual fact, it was found that the build-up of glucose inhibits synthesis and this is verified by these other workers with amylomaltase. If this reaction involved a simple balance between hydrolysis and synthesis, the removal of glucose as it is formed should favour hydrolysis, but this work with glucose oxidase certainly disproves this, as it favours synthesis of higher oligosaccharides. These experiments with glucose oxidase supply excellent evidence for the actual presence of the enzyme-substrate complex mechanism postulated earlier :-



i) $\underset{\text{donor}}{G\text{-o-}X} + \text{Enz.-H} \rightleftharpoons \underset{\text{complex}}{\text{Enz.-G}} + X\text{-o-}H$

ii) $\underset{\text{complex}}{\text{Enz.-G}} + \underset{\text{acceptor}}{H\text{-o-}R} \rightleftharpoons \text{Enz.-H} + \underset{\text{product}}{G\text{-o-}R}$

From these equations, and employing the Law of Mass Action, /

/ it can be stated that the production of product, G -o- R, will be dependent on the proximity of H -o- R and Enz.-G, ie. their relative concentration. If a concentrated solution of H -o- R is present, as in the standard reaction mixtures used, it will be the concentration of Enz.-G which will determine the extent of G -o- R production.

In step i) G -o- X + Enz.-H ⇌ Enz.-G + X -o- H, if X = glucose moiety, G -o- X = cellobiose, therefore X -o- H represents glucose. Thus if glucose oxidase is added it will result in the removal of the glucose which will swing the direction of this reaction to the right. This will result in a considerable increase in the formation of the Enz.-G complex. Therefore it can be expected that in step ii) the increase in Enz.-G will swing that reaction to the right, increasing the formation of product, G -o- R, ie, the trisaccharide if the acceptor is a disaccharide such as cellobiose.

From these facts it can be seen that glucose oxidase will increase the synthesis of oligosaccharides or the hydrolysis of cellobiose, depending on the ratio of the water:cellobiose acceptors, ie. the cellobiose concentration.

The transglycosylation powers of Bromus enzyme preparations seem to be extended to the formation of fructosans from a sucrose donor, with a further sucrose acceptor. The mechanism involved may well be the same as for glucosyl transfer.

i)	G -o- F sucrose	+	Enz.-H	⇌	Enz.-F	+	G -o- H glucose
ii)	Enz.-F	+	G-o-F-oH sucrose	⇌	Enz.-H	+	G -o- F -o- F fructosan, trisaccharide

This synthesised trisaccharide is a member of the fructosan series :- G -o- F, sucrose,

G -o- F -o- F, ... G -o- F -o- F -o- F, etc.

Chromatographic evidence suggested that several of these fructosans are formed but no detailed characterisations were made. It is interesting to note that high concentrations of fructosans were detected in Bromus grains and that these fructosans fluctuated considerably during germination, implying the obvious importance of the enzyme mechanism described above, (refer back to pages 43-44 for data on fructosans in grain). Similar transfructosidase enzyme systems have been recorded by other workers. Such an enzyme was studied in *Agave vera-cruz*(Mill.) by Bhatia *et al*.(1955). They found that the stem of *Agave* contains a transfructosidase capable of building glucofructosans from sucrose alone, requiring no primer, as was also found in *Bromus* . As with *Bromus* this *Agave* enzyme gave no synthesis products with raffinose but did yield hydrolysis products. It appears that no dialysable co-factors are involved. The pH optimum is 5.6 - 5.8, which is only a trace higher than in *Bromus*, but it requires a much higher sucrose concentration,(30%). This is in parallel to the facts noted in transglucosylation studies with cellobiose, where *Bromus* is able to synthesise oligosaccharides from much lower substrate concentrations than is barley (Anderson & Manners, 1959 ; see also page 94).

SECTION B.

Nucleotide - Transosylase

The discovery of a sugar nucleotide, uridine diphosphate glucose(U.D.P.G.) in yeast by Leloir *et al*.(1950), during their researches into galactose utilisation, introduced completely new concepts into carbohydrate biochemistry. Since their discovery, sugar nucleotides have been implicated in innumerable metabolic reactions. Many examples of interconversions of these nucleotides are now known; Leloir (1951) and Trucco(1954) have demonstrated the interconversion of U.D.P. -D-Glucose to U.D.P.-D-Galactose in the presence of U.D.P.-D-4-epimerase from yeast. U.D.P.G. is often oxidised to U.D.P.-D-gluconic acid which has been shown to be involved in several metabolic pathways. This oxidation requires N.A.D.(Strominger,1954). Similar interconversions have been found in higher plants and have been fully reviewed by Hassid *et al*.(1959). Sugar nucleotides function as glucosyl donors in many transglycosylation reactions (Peaud-Lenoel,1959) which lead to the synthesis of glycosides, oligosaccharides and polysaccharides.

A considerable amount of research involving these sugar nucleotides has been concerned with sucrose biosynthesis. Cardini & Leloir(1953)(1955) and Cardini *et al*.(1955) have extracted enzymes from wheat germ which catalyse the synthesis of sucrose and sucrose phosphate from U.D.P.G. and fructose or fructose-6-phosphate. Similar enzymic activity has been found in green peas(Bean & Hassid,1955), in leaves of/

/ *Impatiens holstii* (Ganguli & Hassid, 1954), and in leaves of *Canna indica* (Putman & Hassid,1954). A detailed study of the role of the scutellum of cereal grains in biosynthesis of sucrose has been found to involve U.D.P.G. - F-6-P transglucosylase (Edelman *et al.*,1959).

The importance of sugar nucleotides in polysaccharide synthesis has also received considerable attention in the last decade, especially over the last few years. U.D.P.acetylglucosamine has been implicated in chitin synthesis in *Neurospora crassa* (Glaser & Brown,1957), U.D.P.G. itself has been implicated in glycogen synthesis by liver extracts(Leloir & Cardini,1957), in cellulose synthesis by *Acetobacter xylinum* (Glaser, 1958), and in the synthesis of a β1:3 linked glucosan in mung bean(*Phaseolus aureus*)seedlings (Feingold *et al.*,1958).

This role of U.D.P.G. in glucosyl transfer has necessitated an examination of the possibility of intervention of U.D.P.G. as a glucosyl donor in transglucosylation in *Bromus* spp.. The importance of the possible involvement of this sugar nucleotide should be obvious when one finds just how important a role it appears to play in plant carbohydrate metabolism in general.

<u>Experimental</u>. Using a refrigerated centrifuge, a 'particulate fraction' was prepared from *Bromus inermis* seedlings by fractionation of a cell homogenate in phosphate buffer. The tissue (50g. fresh weight) being examined was ground in an M.S.E. top-drive homogeniser at 0°C. until it was completely homogenised. This grinding was carried out in 0.01M. sodium potassium phosphate buffer, pH=7.0. If roots were being treated it was necessary to use a mortar and pestle, grinding the tissue in the buffer plus silver sand(ice cold): the grinding was completed in as short a time as possible, viz. 5 - 10 minutes in a cold room. The extract was filtered through muslin and then centrifuged at 5,000g for 5 minutes at -5°C. The supernatant was retained and recentrifuged at 20,000g for 25 min. at -5°C. The precipitate was resuspended in the phosphate buffer and again centrifuged at 20,000g for 25 min. at -5°C. The precipitate so obtained was suspended in 3.0 ml. Tris buffer, 0.05M. at pH = 7.12. This suspension was used for the preliminary investigations and was called 'particulate transferase' after Feingold *et al.*(1958). In this investigation the fraction was termed Fraction 1, for further reference. If this fraction could not be utilised immediately it was stored at below -15°C., in a deep freeze.

As the 20,000g fractionation could only be prepared in 28 ml. (4 x 7ml.) quantities,it was replaced by a 60 ml. fractionation at 16,000g ; centrifugation time being increased from 25 to 35 minutes.

In later preparations the tissue grindings were carried out in a cold room using a mortar and pestle <u>without sand</u>. This change was due to the problem of localised heat

development when using the homogeniser.

An attempt was made to purify the extract by treatment with digitonin or detergent(Tween 20), followed by ammonium sulphate fractionation. Digitonin treatment involved shaking 3 ml. of Fraction 1 with 6 ml. of 1% digitonin (prepared as shown on the next page), for 10 min. then centrifuging at 20,000g for 25 min. The remaining precipitate was termed Fraction 2. The supernatant was fractionated with an increasing concentration of ammonium sulphate obtained by adding solid ammonium sulphate. The fraction precipitating out between 35-50% saturation was collected and redissolved in 0.5ml. of 0.1M Tris buffer, pH=7.12 and was termed Fraction 3. The remaining solution was retained, Fraction 4, as was the precipitate immediately after digitonin treatment. With the detergent treatment the same routine was used, except that 3 ml. of particulate Fraction 1 was treated with detergent for 10 min., the overall detergent concentration being 0.2%.

Substrates. Uridine diphosphate glucose,sodium salt, was obtained from Sigma Chemicals Ltd. Cellobiose was bought from Messrs.Lights & Co.Ltd.

Digitonin Solution Preparation. Digitonin was obtained in the solid form from Messrs.Lights Ltd. A 2.0% solution was prepared by suspending 2g. of the solid in a few ml. of distilled water and then about 20 ml. of 5N sodium hydroxide was added with vigorous shaking until all the digitonin was dissolved. The solution was carried out in 5N. HCl and diluted to 100 ml. with distilled water, making the final sodium

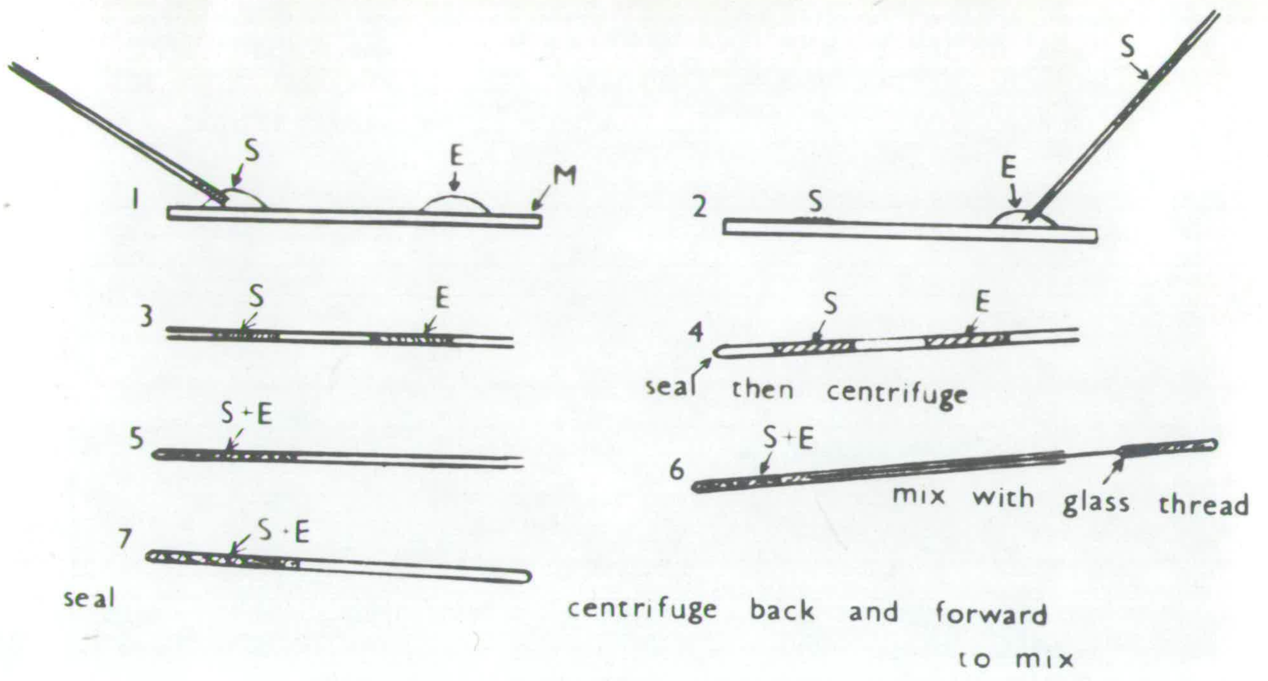
chloride concentration approx. 1.0M. The digitonin remains soluble under these conditions. The digitonin prepared in this manner was used to solubilise preparations after a method of Kaplan(1955). The method of solubilisation relies on the fragmentation of phospho-lipoproteins. Preparations after digitonin solubilisation were, however, very labile indeed, required storage at low temperatures over short periods and even then were only stable for 1-2 hours.

Control Sugar Solution Preparation. Apart from the usual mixture of glucose, laminaribiose, cellobiose,etc., a solution containing a series of laminariodextrins was prepared by hydrolysing a sample of pure laminarin with N. H_2SO_4, by the technique detailed in the next paragraph.

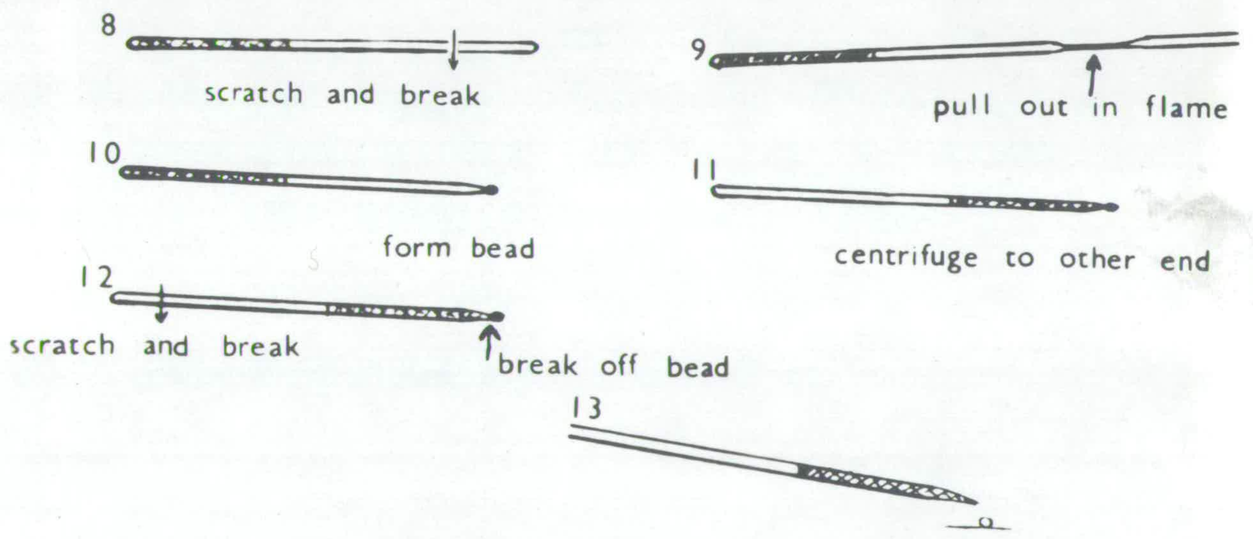
Determination of Enzymic Activity. The enzymic powers of the various fractions which were thought to be capable of transferring glucosyl groups from U.D.P.G. to form β-linked oligosaccharides and polysaccharides were studied using reaction mixtures containing U.D.P.G. substrate, Tris buffer (pH=7.12), Mg ions, glucose or cellobiose 'activator'(see Feingold *et al.*(1958)), plus particulate transferase, Fraction 1. Incubations were at 25°C. over periods of 30-50 minutes in melting point tubes after the method of Porter & Hoban(1954) as shown on ~~page~~ Fig. 12. Enzymic de-activation of reaction mixture was by boiling at 100°C. for 2-3 minutes. The precipitated material was removed by centrifugation in the incubation tubes. This precipitate was washed at least three times with distilled water and retained for hydrolysis. The

/ supernatant was chromatographed on Whatman No.1 paper,at 25°C. using descending technique in butanol:acetic acid:water (4:1:5) as was used for the other transferase studies.
The possible presence of synthesised polysaccharide was looked for by chromatographing an acid hydrolysate of the washed precipitate remaining after incubation. This hydrolysis was carried out with N.H_2SO_4 at 100°C. for 25 min.. The hydrolysate was neutralised with NaOH after cooling and the sodium sulphate was removed by precipitation with four volumes of ethanol. This alcoholic extract was concentrated before chromatographing.

The Use of Melting Point Tubes as Reaction Vessels.



INCUBATE AT 25°C



now use as micro-pipette for chromatography

M = microscope slide

S = substrate

E = enzyme

TRANSFORMATION OF U.D.P.G..

Preliminary studies with this system involved the use of a reaction mixture containing :-

1.9 mg. U.D.P.G.(sodium salt),

1.0 mg. glucose or cellobiose,

1.0 mg. Magnesium chloride,

and 0.32 ml. particulate transferase,Fraction 1, already in buffer at pH = 7.12.

Three stages of grain developments and seedling growth were examined :-

1. Ungerminated grain,
2. 2-day Seedlings,
3. 3-day Seedlings.

1. Ungerminated Grain. No synthesis of oligosaccharides or polysaccharides could be detected in extracts prepared from ungerminated, matures grains of B.inermis.

2. 2-Day Seedlings, grown under sterile conditions at 25°C. Three different reaction mixtures were run, with contents as listed in the Table below.

TABLE 26.

Tube No.	Contents
1	Particulate Transferase + U.D.P.G. + Mg^{++} + Glucose + buffer
2	as above but with no U.D.P.G.
3	Particulate Transferase + Mg^{++} + buffer.

The quantities of each component in the tubes in Table 26 are as listed at the top of the page.

No synthesis products were detected in tubes 2 and 3, but in tube 1 several oligosaccharides are present after the incubation (50 minutes at 25°C as stated in the method, page 128). A full chromatographic characterisation of the oligosaccharides is given in Table 27, below.

TABLE 27 - Oligosaccharide Characterisation - from Supernatant from Tube 1 Incubation.

Spot No.	Rf	log1-Rf/Rf	Tentative Identity
1	0.1734	0.6783	Glucose
2	0.1111	0.9031	Laminaribiose
3	0.0900	1.004	Cellobiose(reference)
4	0.0711	1.1160	Laminaritriose
5	0.0472	1.3051	Tetrasaccharide
6	0.0159	1.7916	tetrasaccharide (or pentasaccharide)

See graphic relationship - log 1-Rf/Rf : saccharide level for complete characterisation of higher oligosaccharides, page 89, and Fig. 11.

3. 3-Day Seedlings. A series of three reaction mixtures was again run, as in 2-day Seedling set. Again, the reaction mixture including U.D.P.G. resulted in the formation of a series of oligosaccharides, whilst those without U.D.P.G. failed to form any oligosaccharides. A full characterisation of synthesised oligosaccharides is given in Table 28, on the next page.

TABLE 28 - Oligosaccharide Characterisation - from Supernatant U.D.P.G. mixture + 3-day Seedling Enzyme Preparation.

Spot No	Rf	Log 1-Rf/Rf	Tentative Identity
1	0.0900	1.004	Cellobiose (reference)
2	0.0737	1.0994	Laminaritriose
3	0.0583	1.2079	mixed trisaccharide
4	0.0400	1.3797	mixed trisaccharide
5	0.0183	1.7220	tetrasaccharide
6-8	no distinct separation, therefore no accurate values of Rf obtained, but there definitely traces of oligosaccharides above the hexasaccharide level.		

A more accurate characterisation of some of the above oligosaccharides can be obtained in Table 16 , ; or by extrapolation from Fig. 11 , .

No sugars could be detected in the hydrolysates of precipitates from any of the already mentioned reaction mixtures, suggesting that no insoluble polysaccharide material had been synthesised. It is also possible that the detection methods used were not sensitive enough to detect synthesised material. There is no question that, if accurate results are to be obtained in this form of study, labelled U.D.P.G. must be used. As this was impossible in this study, no attempt was made to try to get these transferase results onto a quantitative basis after preliminary attempts at determinations of U.D.P. release, increase in oligosaccharides(by reducing power changes in eluted sugars), etc. proved that techniques available were not sensitive enough to detect the minute changes,(at the μg. level).

Attempts were also made to study the effect of solubilisation of the particulate preparations but no satisfactory results were obtained from Fractions 2-4, inclusive (see enzyme preparation). This is almost certainly due to the extreme lability of these preparations (see Feingold _et al._, 1958), plus the detection limits of the techniques used.

Magnesium Requirement. In the incubations with particulate transferase and buffered U.D.P.G. + activator substrate, it was found that if no extra Mg^{++} ions were added in the form of $MgCl_2$ or $MgSO_4$, the synthesising powers were greatly reduced though synthesis was still detectable to the trisaccharide level ; if 10^{-2}M E.D.T.A. was added synthesis was completely inhibited.

Activator Requirement. During preliminary studies it was found that incubation of particulate transferase with U.D.P.G. resulted in the synthesis of oligosaccharides without the addition of any primer or activator except Mg++ , but that synthesis was improved by the addition of glucose or cellobiose.

During preliminary studies on glucosyl transfer from U.D.P.G. it seemed possible that substrate concentration might be a limiting factor. In the following series of incubations, the substrate concentrations were raised.

Reaction Mixture used was :-

5mg. U.D.P.G.,

3mg. cellobiose,

5mg. $MgSO_4$, plus buffered transferase

in a total volume of 0.75 ml.

The Complete reaction mixture plus a set of controls were incubated (for 50 minutes at 25°C.) as listed in the Table below.

TABLE 29. REACTION MIXTURES

Tube No	Contents
1	5mg.U.D.P.G. + 3mg.cellobiose + 5mg.$MgSO_4$ + buffered transferase
2	as above minus Mg^{++}
3	as above(1) minus cellobiose
4	as in 1 minus U.D.P.G.
5	enzyme + Mg^{++} only

The oligosaccharides synthesised and consequently detected in the supernatant of the reaction mixture as listed in the table below.

TABLE 30. Oligosaccharide Synthesis Pattern.

Tube No	Glucose	Cellobiose	Trisacch. level	Tetrasacch. level	U.D.P.G. left	Hexasacch. level
1	+	++++	++	-	+++	+
2	+	++++	t	++	++	-
3	+	-	+	-	+++	-
4	+	++++	-	-	-	-
5	t	-	-	-	-	-

The above table gives an approximate impression of the pattern of oligosaccharide production. The spot identified as hexasaccharide was often very badly streaked, but usually had an Rf value of under 0.012. If a polysaccharide was being synthesised these supernatant oligosaccharides could be intermediates in the synthesising pathway but equally well they could represent degradation products/

from the polysaccharide. After washing several times with distilled water the precipitate remaining after incubation was hydrolysed in acid by the method already described. It was found that in some of the U.D.P.G. containing mixtures a glucose residue- containing polysaccharide had been synthesised which released free glucose on hydrolysis. Details of the products of hydrolysis of the precipitates from the five reaction mixtures tabulated on the preceding page are listed in Table 31 , below.

TABLE 31 - Products of Hydrolysis of Precipitates from Reaction Mixtures in Table .

Tube No./Spot No.	1	2	3	4	5
1	++	t	t	-	t?
2	++	t	t	t	+
3	t	t	-	t	+
4	+	t	-	t	+
5	t	t	-	-	t

A probable characterisation of the above spots is given in the next table.

Special Observations :- Only spots 1 and 2 were visible with the naked eye after aniline hydrogen oxalate spraying. Spots 3 and 4 were detectable only under U.V. but were also noted when the chromatograms were withdrawn from the tank, still wet with butanol:acetic acid:water solvent. They were distinctly hydrophobic in nature and formed spots rather like grease marks. Spot 5 was very distinct when seen under U.V.

TABLE 32 - Partial Characterisation of Hydrolysis Products Listed in Table 31 .

Spot No. :-	1	2	3	4	5
Rf	0.180	0.226	0.0914	0.0665	streak
log1-Rf/Rf	0.700	0.5353	0.9975	1.1473	?
Possible Identity	Glucose	?	hydrophobic aglycone ? ?		?

From the above results and those on the previous page it seems that using these enzyme preparations Mg^{++} ions are not a limiting factor, sufficient probably being present in the enzyme preparation itself. The addition of cellobiose, however, definitely increases the extent of synthesis.

Location of U.D.P.G.- Transferase System.

Unlike the cellobiase/transferase system studied earlier, polysaccharide synthesis involving U.D.P.G. cannot be detected in ungerminated mature grains. This seems to suggest that U.D.P.G. glucosyl transfer cannot take place in areas of powerful degradation, eg. aleurone-endosperm of germinating grain. A series of experiments was performed to try to locate the position in the grain of the U.D.P.G. transferase described in the last few pages.

A. Seedlings were separated from remains of grain after 3 days germination and it was found that no synthesising activity could be detected in the grain itself but a definite synthesis of oligosaccharides could be detected in preparations from the seedlings.

B. An attempt was made to fractionate the seedling into root, shoot and scutellum. Slight activity was detected in the extract from the root but there was no activity in shoot or scutellum preparations. This negative result is by no means certain as determinations were at the absolute limits of the detection methods used. One set of results suggested that the active synthesising region occurs about 1-2 cms. back from the root tip but labelled compounds must be employed if this is to be verified. Another factor which must be taken into consideration is the extreme thermo-lability of this enzyme system, a fact which accounted for several failures during the early stages of this study. This lability problem would be increased during the lengthy procedure of embryo fraction, enzyme extraction and fractionation in this latter part of the study.

Electron Microscopic Examination of the Particulate Transferase Preparation.

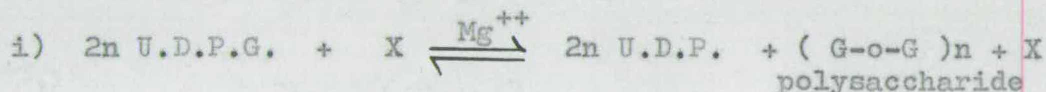
The enzyme extracts fractionating at 500,000g min. (20,000g for 25 min.) were fixed in veronal buffered 2% osmic acid, dehydrated and then embedded in a methy-butyl methacrylate medium. Sections of 300Å were cut and mounted on carbon coated grids. The electron micrographs obtained indicate that the preparation used in the enzymic studies was indeed of a particulate nature, containing organelles of a mitochondrial nature, about 1 micron across. Resolution of detail was poor due to the extraction methods of the enzyme preparation damaging the fine structure. Close examination did however show signs of a double membrane round the particles in places, and internal finger-like processes(cristae) were apparent. There were also traces of material, possibly broken mitochondria, vesicles and larger fragments which were probably aggregated endoplasmic reticulum.
(Electron micrographs are available but have not been included in the thesis to maintain the thesis at a manageable thickness.)

Respiratory Activity. The particulate preparation used in this study was incubated in the presence of sucrose at 25°C. in a Warburg respirometer. Respiratory activity, measured as oxygen uptake and carbon di-oxide evolution was detected.

These preparations required no added cytochrome C as is the case with many animal mitochondrial preparations. Apparently the preparation possesses sufficient endogenous cytochrome C.

Theoretical Consideration of the Observed Nucleotide - Transosylase Activity.

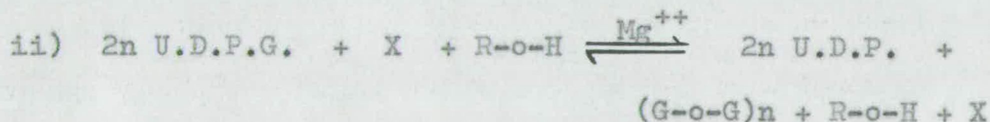
Basically the mechanism of this nucleotide-transosylase involving U.D.P.G. appears to be similar to the glycosidase-transosylase enzymes studied, eg. cellobiase. From other more detailed work with nucleotide-transosylases it appears likely that an enzyme-substrate complex is involved but no absolute evidence about this is available from this work. However, as synthesis of a β-linked polymer involves inversion of the glycosidic bond in the nucleotide, it seems reasonable to postulate that an enzyme-glucosyl intermediate will be necessary. Showing the mechanism as a single equation the reaction would be as follows :-



i) 2n U.D.P.G. + X $\overset{Mg^{++}}{\rightleftharpoons}$ 2n U.D.P. + (G-o-G)n + X
polysaccharide

where X represents the acceptor molecule.

The nature of the glucosyl acceptor is unknown, but a carbohydrate activator is required which does not, however, appear to be incorporated into the final synthesis product. A refined equation for the reaction might therefore be :-



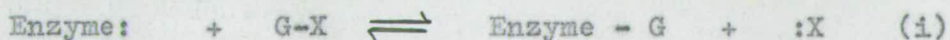
ii) 2n U.D.P.G. + X + R-o-H $\overset{Mg^{++}}{\rightleftharpoons}$ 2n U.D.P. + (G-o-G)n + R-o-H + X

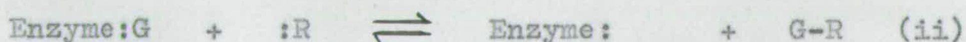
where R-o-H represents the carbohydrate molecule, R may represent a single glucosyl group or more than one linked glucoside ; X as already stated is the acceptor molecule, if it is in fact different from R. It also is probable that this action is irreversible, unlike the basically hydrolytic / pathway of glycosidase-transosylase. Further details of this nucleotide- transosylase system will be considered in the discussion,(in the next few pages).

DISCUSSION.

In discussing the relationship between the two synthesising mechanisms studied in this thesis, it is perhaps best to consider their similarities and differences in action from the theoretical aspect and then to consider their possible roles in the living plant.

Both systems involve a transglycosylation reaction, where a glucosyl group is transferred(except with fructosans) from a donor molecule to an acceptor molecule which may be glycone or aglycone, resulting in the synthesis of oligosaccharides or even polysaccharides. It was shown by Cohn(1949), that the nature of the transferred radical is definitely glycosyl, that is to say, the broken bond is between C^1 of the transferred residue and oxygen and this has been verified with several other studies, Eisenberg(1959), Halpern & Leibowitz(1959), and Mayer & Larner(1959). With this glucosyl transfer, the synthesis of β-linked oligosaccharides will require an inversion of the glycosidic bond as the glycosidic configuration in U.D.P.G. itself is in the α-D-form. As cellobiose already has the β-configuration no inversion is necessary. In both mechanisms of transosylase studied it has been assumed that the exchange reaction involves the existence of a glucosyl-enzyme compound,


Enzyme: + G-X ⇌ Enzyme - G + :X (i)


Enzyme:G + :R ⇌ Enzyme: + G-R (ii)

This is not absolutely necessary, but it is required that the glucosyl residue is fixed in such a way that its 'life' is/

/ long enough to permit time for an exchange of donor residue at its binding site. If there is an enzyme-substrate intermediary, the arrangement of donor and acceptor on the enzyme surface will determine whether there is an inversion or retention of the original bond configuration, therefore in this respect at least the two mechanisms will be distinct (Koshland,1954; Jermyn,1957).

Another outstanding difference between the two enzyme systems is the extreme lability of the U.D.P.G. enzyme, a feature common to enzymes involved in synthesis from U.D.P.G., Glaser(1958), Feingold et al.(1958), etc.. On the other hand, the cellobiase-transferase enzyme(s) is extremely stable, remaining active for over a week at room temperature and many months at $4^{o}C$.. The extreme stability of this enzyme system probably acts as an extra point in favour of the view that it is essentially a hydrolytic enzyme, capable of catalysing synthesising reactions under suitable substrate conditions, a view which is held by many workers (see page 114).

It has also been shown that the U.D.P.G. enzyme has a divalent ion requirement (Mg^{++}), another feature common to U.D.P.G. utilising systems and unnecessary for the cellobiase-transferase system.

It has been established beyond doubt that β-linked oligosaccharides or pentose monosaccharides can act as glucosyl acceptors in cellobiase-transferase, but as yet no conclusive evidence has been presented regarding the possible nature of the glucosyl acceptor in the U.D.P.G. mechanism. It has been shown in this work with Bromus and also by Feingold et al. (1958), that glucose,cellobiose and other carbohydrates are

necessary for the synthesis to occur from U.D.P.G. substrates, but it has also been demonstrated by Feingold *et al.*(1959), that these sugars are not incorporated in the final synthesis products. This has not been established in *Bromus* because of the absence of availability of suitable techniques. During work on pentose transfer from U.D.P.-Xylose, Feingold *et al.* (1959) postulated an aglycone acceptor(hydrophobic), but evidence for this is very meagre in *Bromus*(the identification of a substance running on chromatograms which repelled the acidic solvents and gave no reaction with the sugar spray, aniline hydrogen oxalate).

The actual 'structures' of the two enzyme extracts used were also different, the cellobiase-transferase preparation is a salt-soluble protein extract whereas the U.D.P.G. enzyme is at least particulate in origin, although enzymes could be released from the organelles by various solubilising treatments, with varying degrees of success. The particulate nature of the U.D.P.G. preparation was verified by electron microscopic studies which demonstrated that the particles were just under 1 micron in diameter, but appeared to ressemble small mitochondria. They were certainly shown to possess a double membrane but the isolation methods for enzymic studies were not suitable for the preparation of high quality electron micrographs. Most of the particulate organelles were found to be intact.

If the two mechanisms are considered from the kinetic viewpoint, the U.D.P.G. pathway appears to be the most suitable for synthesis. No direct values for these two pathways are available but studies in the thermodynamics of sucrose and

/ glycogen synthesis agree with the preference for U.D.P.G. enzymes in the synthesis. In studies on sucrose synthesis, a more favourable equilibrium in the case of U.D.P.G.- sucrose transosylase is present suggesting that it plays the main role in the synthesis of sucrose whereas pathways like sucrose phosphorylase are likely to play the role in sucrose catabolism.

i) Sucrose phosphorylase, Doudoroff et al. (1947),

$$\frac{[\text{Sucrose}]\,[P_i]}{[\text{G-1-P}]\,[\text{Fructose}]} = 0.05 \qquad \Delta F = +\,1700 \text{ cals.}$$

ii) U.D.P.G.-Sucrose Transosylase, Leloir(1956),

$$\frac{[\text{Sucrose}]\,[\text{U.D.P.}]}{[\text{U.D.P.G.}]\,[\text{Fructose}]} = 5.00 \qquad \Delta F = -1000 \text{ cals.}$$

In the transfer of glucose from U.D.P.G. to glycogen the ΔF is about -3,000 cal., Robbins et al.(1959), therefore there would be a 99.0% conversion of U.D.P.G.glucose to glycogen. These facts seem to establish that nucleotide-transosylases constitute the most favourable way for poly-saccharide synthesis to take place. It is also interesting to note that U.D.P.G. is involved in many synthesising reactions and all these pathways can be traced back directly to sucrose as the centre of carbohydrate metabolism in the plant.

With these thermodynamic facts in mind, the possible roles of the two synthesising mechanisms will be considered. When studying this problem one is immediately confronted by several difficulties. One of the most outstanding problems which always arises in such a biochemical study is whether the ~~are~~ reactions observed genuinely occur in vivo, or whether they/

/ are simply *in vitro* reactions created by the presence of suitable conditions which may not be available in the plant, eg. the substrates may be isolated in the cell. From the work at present carried out in the field of polysaccharide synthesis, it seems probable that the pathway involving U.D.P.G. does occur in the plant. On the other hand, there is considerable doubt regarding the validity of the cellobiase-transferase mechanism. With its close association with endo- and exo-glucanase action, ie, breakdown of the final product of their joint action, cellobiose (and laminaribiase with laminaribiose), yielding glucose, the normal action of cellobiase is very probably hydrolytic. The interesting difference between this enzyme(s) and other hydrolytic enzymes capable of synthesis is that in preparations from *Bromus* it can catalyse synthesising reactions even with relatively low substrate concentrations, i.e. over 10 times as low as is found with most of the other examples of this type of reaction. These low concentrations can certainly be expected in plant cells and it is quite reasonable to suppose that these enzymes may play a role in carbohydrate metabolism by controlling the pool of oligosaccharide precursors available for polysaccharide synthesis. Evidence from the work on U.D.P.G. indicates the need for β-linked oligosaccharides in the synthesising mechanism although their exact role is unknown at present (Feingold *et al.*, 1958; and this work). It is also interesting to note that excised *Bromus* embryos show extensive growth on culture media containing only cellobiose and mineral salts, suggesting that cellobiose can be rapidly metabolised by embryos to yield precursors for synthesis as well as substrates for respiration.

The pathways so far investigated involving U.D.P.G. have become very numerous and it appears that the energy required for polysaccharide synthesis by this pathway comes via A.T.P. which is used in the synthesis of U.D.P. and especially U.T.P.. Glucose enters this pathway in the form of G-1-P which combines with U.T.P., in the presence of U.D.P.G.-pyrcphosphorylase, to form U.D.P.G.. All the enzymes required for the synthesis of U.D.P.G. have been detected in cereal grains,(Edelman et al., 1959). Along with the detection of U.D.P.G. and intermediates in many seedlings, Ginsburg,(1958), this gives added support to the evidence presented in this thesis and from other workers, for the involvement of U.D.P.G. in polysaccharide synthesis. At this stage it is worth remembering that evidence presented in this thesis suggests that these synthesising enzymes involving U.D.P.G. are found in particulate organelles, possibly of a mitochondrial nature. It is therefore reasonable to assume that these organelles may possess respiratory activity, a fact borne out by the Warburg results(page138), and consequently provide a supply of A.T.P. and G-1-P. Evidence from optical microscope studies of parenchymatous cells in wounded Coleus stem suggests that a peripheral granulation of the cytoplasm precedes and defines the site of formation of the secondary wall. These optical studies have since been substantiated by the electron microscopic findings of Hepler & Newcomb(1963), which suggest that these granulations are due to a concentration of mitochondria, endoplasmic reticulum, Golgi bodies and vesicles. These results are certainly in agreement with the suggestion that mitochondria could indeed be involved in the synthesis of wall material.

Present evidence seems to suggest that the U.D.P.G.-transosylase enzyme system is present in the growing seedlings of <u>Bromus</u> and that the resultant polysaccharide material is possibly of a hemicellulosic nature, containing glucose residues linked mainly by β1:3 bonds but with occasional β1:4 bonds. This suggests, but does not prove, that it is involved in the synthesis of the hemicellulosic component of cell walls. One matter which has been partly considered earlier in this discussion is whether or not the different systems brought together <u>in vitro</u> are in fact together <u>in vivo</u>. This problem arises when U.D.P.G.-transosylase enzyme, found in a cytoplasmic particulate fraction, is considered in respect of its possible role in cell wall metabolism. As has already been pointed out, the location of the enzyme could be favourable in respect of precursor and energy supply. The main problem is the mechanism of association between the cytoplasmic components of the cell and the wall itself, but there is now a multitude of evidence supporting the close association between cytoplasm and cell wall (Newcomb, 1963). This is especially true in the areas of plasmadesmata and pit fields. Esau <u>et al</u>.(1962) have shown that in sieve plates the endoplasmic reticulum becomes localised in the areas of pore sites. This localisation is followed by a series of structural changes which seem to involve the dissolution of some of the original wall and the synthesis of new material. Callose, in the form of platelets, develops below the areas where the endoplasmic reticulum is in contact with the wall. The pore is created by the breakdown of the middle lamella and part of the wall. The platelets of callose line the pore in the sieve plate. It is believed(Esau <u>et al</u>. 1962) that a plasmadesma precedes each pore in the sieve plate.

This evidence from callose formation gives added support to the possible role of the cytoplasmic fraction found to be active in polysaccharide synthesis(this thesis), especially as callose has been shown to be a β 1:3 linked glucosan. It is also interesting to note that it is likely that hydrolytic and synthesising enzymes appear to be functioning together in callose build-up on the sieve plate, a similar condition to that postulated to explain the presence of endo-β-glucanase activity in the developing grain of Bromus ramosus, ie. at a time when hemicellulose deposition must be at its peak, (section B of PART II of this thesis, page 65). A further fact of value in considering callose is that Currier(1957) has established the presence of a substance ressembling callose in the primary pit fields of most parenchymatous cells. The exact significance of plasmadesmata is however not as yet understood, and much evidence exists for the deposition of wall materials in regions where plasmadesmata are not found (Setterfield & Bayley, 1961; Wardrop, 1962; Siegesmund, 1960). Recent electron microscopic studies(data only available at time of final typing of this thesis) have now provided excellent evidence in favour of the involvement of cytoplasmic organelles in cell wall formation. It has been shown, Ledbetter(1964), that glutaraldehyde fixation of tissues displays small tubular structures localised in the cellular cortex immediately beneath the plasmalemma. These tubules are always in positions adjacent to areas of the wall which are undergoing thickening. It has been shown that newly deposited cellulose microfibrils are orientated parallel to these cytotubules. These cytotubules are 230 -270Å in diameter, are of inderminate length and appear to be made up of

(in cross-section) 13 sub-units. These cytotubules have also been found to be involved in the mitotic spindle in the dividing cell. When present in the spindle none can be detected in the peripheral cortex of the cell. Newcomb(1964) has shown that these cytotubules lying next to the cell wall are sometimes found in association with stainable vesicles. He suggests that the vesicles are Golgi vesicles. This recent evidence provides conclusive evidence for an involvement of a cytoplasmic organelle, the cytotubule, in the deposition of cellulose microfibrils. It seems probable that other cytoplasmic organelles, such as the particulate preparation possessing the U.D.P.G.-transferase enzymes, might be responsible for providing precursors, enzymes,etc. for cellulose and hemicellulose formation.

Kivilian et al.(1961) established that cell wall preparations were 10 times as active in U.D.P.G.-pyrophosphorylase(measured as hydrolytic activity as they were unable to detect synthesis) as the soluble or particulate fractions of the cell. Whether this means that these enzymes can be found in the wall itself, free from cytoplasmic organelles or whether particles concerned with synthesis are so intimately associated with the cell wall that the techniques of Kivilian et al. failed to separate them has not been established. It should be noted that the techniques employed in the isolation of the wall fractions would almost certainly inactivate the labile U.D.P.G.-transosylase enzymes if they are present and also that wall preparations have certainly been shown to be low in cellobiase-transosylase activity, Dixon(1963). Many of the results suggesting the presence of protein in the cell wall are almost certainly due

/ to the presence of plasmadesmata or simply due to contamination during isolation. Considerable work has been carried out in studying the possibility of enzymic proteins being adsorbed onto the walls of cells,(Mertz,1961; Lamport & Northcote, 1960). It has been found(Jansen et al.,1960) that Avena coleoptile walls are capable of firmly binding 200 times the normal 'native' amount of pectin-esterase, which can withstand exhaustive water washing, but which can be easily removed with 0.15N NaCl at pH=4.5, implicating ionic bonding. Other enzymes have been shown to behave similarily. There are however several reports of enzymes present in the cell wall which cannot be removed by washing. Many of the results giving unequivocal evidence in favour of the presence of protein in the cell wall have indicated the involvement of hydroxyproline. Hydroxyproline- containing proteins have been found in many walls of different types of plant tissue (Steward, 1956-1960). This amino acid is not found free in plant cells except perhaps in trace quantities. It appears, however, that hydroxyproline-containing proteins are metabolically inert(Steward,1960). Present researches seem to indicate that these proteins may be associated with polysaccharides in the wall, thereby taking on a structural function. An alpha cellulose has been isolated which contains 1.0% of bound hydroxyproline,(Lamport,1962), and it is possible that such a protein could be involved in controlling cell wall growth by forming cross linkages between cellulose microfibrils. This will be considered in more detail in PART I section A, of this thesis, which deals with endosperm wall structure and in the general discussion at the end of the thesis. A further /

/ consideration of the presence of enzymes in the cell wall is that the synthesis of enzymes within the wall would imply the presence of a complex system involving R.N.A. beyond the cytoplasm : this is considered unlikely by many workers, (Newcomb,1963).

The apparent failure to detect an enzyme or enzymes capable of synthesising the purely β1:4 linked glucose polymer, cellulose, is unfortunately in agreement with results of many other workers, whose studies were considered earlier in this thesis. Several possibilities exist to explain this failure to obtain an <u>in vitro</u> synthesis of cellulose. The enzymes responsible may be highly labile; the action may require co-factors which have not been present; the glucosyl donor may not in fact be U.D.P.G. ; the glucosyl acceptor may be some compound not already used; it may in fact be that the synthesis of a large molecule like cellulose may require the presence or concomitant synthesis of other structural molecules(compounds such as hydroxyproline ?) or in fact the formation of cellulose microfibrils may not involve a prior formation of the β-glucosan chain. On the basis of what is already known about cellulose synthesis and its microfibrillar organisation, it is believed that glucosan chains and microfibrils are formed simultaneously by the addition of individual glucose residues at the microfibrillar tip, rather than by organisation of preformed glucosan molecules into microfibrils,(Roelofsen,1959). Evidence in favour of this comes from several sources. Firstly, it was noted that dispersed molecules of cellulose would not recombine into native microfibrils, Hermans(1949),Roelofsen(1959). Under the electron microscope the tip of growing microfibrils

in Acetobacter xylinum cultures appear smooth and tapered, not frayed, and there is no evidence of an amorphous precursor, Colvin et al.(1957), Colvin et al.(1961). Similarily, abundant supplies of oligoglucosides have never been found in A.xylinum,Colvin(1959). Also microfibrils are formed readily by A.xylimum in stiff gels of carboxymethyl cellulose which would interfere with the migration of large cellodextrin molecules, Colvin & Beer(1961). This simultaneous formation of β-glucosan chain and microfibril presents a very important enzymic problem,(Roelofsen,1959). If it is the mechanism of cellulose synthesis U.D.P.G. could act as primary glucosyl donor but no cellodextrin intermediates would be expected. In the case of extracellular synthesis of cellulose by Acetobacter xylinum, it appears that lipid-bound glucose is transported across the cell wall then transferred to the growing tip of a microfibril by an extracellular enzyme,(Khan & Colvin,1961). In this case U.D.P.G. can supply the glucose to an aglycone acceptor(the lipid component) and although these data are from work with a bacterium it is possible that a similar mechanism may be involved in the higher plants. Could the acceptor(aglycone) postulated in this thesis and also by Feingold et al.(1959), supply the lipid component used for transporting the glucosyl group onto the polysaccharide chain ? At the time of writing this final part of the thesis, workers in America, Hassid et al.(1964), have shown that the nucleotide G.D.P.G.(guanosine diphosphate glucose) can act as a glucosyl donor in the presence of a particulate cytoplasmic fraction /

/ similar to that used in the earlier U.D.P.G. experiments, (Feingold *et al.*,1958). They have found that with G.D.P.G. as glucosyl donor the final product is cellulose. This finding is indeed a milestone in carbohydrate chemistry but with all the evidence from other workers regarding the apparent involvement of U.D.P.G., it must at present be assumed that there is more than one pathway of cellulose biosynthesis.

Concluding, it therefore seems that these studies with *Bromus* have again failed to establish an *in vitro* synthesis of cellulose from U.D.P.G., using cell free preparations from a higher plant. They have, however, presented evidence of the synthesis of a polysaccharide material, insoluble in water at 20°C. and in 70% ethyl alcohol, from U.D.P.G. which possesses characteristics of the hemicellulosic material already detected in plant cell walls. Evidence is also in favour of the cellobiase-transferase system playing a minor role in polysaccharide synthesis, probably in the formation of precursors of the U.D.P.G. pathway. The main region of activity of both systems in the young seedling appears to be in the growing root, although cellobiase-transferase is very widespread.

GENERAL DISCUSSION.

Most points for discussion have already been summarised in the relevant surveys at the ends of Parts II and III of the thesis, and this general discussion will therefore be an attempt to summarise some of the findings which, to the writer at least, seem the most interesting.

The researches carried out can be divided into two broad classes; first came the examination of the hydrolases of the Bromus grain, particular reference being paid to the hemicellulases. A second facet of the work involved a detailed study of the transosylase mechanisms of the grain, an attempted investigation into the synthesis of hemicelluloses and their precursors.

The results of this preliminary aspect of the study establish that Bromus is a very rich source of hemicellulosic materials and of the hydrolases responsible for their degradation. This finding is in complete agreement with other workers, MacLeod and Sandie(1961). It has been established that, as in barley , MacLeod and Millar(1962), the main source of hydrolytic enzymes responsible for the degradation of the endosperm reserves is the aleurone layer, and that the release of these hydrolases can be induced by the addition of gibberellic acid. Previous workers have suggested that this effect of gibberellic acid is a parallel of the true control of release in intact germinating grain. It has been established that a substance does move down the aleurone from the embryo and that this substance induces hydrolase release,(Schander, 1934; MacLeod and Millar, 1962). It is believed that this substance is an endogenous gibberellin and in fact gibberellins have been located in the embryo, MacLeod et al.,(1963).

A critical examination of hydrolase release during germination and after treatment with gibberellic acid was made and it was established with reasonable certainty that all hydrolases are not released simultaneously in the grain. It is therefore reasonable to imagine that gibberellic acid does not act directly on a lysosome-like structure of the de Duve type to cause hydrolase release, as suggested by MacLeod and Millar(1962).

Slices of aleurone-endosperm, treated with gibberellic acid, show behaviour similar to that observed in the intact germinating grain in that endo-β-glucanase is released before α-amylase but with gibberellic acid added the release is earlier and the rate of release faster. This finding of different times for release of hydrolases in Bromus has been completely verified in barley by J.H.Duffus(this laboratory) and the joint results are included in a current publication, MacLeod et al.(1964).

Probably the most interesting aspect of the study on hemicelluloses and hydrolases has been the attempt to correlate the biochemical findings with ultrastructural observations. The ultrastructural investigations were never intended to form a major part of the work but after clearing many early obstacles (in methods) satisfactory micrographs were obtained and subsequent investigations have provided very interesting findings. Several unusual inclusions were found in aleurone cells and some of these appear to be involved in the release of hydrolases. The structure of these inclusions was investigated in full in Part I(section A) and some speculations have been put forward suggesting the possible roles of these 'organelles' in hydrolase release.

Although the theory of enzyme release being a result of the rupture of a lysosome-like structure has been disproved by/

/biochemical studies, it is possible that a vesicular organelle (such as the vacuolar deposit found in aleurone cells) may be involved, but that the gibberellic acid action is prior to the formation of such a 'bag' of enzymic protein and its final release is controlled by another factor. This secondary factor could come from the starchy endosperm as it has been found that slices of aleurone-endosperm treated with gibberellic acid will secrete enzyme directly, but isolated(pure) aleurone treated with gibberellic acid, though capable of producing large quantities of enzyme intracellularly does not secrete, MacLeod et al.(1964). These biochemical findings are supported by ultrastructural observations of aleurone tissue after the two treatments(see Part II, section D).

The biochemical aspects of this release problem are being investigated further in this department by J.H.Duffus whose work is at present in the press, MacLeod et al.(1964). Similarly, the writer is at present carrying out further ultrastructural studies in the germinating and G.A.-treated grain in an attempt to obtain a complete correlation between ultrastructural and biochemical findings.

In early studies on hemicellulase activity of Bromus enzyme preparations, MacLeod and Sandie(1961), detected the presence of a powerful transglycosylation activity. It was found that synthesis of gluco-oligosaccharides from a cellobiose substrate occurred using relatively low substrate concentrations. These findings were investigated fully, occupying, in fact, the major part of this thesis topic. Synthesis from very low substrate concentrations(less than 0.5% cellobiose) was detected and this may well be of biological importance, as such concentrations

are much more likely to occur *in vivo* than the usual high concentrations (10-30%) required for similar *in vitro* synthesis studies.

It was found that a series of β-linked gluco-oligosaccharides, up to hexasaccharide level, was synthesised from a cellobiose substrate. Synthesis of β-1:3, 1:4 and 1:6 bonds were obtained but never β1:2, a finding in agreement with other workers, Anderson and Manners(1959). The present investigation suggests that this synthesis mechanism involves glycosidase- transosylase reactions, ie. involving enzymes which are essentially hydrolytic in action, synthesis being induced by high substrate concentrations.

In transglucosylation reactions it was found that hexose acceptor molecules had to be at the disaccharide level,(eg. cellobiose and laminaribiose), yet pentose monosaccharides (eg. xylose and arabinose) could act as glucosyl acceptors producing 'mixed' oligosaccharides as synthesis products. In all cases(except one) of transglycosylation reactions examined, the donor molecule had to be at least at the disaccharide level (eg. cellobiose and laminaribiose). The exception was the apparent synthesis of a pentose disaccharide(tentatively characterised as xylobiose) from a pure xylose substrate.

It appears that these transglycosylation reactions involve the intermediary formation of an enzyme-glycosyl complex.

The general evidence from this present study appears to suggest that the enzymes involved in these *in vitro* reactions almost certainly perform an essentially hydrolytic action in the living cell and such *in vitro* synthesising activities are probably of little value in the plant itself. This will certainly be true in the aleurone which is the main source of these enzymes.

It is unlikely that these 'transferase'enzymes are responsible for the synthesis of long-chain polymers(eg. cellulose or hemicellulose) in cells which are not growing. It is possible that they could produce a range of low-molecular precursors for another polymer-synthesising enzyme system. This could be the role of the glycosidase-transosylase enzymes detected in the embryo and growing seedling.

The other transosylase mechanism investigated in seedling preparations involved uridine diphosphate glucose(U.D.P.G.) as a glucosyl donor. This enzyme system was found to be extremely labile and required a divalent cofactor(Mg^{++}). It was particulate in nature and attempts made to 'extract' the enzymes from the 'mitochondrial-like' particles produced extremely labile preparations. Evidence was obtained for the synthesis of a hemicellulose-like polymer containing β1:3 and β1:4 linkages but techniques available for studying this system were very limited and further researches to confirm the present findings would require the use of C^{14} in the glucose moiety of U.D.P.G.. This glucosyl transfer from U.D.P.G. appears to require the presence of other glucosyl compounds(eg. glucose and cellobiose). If this reaction is similar to that studied by Feingold et al.(1958), these glucosyl compounds are not actually incorporated in the synthesised polymer and therefore they cannot be termed glucosyl acceptors. U.D.P.G. has been shown to be involved as a glucosyl donor in several carbohydrate biosyntheses and recently G.D.P.G. has been shown to be a glucosyl donor in cellulose synthesis in higher plants, Hassid et al.(1964).

Throughout these studies attempts have been made to tie together biochemical and biological findings. This has sometimes, but not always, been successful. A few examples of this correlation might be suitable to conclude this thesis -

1. Time courses of hydrolase release, wall dissolution, hemicellulose content changes are all in agreement and these in turn fit into the pattern of sugar production and embryo growth.

2. Embryo nutrient requirements; enzymic potentialities and free sugar availability show a close correlation.

3. Ultrastructural changes appear to agree with theories (based on biochemical work) regarding hydrolase production.

4. Ultrastructural, histochemical and gross chemical studies of endosperm cell walls suggest that hericellulosic polymers may have a fibrillar organisation similar to that of cellulose.

* * * *

The solving of some problems invariably produces others and this work forms no exception. Future studies should therefore include -

1. further ultrastructural studies(a wide field),
2. fractionation of aleurone cell organelles using ultracentrifuge techniques to help establish the correlation between biochemical properties and ultrastructure,
3. hemicellulose biosynthesis studies using labelled glucosyl donors (eg.U.D.P.G.,G.D.P.,A.D.P.G.).

APPENDIX 1

Acid Dehusking of Grain.

Following the technique of Pollock *et al.*(1955) for eliminating barley dormancy, samples of *Bromus* grain were immersed in 50% sulphuric acid. As well as breaking dormancy this treatment also removes most of the husk. The normal recommended time of treatment was 4 hours for barley and a simple experiment verified that this was also true for *Bromus* :-

Period in Acid hr.	% Germination	Dehusking
1	80.0	high % husk still present
2	99.0	still some husk present
3	100.0	"
4	100.0	only trace of husk present
4.5	80.0	"

The acid treatment was carried out at room temperature, the grains being washed several times after treatment. The washing included a wash in a suspension of calcium carbonate to neutralise any remaining acid.

This treatment serves three main functions :-

1. dehusking of grain,
2. breaking of dormancy,
3. sterilisation of grain.

APPENDIX 2

Absorption Spectrum of β-Glucan:Iodine Complexes.

A series of simple spectrophotometric studies was made with solutions of β-glucan and its apparent complex with iodine. As this work is not really relevant to the topic of the thesis only a brief mention of the results shall be made.

It appears that β-glucan does complex with iodine and that this complex has certain absorption peaks which are unlike that of starch. There appears to be a peak at about 320 mμ., ie. between the two common peaks of starch, (ie. 280 mμ and 350 mμ.).

If any further information is required it can be obtained from the author.

* * * *

APPENDIX 3.

Basic Factors Affecting Degree of Extraction of βGlucan.

The factors listed below should be borne in mind if one intends to prepare β-glucan according to the method outlined in Part II.

1.. A hand mill used for grinding grain gives a higher yield, of higher viscosity β-glucan than an electric type of mill(eg. coffee mill).

2. The volume of water used at the 40°C extraction should be large, at least twice the amount recommended by Preece *et al*..

3. Careful control of this temperature is required to prevent extraction of starch, and careful fractionation with ammonium sulphate is required to keep it pentosan free.

PUBLICATIONS.

A considerable portion of the information given in Part I of this thesis,(that referring to ultrastructural studies), has been included in a publication -

" Ultrastructure of Caryopses of the Gramineae - I. Aleurone and Central Endosperm of Bromus and Barley " by A.M.MacLeod,C.S.Johnston and J.H.Duffus,(J.Inst.Brew.,1964, 70,303-7.)

This material also formed the basis of a lecture given by the author at the 1964 Research Symposium of the Botanical Society of Edinburgh(held in Glasgow that year).

Information dealing with the time courses of hydrolase release in germinating and gibberellic acid treated grain, Part II,section C of thesis, has been included in a further edition of the J.Inst.Brew., which should be issued just after the date of submission of this thesis.

Work at present being carried out on aleurone ultrastructure changes in germination is expected to form a sequel to the first such paper(above) in the J.Inst.Brew..

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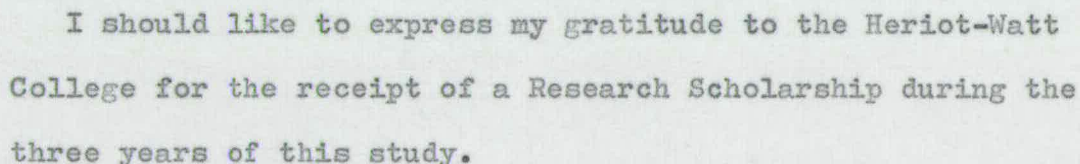


I should like to express my gratitude to the Heriot-Watt College for the receipt of a Research Scholarship during the three years of this study.

SYMBOLS USED IN PLATES.

D.B.	-	dense body
E.R.	-	endoplasmic reticulum
I.	-	inclusion (vacuolar)
I.S.	-	intercellular substance(if not a distinct M.L.)
M.	-	mitochondrion
M.L.	-	middle lamella
N.	-	nucleus
N.E.	-	nuclear envelope
NL.	-	nucleolus
P.	-	plastid(or proplastid)
Pd.	-	plasmadesmata
P.M.	-	plastid membrane
Pl.	-	plasmalemma
Pr.	-	'protein', of vacuolar deposit(aleurone) or storage protein of endosperm
S.	-	starch grain
S.M.	-	'sac membrane' round endosperm storage 'protein'
U.B.	-	unidentified body (vesicular)
V.	-	vacuole (or transparent vacuolar inclusion)
V.M.	-	vacuolar-type membrane (single)
W.	-	wall
W.H.$_{1-3}$	-	believed to be hemicellulosic wall(endosperm) see text

EXPLANATION OF PLATES 1-3.

PLATE 1. Aleurone of *Bromus*, fixation in unbuffered $KMnO_4$.

Tissue from grain soaked intact for 18 hr..

PLATE 2. Aleurone of *Bromus*, fixation in veronal buffered $KMnO_4$, 2 hr.; tissue from dry, unimbibed grain.

PLATE 3. as in plate 2.

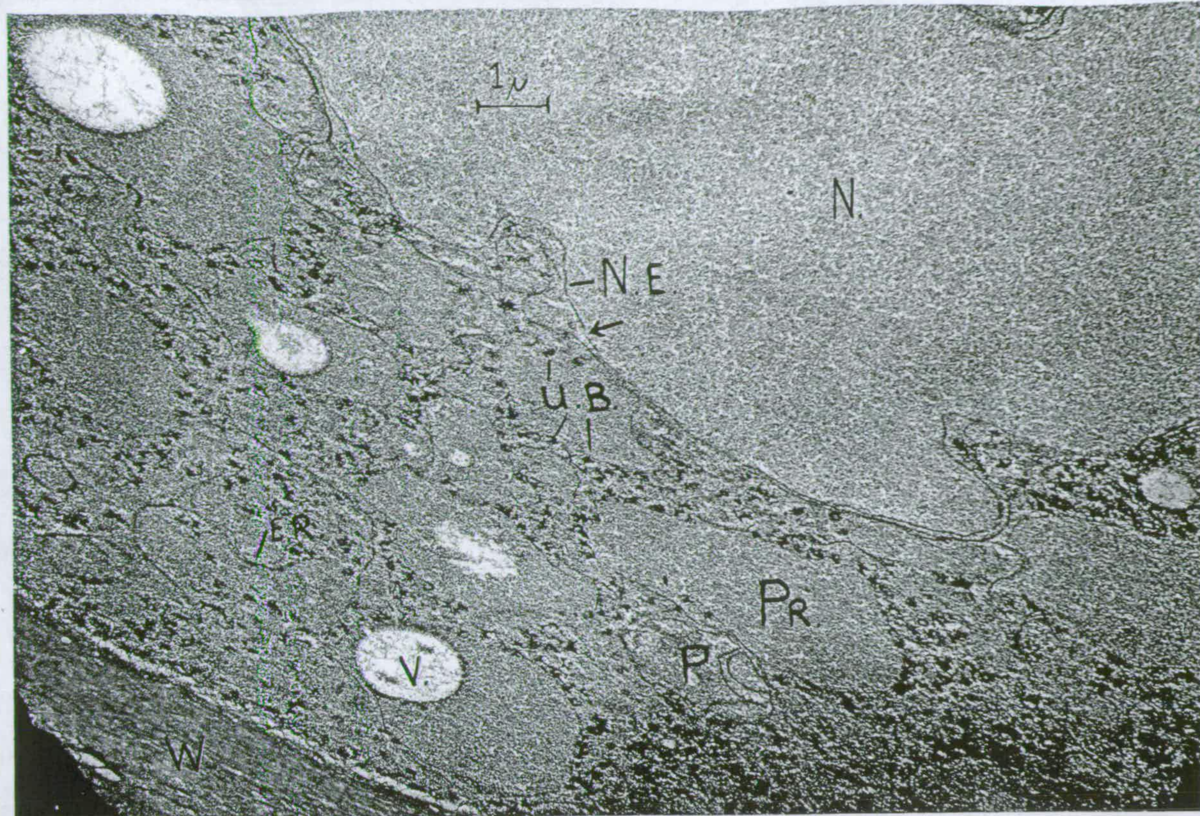


PLATE 1

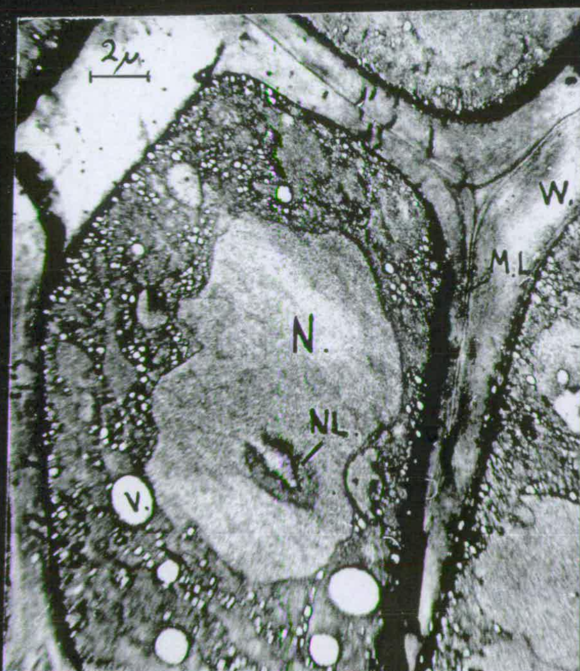


PLATE 2

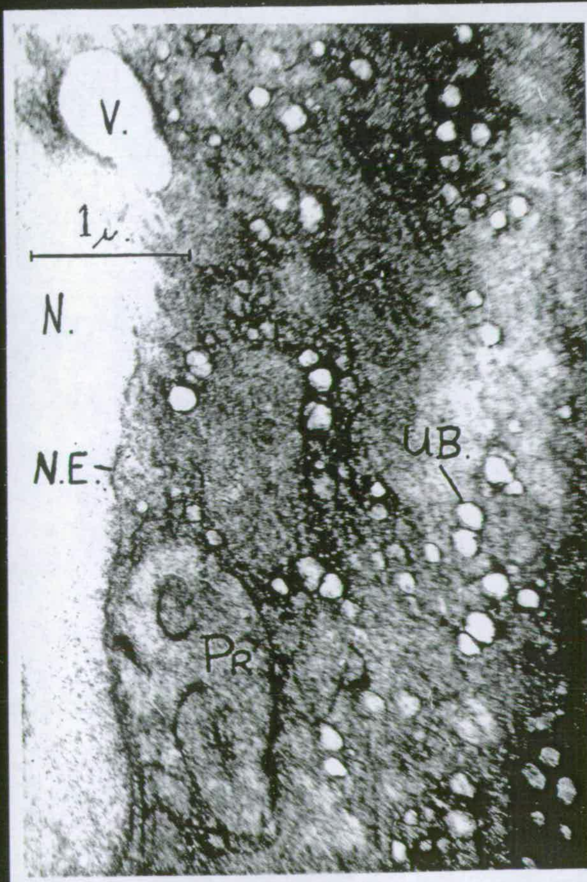


PLATE 3

EXPLANATION OF PLATES 4 and 5.

Both plates are of barley aleurone from slices of aleurone-starchy endosperm removed from dry grain then soaked overnight.

Fixation in veronal buffered $KMnO_4$ (pH 7.4), for 2 hours.

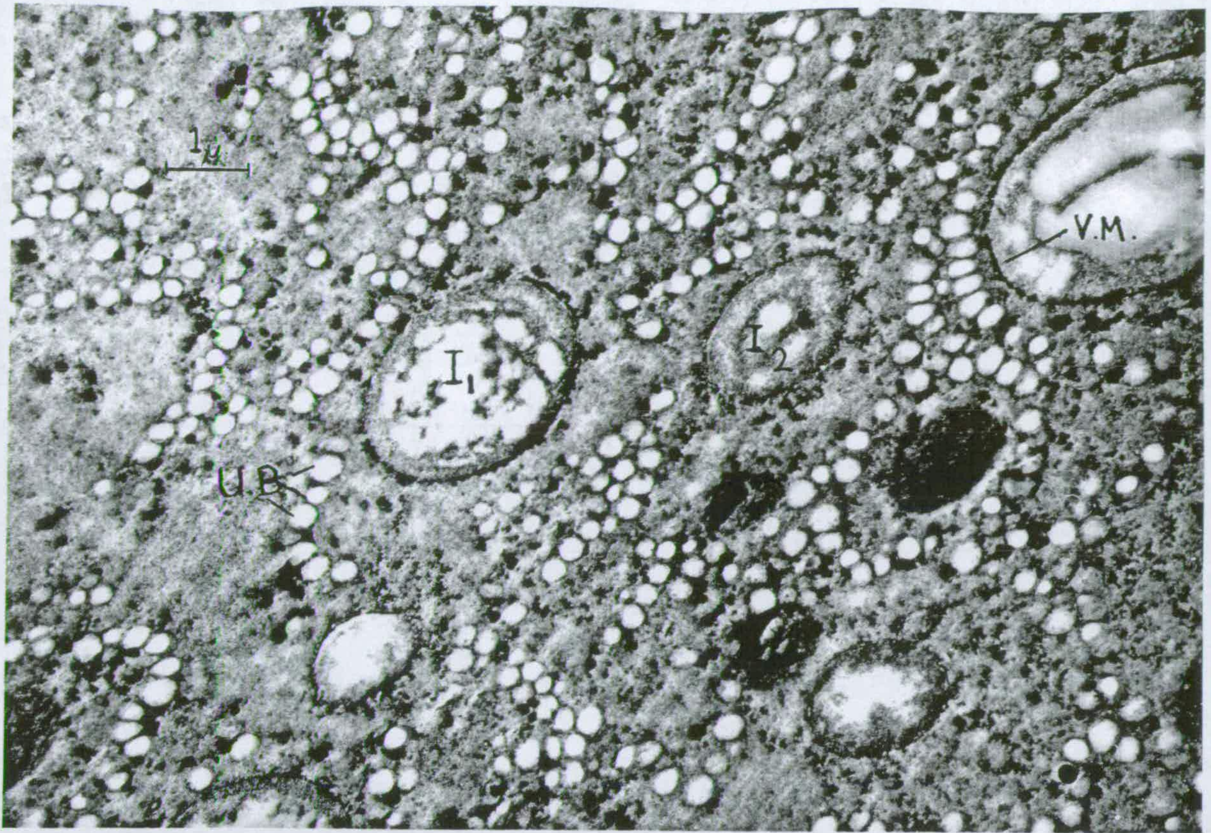


PLATE 4

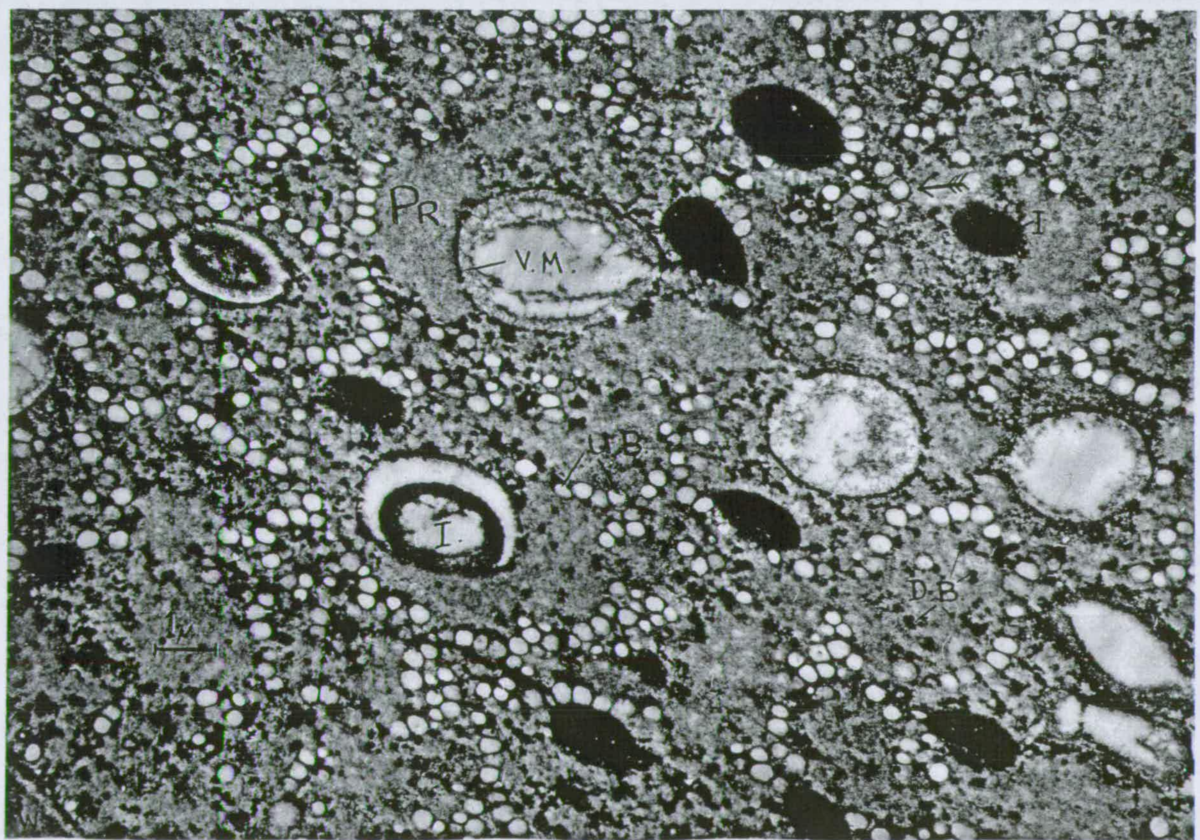


PLATE 5

EXPLANATION OF PLATES 6 & 7.

As in plates 4 & 5, these two micrographs are of Barley aleurone from slices from dry grain which have been soaked overnight.

Fixation in veronal buffered $KMnO_4$ for 2 hours.

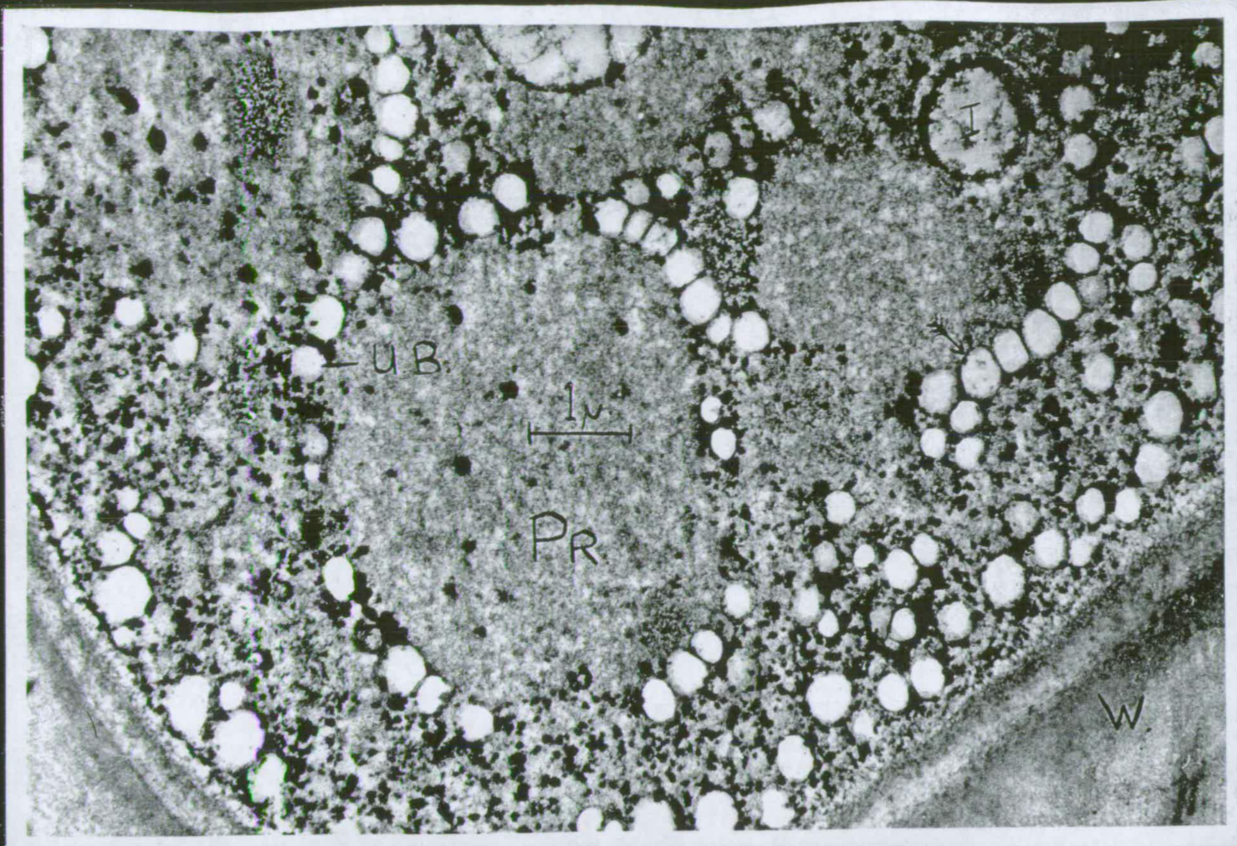


PLATE 6

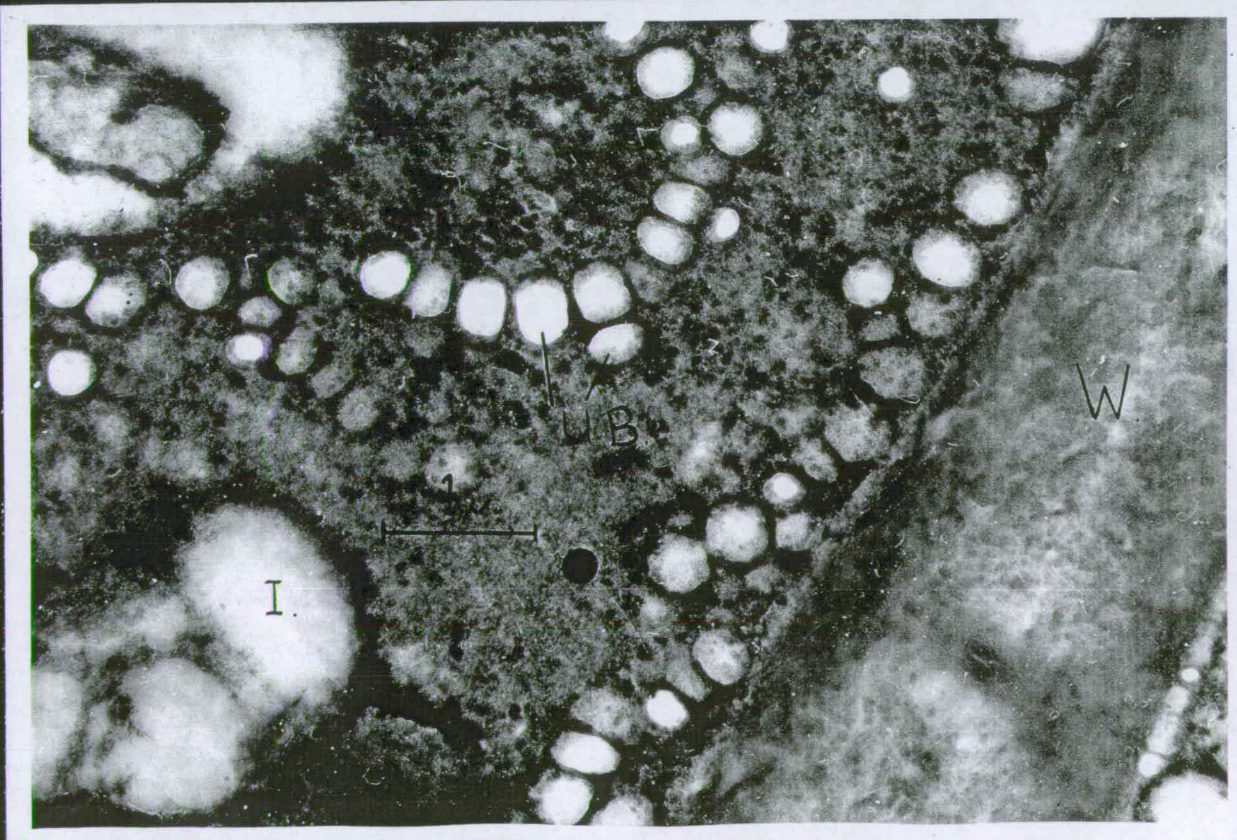


PLATE 7

EXPLANATION OF PLATES 8-13.

PLATES 8-12. Aleurone of *Bromus*, from imbibed(18hr.) grain.

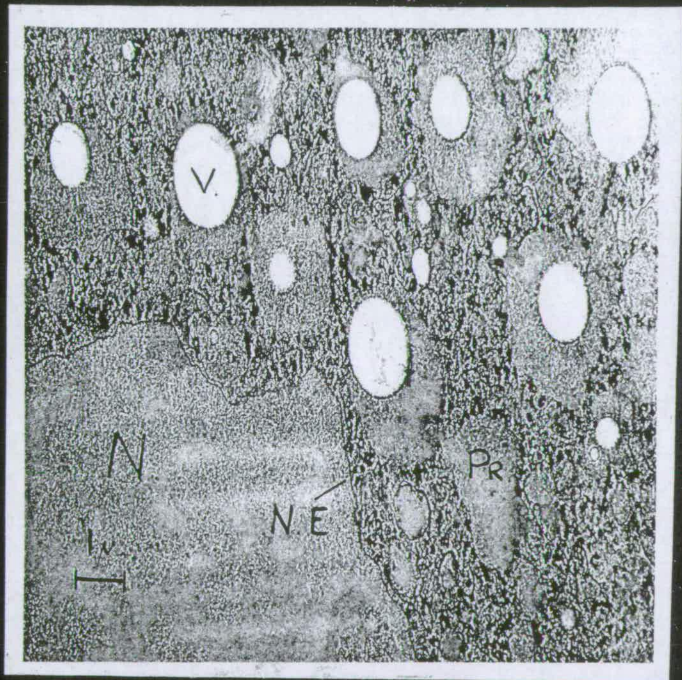
Prefixation in neutral formalin followed by fixation-staining in buffered $KMnO_4$, for 2 hr..

PLATE 13. Aleurone of barley, from slice of endosperm-aleurone cut from dry grain then soaked overnight.

Fixation in buffered $KMnO_4$ for 2 hours.

PLATE 8

PLATE 9



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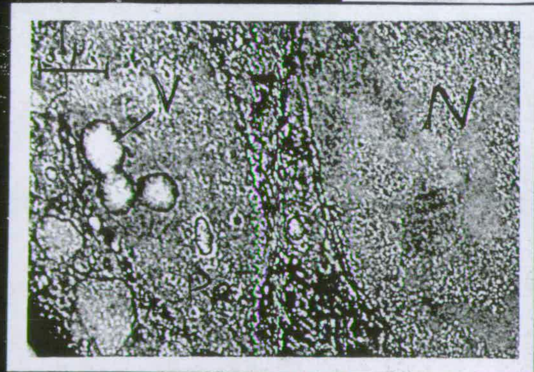


PLATE 10

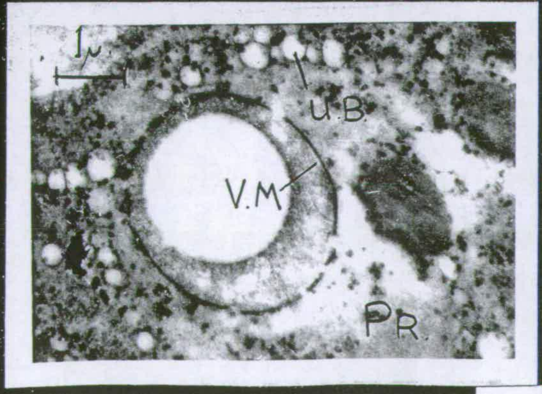


PLATE 13

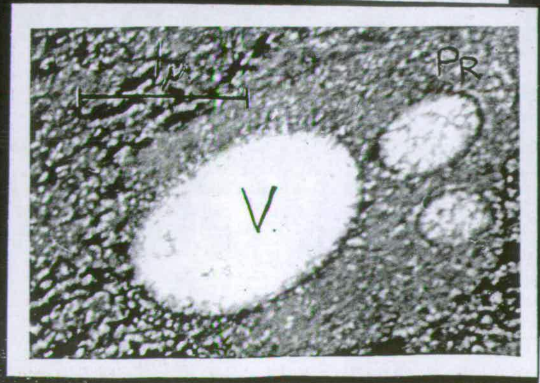


PLATE 11

EXPLANATION OF PLATES 14-16.

PLATES 14 - 16. Starchy endosperm of *Bromus*, slice from 18 hr. imbibed, intact grain.

Fixation in buffered $KMnO_4$, for 2 hr..

Plate 16 is from a disrupted section of endosperm.

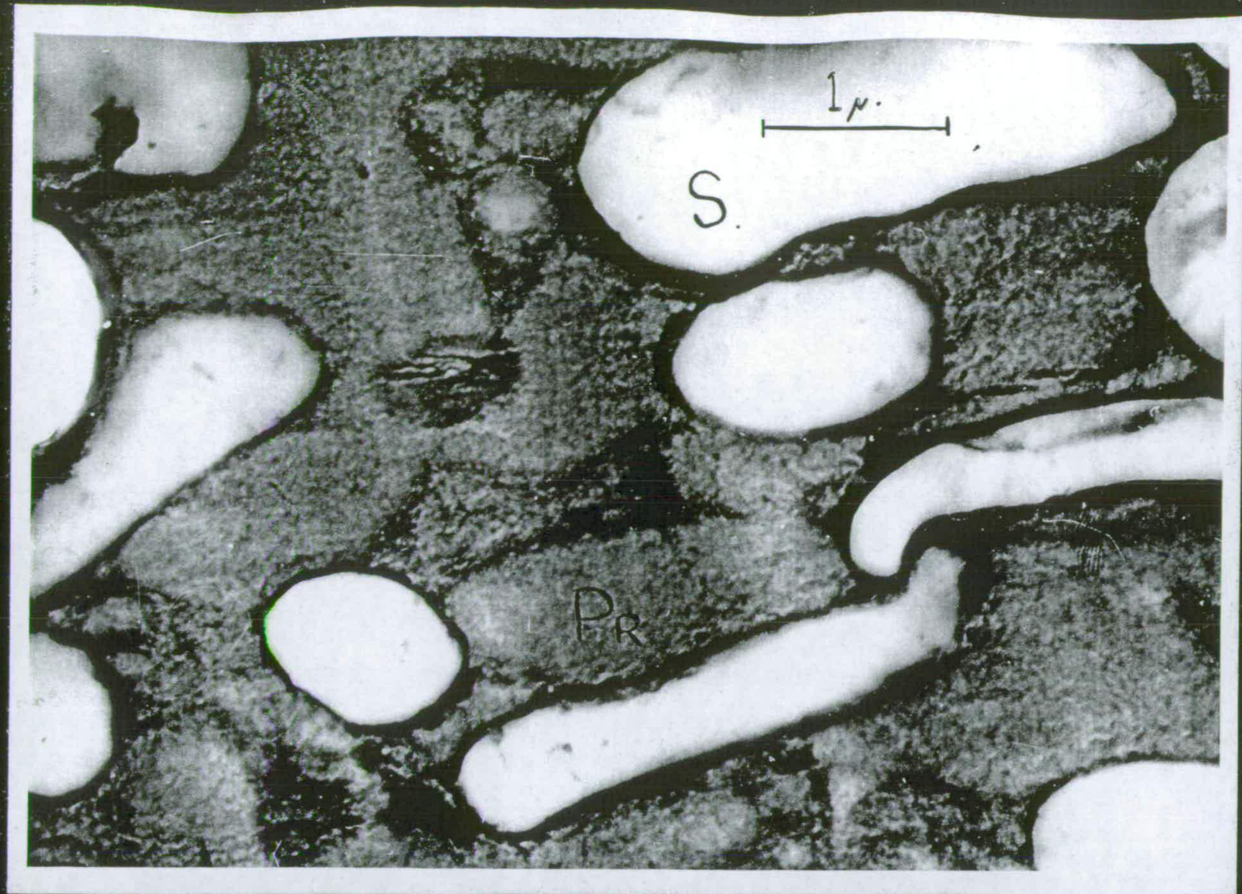


PLATE 14

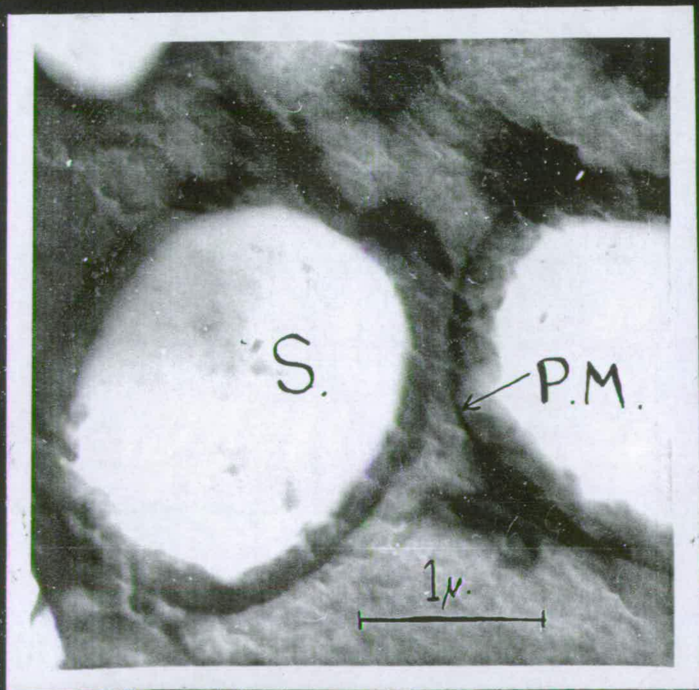


PLATE 15

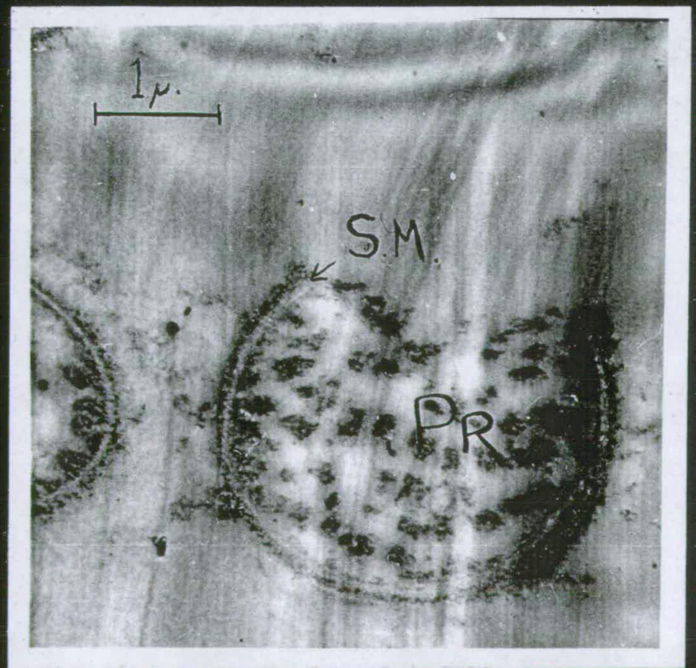


PLATE 16

EXPLANATION OF PLATES 17 & 18.

PLATE 17. Aleurone of *Bromus*, from intact, imbibed grain.

Fixation in buffered $KMnO_4$, 2 hours.

PLATE 18. as with Plate 17.

Prefixation in neutral formalin followed by fixation-staining in buffered $KMnO_4$.

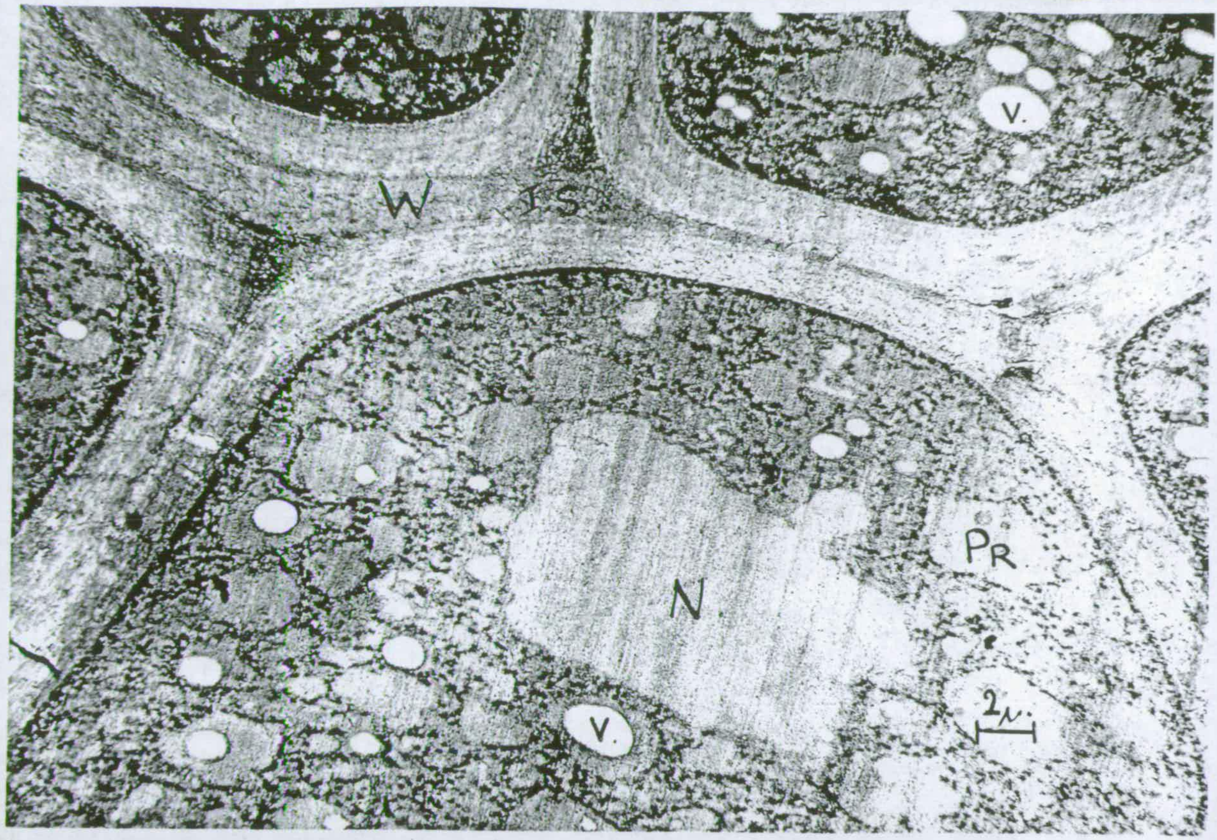


PLATE 17

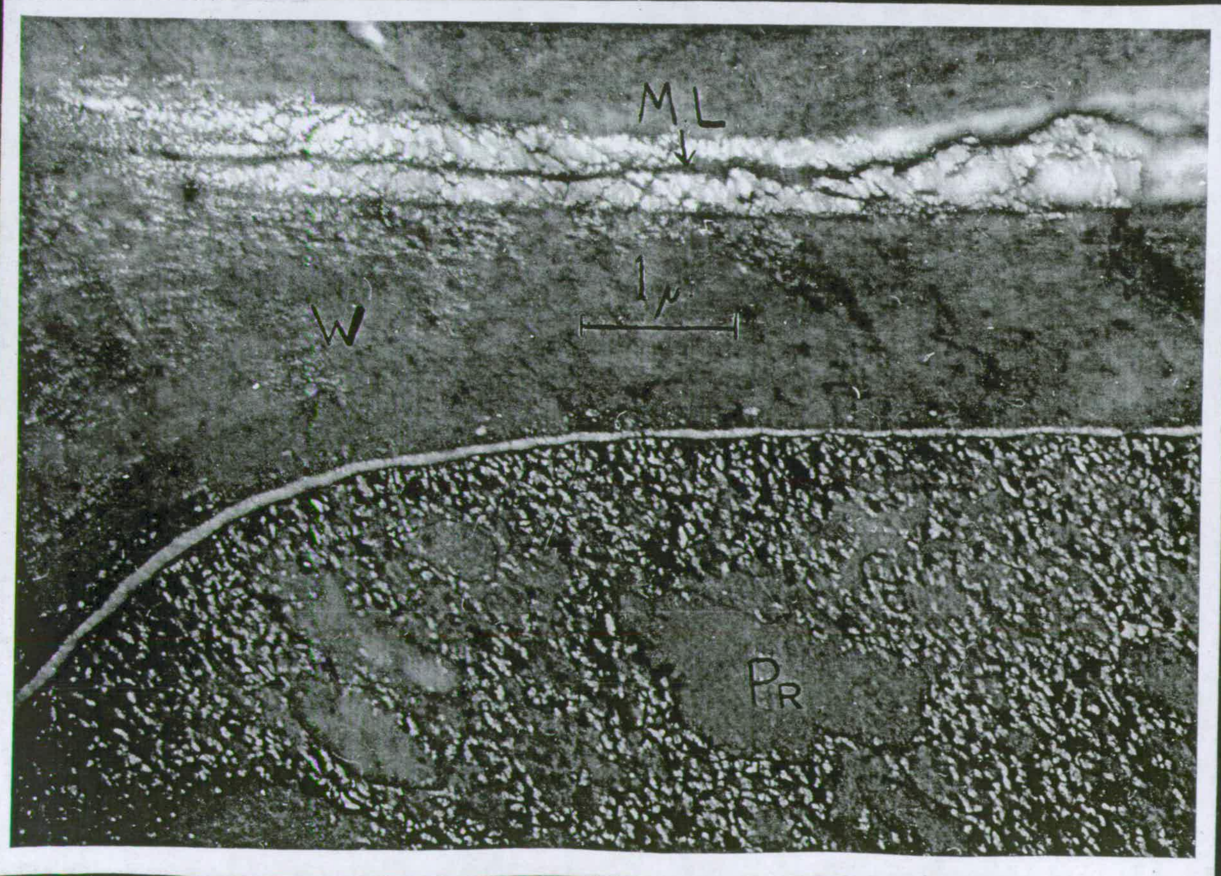


PLATE 18

EXPLANATION OF PLATES 19 - 21.

PLATE 19. Cell wall from *Bromus* aleurone, slice from imbibed, intact grain(18 hours.)

Fixation in buffered $KMnO_4$ for 2 hours.

PLATE 20. As above but fixation period extended to 4 hours.

PLATE 21. Wall between aleurone cell and starchy endosperm cell of *Bromus*, from imbibed intact grain.

Fixation in buffered $KMnO_4$ for 2 hours.

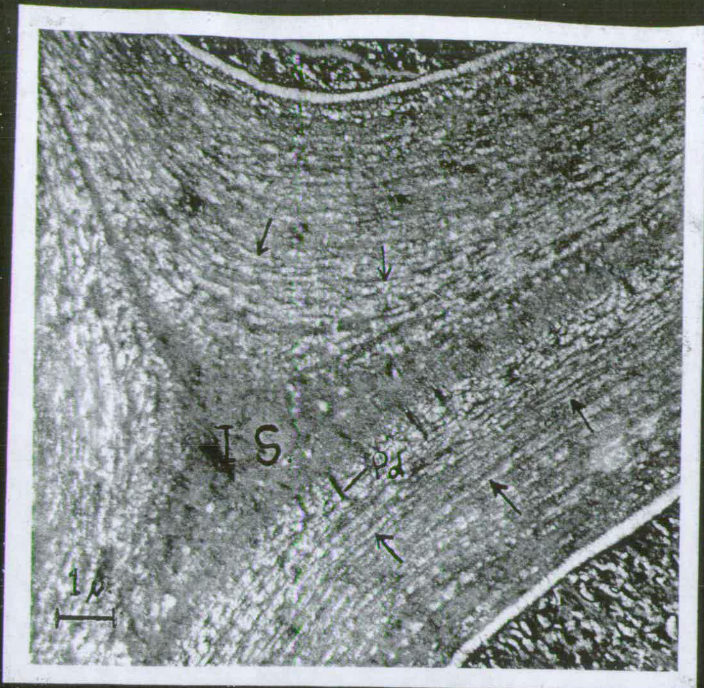
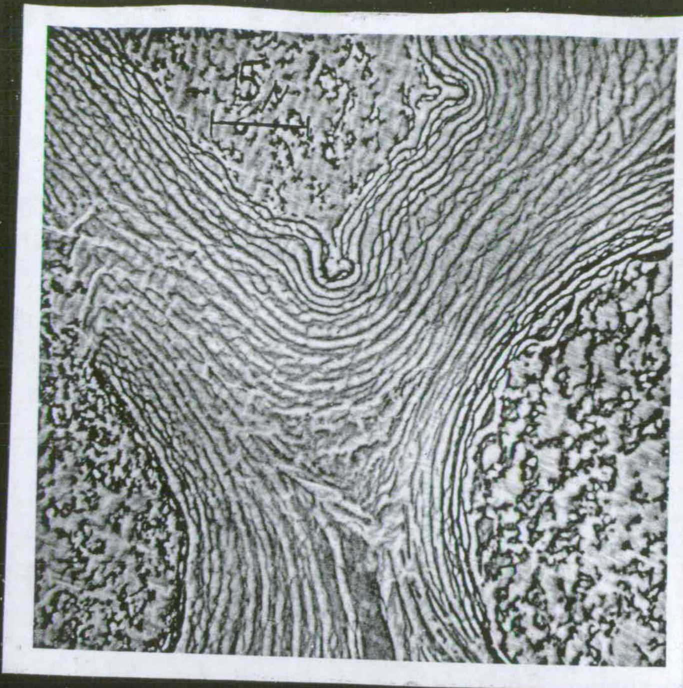


PLATE 19



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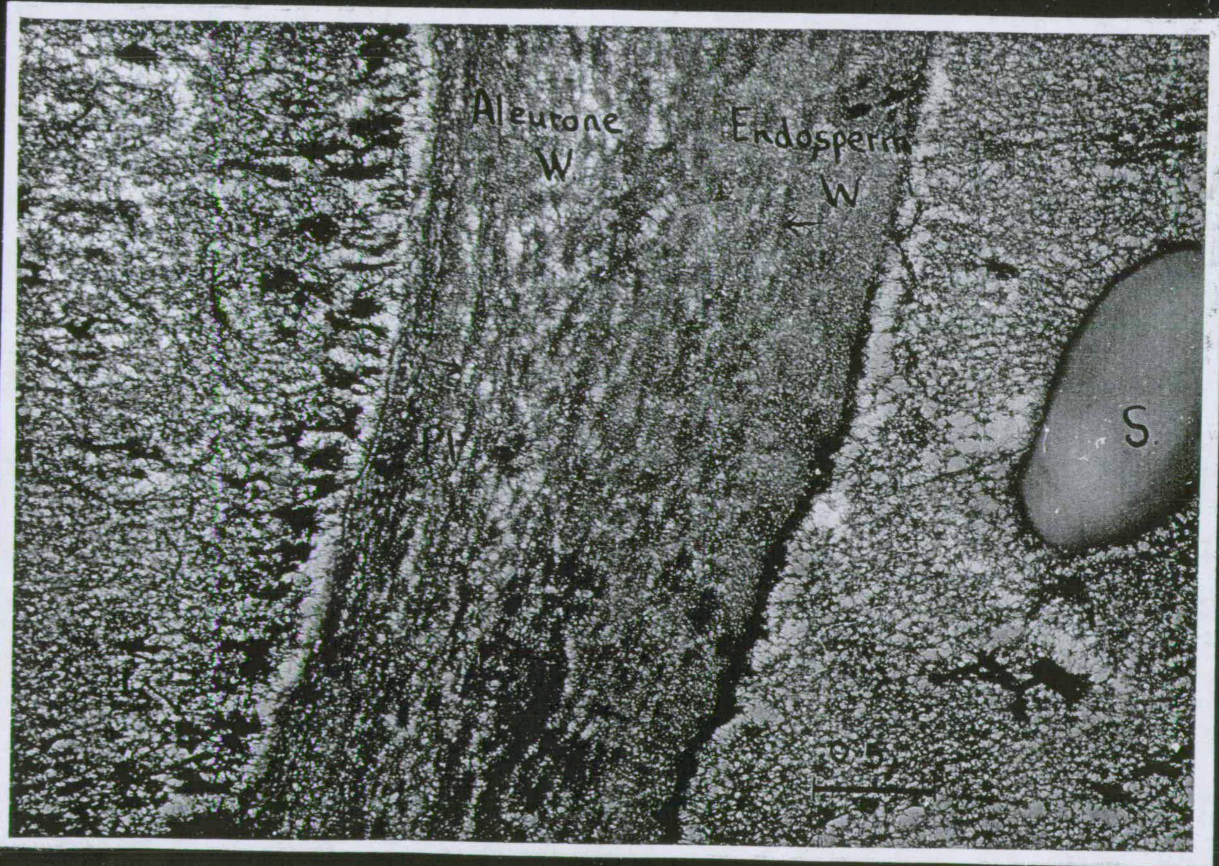

PLATE 21

EXPLANATION OF PLATES 22 & 23.

PLATES 22 & 23. Starchy Endosperm cells from *Bromus*.

Slices from imbibed, intact grain.

Fixation in buffered $KMnO_4$ for 2 hours.

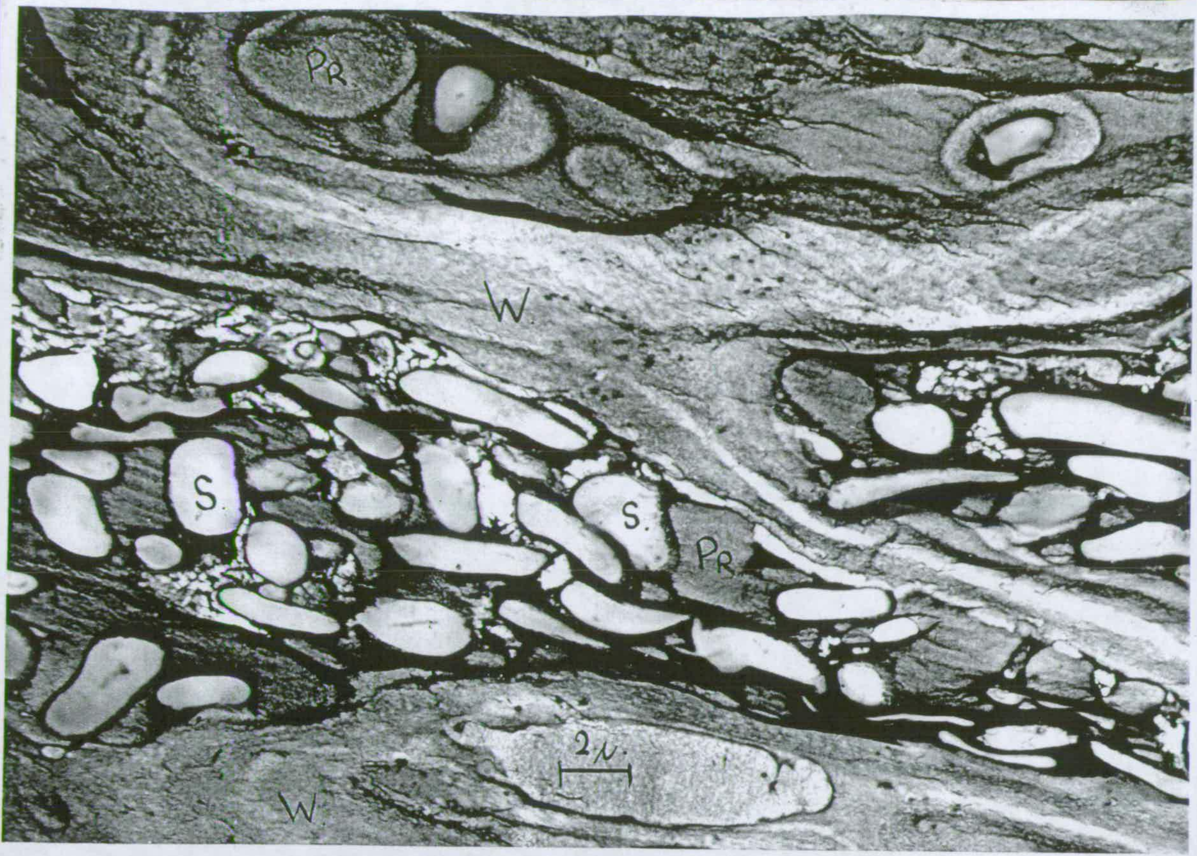

PLATE 22

PLATE 23

EXPLANATION OF PLATES 24 & 25.

PLATE 24. Starchy Endosperm of Bromus, slices from imbibed, intact grain.

Pre-Fixation in neutral formalin followed by fixation-staining in buffered $KMnO_4$.

PLATE 25. as in plate 24(above) but

Fixation in buffered $KMnO_4$ for 4 hours.

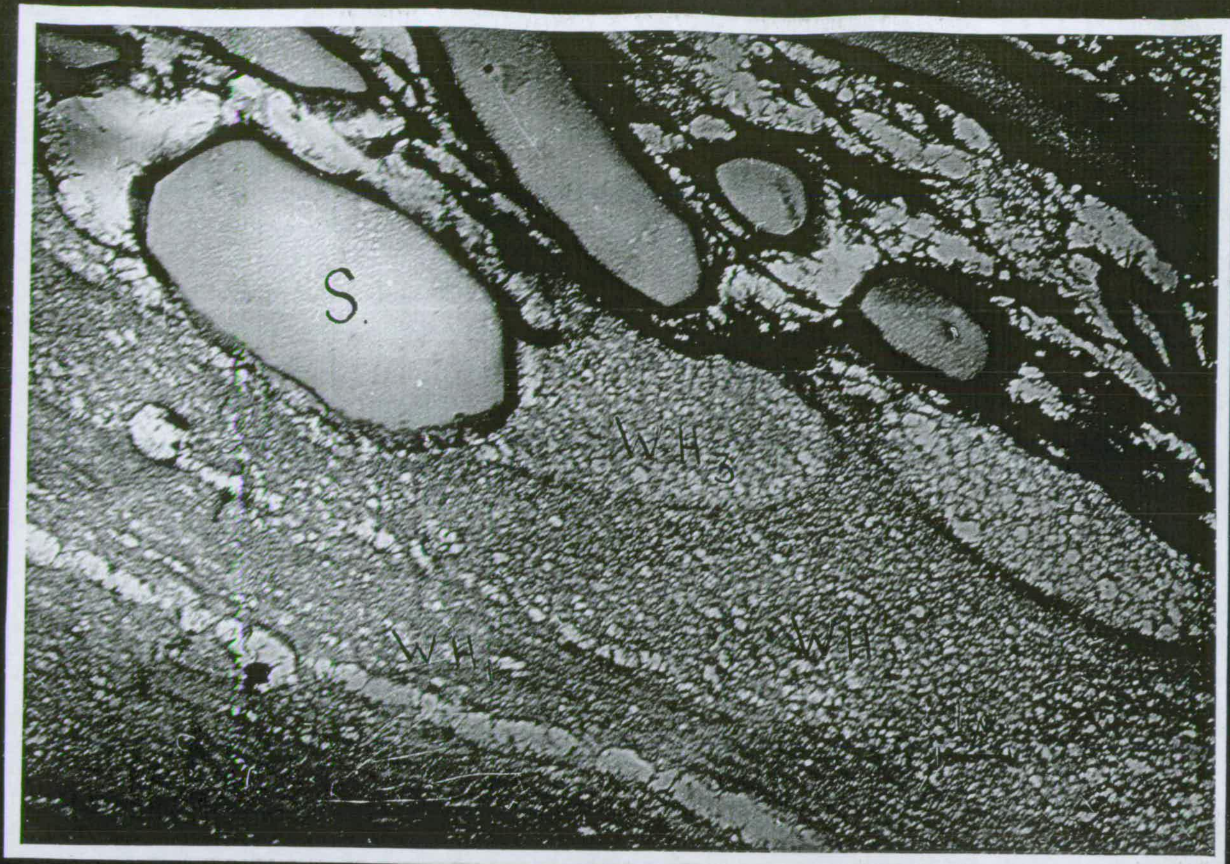

PLATE 24

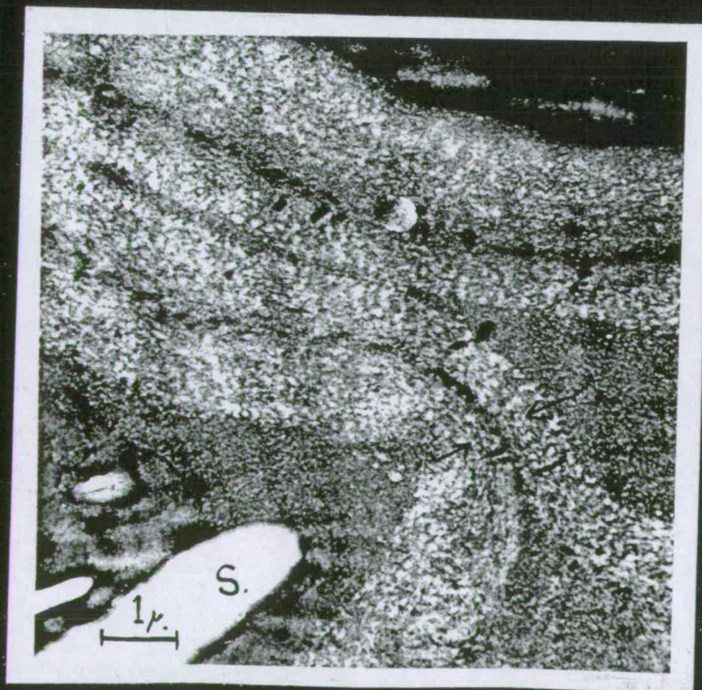

PLATE 25

EXPLANATION OF PLATES 26 - 28.

PLATES 26 & 27. Surface View - Cell wall aleurone of *Bromus*.

Direct examination of wall surface. Metal shadowed, gold-palladium alloy at 15°.

PLATE 28. Surface view - Cell Wall outer starchy endosperm cell of *Bromus*.

Direct examination of wall surface. Metal shadowed, gold-palladium alloy at 15°.

PLATE 26

PLATE 27

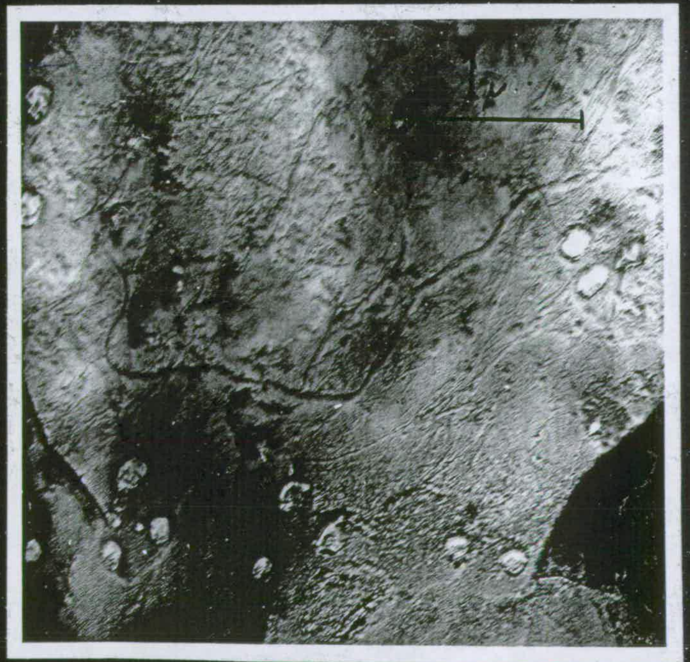

PLATE 28

EXPLANATION OF PLATES 29 & 30.

PLATE 29. Starchy endosperm cell of barley.

Slice from dry grain; imbibed overnight

Fixation in buffered $KMnO_4$ for 30 minutes.

PLATE 30. Section through aleurone cell wall of barley - after 3 days germination period at 25°C.

Fixation in buffered $KMnO_4$ for 1 hour.

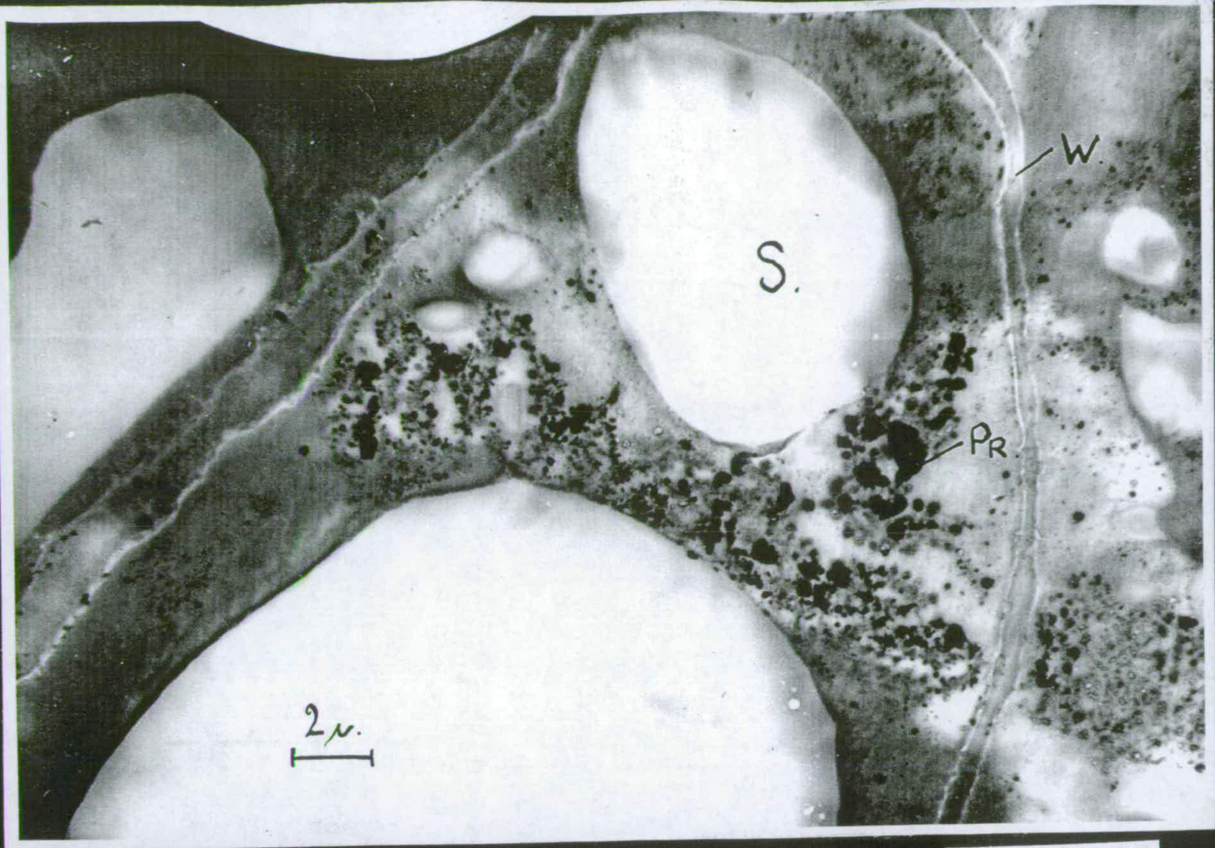

PLATE 29

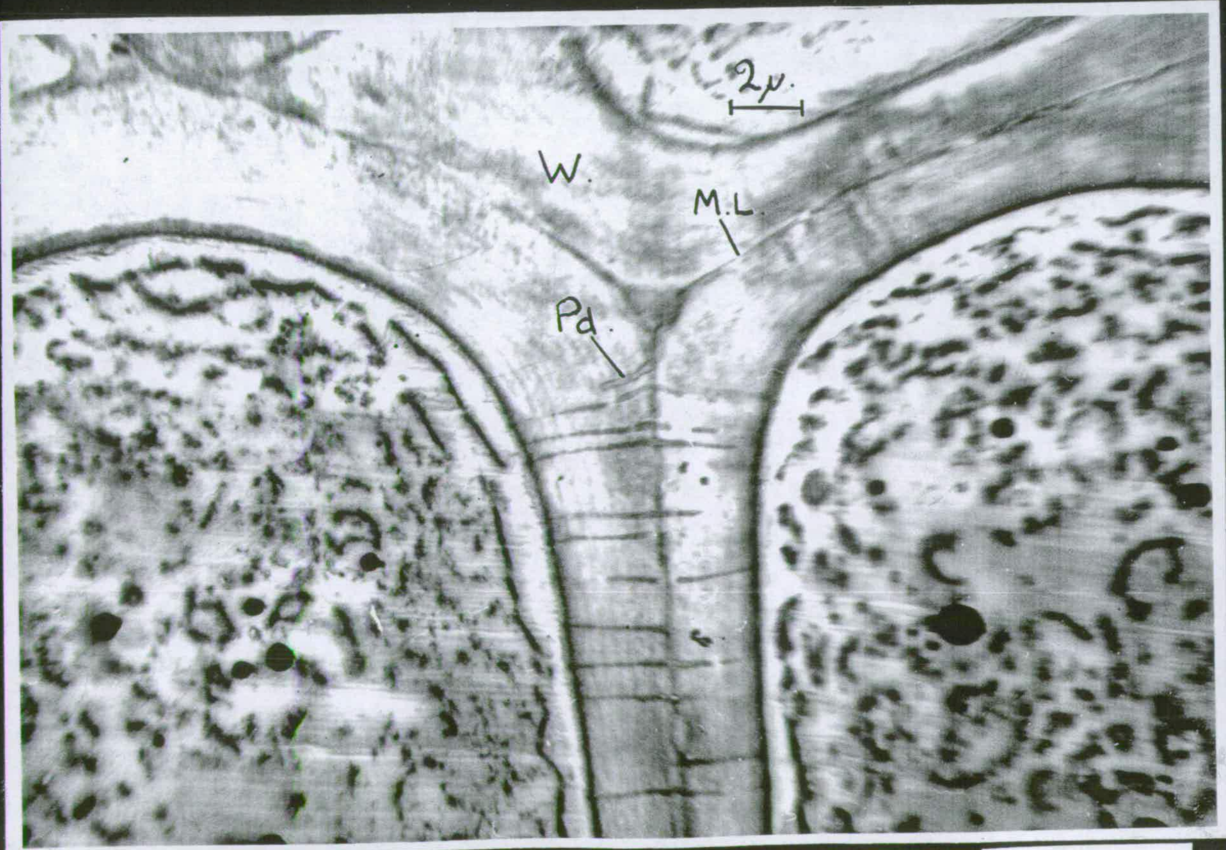

PLATE 30

EXPLANATION OF PLATES 31 - 34.

PLATE 31. Aleurone Cell Wall - from 3 day germinated barley grain, showing plasmadesmata. Fixation in buffered $KMnO_4$, for 1 hour.

PLATE 32. Higher magnification of tissue seen in Plate 31.

PLATE 33. Section through aleurone cell wall in germinating (3 day) barley; as in other two plates(above). This section passes across surface of one wall, showing plasmadesmata in cross-section.

PLATE 34. Higher magnification of plate 33.

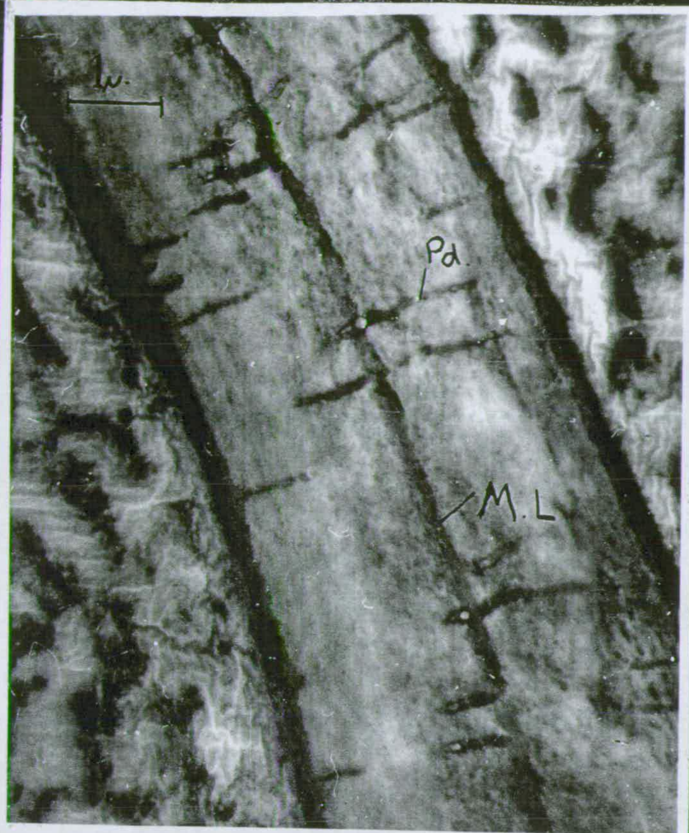

PLATE 31

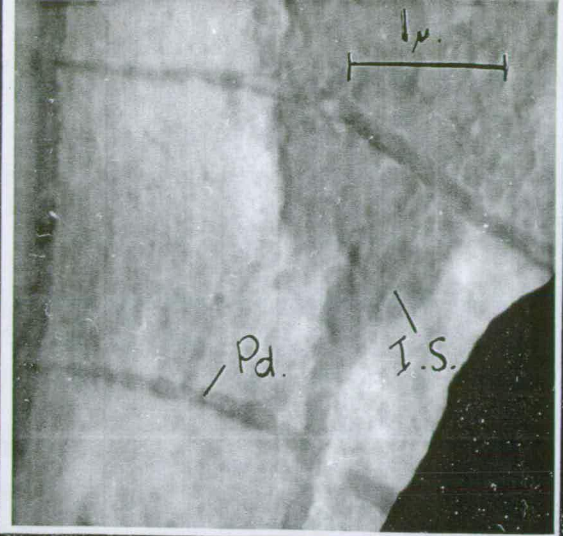

PLATE 32

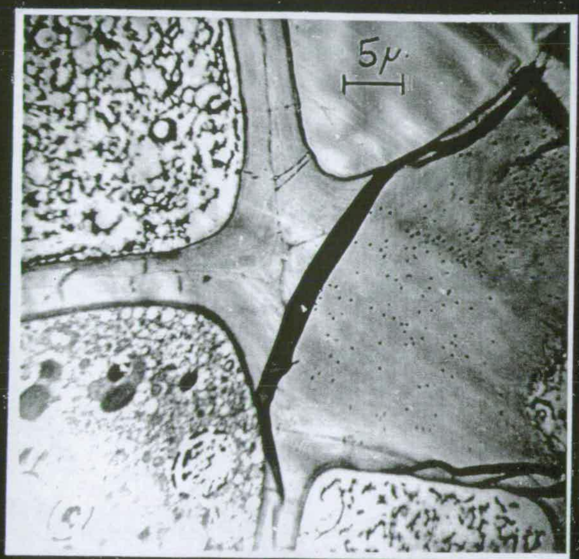

PLATE 33

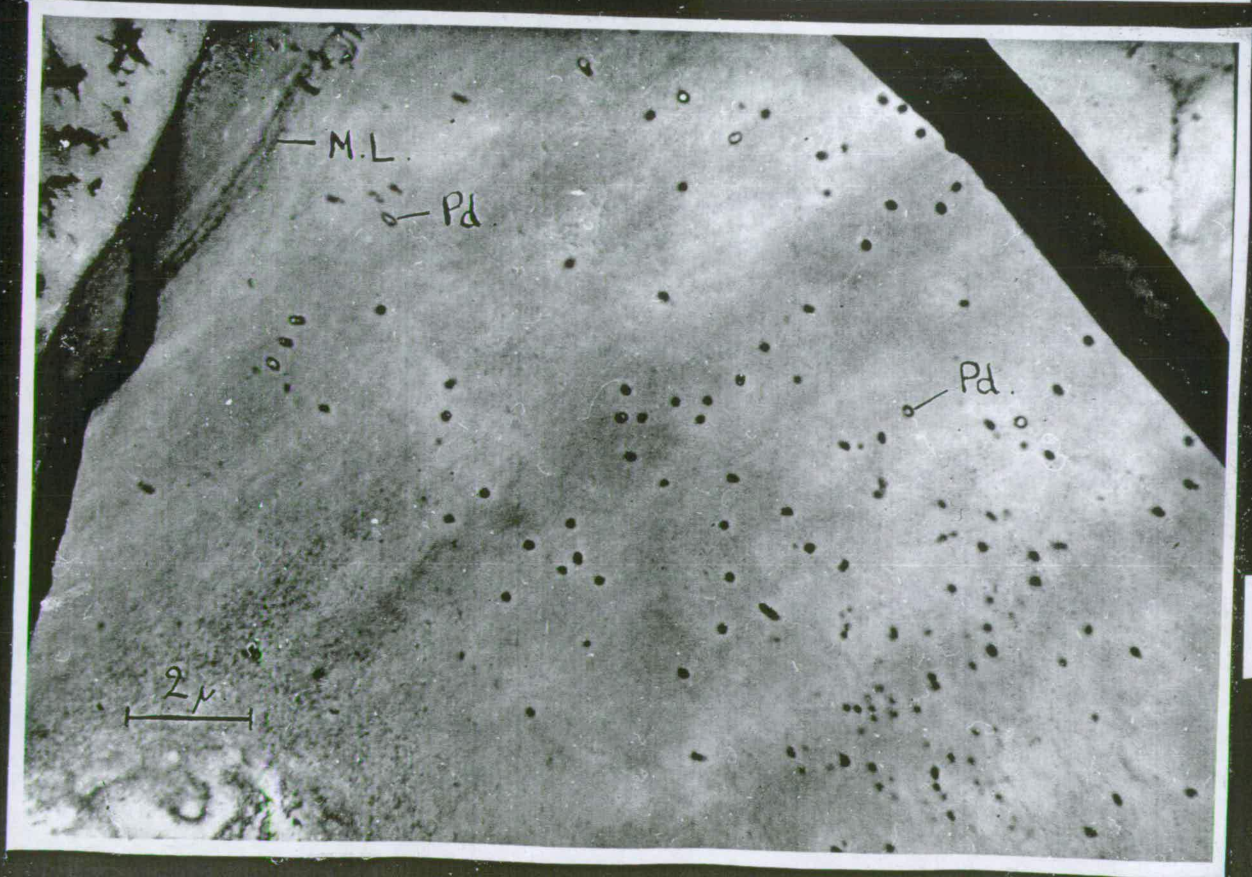

PLATE 34

EXPLANATION OF PLATES 35 & 36.

PLATES 35 & 36. Aleurone of barley after treatment of slices of aleurone-endosperm with gibberellic acid for 30 hours at 25°C..

Fixation in buffered $KMnO_4$ for 30 min..

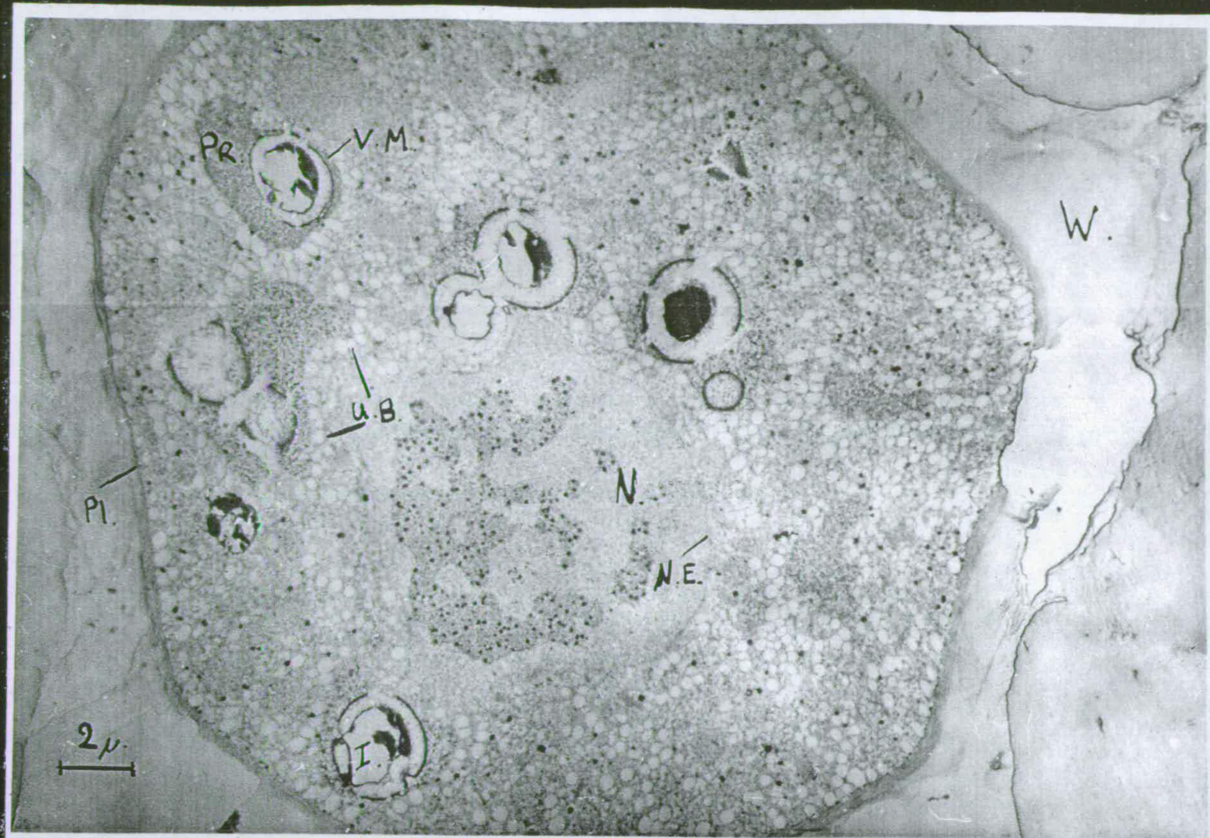

PLATE 35

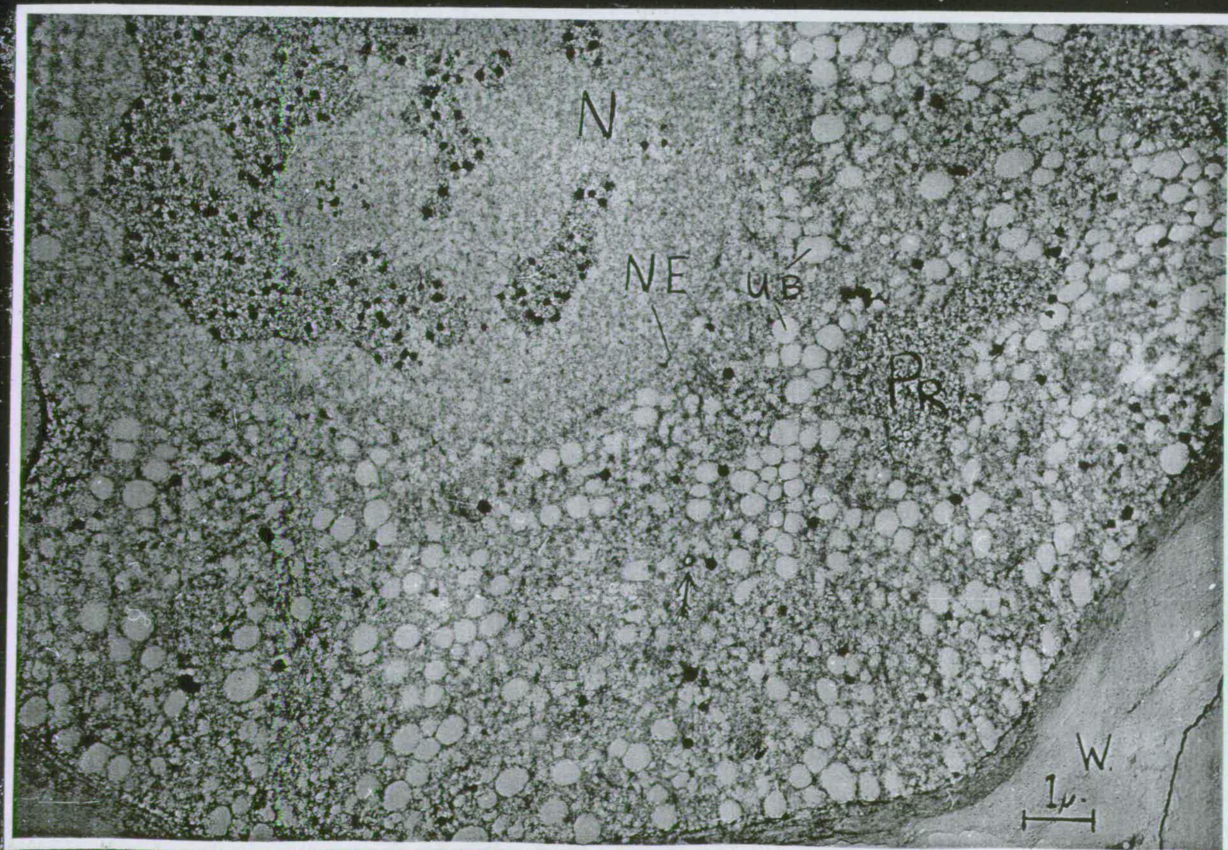

PLATE 36

EXPLANATION OF PLATES 37 & 38.

As with the previous two plates these are of aleurone cells from G.A. treated slices of Aleurone-Endosperm.

Fixation also in buffered $KMnO_4$ for 30 minutes.

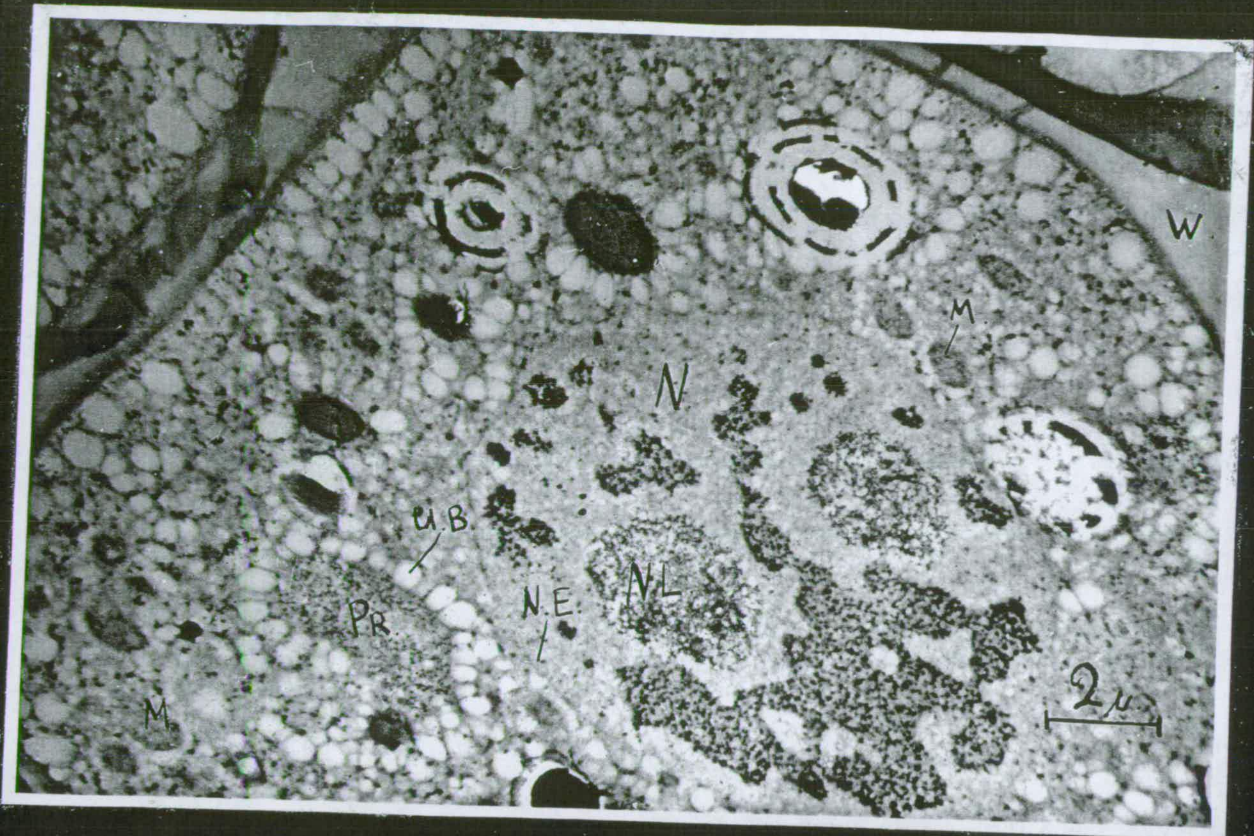

PLATE 37

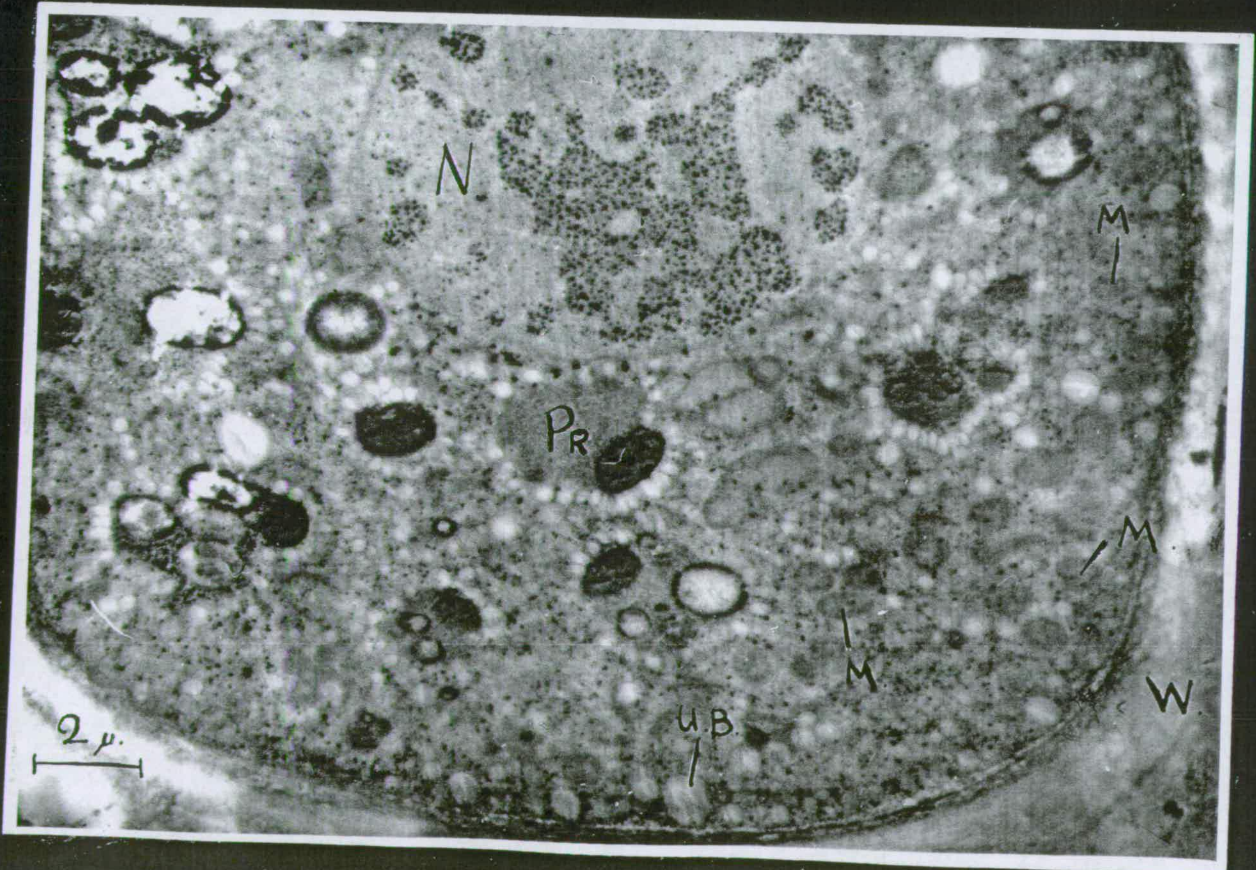

PLATE 38

EXPLANATION OF PLATES 39 - 41.

PLATE 39. Detailed portion of cytoplasm of aleurone cell from barley slice after G.A. treatment as in plates 35-38.

Fixation in buffered $KMnO_4$ for 30 minutes.

PLATES 40-41. Aleurone Cell from G.A. treated isolated aleurone tissue(see text). G.A. treatment was for 30 hours at 25°C..

Fixation in buffered $KMnO_4$ for 1 hour.

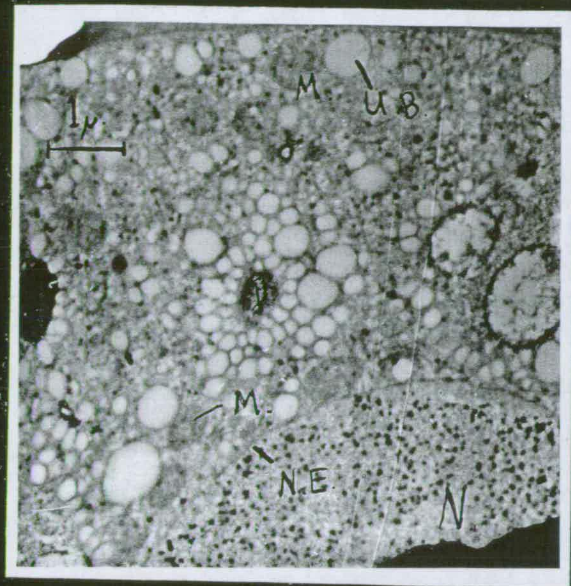

PLATE 39

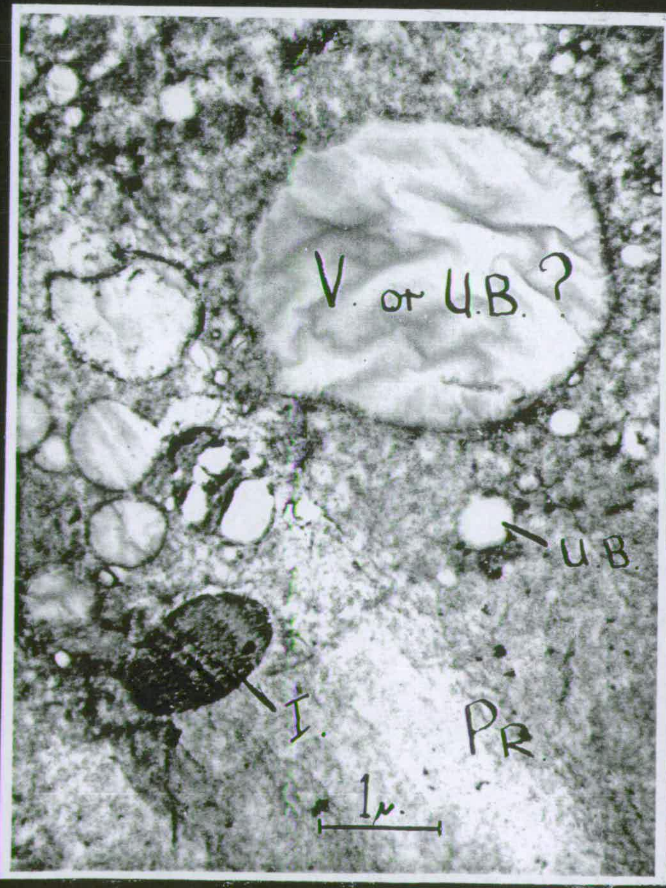

PLATE 40

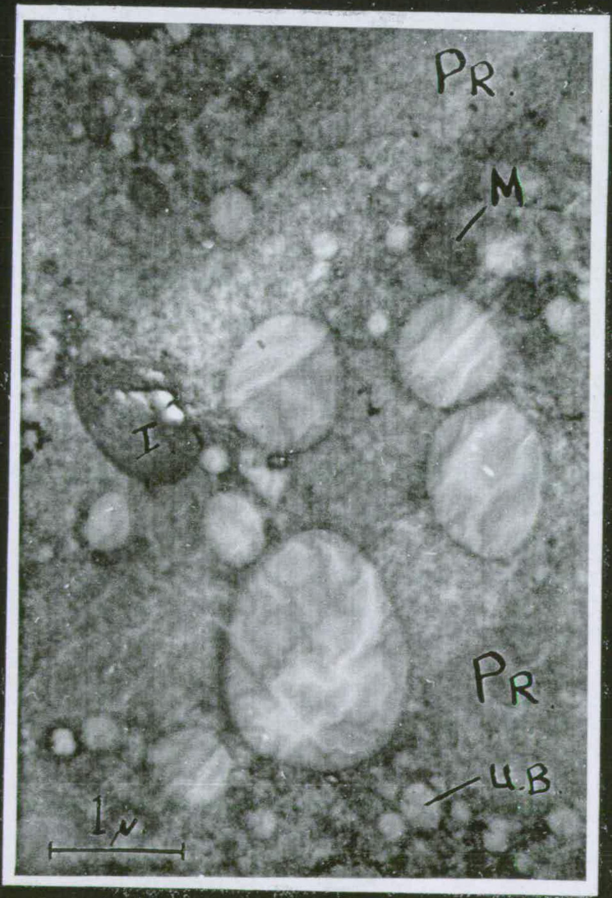

PLATE 41

BOTANICAL SOCIETY OF EDINBURGH

(Incorporating The Cryptogamic Society of Scotland)

RESEARCH SYMPOSIUM

DEPARTMENT OF BOTANY

University of Glasgow

PROGRAMME

THURSDAY, 1st OCTOBER, 1964

9.30 a.m. Welcome by Professor P.W. Brian, Sc.D., F.R.S.

9.35 a.m. Introductory remarks to the morning session by the Chairman, Dr. W.W. Fletcher (Royal College of Science and Technology, Glasgow).

9.40 a.m. The effect of hydroxybenzenitriles on micro-organisms.
- J. E. Smith, B.Sc., Ph.D. and W.W. Fletcher, B.Sc., Ph.D., F.L.S., (Department of Microbiology and Biology, Royal College of Science and Technology, Glasgow)

10.00 a.m. Hydroxybenzenitriles and plant metabolism.
- D. Paton, B.Sc., and J.E. Smith, B.Sc. Ph.D., (Department of Microbiology and Biology, Royal College of Science and Technology, Glasgow)

10.20 a.m. Questions and discussion.

10.40 a.m. Coffee and demonstrations.

11.10 a.m. Studies on the uptake and translocation of amino triazole C^{14}.
- R.C. Kirkwood, B.Sc., Ph.D., (Department of Microbiology and Biology, Royal College of Science and Technology, Glasgow)

11.30 a.m. The effect of herbicides on Pteridium aquilinum. L. Kuhn E.B.
- David J. Martin, B.Sc., M.Sc., Ph.D. (Department of Botany, West of Scotland Agricultural College, Glasgow)

11.50 a.m. Respiratory adaptations to submersion in Mesophyte roots.
- R.M.M. Crawford, B.Sc., Ph.D., (Department of Botany, University of St. Andrews)

12.10 p.m. Questions and discussion.

12.30 p.m. L U N C H

2.00 p.m. Introductory remarks to the afternoon session by the Chairman, Dr. G. Bond.

2.05 p.m. Steroid growth factors for Phytophthera.
- C.G. Elliott, M.Sc., Ph.D., (Department of Botany, University of Glasgow)

2.25 p.m. Some observations on the germination and post germ tube growth of the zoospores of Phytophthora infestans.
- D.D. Clarke, B.Sc.

2.45 p.m. Questions and discussion.

3.00 p.m. Tea and demonstrations.

3.15 p.m. Tour to Botanic Gardens, Glasgow. The Fossil Grove, Possil Park and Garscube Estate.

5.45 p.m. Return to Queen Margaret Hall, Clevedon Road.

6.00 p.m. D I N N E R

7.45 p.m. Reception in the College Club at the invitation of The University Court.

FRIDAY, 2nd OCTOBER, 1964

9.30 a.m.	Introductory remarks to the morning session by the Chairman, Professor J. Macdonald.
9.35 a.m.	Studies on Ferrobacillus a bacterium in acid mine waters. - Marcus Allan, B.Sc., (Department of Microbiology and Biology, Royal College of Science and Technology, Glasgow)
9.55 a.m.	Biological fixation of nitrogen - how widespread is it ? - G. Bond, B.Sc., Ph.D., D.Sc. (Department of Botany, University of Glasgow)
10.15 a.m.	Questions and discussion.
10.40 a.m.	Coffee and demonstrations.
11.10 a.m.	The effect of temperature and dextrose levels on symbiotic growth of Orchis purpurella. - H.G. Harvais, B.Sc., (Department of Botany, University of Aberdeen)
11.30 a.m.	The production of pectic enzymes by orchid endophytes. - G.Hadley, B.Sc., Ph.D., and M.Perombelon, B.Sc., (Department of Botany, University of Aberdeen)
11.50 a.m.	Possible growth effects induced by mycorrhizal Endogene spp.. - T.H. Nicolson, B.Sc., Ph.D., and M.J. Daft, B.Sc., Ph.D., (Department of Botany, Queen's College, University of St. Andrews, Dundee)
12.10 a.m.	Questions and discussion.
12.30 p.m.	L U N C H
2.00 p.m.	Introductory remarks to the afternoon session by the Chairman, - Dr. Macleod.
2.05 p.m.	Fine structure of grass endosperm. - Clifford Johnston, B.Sc., (Department of Brewing, Heriot-Watt College, Edinburgh)
2.25 p.m.	Gibberellic acid and barley germination. - John Duffus, B.Sc., (Department of Brewing, Heriot-Watt College, Edinburgh)
2.45 p.m.	Questions and discussion.
3.00 p.m.	Tea and demonstrations.
3.30 p.m.	Interactions of kinetin and temperature on virus-infected plants. - M.J. Daft, B.Sc., Ph.D., (Department of Botany, Queen's College, University of St. Andrews, Dundee)
3.50 p.m.	The root-shoot potassium relations in intact young Castor-oil plants. - Nathan I.C. Nwachuku, B.Sc. (Department of Botany, University of Aberdeen)
4.10 p.m.	Questions and discussion.
4.30 p.m.	Votes of thanks. Announcement of future arrangements.

DEMONSTRATIONS

Dactuliophora tarrii, a new fungal pathogen of legumes in Africa.
- Joseph Mukiibi.
  (Department of Botany, University of St. Andrews)

Variants of Phytophthora cactorum.
- D. Shaw, B.Sc.,
  (Department of Botany, University of Glasgow)

The use of disc-electrophoresis in the analysis of plant proteins.
- A.M.M. Berrie, B.Sc., Ph.D.,
  (Department of Botany, University of Glasgow)

Several methods for measuring and recording soil moisture tensions.
- J.A. Rogers and Mr. Legge
  (Hill Farming Research Organisation, Edinburgh)

Cytological demonstration.
- Janet Taylor, B.Sc.,
  (Department of Botany, University of St. Andrews)

Blind Seed Disease.
- M. Noble, B.Sc., Ph.D.,
  (Scientific Services, East Craigs, Edinburgh)

GENERAL INFORMATION

Papers will be read and demonstrations set out in the Department of Botany on University Avenue.

Dinner, bed and breakfast will be available in the new Queen Margaret Hall, Clevedon Road, Kirklee, Glasgow, and buses will be on hand for transport to and from the Department of Botany and for the excursion. Arrangements will be made for lunch in the vicinity of the University on both days.

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JULY–AUGUST, 1964.

ULTRA-STRUCTURE OF CARYOPSES OF THE GRAMINEAE

I. ALEURONE AND CENTRAL ENDOSPERM OF *BROMUS* AND BARLEY

BY

Dr. ANNA M. MacLEOD, F.R.S.E., C. S. JOHNSTON, BSc.
and J. H. DUFFUS, B.Sc.

ULTRA-STRUCTURE OF CARYOPSES OF THE GRAMINEAE
I. ALEURONE AND CENTRAL ENDOSPERM OF *BROMUS* AND BARLEY

By Dr. Anna M. MacLeod, F.R.S.E., C. S. Johnston, B.Sc., and J. H. Duffus, B.Sc.
(*Heriot-Watt College, Edinburgh*)

Received 18*th March*, 1964

Sections of ungerminated caryopses of barley and of *Bromus* examined by electron microscopy are found to contain in the cells of the aleurone layer the usual membranes and cell organelles; additionally there are present vacuoles with (in barley only) characteristic electron-dense inclusions. Large numbers of unidentified bodies, 0·1–0·4 μ. in diameter, are also found in the aleurones of both species. The aleurone cell walls have a fibrillar cellulose-like structure; the walls of the central endosperm are less highly organized. Starch grains and deposits of material of medium electron density, probably proteinaceous in nature, are the only observed inclusions in the central endosperm cells.

Introduction

Results of recent biochemical studies[1,8] have focused attention on the properties of the aleurone layer of cereal grains. This tissue is now known to be implicated in the liberation of certain of the hydrolytic enzymes which participate in modification of the reserve material of the central endosperm; it is also the region of the grain which responds to addition of gibberellic acid. Calculation suggests that more than 80% of the total α-amylase[1] and endo-β-glucanase[7] of barley malt is derived from the activities of the aleurone cells and, though comparable figures are not available for proteinase and endopentosanase, it is clear that these enzymes also can be produced in substantial amounts by the aleurone.[8] The recent demonstration[5] that gibberellic acid is present in ripening barley grains has given added weight to the hypothesis that, during germination, endogenous gibberellic acid interacts with the aleurone to catalyse the mobilization of hydrolytic enzymes. Although the biochemistry of the aleurone-gibberellin interaction is not yet fully understood, there can be no doubt at all about the importance of the aleurone in the metabolism of germinating barley.

Other grass seeds—wheat,[3] oats[12] and certain species of *Bromus*[6]—respond to additions of gibberellic acid in a manner generally similar to that observed with barley and, in *Bromus* at least, it is also the aleurone layer of the seed which responds to the hormone. To supplement the biochemical studies of

the effects of gibberellic acid on barley, a survey is now being made of the fine structure of the aleurone and starchy endosperm cells of barley and of *Bromus* during germination without additives and in endosperm slices treated with gibberellic acid. *Bromus* has been chosen for study in conjunction with barley because, although this grass is phylogenetically distinct from the Hordeae, it resembles barley rather closely in the general pattern of evolution of cytolytic enzymes during germination. It is dangerously easy to mistake artefacts for reality in interpreting electron micrographs and it is hoped that, by examining two apparently unrelated species of the Gramineae, this danger may be minimized.

The present Communication is a record of observations made on the ultra-structure of endosperm prior to germination; changes taking place in aleurone and in starchy endosperm in response to germination and in response to addition of gibberellic acid will be reported later.

Technique

The barley used was Proctor from the 1963 harvest. Slices of endosperm, 2 mm. thick, were cut from dehusked grain, halved through the furrow and steeped in water at 25° C. overnight. These endosperm fragments were then ground gently in a glass homogenizer until all the starchy tissue was suspended in the distilled water used as grinding medium. The aleurone was separated by passing the mixture through a sieve and washing the residue thoroughly: the isolated aleurone was then processed in the manner described below. Preparations were also made from slices containing both aleurone and starchy endosperm; these gave electron micrographs which were similar to those from the isolated aleurone, and it thus appears that the separated aleurone is essentially unchanged in its ultrastructure.

For *Bromus*, caryopses of *B. inermis* Leyss were freed from husk by treatment with 50% sulphuric acid, washed and stored moist at 25° C. overnight. 1-mm. slices were cut transversely from the endosperm and smaller (approximately 0·5-mm.) fragments were cut from these slices to include both aleurone and starchy endosperm. It will be realized that significant changes may have taken place during the 18-hr. equilibration of the grain, or grain fragment, with water, but it has as yet proved technically impossible to prepare electron micrographs from unimbibed material.

Processing.—The isolated aleurone, or the endosperm fragments, were normally fixed at 5° C. in 0·6% buffered potassium permanganate over a range of times extending from 30 min. to 4 hr. The fixed material was dehydrated in increasing concentrations of ethanol and embedded under reduced pressure in methacrylate (88% butyl methacrylate + 12% methyl methacrylate polymerized at 60° C. in presence of 1% dichlorobenzoyl peroxide). The polymerized blocks were trimmed and sections were cut on to 15% ethanol by a *Cambridge* ultramicrotome. The sections were usually of 500 Å thickness, though on occasions sections of 250 Å were examined. Any ripples present in the sections were evened out by treatment with xylol vapour and the sections were mounted on uncoated grids and subsequently coated with a thin layer of carbon on one side only. The specimens were examined and photographed in an *Akashi* TRS 50 E1 electron microscope fitted with a specially modified D.C. filament and a 50 kv. E.H.T.

Explanation of Figures

Fig. 1.—Aleurone of *Bromus*. (*a*) fixation in unbuffered $KMnO_4$; (*b*) pre-fixation in neutral formalin followed by fixation in buffered $KMnO_4$; (*c*) as in (*b*).

Fig. 2.—Aleurone of *Bromus*. (*a*) fixation in unbuffered $KMnO_4$; (*b*) prefixation in neutral formalin followed by fixation in buffered $KMnO_4$; (*c*) as in (*b*); (*d*) prolonged fixation (4 hr.) in buffered $KMnO_4$.

Fig. 3.—Barley aleurone. (*a*), (*b*) and (*c*) fixed in buffered $KMnO_4$.

Fig. 4.—Starchy endosperm of *Bromus*. (*a*) fixation in unbuffered $KMnO_4$; (*b*) prefixation; (*c*) fixation in unbuffered $KMnO_4$.

(D.B.—dense body; E.R.—endoplasmic reticulum; I—inclusions, mainly vacuolar; I.S.—intercellular substance; M—mitochondrion; M.L.—Middle lamella; N—nucleus; N.M.—nuclear membrane; P—plastid; Pr—medium density deposit, probably protein; S—starch grain; S.M.—membrane surrounding "protein" sac; U.B.—unidentified body; V—vacuole; V.M. vacuolar membrane; W—wall (in general); W_{1-3}—three apparent layers of endosperm wall.

Observations

Internal Structure of the Aleurone Cell

The general structure of a typical aleurone cell from *Bromus* is shown in Fig. 1. In addition to structures which are normally found in undifferentiated plant cells—*e.g.*, nucleus, mitochondria, plastids, endoplasmic reticulum and plasmalemma—there are also small vacuoles which here are very distinct indeed, and which are enclosed in sharp and well-preserved membranes. Other less familiar inclusions are also present and not all these organelles can as yet be identified with certainty. However, the descriptions which follow are based on results obtained from a large number of different preparations, and we are reasonably confident of the authenticity of these unfamiliar inclusions.

Nucleus.—The nuclei are from 15 to 20 μ. in diameter and on occasions the presence of nucleoli has been demonstrated. The nuclear envelope is distinctly a double layer: the outer membrane passes into the cytoplasm and is apparently continuous with the endoplasmic reticulum (Fig. 1 *a*). Holes, about 500–800 Å in size, are visible in the inner membrane as indicated by arrows in Fig. 1 *a*.

Mitochondria.—In *Bromus*, membrane-enclosed organelles (M) of the mitochondrial type, 0·7–1·4 μ. in diameter, can be seen in aleurone cells and a close examination shows that they are of the same internal structure as those reported from most other plant cells. The number of characteristic, easily-recognized mitochondria present seems to depend on the developmental stage of the cell, and many of the cells of *Bromus* and most of the barley aleurone cells (at the stages under discussion) contained no typical mitochondrial structures. It is possible that there is some connection between the appearance of mitochondria and the small vacuoles (V) which are often present in fairly large numbers (Fig. 2 *b*). This possibility will be considered more fully later.

Plastids.—Structures resembling plastids (P) have been detected in some aleurone cells (Fig. 1 *a*), but it is possible that these are simply mitochondria of the type reported by Hrsel *et al.*[4] to be present in the scutellum of wheat.

Vacuoles and vacuolar inclusions.—In all specimens of *Bromus* and of barley examined (Fig. 1; Fig. 2, *a* and *b*; Fig. 3), several small, regularly-shaped vacuoles (V) are found. These vacuoles are bounded by a distinct membrane and they are usually associated with regions of medium-electron-density material which is probably proteinaceous in nature. In *Bromus* this presumptive protein (Pr) is also enclosed in a membrane which is apparently continuous with the endoplasmic reticulum (Fig. 1 *a*), but in barley (Fig. 3, *a*, *b* and *c*) no evidence of such membranes has been found. It is possible that this medium-electron-density deposit may be a region of protein synthesis, especially where it is associated with the endoplasmic reticulum.

From Fig. 1, *a* and *c*, it seems probably that the vacuoles increase in number by a "budding" process which results in the formation of a large number of smaller vacuoles (Fig. 2 *b*). Examination of Fig. 2 *b* shows that there is often a considerable number of these smaller vacuoles present within one cell. It may be suggested that these "newly-budded" vacuoles are associated with respiration, in analogy with observations recently made by Polakis *et al.*[10] for yeast.

In all the specimens of *Bromus* examined, most of the vacuoles contained only an almost electron-translucent matrix (Fig. 1 *a*) whereas barley (Fig. 3) contained additionally many vacuoles with almost spherical electron-dense inclusions (I). There appears to be a gradation in the electron density of these vacuolar inclusions (I, 1–3 in Fig. 3 *a*). The significance of these inclusions of varying density is unknown: a gradation in development could be inferred, but there is as yet no evidence to indicate the direction of any possible trend. Similar vacuolar inclusions have been noted in wheat embryos[11] and in the aleurone of ripening wheat.[2] Buttrose[2] has provided convincing evidence for linking these vacuolar inclusions with the "aleurone grain" familiar from optical microscopy.

Unidentified bodies.—These small structures, 0·1–0·4 μ. in diameter and labelled U.B. are present in both barley and *Bromus*. In barley they are regular structures, 0·3–0·4 μ., and are electron-translucent. In *Bromus* they are smaller than 0·3 μ. and appear to be electron-dense (Fig. 1 *a*), though in other respects they are similar to those of barley, being vesicular in structure and provided with a definite limiting membrane (Fig. 2 *c*). In both *Bromus* and barley, these unidentified bodies are associated with the medium-density deposits (Pr) shown in

Fig. 1 *a* and in Fig. 3 *c*. They are also concentrated round the periphery of the cell, adjoining the plasmalemma (Fig. 1, *b* and *c*).

The differences in electron-density do not appear to be directly due to hydration, as was suggested by Buttrose,[2] since the U.B. remain electron-translucent in isolated barley aleurone which has been soaked in water for up to 24 hr., without influence from the embryo.

Dense bodies.—These small inclusions (D.B.) are visible only in the aleurone cells of barley (Fig. 3 *c*). They are widely dispersed throughout the cytoplasm, but they do not regularly appear to be associated with the U.B. Their significance is unknown, but similar structures have been detected in cells of cereal embryos.[11]

Internal Structure of the Starchy Endosperm Cell

This part of the investigation is still in an initial stage: indeed, little of interest can be expected until the starch-filled cells come under the influence of hydrolytic enzymes from the aleurone. However, Fig. 4 (*a* and *b*) shows the typical starch granules together with membrane-enclosed deposits of medium-density material which is probably proteinaceous in nature. Fine detail of the cell structure is lacking and no organelles resembling mitochondria have been detected. As would be expected from results with optical microscopy, which indicate only occasional remains of degenerating nuclear material, no evidence was found for the presence of a nucleus. The starchy-endosperm cells adjoining the aleurone layer contain a large number of small (less than 1·5 μ.) starch grains as well as the normal (1·5–4 μ.) grains, whereas few of the smaller grains are found in the inner tissue of the caryopsis.

Comparison of Cell-wall Structure in Aleurone and in Starchy Endosperm

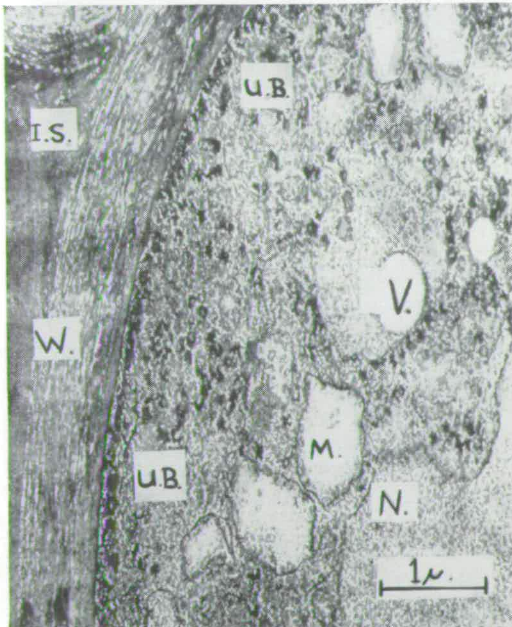
Aleurone wall.—The structure of a typical aleurone cell wall can be seen in Fig. 1, *b* and *c*. The wall has a fibrillar structure similar to that seen in most normal plant cells, and, taking its appearance in conjunction with results of earlier biochemical studies, it is reasonable to attribute this largely to cellulose. The fibrillar structure can be clearly demonstrated by prolonged treatment of the tissue with potassium permanganate, which results in oxidation of interfibrillar material and simultaneously induces separation of the resistant fibres (Fig. 2 *d*). The presence of a definite middle lamellar region has also been demonstrated in the aleurone.

Starchy-endosperm wall.—In Fig. 4, *a*, *b* and *c*, it can be seen that the endosperm wall of *Bromus* is exceedingly thick, though it has a rather less well-defined structure than that of the aleurone. Nevertheless, there is a considerable degree of organization, possibly fibrillar, still present. The distribution of cellulose in the caryopsis of *Bromus* is not fully known, though preliminary studies indicate that true cellulose is not present in large amounts in the wall of the starchy endosperm cell: the organized material, which is birefringent, may therefore be hemicellulose, which is certainly present in high concentrations in *Bromus*. Close examination of the micrographs (Fig. 4 *c*) suggests that this wall is in fact made of more than one distinct layer: there is a well-defined outer region within which can be seen one or possibly two more amorphous layers, the innermost of which appears to extend into the lumen of the cell as a form of matrix round the starch granules and other inclusions. Eventually, it should prove possible to relate these morphological observations to earlier biochemical[9] work in which cell-wall material has been fractionated into water-soluble, alkali-soluble and encrusting hemicellulose.

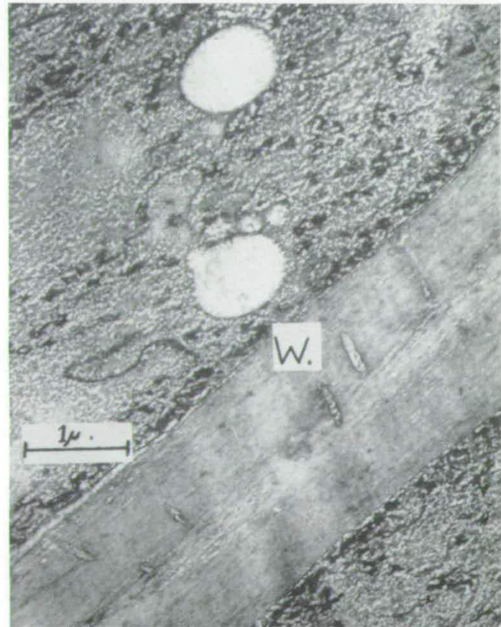
Discussion

Cereal grains and grass seeds are rather refractory objects as regards processing for electron microscopy, and the methods used (permanganate fixation followed by embedding in methacrylate) can lead to implosion of vesicles and formation of various types of artefact: these methods, however, are the only ones which, in our hands, have given consistently successful preparations. Despite the use of permanganate and methacrylate, membranes have been well preserved (Figs. 1–4) and the familiar subcellular organelles present a normal appearance: it is therefore reasonable to assume that the unfamiliar unidentified bodies are also authentic cell components.

(a)

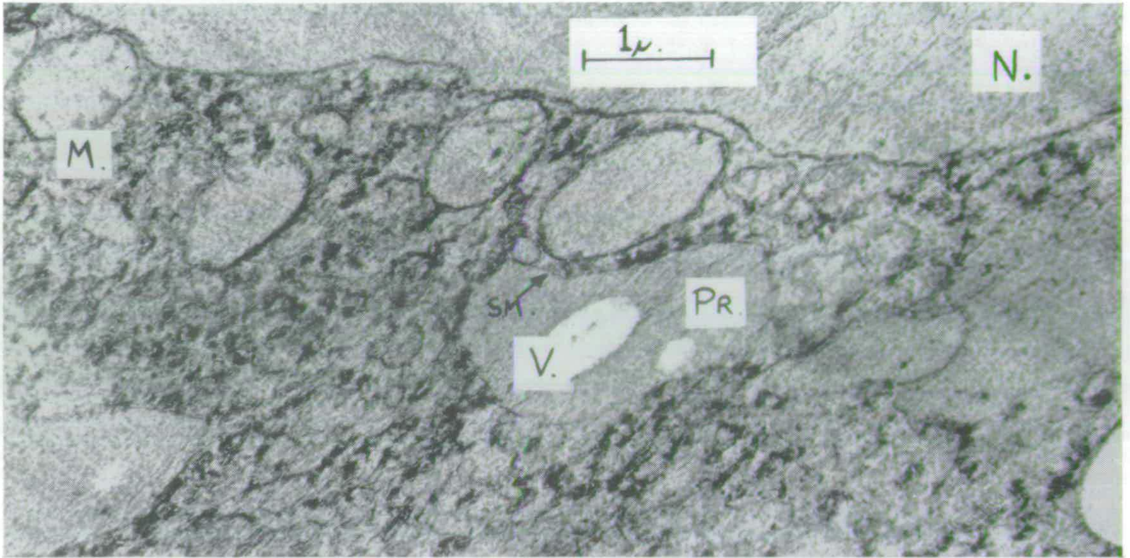


(b)

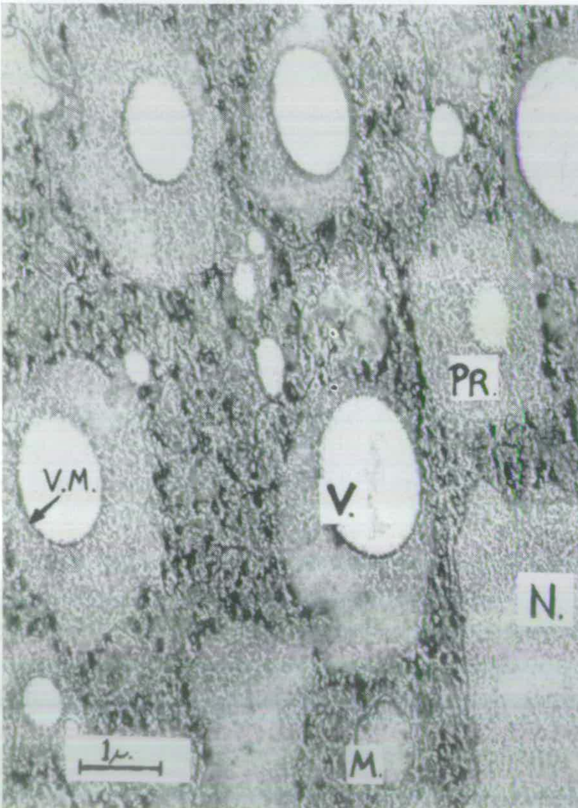


(c)

Fig. 1.—Aleurone of *Bromus*.



(a)



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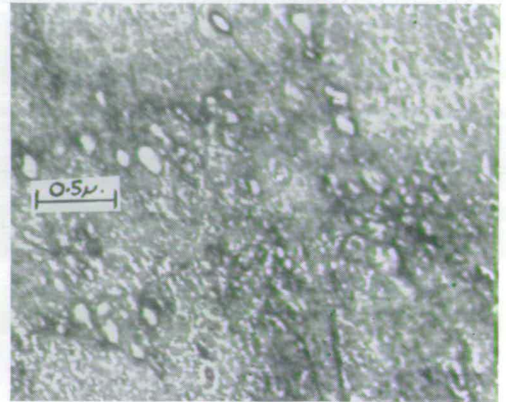
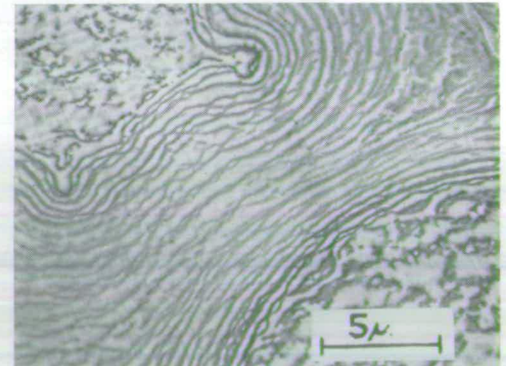


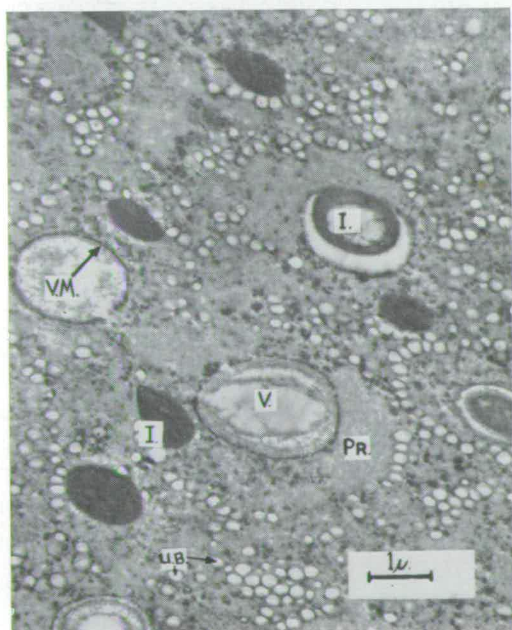
(c)



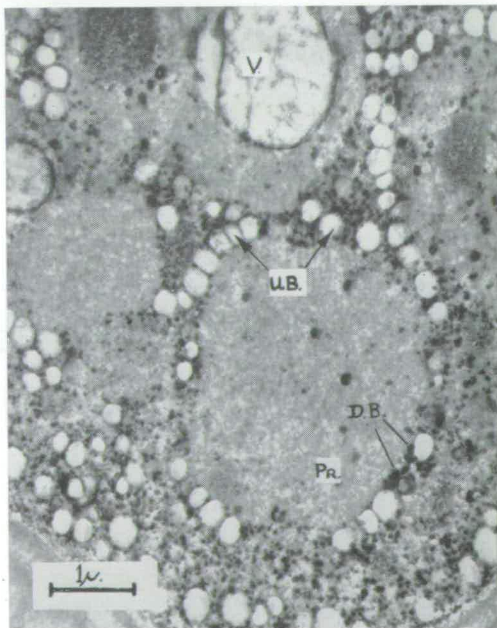
(d)

Fig. 2.—Aleurone of *Bromus*.

(*a*)



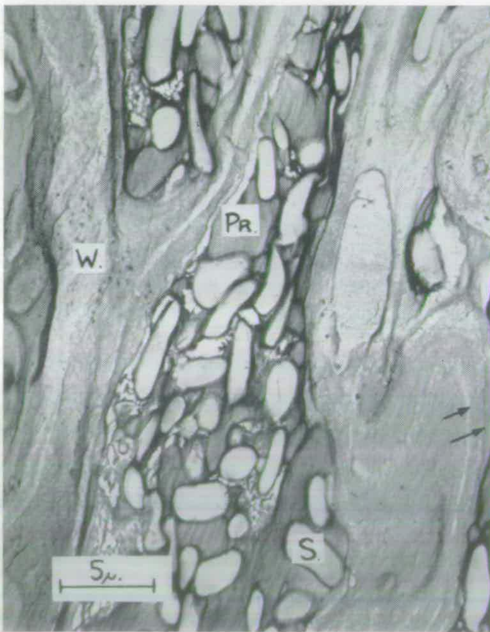
(*b*)



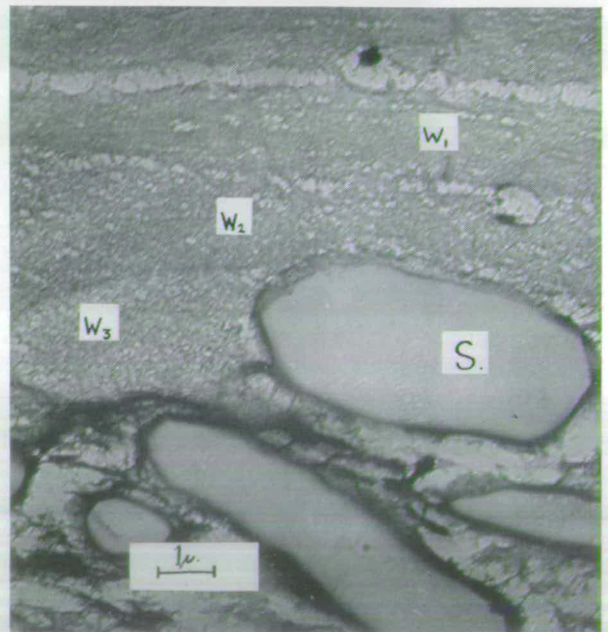
(*c*)

Fig. 3.—Barley aleurone.

(a)



(b)



(c)

Fig. 4.—Starchy endosperm of *Bromus*.

For preparations from developing wheat caryopses, Buttrose[2] has additionally used fixation with osmic acid and araldite embedding, and the fine structure of aleurone from ripening wheat appears to be essentially similar to that now reported for barley and for *Bromus*. In wheat there are also unidentified bodies which vary in electron density at different stages of ripening and which become electron dense after steeping for 24 hr.: this variation is attributed by Buttrose to differences in degree of hydration. We do not feel that this is the whole explanation, at least for barley, since the U.B. from fully hydrated aleurone, maintained separately from the embryo, remain electron translucent after 24 hr.

However, as was indicated in the Introduction, it is not proposed to speculate extensively on the nature and function of any of the less familiar organelles detected in the aleurone. It is hoped that the significance of some at least of these inclusions will become clear when more detailed examination has been made of tissue from germinating or gibberellin-treated material. It is, nevertheless, reassuring to find that *Bromus* and barley yield essentially similar electron micrographs and that both contain aleurone cells which resemble those described by Buttrose for wheat.

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ABSTRACT OF THESIS

Name of Candidate: Clifford Smith Johnston.

Address: 32, Arnott Gardens, Edinburgh, 11.

Degree: Ph.D. Date: 1st. November, 1964.

Title of Thesis: Enzymic Potentialities of Bromus Seeds.

A structural study of the caryopsis of Bromus showed that the walls of the starchy endosperm cells were very thick indeed, and chemical evidence suggested that these walls were rich in hemicellulosic compounds. Electron microscopy indicated that the hemicellulosic walls possessed some degree of fibrillar organisation though this organisation was not so distinct as that of the cellulosic walls of the aleurone.

Bromus was also found to be rich in hemicellulases. In Bromus, as in barley the main source of hydrolytic enzymes is the aleurone and release of hydrolases from the aleurone is induced by the addition of gibberellic acid. It has been demonstrated that during germination endo-β-glucanase is released prior to α-amylase. A similar pattern occured with gibberellic-acid-treated tissue, although enzyme release was earlier and the rate of formation faster. This fact of hydrolases being secreted at different times rules out the possibility of release being due to a sudden rupture of a lysosome within the cell.

With recent attention being focussed on the role of the aleurone in hydrolase production, the ultrastructure of Bromus and barley aleurone was investigated. The cells were found to contain the usual organelles and in addition numerous vacuoles were observed. These vacuoles(after $KMnO_4$ fixation) contained medium-density deposits which are believed to be proteinaceous in nature. In the deposits were inclusions about 1μ in length. Large numbers of unidentified bodies, 0.1μ - 0.4μ, were also found. These vesicular structures were usually in close association with the vacuolar deposits and were also present along the inside of the plasmalemma. Preliminary studies on germinating and gibberellic-acid-treated grains have suggested that these unusual organelles may be involved in hydrolase release.

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The other main facet of the study involved examination of transosylase enzymes in Bromus. A detailed study was made of the glycosidase-transosylase mechanisms, especially those involving synthesis of β-linked gluco-oligosaccharides from β-linked disaccharides (eg. cellobiose). It was found that synthesis occured using very low substrate concentrations, ie. below 0.1%. These enzymes were detected in large quantities in the aleurone and were also present in the embryo. It is believed that they play an essentially hydrolytic role in the non-growing cell. The other transosylase system studied was of the nucleotide- transosylase type, where UDPG was used as a glucosyl donor. This system has been detected in the young seedling and has been shown to be present in a particulate structure of the mitochondrial type. It has been implicated in the synthesis of a hemicellulosic material.

Throughout the study attempts have been made to correlate biochemical findings with structural observations.