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THE BIOSYNTHESIS OF STORAGE GALACTOMANNAN

IN DEVELOPING SEEDS OF FENUGREEK (*Trigonella foenum-graecum* LEGUMINOSAE)

- AND RELATED CHEMOTAXONOMIC STUDIES

by

J. McA. Campbell

Department of Biochemistry,
University of Stirling,
Stirling,
FK9 4LA.

1978

Illustration of Trigonella foenum-graecum L.

Mature plant specimen, approximately life size.

PREFACE

This thesis is a record of research carried out by the author, under the supervision of Dr. J.S.G. Reid, in the Department of Biochemistry between October 1974 and August 1977. It is submitted for consideration in the belief that it is wholly original, except where due reference is made.

I should like to take this opportunity to thank Dr. J.S.G. Reid whose advice, encouragement and enthusiasm throughout the work has been of great value.

I also extend my appreciation to Mr. H. Milne and his staff at the University's greenhouses for their work in the preparation and maintenance of fenugreek plants; Mr. T. Forrest, Department of Biology for technical help and advice in aspects of electron microscopy; members of the Physical Chemistry laboratory, Department of Chemistry for advice on the use of the light scattering instrument; Dr. K.C.B. Wilkie, Department of Chemistry, University of Aberdeen for the supply of reference methylated sugars. Finally I thank the SRC for the award of a research studentship during this research.

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INTRODUCTION

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The leguminous seed endosperm contains galactomannan, a cell wall reserve polysaccharide, as its main carbohydrate store. Cell wall reserve polysaccharides are of very widespread distribution in seeds and are of several distinct types. The present knowledge about their structures and metabolism is very limited indeed, in comparison with starch, the seed's plastid storage carbohydrate. This thesis is a contribution towards an understanding of the biosynthesis of reserve galactomannans and of their taxonomic value.

Cell wall reserve polysaccharides.

The major polysaccharides so far encountered as cell wall reserves in seeds are: galactomannan, glucomannan, mannan, and xyloglucan ("amyloid"). Structurally the first three types have much in common; all are based on a linear main chain of β (1→4) linked D - mannopyranosyl residues. The galactomannans have single-residue α (1→6) linked D - galactopyranosyl residues attached to the main mannan chain (see for example 153, 34); the glucomannans have β (1→4) linked D - glucopyranosyl residues interspersed in the main chain (see for example 123 , 5 ,21); and the mannans have the basic β (1→4) D - mannan structure, yet generally possess a few percent galactose as in the galactomannans (see for example 80 ,178). The xyloglucan is a substituted cellulose molecule; it is a β (1→4) linked D - glucan with α (1→6) linked D - xylopyranosyl side units some of which, in turn, carry β (1→2) linked D - galactopyranosyl residues (see for example 82 ,75).

Some typical examples of the species of seed containing a cell wall reserve polysaccharide are:-

galactomannans: legumes containing an endosperm
e.g. Cyamopsis tetragonoloba (guar), Ceratonia siliqua (carob)

glucomannans: Iris, and Endymion species

mannans: Palm seed endosperms, Strychnos nux-vomica (nux vomica, Loganiaceae)

xyloglucans: Tamarindus indica (tamarind), Tropaeolum majus (nasturtium). In a few cases the glucomannan type of reserve has also been found in plant tissues other than the seed: Amorphophallus tubers, Orchis tubers and Lilium bulbs. There are also other reserve polysaccharides which due either to their limited occurrence (like glucomannans in tubers), or their small amounts in seeds, can be classified as "minor" reserves. Examples of such "minor" reserves are the galactan of Lupinus cotyledons (113) and the cereal endosperm β-D-glucans and arabinoxylans (7).

The major cell wall polysaccharide reserves can be a prominent feature of seed endosperms or cotyledons which contain them. They develop as large secondary cell wall deposits. Up to 40% of the seed weight is contributed by galactomannan in some leguminous endosperms (3), and similarly the xyloglucan of Tamarindus indica cotyledons amounts to 40 - 50% of the seed weight (81). The mannans can also be found in large amounts in seed endosperm, especially in Phytelephas (ivory nut palm) where 50% or more of the seed weight is mannan (6).

The survey of galactomannans in the Leguminosae, by Anderson (3), showed that of 163 species tested, 120 contained endosperm galactomannan. A later survey of xyloglucans (amyloids) in seeds from many plant families, by Kooiman (81), showed that of 2700 species tested, 237 contained xyloglucan. It must be pointed out that in Kooiman's survey on the occurrence of amyloids in plant seeds, several species which gave a negative result within the Leguminosae actually contained galactomannan. Also of the species tested no representative xyloglucan was found in 25 families of the Monocotyledones. Another feature which seemed to arise from this work was that the seeds containing amyloid were grouped together taxonomically i.e. whole tribes were either predominantly amyloid positive or negative. Although a final conclusion

about the occurrence of cell wall reserves cannot be made from these partial lists, it is apparent that they are widespread, and that when present in a seed the polysaccharide often assumes the role of principal carbohydrate reserve.

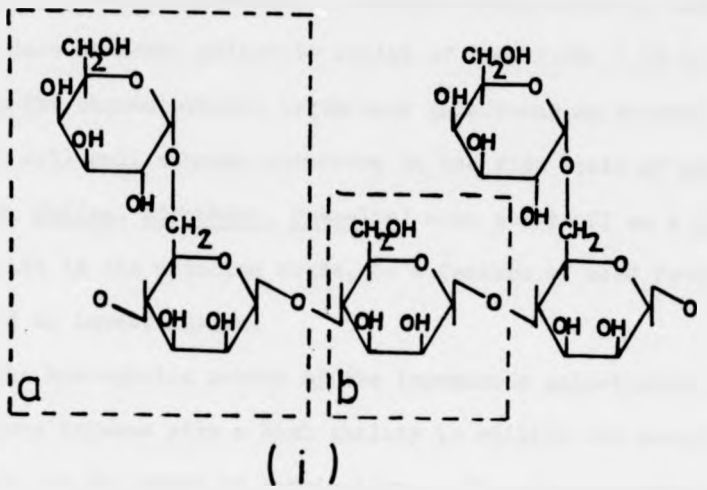
Early microscopic observations in the nineteenth century not only showed the occurrence of major deposits of carbohydrate in the cell walls of certain seeds, but also that they disappeared during germination. Tschirch in 1889 (166) noted that the mucilaginous endosperm of Trigonella foenum-graecum (fenugreek) was dissolved during germination, and Nadelmann in the same year (108) observed the formation of transitory starch granules in the cotyledons during endosperm dissolution. Even earlier, Schleiden in 1838 (145), had shown that the cotyledon cell walls of some seeds were stained blue with iodine-potassium iodide solution. These cell wall materials were called "amyloids". Throughout the nineteenth century various authors observed amyloids in the cell walls of cotyledons or of endosperm, often in large amounts (47, 67 ,135). The presence in the cell walls of seed endosperm or cotyledons of large amounts of carbohydrate material, and their disappearance during germination, led these authors to believe the cell wall materials to be seed reserves. This has been corroborated by later, and more precise, work, for example: galactomannan mobilisation in fenugreek, crimson clover and lucerne (all members of the tribe Trifolieae) by Reid and Meier in 1972 (131) and xyloglucan mobilisation in white mustard by Gould et al in 1971 (58). The mobilisation of four major types of cell wall polysaccharides, mentioned before, in seeds during germination has shown that their primary role in the seed is that of a carbohydrate reserve.

Reserve galactomannans - structure, function, and metabolism.

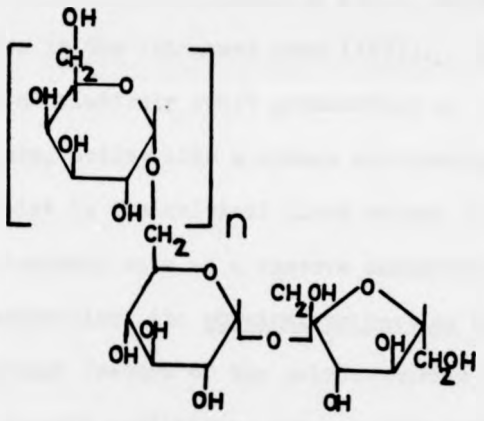
The basic β (1→4) linked D - mannan chain with α(1→6) linked D -

galactose side-chains appears to be the consistent structure of seed galactomannans (152, 34) (see diagram part (i), page 7). One major variation in molecular composition is the ratio of mannose to galactose residues, which can vary from one seed to another. If one excludes the mannan polysaccharide in the ripe endosperms of species within the Palmae family, which have a few percent galactose (mannose: galactose of approx. 50:1 - 100:1), then the range of mannose: galactose ratio in characterised galactomannans of seeds is about 1:1 - 5:1 (83). Galactomannans of different mannose: galactose ratio have markedly different characteristics in water. In the galactomannan molecule the galactosyl residue side-chains prevent the association of the main mannan chains by hydrogen bonding and the polysaccharide is soluble in water. An increase in the mannose: galactose ratio of a seed galactomannan results in a lower solubility in water. As an example, the galactomannan of Ceratonia siliqua, carob, (mannose: galactose ratio of 4.5: 1.0) is only half as soluble in cold water as the galactomannan of Cyamopsis tetragonoboba, guar, (mannose: galactose ratio of 2.0: 1.0) (76). Of course the molecular weight of the galactomannan sample also influences its solubility. Indeed a galactomannan sample may be polydisperse and can be sub-fractionated (76).

As a complete contrast to the galactomannan's hydrophylic nature, the palm seed mannans are insoluble in water. In the palm seeds themselves the very hard endosperm is produced by the deposition of crystalline cell wall reserve mannan, which in its dehydrated state results in this very dry, armoured tissue. An interesting transitional phenomenon is encountered in the seed maturation of several genera within the Palmae (e.g. Cocos, Phoenix, Borassus, Arenga). The immature, unripe palm seed endosperm contains a leguminous-type galactomannan. Yet the cell wall reserve of all ripe palm seeds is, as already stated, a mannan with but a few percent of galactose (i.e. considered a mannan when 95%



(i)



(ii)

Diagram of (i) galactomannan structure,

(ii) raffinose family oligosaccharides.

mannose residues or more). The removal of galactose from the galactomannan during seed maturation occurs concomitantly with the transition of the endosperm from a hydrated, gelatinous phase to a dehydrated, solid mature state. The galactomannans from the unripe seeds of the Palmae family can have mannose: galactose ratios of from 2.00: 1.00 to 2.57: 1.00 (83), with the characteristic leguminous galactomannan structure. Whether the cell wall mannans occurring in the ripe seeds of other plant genera (e.g. Coffea, Strychnos, Phacelia) also start off as a galactomannan polysaccharide in the maturing seeds, is a feature of seed formation which has received no investigation.

The hydrophilic nature of the leguminous galactomannan endows the albuminous legumes with a high ability to utilise the available water supply, at the onset of germination. The imbibed endosperm is soft and mucilaginous, with the endosperm tissue taking up a larger percentage of the water, per dry weight of tissue, than the rest of the seed (about 4 times in the fenugreek seed (127)). Good moisture conditions promote a relatively swift germination of a good percentage. The imbibed endosperm, acting like a sponge surrounding the germinating embryo, keeps it moist in the critical first stages of germination. Although the galactomannan acts as a reserve carbohydrate store to be utilised during germination, its physical properties would certainly seem to be an important feature of the polysaccharide's role in the seed (128). Also, in drought conditions, the gel-like properties of the endosperm cell wall may help prevent desiccation of the embryo and cell death due to the rupturing of the protoplast, which would follow vacuole disintegration. This drought resistance might be brought about as the water loss from the embryo is retarded in passage through the viscous endosperm tissue, as might also be the case in the whole soya bean plant cell wall, which accumulates markedly more hemicellulose in its cell wall during severe dry spells than in normal moisture conditions (29). The

hemicellulose accumulation in large quantities seems to be this plant's adaptation to its environment and therefore the soya bean plant achieves an increased capacity to resist drought.

Although the reserve galactomannan of leguminous seeds has no nutritional value for Man, the applications in which the polysaccharide has been found useful are numerous. The range of uses for galactomannan hinges on its aforementioned solubility characteristics and the manipulation of these characteristics. The industrially important galactomannans are from the guar plant (alternative names are guaran, guar gum) and the carob tree (alternative names are locust bean gum, gum gatto, gum tragon, St. John's bread), where the yields from the seeds approach, or better 40% (on a seed dry weight basis). The viscous solutions of galactomannan that can be made in water have applications in modern industries, usually as thickening and binding agents. Galactomannan in solution is used: as an additive in the paper industry to increase burst and tensile strengths, and to improve paper sizing (159); in adhesives (177); to form emulsions and gels with other solutions (95,103); to stabilise beverage solutions (157); to act as a laxative (59); as an additive in drug preparations (142); as an additive to under-water explosives (26); and as an alternative to the commercial plant gum exudates (expecially gum arabic from Acacia species and gum tragacanth from Astragalus species), in their multiple roles in industries. When it has been desired to gel the thick viscous solutions of galactomannan, then in general an inorganic salt such as borax has been added. The properties of galactomannans have not only been made use of in twentieth century industries, but were utilised in Egypt at least as far back as 5,000 years ago (125). The binding of mummies, in this era of Ancient Egypt, was accomplished by using a paste made from the galactomannan of the carob seed.

It has certainly been the galactomannan's uses in many areas of

industry, that have prompted investigations into the structures of the various seed galactomannans, building up a consistent picture of their structural framework. The alteration of the polysaccharide's physical properties, either by inducing modifications in chemical structure or by finding the correct galactomannan sample, may meet new industrial goals. A very interesting facet of the galactomannan's structural properties has been shown in their interaction with other gel-forming polysaccharides, notably carrageenan. In such cases the polysaccharide mixtures have shown behavioural patterns that are interpreted as being akin to the co-operative binding of protein sub-units into a cohesive quaternary structure (125, 34). These findings might be used to explain some of the nature of polysaccharide component binding in the plant, and bacterial cell wall.

The paucity of information concerning the anabolism and catabolism of the reserve galactomannans is in sharp contrast to the structural information, which has been gathered from many characterisation studies. In particular, the biosynthesis of this polysaccharide, or of any other cell wall reserve polysaccharide, has received very little attention indeed.

The plant species from which the most information has been gathered regarding the metabolism of its seed galactomannan is Trigonella foenum-graecum, fenugreek. This is one of only three seeds which have been the subject of investigation. The other two seed sources are from the guar and carob plants, upon which only germination studies have been carried out.

In the developing fenugreek seed, galactomannan is deposited firstly in the endosperm cells next to the embryo and then progressively outwards to the seed coat, until all of the reserve cells are completely filled (132). In the mature seed all the endosperm cells are filled with galactomannan, except those which are part of the aleurone layer.

Another feature of seed formation during the development of the endosperm cell wall galactomannan is the concurrent appearance, in the embryo and endosperm, of considerable amounts of the raffinose family oligosaccharide stachyose (β-D-fructofuranosyl O-α-D-galactopyranosyl - (1→6) -O-α-D-galactopyranosyl - (1→6) -α-D-glucopyranoside or digalactosylsucrose) (129), see diagram part (ii), page 7. This is a phenomenon that provokes the idea of a link in the biosynthesis of this oligosaccharide and the reserve galactomannan, as the galactosidic linkages in both are α (1→6). From a recent ultrastructural investigation of galactomannan formation in fenugreek (101), it would seem that the deposition ("secretion") of galactomannan is brought about by rough endoplasmic reticulum (ER). Swollen ER intracisternal spaces contain similarly-staining material to the already deposited cell wall galactomannan, and the ER polysaccharide then seems to be expelled outside the plasmalemma.

The degradation of the galactomannan, in germinating fenugreek seeds, has been shown to occur after mobilisation of the oligosaccharides of the raffinose family. The hydrolysis products of the galactomannan are absorbed by the cotyledons in which sucrose increases and starch is formed (128). The aleurone layer of the fenugreek endosperm has been shown ultrastructurally to be responsible for the breakdown of galactomannan (131), and biochemically when the production of α - galactosidase and β -mannosidase was associated with these cells (133). Endo-β-mannanase, the third enzyme required for the complete breakdown of a galactomannan (126), has only recently been shown to be produced by the fenugreek endosperm aleurone layer (134). An α-galactosidase (31) and a β -mannanase (29) had previously been isolated from whole germinating seeds. A feature of the aleurone system in fenugreek is that it is self-regulating, in that it is not controlled by any factors emanating from the embryo. Half-endosperms of ripe fenugreek seeds can be

incubated under germination conditions and the galactomannan will be broken down (131). This presents a generally similar pattern to the system of the cereal aleurone (30), except that no embryonal control is present in the breakdown of fenugreek galactomannan. Metabolic inhibitors, especially cycloheximide and abscisic acid, acting on the aleurone cells, reduce the activity of the hydrolytic enzymes and reduce to the same extent galactomannan breakdown (133).

The mobilisation of galactomannan during the germination of carob seeds is accomplished by all of the endosperm cells (all of them remain viable in the ripe seed), with no participation by the embryo (146). In this seed α-galactosidase, β- mannosidase, and β- mannanase produced in the endosperm are responsible for the polysaccharide's breakdown and the mobilised products are metabolised in the embryo where starch synthesis is observed. Like the fenugreek system the oligosaccharides of the raffinose family are hydrolysed prior to galactomannan breakdown.

In a study of galactomannan, nitrogen, and phosphorus changes in germinating guar seeds (99) all of the endosperm galactomannan disappeared, being then translocated to the cotyledons. After a short lag period, the seedling axis increased rapidly in dry weight upon the translocation of the galactomannan hydrolysis products. Microscopic observations of the guar endosperm, in this study, showed a several-celled outer layer which contained the only metabolically active endosperm cells (by triphenyltetrazolium staining). These cells may relate to the fenugreek aleurone system which contains "aleurone grains", presumably of stored protein in readiness for enzyme production during germination (131).

The mannose: galactose ratio in leguminous seed galactomannans increasingly appears to have a chemotaxonomic value, and may be helpful in the problem of classification in the Leguminosae. It has been shown that member species within a tribe, the Trifolieae, have identical

mannose: galactose ratios(130). In the same report the authors also pointed out, from a review of the available galactomannan structures, that similar mannose: galactose ratios were found in members of the same tribe, throughout the sub-families of the Leguminosae. Certain small differences in galactomannan composition of one seed species, which have been reported by different groups of workers, are probably due to differences in analytical techniques (152).

In another study on the occurrence of galactomannan in species of Sophora, Bailey in 1974 (10) showed that only two members in this genus, of the species tested, contained any galactomannan. He pointed out that this may well corroborate the classification of Yakovlev (180) of these two particular species (Sophora japonica and Sophora affinis), which were placed in a separate genus, Styphnolobium, by Yakovlev. The general feature of a reserve cell wall galactomannan being either predominantly found within a tribe or not (9) and of different mannose: galactose ratios from one tribe to another, would appear to be an associated characteristic of galactomannan.

There still remains controversy over the classification of certain groups within the Leguminosae and several differing classification systems, based on different treatments of plant characters, have been proposed (see for example (77, 52 ,139). In a recent investigation, Bisby and Nicholls (1977) (13) showed that different formulation and/or different selection of the same morphological characters produce markedly different data sets. This report certainly highlights that first stage of taximetrics, namely the formulation, selection and scoring of morphological observations which precede the actual classification from a given multivariate data set.

It would be presumptious, at the moment, to say that more detailed analysis of leguminous galactomannans would produce a clear-cut classification of the Leguminosae, but such data would certainly be

beneficial in attempts to classify this family. This would be in line with several current approaches in the advancing field of chemosystematics, especially at a macromolecular level, which have contributed along with taximetrics to present day systematics (72).

Background to the biosynthesis of polysaccharides.

The plant cell wall reserve polysaccharides are a distinct group of wall polymers, in that they serve as a store of carbohydrates in the seed. Yet there exist links with the structural cell wall polysaccharides, from a structural point of view. Some of the reserve polysaccharides of the seed cell wall are analogous to certain structural wall polysaccharides in vegetative tissue.

Considering two of the major reserve types, glucomannans and xyloglucans, then analogous polysaccharide structures are also encountered in structural components of certain plant cell walls. Glucomannan is present in angiosperm wood (176) and the xyloglucan type has been shown to be an integral feature of sycamore primary cell walls (161). Even the "minor" reserve types have analogues in the structural units of the cell wall: Lupinus galactan relates to the neutral galactan component of pectins (7); cereal arabinoxylans relate to the hemicellulosic arabinoxylans present in many plant walls (176).

On the other hand galactomannan and mannan type reserves do seem to exist uniquely as reserve polymers. As yet no equivalent structural component of the higher plant vegetative cell wall has been isolated. These two polysaccharides may be the result of an evolutionary bias towards a purely reserve function in seeds.

The complete lack of any biochemical information regarding the biosynthesis of the cell wall reserves is not mirrored in the area of the cell wall's structural polysaccharide components. A great deal of

research upon the biosynthesis of plant cell wall structural polysaccharides has generated a convincing, if not complete, picture of their biosynthesis. Generally, the field of polysaccharide biosynthesis and glycosylation reactions (especially of glycoproteins) in animal and bacterial cells has furnished additional material covering the transfer of sugar residues from donor molecules to high molecular weight acceptors. The general pattern, of biosynthesis of the structural polysaccharides of the cell wall, can certainly be useful in interpreting any possible scheme drawn up from data on cell wall reserve polysaccharides. The following paragraphs relate the important features of saccharide biosynthesis which have emerged from research in this field over the last twenty-five years.

A major advance in carbohydrate biochemistry came from Leloir's group in 1950 (23), when they characterised one of the necessary cofactors for the reaction, in the yeast Saccharomyces fragilis of galactose 1 - phosphate transformation to glucose 1 - phosphate then glucose 6 - phosphate, as uridine diphosphate glucose (UDP-glucose). This was part of their continuing study of lactose metabolism in this organism. This work was followed on closely by Park, who identified three uridine diphosphate sugars, from a bacterial source (112), later identified as N-acetyl muramic acid derivatives (158). With the isolation of the first pyrophosphorylase, catalysing UDP-glucose formation from UTP and glucose 1- phosphate (107), the interest in the field of sugar nucleotides (as they became to be known) swelled and especially during the period 1953 - 1962 many were described from natural sources (85). Several transformations were shown to take place at the level of the sugar nucleotide including epimerisation, oxidation, and decarboxylation. All these observations laid the foundation upon which the answering of several questions, concerning polysaccharide biosynthesis and other glycosylation reactions, were attempted.

The idea that sugar nucleotides could act as donors in the

transfer of sugar residues, came first of all from the work of Dutton and Storey (41) who showed in 1953 that glucuronides of phenols were formed in liver from UDP-glucuronic acid. This work was amplified by Leloir's group who showed that trehalose 6 - phosphate was formed from UDP-glucose and glucose 6 - phosphate, with a specific phosphatase being present to release free trehalose (87). Soon after, the utilisation of UDP-glucose in plants for the formation of sucrose (24) and sucrose phosphate (88), was shown. The preceding observations were all carried out without using radioactive tracers, but following the preparation of labelled sugar nucleotides the detection of transfer reactions was facilitated.

It was also corroborated, from a thermodynamic point of view, that sugar nucleotides were superior donors for formation of complex saccharides. It was shown that they have a high negative free energy of hydrolysis. Relative to other compounds containing glycosyl groups, the sugar nucleotides act as superior monosaccharide donors e.g. $\Delta G^{o}_{hydr.}$ of UDP - D - glucose (pH 7.4) is -7600 cal $mole^{-1}$ (89) compared to that of α - D - glucopyranosyl phosphate (pH 8.5) which is -4800 cal $mole^{-1}$ (22).

From the middle fifties to the present day numerous syntheses *in vitro* of polysaccharides have followed on from the use of radioactive sugar nucleotides as sugar donors. The following list (in chronological order) is of polysaccharides that have been formed *in vitro* and characterised totally or partially, using enzymes from plant, animal, and bacterial sources: hyaluronic acid (55); chitin (56); bacterial cellulose (53, 54); liver glycogen (88); callose (43); starch (90,124); paramylon (57); plant cellulose (12); glucomannan (12); pectin (polygalacturonate component) (170); xylan (11); acidic hemicellulose B (79); bacterial mannan (143); several neutral hemicelluloses (18, 68,111); reserve mannan (48).

Of the higher plant systems mentioned in this list, only starch and reserve mannan are storage polysaccharides which can be

utilised as energy sources, at the appropriate time, after their deposition in the storage tissues. No publication concerning a seed cell wall reserve polysaccharide has been reported, as the reserve mannan (salep mannan) is one of the tuber associated glucomannans (mannose: galactose = 3:1) contained in Orchis species.

The bulk of the next part of the text shall deal with the polysaccharides of the plant cell wall, and the information gathered concerning the site of synthesis of these structural macromolecules within the cell. Yet where appropriate, material will be drawn from work with bacterial and animal cells. As mentioned previously, the lack of data on the metabolism of the reserve, cell wall polysaccharides leaves their biosynthesis totally in the dark.

A great deal of work has been done over the last twenty years to implicate specific cellular organelles as the site of enzymes responsible for the biosynthesis of polysaccharides. The actual biosynthetic site of a polysaccharide does not have to reside at the organelle which is active *in vitro* in its synthesis. Thus, for cell wall polysaccharides, the possible migration of organelle specific enzymes and/or polysaccharide to the cell wall has also provoked investigation. The elucidation of the role of a particular organelle species with respect to macromolecular components of the cell wall has relied mostly on the following methodology: enzyme isolation and *in vitro* assay; *in vivo* radioisotope incorporation; autoradiography; electron microscopy, using general techniques and specific carbohydrate staining procedures.

All plant cells produce and secrete an extracellular matrix composed of different macromolecules, most of which are synthesised in the cytoplasm. Thus a normal plant cell devotes a good deal of its metabolic activity towards the synthesis of extracellular polysaccharides without being specialised for this particular function in the same way that certain gland cells, plant and animal, are. Discounting the structural

protein, hydroxyproline rich extensin (84), and the enzymic protein content of the cell wall then the predominant components are the polysaccharides. Virtually all the polysaccharide synthetase enzymes (sugar nucleotide: acceptor transglycosylases, E.C. 2.4) that have been shown to operate in vitro are particulate in nature. Investigation of the cytoplasmic site of synthetase enzyme and/or polysaccharide biosynthesis has strongly implicated the dictyosomes (Golgi apparatus) and the endoplasmic reticulum (ER) in these roles (see for example (27)).

Dictyosomes. It was observed more than 15 years ago by Whaley and fellow workers that hypertrophied dictyosomes were present in the root cap cells of corn (175). They later postulated that these organelles were involved in the secretion of root cap slime (106) and the formation of the cell plate (174). Subsequent autoradiographic investigation of root cap slime synthesis (corn root slime is a mixture of highly hydrated polysaccharides which are particularly rich in uronic acids and fucose (62)) were consistent with this interpretation (109, 118). In these investigations a pulse-chase experiment with ^{3}H-glucose, showed the radioactivity first in the dictyosomes and following a chase of non-radioactive glucose the radioactivity disappeared from the dictyosomes and appeared in the extruded slime.

Similarly, Paull and Jones (115) have shown by autoradiography that fucose became incorporated exclusively into dictyosomes of corn root cap cells and was then transferred to the slime in the periplasmic space. The dictyosomes are also implicated as the site of polysaccharide sulphation in algae which secrete sulphated polysaccharides; again by autoradiographic techniques (42).

Analyses of the carbohydrate content of isolated dictyosomes or secretory vesicles have pointed to this organelle and the ER as the site of synthesis of pectic and hemicellulosic polysaccharides (16, 17). After maize roots were incubated in vivo with ^{14}C - glucose, dictyosome,

ER and plasmalemma fractions were isolated and hydrolysed. It was concluded from the distribution in the sugars found in the two organelles and the plasmalemma that the dictyosomes/ER synthesised pectic and hemicellulosic wall polymers and secreted them at the growing cell wall. The relative specific radioactivity of each organelle, after calculation of the amount of membrane, indicated that the dictyosomes were probably a focal point in the synthesis and transport of pectin and hemicellulose (the dictyosomes showed 100% increase in specific radioactivity compared to the ER).

It is of interest that in this same report no characteristic glucan accumulation was observed in the cytoplasmic organelles. However, the major part of the radioactivity present in the cell wall was in a glucan polymer, and the authors proposed that cellulose was synthesised at the plasmalemma.

Ray et al (122) using marker enzymes and sucrose density gradients to separate cellular organelles, observed that UDP - glucose: β-(1→4)-glucan glucosyltransferase ("cellulose synthetase") was associated with a dictyosome-rich fraction from etiolated pea epicotyls. However, Shore et al (151) demonstrated the high cell surface activity of β-(1→4)-glucan (cellulose) synthetase in pea epicotyls was destroyed by normal homogenisation procedures, and only a small amount of activity (3 - 10% of total) could be detected in particulate fractions. This observation was taken as evidence that the active cellulose synthetase was located at the cell surface <u>invivo</u>. Of course the dictyosomal β -(1→4)-glucan activity may be associated with synthesis of the hemicellulosic glucans (i.e. xyloglucans).

In a completely different system, the unicellular green alga <u>Pleurochrysis scherffeli</u> has been shown ultrastructurally to assemble and transport both its cellulosic and non-cellulosic components of the cell wall scales, in different parts of dictyosomes (20,100).

The secretory vesicles formed by dictyosomes may play a role in cell wall matrix polysaccharide biosynthesis in germinating pollen (168). The isolated dictyosome vesicles of Lilium longiflorium pollen were shown to stain similarly to the amorphous component of the cell wall, by a specific ultrahistochemical procedure. The composition of the polysaccharide present in the vesicles was similar to the pectin and acidic hemicellulose components of the cell wall (amorphous polysaccharides).

A further role proposed for the dictyosome is an involvement in the synthesis and secretion of the glycoprotein extensin (49), a component of plant primary cell walls (84). Pulse labelling of carrot root phloem cells with ^{14}C - proline followed by fractionation of the cytoplasmic organelles showed that the major site of glycoprotein was in the dictyosome fraction. Also, the enzyme UDP-arabinose arabinosyl transferase, involved in the glycosylation of the hydroxyproline residues of extensin, banded together with a specific dictyosome marker enzyme (IDP ase) on sucrose density gradients.

Endoplasmic reticulum (ER). There is much less evidence implicating the ER than dictyosomes in the biosynthesis and secretion of cell wall polysaccharides. Several ultrastuctural studies of cell types known to be involved in the biosynthesis of cell wall polysaccharides have shown characteristic organisation and distribution patterns of ER, near the site of cell wall formation. For example, the deposition of callose during sieve plate formation (110), the formation of thickenings on the wall of primary tracheary elements (32), and the formation of the cell plate following cytokinesis (71). Observations in the electron microscope (101) that rough ER cisternae with polysaccharide contents are present near the forming cell wall in fenugreek endosperm, would seem to indicate that this organelle is responsible for the synthesis and release of the wall galactomannan.

In many cases ER cisternae have failed to give a positive reaction for specific polysaccharide cytochemical stains (e.g. Thiery stain, PATAg) (50,117,138). In these cases this may be because the ER either contains little carbohydrate or it is masked in some way (27).

It has already been mentioned in the preceding dictyosome section that the ER plays a part in the synthesis of pectin and hemicellulose components of the cell wall (16).

Shore et al (151) have shown that when cells have ceased elongating, the cytoplasmic organelle site of β (1→4) glucan synthetase activity alters. In cells that had ceased elongating in pea epicotyls (an actively elongating tissue), either because of normal maturation or treatment with ethylene, the enzymic activity shifted from being purely dictyosome associated to being associated with dictyosomes and ER. This development of ER synthetase activity is a feature also of auxin treatment of this same tissue; auxin stimulates cellulose deposition in vivo and maintains a high synthetase activity in the cytoplasmic membranes (150). Ray (121) has shown that auxin binding sites in maize coleoptiles are mostly located on the rough ER.

It must be considered when one views the two organelle species, dictyosome and ER, that they may function as part of an interassociated system. Mollenhauer and Morre (1976) (105) have observed the presence of transitional regions of ER near the edges of dictyosomes and plasmodesmata in plant cells. Also, direct connections between ER and dictyosome cisternae were occasionally found in isolated membrane preparations. To what extent these electron microscope observations of structural interrelationships of the two membrane components are representative of the situation in vivo, must await further study.

Glycosylated polyprenol phosphates act as intermediates in the synthesis of glycoprotein in mammalian tissues (dolichol, 16 - 21 isoprene units) (see for example (91, 114)) and of extracellular

polysaccharides in bacteria (undecaprenyl, 11 isoprene units) (see for example (179, 70)). That similar systems may exist in the plant cell for polysaccharide and/or glycoprotein synthesis has been proposed (1, 45, 19).

Forsee and Elbein (1975) (45) have shown that cotton fibres contain enzymes which catalyse the incorporation of mannose (Man) and N - acetylglucosamine (GlcNAc) from the corresponding sugar nucleotides, into lipid (polyprenol phosphate), oligosaccharide linked lipids and glycoprotein. No direct evidence was obtained from this study to show that the sugars were transferred from the glucosyl-lipid to the oligosaccharide linked lipids. However, from experiments with p - hydroxymercuribenzoate it was suggested that the polyprenol phosphate was an intermediate in the synthesis of the oligosaccharide linked lipids.

The structures of the oligosaccharide-lipids, $(Man)_{1-8}$ - GlcNAc - GlcNAc - lipid, were similar to the intermediates in the synthesis of the core oligosaccharides of mammalian glycoproteins containing the asparagine -GlcNAc linkage (114). Similarly, the linkage of the glycoprotein oligosaccharide appeared to be a GlcNAc - peptide linkage, which would seem to be the same type as found in the majority of known plant glycoproteins (149).

There is no clear cut evidence, as yet, that lipid "intermediates" participate in the biosynthesis of plant cell wall polysaccharides. However, it would seem probable that lipid-sugars found in plants form part of a mechanism for glycoprotein synthesis analogous to that found in animals (19).

An interesting idea has been proposed by Bowles and Kauss (15) from their investigation of the enzymic and lectin properties of isolated membranes from mung bean seeds. When they analysed the polysaccharides formed in the membrane fractions, after *in vivo* ^{14}C - D - glucose incubation, the sugars were similar to the specificity of

the lectins associated with the membranes (ER and dictyosomes). The authors proposed that this may indicate that lectin-cell wall polysaccharide complexes occur in the plant membrane system. Such a device could bind the secretory polysaccharides to the membranes during synthesis and transport. Thus the milieu of the membrane site of polysaccharide synthesis, with regard to lipid and binding proteins, may play a part in polysaccharide biosynthetic systems.

Seed formation.

It has often been tacitly assumed that starch forms the only important carbohydrate reserve in developing seeds, as becomes apparent from a recent review of seed formation by Dure in 1975 (40). This author made no mention of cell wall reserve deposition being a normal feature in the formation of many seeds, but he did admit that the literature was greatly influenced by research on seed species of human and nutritional value (i.e. containing starch, protein and oil). In fact the assumption was made in this review that legumes do not contain an endosperm. This is not a solitary occurrence of such a misconception, as can be seen in the well known taxonomic work by Hutchinson (77), wherein the seeds of the Leguminosae are classified as non-endospermic. Unlike those legumes where the main stores are held within the cotyledon cells, the leguminous seeds with an endosperm invariably contain a cell wall reserve galactomannan (3).

Of course all seeds undergo a period of endosperm development at the early stages of seed formation. During seed maturation the extent of endosperm consumption by the embryo varies among angiosperm species. The general pattern of endosperm tissue expansion follows from a build up of nutrients which have passed from the surrounding tissues of the ovule. The vegetative plant supplies a flow of essential precursors through the ovarian vascular elements contained within the funiculus (a stalk-like

tissue joining the forming seed and the ovary). This at first results in a rapid development of the testa components (the outer integument of the ovule) and the usually triploid endosperm tissue. Those seeds in which the endosperm expands (in size and cell number) and retains the role of a reserve tissue in the ripe seed are called albuminous, and those in which the endosperm reserves are absorbed by the cotyledons during seed maturation are designated exalbuminous.

The pattern of endosperm development in angiosperm seeds, and in the Leguminosae in particular (120), exhibits a range of variation in regard to the extent to which cell formation takes place. Quite often the early phase of endosperm development shows a free nuclear condition. This can sometimes have haustorial activity bringing about the disorganisation of the nucellar tissue (the central core of the ovule), e.g. Glycine javanica and Terammus labialis. The transition to a cellular state in the endosperm may be complete, or a portion may retain its free nuclear organisation; the latter case is encountered in species of Cyamopsis, Crotalaria, Cassia, and Parkinsonia (Leguminosae). The transition in fenugreek is complete and the cellular state is accomplished when plasmalemma and primary walls are laid down, prior to the deposition of reserve galactomannan (approx. four weeks after fertilisation). There are strong indications from statistical correlations with many undoubtedly primitive characters that, among dicotyledons, the nuclear endosperm is primitive (156).

The albuminous legumes more closely resemble the cereal monocotyledons, which retain a starchy endosperm store for growth of the germinating embryo, than do the exalbuminous legumes. In exalbuminous seeds after endosperm reserve consumption the embryo builds up its own large stores of starch (or protein/oil) in the cytoplasm of the cotyledons (cf. peas and beans). We see in the exalbuminous legumes, and cotton as another example, a somewhat strange phenomenon where there are nutritive

tissues expanding in one zone of the seed while being destroyed in the areas surrounding the embryo. Hence the endosperm (and nucellus) is a transient tissue in some cases, but a persistent one in others. It would seem that those seeds which completely consume the endosperm during embryogenesis expend a great deal more energy in forming seeds than do the albuminous seeds (legumes and monocotyledons) that enter dormancy with most of the seed's nutrition still polymerised in the endosperm. Since in germination the endosperm hydrolysates move mostly to the developing axis, the albuminous species avoid a repackaging step in the cotyledons. It might be that the process of retaining an endosperm reserve tissue throughout seed formation offers a distinct evolutionary advantage (40). The opposite point of view can be taken when considering the seed from the standpoint of germination, in that an abundant store in the cotyledons could be a selective advantage (155).

The embryo proper develops a little later than the endosperm, and by the end of their maturation the funiculus cells degenerate, making the seed a closed system nutritionally. The desiccation of the ovule (seed ripening process) then ensues as water is lost to the environment and the seed coat (testa) sclerifies and dies, providing a protective, armoured casing around the endosperm/embryo.

Several patterns of cell wall reserve polysaccharide deposition, during seed formation, are encountered from plant species to species (81, 66). The deposition of galactomannan in fenugreek endosperm reaches a point when the cell lumina are completely filled by the wall thickenings (132) and are at this point non-living storage centres (131). This extreme is not seen in the endosperm of another galactomannan-containing legume, Ceratonia siliqua (carob), where the endosperm cell walls do become enlarged but the cells retain a viable protoplasm (146). Mannan deposition in the seed endosperm cell wall can be quite advanced (the wall occupying more than half of the cell volume) yet in all cases

the endosperm cells remain viable in the ripe seeds. Variations are encountered in the intercellular connections which are maintained through these thickened walls (98). In Phoenix dactylifera (date palm) several distinctly shaped "pits" (depressions in the wall thickening) allow plasmodesmata to penetrate through thin primary cell walls, and in Strychnos nux-vomica (nux vomica) the endosperm cell wall thickening is interrupted by numerous fine channels, each containing a plasmodesma.

The xyloglucan, or amyloids, can be present in the endosperm or the cotyledons as much thickened cell wall stores (81). Looking at the case of one "minor" reserve polysaccharide, the galactan present in Lupinus alba, we see again much enlarged cotyledon cell walls but in addition viable cells containing copious oil and protein reserves.

It can be seen from these few examples that variations occur not only in the tissue site of cell wall reserve deposition, during seed formation, but also in the mode and extent of deposition.

The investigation.

The larger portion of this investigation was concerned with the biosynthesis of galactomannan in Trigonella foenum-graecum (L) fenugreek, during seed formation. The evaluation of the synthetase system in the developing seed and the possible cytoplasmic organelle site of synthesis of the polysaccharide were the major aims of this enquiry. The need for synchronous seed material, at various seed maturation stages, was met by tagging of flowers and harvesting of seeds at the appropriate time. After a maturity parameter table had been established any seed sample's age could be calculated a priori.

The remainder of the investigation dealt with a survey of the mannose: galactose ratios in the Genisteae tribe of the Leguminosae, along with a structural characterisation of Laburnum anagyroides seed galactomannan which had not been previously undertaken. The

characterisation of the type of glycosidic linkages in the L.anagyroides galactomannan was accomplished using the "classical" structural methods of polysaccharide chemistry (periodate oxidation and methylation analysis).

The fenugreek plant (see Frontispiece) is a member of the Trifolieae tribe, sub-family Papillionaceae (Leguminosae). Other genera within this tribe are Trifolium (clovers), Medicago (lucerne), Melilotus (sweet clovers), and Parochetus. The genera of the Trifolieae are very consistent (147) and fenugreek shares in common with the others: the same mode of embryo development (119); many anatomical details (77); similar growth habit. Fenugreek is an annual, herbaceous plant with a relatively long flowering period. The small, white flowers develop from the shoot tips and a single plant may then have pods, at all stages of ripeness, arranged in order of increasing maturity down the stem. The plant is widely grown throughout N. Africa, the Middle East, India and Pakistan. The ripe, brown seeds are harvested from the long pods and used after grinding, as a flour included in maize bread, used directly as a spice, and for various medicinal purposes (usually as a treatment for digestive disorders) (33).

The Medicago and Trifolium genera are very important pasture legumes grown as green fodder, hay and for silage; notably lucerne (or in U.S., alfalfa), crimson clover and red clover. Lucerne, Medicago sativa. is the foremost forage plant grown on a world basis along with other Medicago species. From available data on world production, the lucerne hay production around 1954 of 75 million tons (from 50 million acres, (14)) approaches the enormous world production of the cereals, for example, wheat at 150 million tons (from 300 million acres, (144)). The Trifolieae therefore, represent an economically very important tribe of legumes. Analysis of the biochemistry of the endosperm in lucerne and clovers is hampered by their minute seeds. Fenugreek with its developing seed reaching an approximate diameter of 0.7 mm when mature, is a much better

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system to manipulate in an investigation of this tribe.

The galactomannan of fenugreek has been shown to have a mannose: galactose ratio of from 1.20 : 1.00 to 1.00 : 1.00 (see for example (4,129)), or $^{a}/_{b}$ = 5 to b = 0 in diagram, part (i) page 7. With such an extremely low mannose: galactose ratio a marked solubility in water is endowed upon this galactomannan, enabling the seed to have a high capacity of imbibing water. It may be in fenugreek and the other Trifolieae genera (all having a low mannose: galactose ratio) that this role for the galactomannan has originated in their indigenous habitats, the dry Eastern Mediterranean countries (65).

The chemotaxonomic study (and identification of structure) within the Genisteae tribe was pursued to assess the value of mannose: galactose ratio in the classification of the Leguminosae. Seeds from all four sub-tribes were tested to see whether they would fit into the general picture of tribal mannose: galactose ratio consistency, which has been proposed in leguminous seeds (130) and been shown to be the case in the Trifolieae (130).

It should be pointed out that the identification of the galactomannan structure of L.anagyroides was not contemplated from the outset of this research. It was a means of adapting to circumstances, namely a disaster befalling fenugreek plant specimens in January 1975 when all the greenhouse grown plants under the care of greenhouse staff, quite unexpectedly, died overnight. This state of affairs would have left a period of at least 8 - 10 weeks before any developing seed could be harvested, and so the characterisation of one of the galactomannans of the tribe Genisteae - none of which had previously been studied - was undertaken. Although embarked upon as a useful means to occupy the time interval until fenugreek samples were available, this work has, in keeping with the chemotaxonomic study, provided a more widespread coverage of the nature of leguminous galactomannans.

MATERIALS AND METHODS

CONTENTS

GENERAL METHODS

Plant material.

All fenugreek seed samples were harvested either from plants grown in a controlled growth room or from greenhouse plants, grown under natural and supplementary light sources. The growth room plants were sown in a general seed compost and grew with a 12 hour day period in high light intensity, at a temperature of 12.5 - 15.5°C. As soon as flowering commenced, approximately eight weeks from sowing, a nutrient solution (Vitafeed, Vitax Ltd.) was applied once a week to the plants.

Isolation and hydrolysis of the galactomannan.

Selected seed samples were removed from their pods and the embryos dissected out of the seeds. The endosperm tissue was stripped from the surrounding testa and dropped into boiling 70% MeOH (5ml) contained in a 12ml conical centrifuge tube, kept in a 70°C water bath. The endosperm pieces were squashed against the glass walls with a glass rod, during the immersion in the water bath. After 15 minutes the tubes were removed and centrifuged (approx. 1,000g). The supernatant was removed, a further 5ml of 70% MeOH was added to the precipitate and a second extraction followed. After the second centrifugation, the galactomannan precipitate was taken up in a little water and deep frozen in liquid nitrogen, ready for freeze-drying. Combined supernatants were either analysed for low molecular weight carbohydrates after concentration or were discarded. All solvent evaporation was accomplished using a Buchi rotary evaporator at 40°C, for this step and for subsequent solvent removal steps.

The freeze-dried galactomannan, white and flocculent, was weighed and hydrolysed by the 72 - 4% H_2SO_4 method (141). A sample (1 - 5mg) was dissolved in 0.1ml 72% H_2SO_4 at 30°C for 45 minutes. Then

2.8ml of water was added and the solution was autoclaved at 110°C for 1 hour. After cooling, the hydrolysate was neutralised using barium carbonate and the insoluble barium sulphate removed by centrifugation. An internal standard of myo-inositol was added in certain cases as an aqueous solution during dilution of the 72% H_2SO_4, particularly when the sample was taken for gas-liquid chromatography analysis. After neutralisation with barium carbonate the hydrolysate was analysed by paper chromatography and/or gas-liquid chromatography.

Gas-liquid chromatography (GLC).

Estimations by GLC were performed using a Pye 104 chromatograph with dual flame ionisation detectors on glass columns (1.5m x 0.6mm). Sugars from hydrolysates were analysed as their alditol acetate derivatives (2), after reduction by sodium borohydride, the removal of borate as its methyl ester, and acetylation at 120°C for 3 hours using acetic anhydride. Such derivaties were separated using system:-

(A) 3% ECNSS-M (copolymer of ethylene glycol succinate polyester and nitrile silicone polymer) on Gas Chrom Q 100 - 120 mesh operated either isothermally at 200°C, or programmed from 170°C to 190°C at 40°C/min with 10 minute periods at the beginning and end of the programme. The nitrogen carrier gas rate was 40ml/min throughout.

The methyl glycosides of the methylated sugars from methanolysis of the permethylated Laburnum anagyroides galactomannan (See Section 2), required a different liquid phase for separation. These derivatives were separated using system:-

(B) 3% PPE (polyphenyl ether) on Gas Chrom Q 100 - 120 mesh operated at 200°C isothermally with a carrier gas rate of 50ml/min.

Paper chromatography.

Descending paper chromatography on Whatman no.1 paper was carried out for low molecular weight sugar separation. The solvent systems used were:-

(A) Ethyl acetate : pyridine : water = 8:2:1 (73)

Separation was complete after 24 hours.

(B) Ethyl acetate : pyridine : water = 2:1:2 (78)

Separation was complete after 20 hours.

(C) n-Propanol : ethyl acetate : water = 7:1:2 (148)

Separation was complete after 42 hours.

After air drying of chromatograms the sample components and appropriate standards were detected using the following dip reagents:-

(1) Alkaline silver nitrate (165).

(2) p-Anisidine HCl (74).

Liquid scintillation counting (LSC).

^{14}C measurements were made using a Philips liquid scintillation counter standardised by the channels-ratio method. Aqueous samples were counted in a cocktail, containing butyl-PBD scintillant, whose components were:-

Methanol 500ml; Triton X 100 1,500ml; Toluene 3,000ml; 31.5g butyl-PBD (PBD = 2 phenyl - 5 -(4 biphenyl) - 1, 3, 4 oxadiazole).

Aqueous samples (1ml) readily became soluble in 10ml scintillation cocktail especially if the counting vials were pre-cooled (6°C for 10 minutes; operating temperature of Philips counter is 13°C). Efficiency of counting was ≥ 85%.

Section 1: The Biosynthesis of Fenugreek Galactomannan

Maturity parameter data.

Flowers were tagged on the day of anthesis, when petals are fully reflexed, by attaching a gummed label to the stem directly below the flower. Seed samples were harvested from labelled pods to give

data on: (a) seed weight (fresh and dry); (b) α - galactosidase levels in the embryo and the endosperm; (c) galactomannan and stachyose content of the endosperm. Analysis of seed weights was used to derive the absolute water content and the percentage moisture content, on a fresh weight basis. These parameters were used to evaluate the maturity of unlabelled seed samples.

(a) Seed weight: Pods or individual seeds were kept fresh in water, before weight analysis. Seed samples (6 on average) were surface dried on filter paper, and placed in pre-weighed aluminium foil packets (50 - 150mg), and the opening closed. The fresh weight of the seeds was determined after the foil packet plus contents were weighed on a five point balance. After this the packets were opened and placed in an oven at 105°C for 22 hours (136), then removed and re-sealed so that the dry weight could be determined. Absolute water values (fresh weight - dry weight) expressed as a percentage of the fresh weight, provided the % moisture content values.

(b) α - galactosidase assay: The preparation of hydrolase activity involved grinding isolated embryos or endosperms in a Potter homogeniser with McIlvaine buffer (pH5.0), 1 seed/ml of buffer (133). The assay consisted of 0.1ml of the enzyme, 0.05ml of 0.01M p - nitrophenyl α - D - galactopyranoside, and 0.25ml of McIlvaine buffer (pH5.0), all incubated at 30°C for 15 minutes. The incubation was terminated by adding 0.1M sodium carbonate (5ml), and the release of p - nitrophenol from the substrate was monitored at 400nm against enzyme blanks, to which the substrate was added at the end of the incubation period. For analysis of dry, ripe seeds the glassy endosperm and testa component, freed from the embryo, was laid on wet filter paper. When the endosperm tissue had swollen, one could remove the endosperm from the surrounding testa which contributes to and invalidates the absorbance reading for the endosperm, if still attached.

(c) Galactomannan and stachyose content of the endosperm: Endosperm tissue from seed samples (6 seeds) was treated with 70% MeOH in the standard isolation procedure for galactomannan. The isolated galactomannan was freeze-dried and weighed. The low molecular weight soluble sugars were concentrated to dryness. Sample aliquots (50%) were applied as strips onWhatman no.1 paper, and developed in solvent (B), from which the stachyose band was eluted with a fixed volume of water. Stachyose was determined after hydrolysis of the sugar and estimation of the fructose released (137). A 0.5ml portion of the stachyose sample was pipetted into a spectrophotometer tube after which 3.5ml 30% HCl and 0.5ml resorcinol-thiourea were added. The solution was mixed gently and the tube placed in a water bath at 80°C for 10 minutes. The tube was removed and cooled by immersion in tap water in the dark. The absorbance at 520nm was recorded along with standard sucrose solutions treated in a similar manner.

"Semi in vivo" monosaccharide incorporation.

All the following operations were performed aseptically within a laminar flow cabinet, with previously sterilised glassware. Seeds (4 - 5) were selected and the embryos removed. After stripping the testa from the endosperm tissue, the latter was floated as endosperm halves on the radioactive monosaccharide solution contained in a cavity block. The solutions consisted of 0.5 µCi of D - (U - ^{14}C) mannose or D - (U - ^{14}C) galactose (total volume of 0.5ml), at 0.33mM and 2.3 x 10^{-2}mM respectively. The cavity blocks were held in petri dishes containing a filter paper previously soaked with 4ml sterile water. The petri dishes were then incubated at 26°C for 22 hours in the dark. At the end of the incubation the endosperm tissue and remaining solution were transferred by Pasteur pipette to a conical centrifuge tube containing boiling 70% MeOH (2ml). To remove traces

of soluble galactomannan and sugars, the cavity blocks were flushed with water (1ml) which was added to the centrifuge tube. Sufficient absolute methanol was added to the tube to maintain the methanol concentration at 70%, and the standard galactomannan isolation procedure was carried out. The galactomannan, after freeze-drying, was weighed and hydrolysed by the standard method. An aliquot (1ml) of the neutralised hydrolysate was counted in 10ml scintillation cocktail.

The low molecular weight sugars from the incubation, i.e. the 70% MeOH soluble fraction, were analysed as strip samples by paper chromatography in solvents (A) and (B). Mono - and oligosaccharide components of the sample were cut out corresponding to standard sugars run on the same chromatogram, and located using detection reagent (1). The areas of paper were cut up and placed in counting vials and 1ml of water added. The vials were left for 1 hour and then counted.

Isolation and assay of galactomannan synthetase (GDP-mannose: galactomannan mannosyltransferase).

Selected seed samples were dissected and the endosperms dropped into 0.05M Tris HCl buffer (pH8.9) containing 5mM dTT and 5mM Mg^{2+} kept in an ice-cold mortar (usually 12 seeds/ml). The tissue was ground with sand (approx. 30mg) and PVP (insoluble polyvinyl polypyrrolidone; approx. 10mg) for 2 min (122). All subsequent operations were carried out at 4°C. The homogenate, minus an aliquot for the determination of the enzyme activity in the total homogenate, was centrifuged at 1,000g for 10 min. The 1,000g supernatant was centrifuged at 100,000g for 45 min, and the pellet which formed was taken up in isolation buffer, to give the particulate enzyme. The 100,000g supernatant was used as the soluble enzyme. The grossly particulate 1,000g pellet, or "cell wall fraction", was washed twice in buffer and then further homogenised in a mortar on ice. This homogenate was used as the cell wall enzyme.

Homogenisation using a Potter homogeniser was not practicable as the solution had too high a viscosity.

The standard assay system used for the particulate enzyme was as follows:-

100µl fenugreek galactomannan solution (1mg/ml); 24µl UDP - galactose (8.1×10^{-4}M); 8µl GDP - (U - ^{14}C) mannose (8.1×10^{-4}M; 0.1µCi); 50µl enzyme preparation; made up to 0.5ml with 0.05M Tris HCl buffer, 5mM dTT, 5mM Mg^{2+} and all contained in a 12ml conical centrifuge tube. The tube was maintained at 30°C for 45 min and then transferred to a boiling water bath for 10 min (tube capped with a marble). Fenugreek galactomannan solution (0.5ml ≡ 500µg polysaccharide) was added to the tube followed by methanol to bring the methanol concentration to 70%. The contents were extracted twice via the standard extraction procedure (70% MeOH), to remove the low molecular weight material. The precipitate was taken up in water and freeze-dried. After hydrolysis an aliquot (1ml) was counted by LSC.

The soluble enzyme activity was assayed by the standard method. The assay of both the cell wall enzyme and the total homogenate differed from the standard assay. The system used for these two enzyme sources was:-

100µl fenugreek galactomannan (1mg/ml); 24µl UDP - galactose (8.1×10^{-4}M); 8µl GDP - (U - ^{14}C) mannose (8.1×10^{-4}M; 0.1µCi); 1ml cell wall preparation or 0.25ml total homogenate.

Both incubation tubes were shaken in the 30°C water bath so that the enzyme and assay contents were kept in contact during the incubation. The analysis of radioactivity incorporated into 70% MeOH insoluble product was as for the particulate enzyme.

Analysis of UDP - galactose: galactomannan galactosyltransferase activity in endosperm cell fractions was also carried out using UDP - (U - ^{14}C) galactose (5.4×10^{-4}M), and non-radioactive GDP -

mannose (5.4×10^{-4}M) in the standard assay system. Proportions of the radioisotope to the other sugar nucleotide were identical to the system for mannosyltransferase. The 70% MeOH soluble contents, at the end of the assay, were also examined by paper chromatography in solvent (B). After elution of the stachyose zone it was applied to chromatography paper and developed in solvent (C), to detect any galactinol present.

The preparation of galactomannan primer (4).

Ripe seeds (20g) were milled, added to water (1 litre) at 90°C, and stirred for 2 hours. The mixture was centrifuged. Two volumes of E+OH were added to the supernatant with stirring and the resultant stringy precipitate collected by centrifugation. The residue from the first extraction was re-extracted in water (1.5 litres). After centrifuging off the residue the supernatant was again treated with 2 volumes of E+OH. The combined galactomannan precipitates were taken up in water (100ml) and freeze-dried. The galactomannan collected amounted to 3.13g, giving a 15.6% yield. A solution of galactomannan at 1mg/ml was produced by adding finely divided galactomannan to water at 60°C, with constant stirring for approx. 6 hours. This solution was then used as a source of primer in the in vitro galactomannan synthetase assay.

Identification of the synthetase in vitro product.

The following tests were applied to the synthetase in vitro product, the isolated fenugreek galactomannan and to a galactoglucomannan from spruce (92) containing 11% galactose, 21% glucose, 60% mannose, 3% arabinose, 5% xylose.

(a) Borate gellation (63, 3): A 0.5% solution (usually 0.5ml) of the polysaccharide and an equal volume of saturated borax ($Na_2B_4O_7$) were mixed, and upon standing a clear gel was formed with galactomannan.

Precipitation was complete, as no polysaccharide could be recovered by methanol precipitation of the supernatant after centrifugation of the gel at 20,000g. Spruce galactoglucomannan was treated in the same way, but gave no gel. A mixture of fenugreek galactomannan and spruce galactoglucomannan was tested: the centrifuged gel was washed twice with water, decomplexed in glacial acetic acid, precipitated with methanol (to give a final methanol concentration of 70%), then hydrolysed and examined by GLC. The supernatant was treated with methanol and the resultant precipitate hydrolysed, then examined by GLC.

The standard galactomannan synthetase incubation was carried out and the reaction terminated by heating at 100°C for 10 minutes. Fenugreek galactomannan solution (0.5ml of 0.5%) was added, followed by saturated borax (0.5ml). The gel which formed was centrifuged, washed twice with water, sonicated and counted by LSC. Any high molecular weight radioactive material remaining in solution after borax gellation was recovered by treatment of the supernatant with methanol, and examination of any precipitate by LSC.

(b) Ricinus communis lectin (RCAII) precipitation (169): Either non-radioactive fenugreek galactomannan (100µg) or the synthetase incubation mixture (normally containing 100µg fenugreek galactomannan primer) was placed in a water bath at 25°C. The synthetase incubation mixture, having been kept at 100°C for 10 min to stop the reaction, was cooled down on ice before being placed in the 25°C water bath. Sodium chloride (0.5ml, 0.4M) and 0.1M sodium phosphate buffer, pH 7.4 (0.5ml) were added and the volume made up to 2ml with water. After mixing 1ml of Ricinus communis lectin (RCAII; agglutinin 120, Miles Yeda Ltd., Miles Laboratories, UK) solution (0.75mg/ml in 0.1M NaCl, pH 7.0) was added and the mixture incubated for 15 min. Centrifugation (approx. 2,000g) gave a dense, semi-opaque precipitate. The precipitate was washed in water, and collected by centrifugation; in the case of the synthetase

assay mixture the washed precipitate was counted by LSC.

(c) Metal ion precipitation: Transition elements have been shown to form insoluble complexes with galactomannan molecules (26). Fenugreek galactomannan, spruce galactoglucomannan and a mixture of both polysaccharides, as well as the synthetase in vitro product were tested. Polysaccharide solutions were at a concentration of 1%. Transition element solution (0.1ml; 10mM) was added to 1ml of polysaccharide solution. Complexing was induced by adding 20% (w/v) NaOH (22µl). Flocculent or gel-like precipitates were formed with characteristic colourations due to thetransition metal ion. Decomplexing of the precipitates was brought about by boiling in glacial acetic acid for approx. 30 minutes: this left behind a characteristic fluffy, white, polysaccharide precipitate. Precipitates were analysed, after hydrolysis, by paper chromatography (Solvent (A)). The precipitated synthetase product was counted either as the sonicated precipitate or as an aqueous aliquot of the hydrolysate from the decomplexed precipitate. Any polysaccharide remaining in solution after metal-ion precipitation was recovered by treatment of the soluble fraction with methanol, and then analysed, after hydrolysis, by paper chromatography (Solvent (A)).

Characteristics of the galactomannan synthetase.

All the following experiments were carried out on the particulate enzyme only.

(a) pH profile: Homogenisation of the endosperm tissue was carried out in 0.05M Tris HCl buffer (pH 7.5), 5mM dTT, 5mM Mg^{2+} and the particulate preparation was resuspended in water. The standard in vitro assay mixture (132µl including 50µl of the enzyme preparation) was made up to 0.7ml with a buffer of the required pH value. The buffers used, all containing 5mM dTT and 5mM Mg^{2+}, gave a range of pH values between 5.8 and 9.8 and comprised:-

0.05M sodium phosphate buffer pH 5.8, pH 6.8, pH 7.5;

0.05M Tris HCl buffer pH 7.5, pH 8.4, pH 8.9;

0.05M glycine - NaOH buffer pH 8.9, pH 9.4, pH 9.8.

The incubation and analysis of synthetase activity at the various pH values was carried out in the standard manner.

(b) Effect of cations: Several cations were tested for their effect upon the particulate enzyme activity. The cations were made up in 0.05M Tris HCl buffer (pH 8.9; 5mM dTT) at 20mM, and added to give a final concentration in the standard incubation mixture of 10mM. The following ions as their chloride salts were tested:-

Mg^{2+}, Mn^{2+}, Cu^{2+}, Ca^{2+}, Co^{2+}, and K^{+}.

The stimulation of synthetase activity by magnesium ions was also followed over the concentration range, 0 - 10mM.

(c) Dialysis of particulate enzyme: The resuspended particulate enzyme was dialysed in two ways and the activity of the dialysis residue was tested.

(i) The particulate preparation was placed in Visking dialysis membrane and dialysed overnight against running tap water, at room temperature. Then the membrane was placed in two litres of distilled water adjusted to pH 8.9, and stirred, with one change of water, for 4 hours. The contents of the dialysis sac were then tested for synthetase activity.

(ii) The particulate preparation was dialysed against 0.05M Tris HCl buffer (pH 8.9) over an Amicon PM10 membrane (nominal cut off 10,000 molecular weight) in an Amicon (Model MMCA) micro pressure dialyser/ concentrator, at $4^{o}C$. After 10 volumes of buffer were collected (compared to the original particulate sample) the preparation was concentrated, and assayed for synthetase activity.

(d) Solubilisation of the particulate enzyme: Attempts to solubilise the particulate enzyme were made using a bile salt (deoxycholate, DOC), a detergent (Triton X-100), and a steroid glycoside (digitonin).

DOC and Triton X-100 were tested on the particulate preparation at final concentrations of 0.5%, and 0.1%. The digitonin was added as a 1.6% solution in 0.05M sucrose and treatment lasted 20 minutes at 4°C (60). At the end of a treatment the whole preparation was centrifuged at 100,000g for 45 min and the precipitate and soluble fraction assayed in the usual way.

(e) Detection of a possible lipid intermediate: After a normal assay of a particulate synthetase preparation, the contents were extracted not with 70% MeOH but with chloroform: methanol (3:1). Either 0.5ml of fenugreek galactomannan solution (approx. 0.1%) was added followed by 1.5ml of $CHCl_3$: MeOH (3:1), or else the solvent mixture was added directly to the incubation. The aqueous and non-aqueous phases were separated using a Pasteur pipette, then additional $CHCl_3$: MeOH (3:1) was added to the aqueous fraction and water to the non-aqueous fraction. After this washing procedure the original aqueous phase was treated with sufficient methanol to give a final MeOH concentration of 70%, and any precipitate formed was removed by centrifugation and washed. This was treated in the same manner as other 70% MeOH insoluble products of the *in vitro* synthetase assay. The original non-aqueous phase was added directly to a counting vial and counted in the usual manner, or the chloroform was evaporated in the counting vial before counting.

(f) Effect of substrate concentration: The reaction velocity was followed as the concentration of the substrate, GDP - (U - ^{14}C) mannose, was altered. With each increase in concentration of GDP - (U - ^{14}C) mannose a concomitant increase in UDP - galactose was made in the reaction mixture, to keep the ratio the same as in the standard assay. Measurement was made using amounts of GDP - (U - ^{14}C) mannose corresponding to 0.1, 0.3, 0.4, 0.6, and 0.8μCi (from a 0.81mM solution, at 12.5μCi/ml), in a constant total volume of 0.5ml reaction mixture. A double reciprocal plot of velocity against concentration (93) was constructed.

Subcellular and temporal distribution of galactomannan synthetase activity.

Analysis of the particulate, soluble, cell wall and total homogenate galactomannan synthetase activities was made over the galactomannan-deposition phase of seed maturation.

The detection of subcellular sites of synthetase activity was performed using the following techniques.

(a) Sucrose density gradients: All enzyme extracts to be placed on sucrose gradients were prepared in 0.05M Tris HCl buffer (pH 8.9) containing 5mM dTT, 5mM Mg^{2+}, 0.4M sucrose unless stated otherwise. For separation by velocity sedimentation the sample was placed on a 15 - 35% sucrose gradient (16ml) in 0.05M Tris HCl buffer (pH 8.9) usually containing 5mM dTT and 5mM Mg^{2+}, produced in an MSE (no.36657) gradient maker. All operations including centrifugation steps were carried out at 4°C. The tubes were centrifuged in a 3 x 25ml swing out rotor on an MSE 65 high speed centrifuge at 10,000rpm (approx. 10,000g) for 30 min. At the end of this time the tubes were removed and fractions taken from the bottom of the gradient by inserting a needle, connected to a peristaltic pump, through the gradient to the bottom of the tube. Very little disturbance in gradient density profiles resulted from any of these operations.

For isopycnic sedimentation the sample was placed on a 20 - 50% sucrose gradient (16ml) in the same buffer as for the velocity separations. The tubes were then centrifuged in a 3 x 25ml swing out rotor on an MSE 65 high speed centrifuge at 20,000rpm (approx. 40,000g) for 2.5 hours (151).

All collected fractions were analysed for (i) synthetase activity, (ii) protein content, (iii) refractive index. Most gradients were assayed for (iv) the marker enzymes NADH - cytochrome C reductase and IDP ase. In a few samples the absorbance at 260nm was also recorded from the individual fractions of the gradient.

(i) Synthetase activity. Approximately 10% samples were taken from the gradient fractions and incubated in the normal fashion. Any pelleted material at the bottom of the gradient (on the tube walls), was resuspended in buffer and a suitable aliquot removed for assay of synthetase activity.

(ii) Protein content. A preliminary scan of the fractions in a spectrophotometer at 280nm, in some gradients, gave a profile of the protein content. All gradients were analysed for protein by the method of Lowry (97); the aliquots taken were no larger than 100µl, as larger amounts of sucrose can interfere with the assay (51). Bovine serum albumin was used as the standard protein solution.

(iii) Refractive index. The refractive index was recorded manually using an Abbé refractometer (Hilger Watts Ltd.). Conversion of refractive index values to density values, as g/ml, was brought about using published data (154, 173).

(iv) Marker enzymes. NADH - cytochrome C reductase was measured by following the increase in absorbance at 550nm at 25°C when 50µl aliquots (approx. 5% of gradient fractions) were added to 2.5ml of a solution containing 50mM sodium phosphate buffer (pH 7.5), 0.2mM NADH, 0.7mM KCN, and 30µM cytochrome C. The change in absorbance was followed using a Pye Unicam SP1800 spectrophotometer.

Latent IDP ase activity, after storage at 4°C for 3 days (122), was measured with 0.1ml aliquots (approx. 10% of gradient fractions) incubated for 1 hour at 35°C in a reaction medium (0.1ml) containing 75 mM Tris HCl buffer (pH 7.8), 2.5mM inosine diphosphate, and 1.0mM $MgCl_2$. The P_i released was measured with the ferrous sulphate - ammonium molybdate reagent (163).

Fractions of high galactomannan synthetase activity from a velocity sedimentation gradient were removed and processed for electron microscopy (see Microscopy section, page 46).

(b) Differential pelleting: Endosperm tissue was homogenised in the normal galactomannan synthetase isolation buffer, plus 0.4M sucrose. The 1,000g supernatant was taken for successive centrifugations at the following 10^{-3} x g values:-

10, 25, 40, 75, and 100 - all at 4°C.

The pelleted material at each step was resuspended in buffer (plus 0.4M sucrose), and assayed by the standard method.

Microscopy.

Endosperm tissue taken from seeds during galactomannan deposition (from 30 - 50 days after anthesis) was observed in the light and electron microscopes. The examination of particulate material from endosperm tissue homogenates fractionated by sucrose density gradient centrifugation was carried out using the electron microscope.

(a) Light microscopy: Seeds were placed in Ames O.C.T. Compound (R.A. Lamb, London), on cryostat mounting blocks and deep frozen in liquid nitrogen. Sections (approx. 20µm) were cut using the cryostat (Brights Ltd.) and transferred to microscope slides, which were then immersed in degassed, cold 60% E+OH. Sections were either viewed unstained by Nomarski interference contract (Olympus, model BHB), or after periodic acid - schiff (PAS) staining (128).

(b) Electron microscopy: (i) Endosperm tissue. Seeds were cut transversely into approx. 1mm slices, and the intact pieces fixed with glutaraldehyde and osmium tetroxide using the following procedure. The tissue pieces were immersed overnight in 3% (w/v) glutaraldehyde in 0.05M sodium cacodylate buffer (pH 7.2), washed with three changes of the cacodylate buffer over 24 hours, then postfixed with 2% osmium tetroxide in the same buffer. The fixed material was washed with buffer (3 x 10 min) and dehydrated in the following ethanol series:-

10, 30, 50, 70, 90, 96, 2 x 100% E+OH, over a 72 hour period.

The preceding steps were carried out at 4°C in a cold room.

The fixed, dehydrated tissue pieces were then treated successively with ethanol/propylene oxide (1:1; 1 x 10 min), and propylene oxide (3 x 10 min) at room temperature. The last volume of propylene oxide then had an equal volume of propylene oxide/Araldite (1:1) added to it, to give propylene oxide/Araldite (2:1; Araldite = Durcupan ACM, Polaron Ltd., UK). The pieces were left in propylene oxide/Araldite (2:1) for 2 hours then treated successively with propylene oxide/ Araldite (1:2; 24 hours), and Araldite (24 hours in an evacuated desiccator); finally they were embedded in Araldite (2 days at 60°C). Ultrathin sections were cut with glass knives on an ultramicrotome (LKB), transferred to copper grids and stained with 2% uranyl acetate followed by Reynolds lead citrate. The sections were examined with either a Corinth 275 or a Jeol JEM - 7 electron microscope.

(ii) Density gradient pellet. Galactomannan synthetase containing fractions from a velocity (rate sedimentation) sucrose density gradient centrifugation were pooled (1.04 - 1.07g/ml density fractions). To this sample 25% (w/v) glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.2) was added (0.1 volume per volume of sample), mixed gently and left to stand at 4°C in a cold room overnight (44). The following operations were also carried out at 4°C. The sample was diluted with an equal volume of 0.1M sodium cacodylate buffer in 0.25M sucrose (pH 7), transferred to a 3ml centrifuge tube, and centrifuged at 10,000g for 30 min. The supernatant was carefully removed and the sample pellet gently resuspended in 0.1M sodium cacodylate buffer in 0.25M sucrose (pH 7; 0.2ml). The resuspended pellet was transferred to a conical Beem capsule, and centrifuged at 10,000g for 30 min cushioned upon a rubber bung with a conical hole removed. The compacted pellet, at the tip of the capsule, was washed free of glutaraldehyde with 0.1M sodium cacodylate buffer in 0.25M sucrose (pH 7; 5 changes in 1 hour), without disturbing the pellet. After the removal of the last wash, 1% osmium

tetroxide in 0.1M sodium cacodylate buffer (pH 7.2) was added and left overnight. The osmium tetroxide was removed and replaced with cacodylate buffer, washing down the capsule sides carefully. After a second wash with buffer the pellet was dehydrated and embedded *in situ*, as in (b)(i) above. Ultrathin sections were cut with a diamond knife on an ultramicrotome (LKB) and placed on copper or gold grids.

The sections on the copper grids were stained with 2% uranyl acetate followed by Reynolds lead citrate.

The gold mounted sections were used in staining sections by the Thiery reaction (164) used for selectively staining polysaccharides. The following sequence of operations was carried out in glass cavity blocks, holding approx. 0.5ml of solution, at room temperature. The gold grid was placed on 1% periodic acid for 20 min, then thoroughly washed with water (5 changes over 30 min), and transferred to 0.2% thiocarbohydrazide in 20% acetic acid (3 hours). The grid was washed with 10% acetic acid (5 x 10 min), with water (5 x 10 min), and transferred to 1% silver proteinate (Polaron Ltd., UK) and kept for 30 min in the dark. As a control, a grid was carried through the whole staining procedure but missing out the periodic acid step. Grids were dried and examined with a Jeol Jem - 7 electron microscope.

Section 2: Chemotaxonomic Studies

A structural characterisation of *Laburnum anagyroides* galactomannan along with the determination of mannose: galactose ratios of the galactomannans from species within the *Genisteae* tribe was carried out. *L. anagyroides* seed samples, when fully ripened, were harvested from trees known to be of this species within the Stirling University campus. Seed samples used in the investigation of the

Genisteae species were obtained from the Botanical Gardens of the Universities of Copenhagen, Denmark, and Coimbra, Portugal and from The Royal Botanical Gardens, Kew, England.

Isolation of galactomannans.

(a) Laburnum anagyroides: Seeds (26.05g; average weight 23mg) were gathered, surface sterilised in sodium hypochlorite, washed in water and then swollen overnight in water after removal of all inferior seeds. The swollen seeds were then dissected to remove the embryo leaving the endosperm and testa. The endosperm tissue was then stripped from the testa and placed in boiling 70% MeOH (300ml), which was changed until a negative Molisch test was observed with the soluble fraction i.e. the supernatant after centrifugation. The insoluble material was taken up in water and freeze-dried, after which 1.75g was present (approx. 7% yield, based on total seed weight). The protein content of the extract was estimated by the methods of Waddell (171) and Lowry (97).

(b) Genisteae species: Seeds (0.03- 0.3g, depending upon the availability) were placed in water at 80°C until they were swollen. The seeds were squashed and then sonicated, three times, with removal of the supernatant at each stage. Fehlings solution was added to the combined supernatants and the precipitate collected. The precipitate was dissolved in 2M acetic acid, and the decomplexed polysaccharide precipitated with ethanol, washed in ethanol and freeze-dried.

Light Scattering.

All solutions of the L. anagyroides galactomannan were made in filtered water and then passed through MF - Millipore filters, housed in a clean syringe, directly into the light scattering cells. These precautions were made to remove as many as possible of the dust particles which are a serious drawback to all light scattering experiments (160). All glassware had been cleaned using chromic acid soaking, followed by

rinsing in filtered water. The scattering intensities of various concentrations of L. anagyroides galactomannan solutions were measured on a SOFICA Photo-Gonio Diffusometer. Presuming that the molecular weight was less than 1×10^5, the dissymmetry effect was taken to be negligible and all readings were taken at a fixed angle of 90°. The machine was normalised using a standard benzene solution and a light source giving a monochromatic unpolarised beam of light of $\lambda = 436nm$ (violet filter). The weight average molecular weight, M_w, was calculated from the equation:-

$$\frac{1}{M_w} = k \left(\frac{dn}{dc}\right)^2 \left(\frac{c}{I_\theta}\right)_{c \to o}$$

k = callibration constant for standard benzene (42.221)

$\frac{dn}{dc}$ = change in refractive index with respect to changes in concentration. This was measured on a Brice-Phoenix refractometer.

$\left(\frac{c}{I_\theta}\right)_{c \to o}$ = intercept at c equal to zero of the line formed from a plot of $\frac{c}{I_\theta}$ versus c.

c equals the concentration of galactomannan solution.

I_θ equals the scattering intensity.

Methylation and analysis.

A sample (0.5g) of the L. anagyroides endosperm material was treated once by the method of Haworth (64) and twice by the method of Hakomori (61), to effect complete methylation.

The Haworth methylation started with the addition of nitrogen gassed 40% NaOH (20ml), under a nitrogen atmosphere, until the material was dissolved (stirred for 2 hours). Dimethyl sulphate (3 x 6ml) was added and left stirring for 3 hours. The mixture was then removed and dialysed overnight against tap water, then distilled water.

The dialysis residue was freeze-dried and then swollen in dimethyl sulphoxide (DMSO) at $50^\circ C$ for 2 hours (61). This step and all subsequent reactions took place in a nitrogen atmosphere, in a three necked flask. A dimethylsulphinyl carbanion solution (produced by adding

0.7g dry NaH to DMSO (7.5ml), under nitrogen at 60°C for 2 hours) was added and stirred for 30 min at 25°C. The mixture was left for 30 min, and then cooled to 20°C in an ice and water bath. Methyl iodide (1.8ml) was added over a 5 - 6 min period, maintaining the temperature below 25°C, until the solution became a clear yellow (2 hours). At this point excess methyl iodide was removed by rotary evaporation at 40°C. The solution was then dialysed and the residue was shown to be not totally DMSO soluble. The Hakomori step was repeated once more and the material was shown to be totally methylated, as seen from the total absence of any absorption in the i.r. spectrum characteristic of free hydroxyl groups (no absorption at 3400 - 3600 cm^{-1}, for the chloroform solution of the methylated galactomannan).

A portion of the methylated galactomannan was then treated with 90% (v/v) HCOOH (10ml) for 1 hour, followed by 0.5M H_2SO_4 for 7 hours, both at 95°C under reflux. The resultant permethylated sugars were converted to their alditol acetates and analysed by GLC on column (A). A further sample (1.5mg) was methanolysed, using 3% methanolic HCl (5ml) for 24 hours under reflux. The resultant methyl glycosides of the permethylated sugars were analysed directly by GLC on column (B), after reduction to dryness and dissolving in a minimal volume of methanol. The glycitol acetates of the following sugars were used as standards for column (A): 2, 3, 4, 6 - tetra - O -methyl - D - galactose; 2, 3, 4, 6 - tetra - O -methyl - D - mannose; 2, 3, 6 - tri - O -methyl - D - glucose; 2, 3, 6 - tri - O -methyl - D - mannose; 2, 3 - di- O - methyl - D - mannose. The methyl glycosides of 2, 3, 4, 6 - tetra - O -methyl - D - galactose were used as internal standards for [illegible] on column (B).

Periodate oxidation and analysis.

A sample of the [illegible] galactomannan (15mg) was subjected to oxidation by 0.05M sodium metaperiodate [illegible] at 4°C

0.7g dry NaH to DMSO (7.5ml), under nitrogen at 60°C for 2 hours) was added and stirred for 30 min at 25°C. The mixture was left for 30 min, and then cooled to 20°C in an ice and water bath. Methyl iodide (1.8ml) was added over a 5 - 6 min period, maintaining the temperature below 25°C, until the solution became a clear yellow (2 hours). At this point excess methyl iodide was removed by rotary evaporation at 40°C. The solution was then dialysed and the residue was shown to be not totally DMSO soluble. The Hakomori step was repeated once more and the material was shown to be totally methylated, as seen from the total absence of any absorption in the i.r. spectrum characteristic of free hydroxyl groups (no absorption at 3400 - 3600 cm^{-1}, for the chloroform solution of the methylated galactomannan).

A portion of the methylated galactomannan was then treated with 90% (v/v) HCOOH (10ml) for 1 hour, followed by 0.5M H_2SO_4 for 7 hours, both at 95°C under reflux. The resultant permethylated sugars were converted to their alditol acetates and analysed by GLC on column (A). A further sample (13mg) was methanolysed, using 3% methanolic HCl (3ml) for 16 hours under reflux. The resultant methyl glycosides of the permethylated sugars were analysed directly by GLC on column (B), after reduction to dryness and dissolving in a minimal volume of methanol. The glycitol acetates of the following sugars were used as standards for column (A): 2, 3, 4, 6 - tetra - O -methyl - D - galactose; 2, 3, 4, 6 - tetra - O -methyl - D - mannose; 2, 3, 6 - tri - O -methyl - D - glucose; 2, 3, 6 - tri - O -methyl - D - mannose; 2, 3 - di- O -methyl - D - mannose. The methyl glycosides of 2, 3, 4, 6 - tetra - O -methyl - D - galactose were used as internal standards for separations on column (B).

Periodate oxidation and analysis.

A sample of the L. anagyroides galactomannan (16mg) was subjected to oxidation by 0.05M sodium metaperiodate (25ml) at 4°C in

the dark. The sample and a blank, containing the periodate solution but no polysaccharide, were stored in capped, amber-glass bottles. Aliquots were removed periodically to measure (a) periodate consumption, and (b) formic acid production.

(a) A sample (0.5ml) was removed from the galactomannan reaction vessel and from the blank, and diluted 250 times. The absorption at 223nm (38) was recorded along with that of an equi-molecular iodate solution. The uptake of periodate in moles is related to the drop in the absorption reading for the galactomannan vessel with respect to the blank.

(b) A sample (0.5ml) was removed from the galactomannan reaction vessel and from the blank, and the periodate was destroyed by adding two drops of ethylene glycol. The solutions were then titrated against 0.01M NaOH, using phenol red as the indicator, using a micro-burette.

The oxidised polysaccharide, after removal of salts by dialysis, was hydrolysed to detect the presence of any unoxidised sugar residues. The hydrolysate was analysed by paper chromatography (solvents (A) and (B)) and GLC on column (A).

RESULTS AND DISCUSSION

CONTENTS

Section 1: The biosynthesis of fenugreek galactomannan

Parameters of seed maturation

To determine where the formation of the endosperm galactomannan fitted into the general pattern of seed development in fenugreek, it was necessary to establish the most basic parameters of seed formation. It was relative to this framework of events during development that the later biochemical experiments were viewed and appraised.

Flowers were tagged at anthesis, and seed samples taken at intervals thereafter to determine their fresh and dry weights. The youngest developing seeds analysed were taken 10 days after anthesis, as prior to this the ovules were microscopic. The fresh and dry weights of seed samples at different maturity stages are recorded in Table 1 along with the derived absolute water values and percentage moisture contents. A composite view of the physical changes up to sixty days after anthesis is given in Fig. 1. It can be seen that the fresh weight of a seed increases to a value of approx. 33 mg after 50 days of development and then progressively decreases until in the fully ripe seed the fresh weight is on average 15 mg. The dry weight also increases over this same period but reaches a steady level of approx. 13 mg at 55 days.

The earliest samples have a high percentage moisture content, which increases slightly over the first 25 days of development to a peak of 82% and thereafter steadily declines reaching a level of 11% (on average) in the ripe seed. The fresh weight pattern is repeated by the values for absolute water which reach a maximum of 21 mg per seed around 50 days and then decrease to approx. 2 mg in the ripe seed, thereby causing the fall in the fresh weight.

Leguminous seed formation in general is characterised by

Table 1 Maturity parameter data

Seed age (days)	Fresh wt. (mg)	Dry wt. (mg)	Absolute H_2O (mg)	% Moisture	Appearance of Endosperm	Appearance of Seed
11	0.42	0.08	0.34	78.8	liquid	green
15	2.19	0.43	1.76	80.4		
22	2.25	0.40	1.85	82.0		
25	6.80	1.29	5.51	81.0		
30	8.80	1.76	7.04	80.0		
32	9.57	1.82	7.75	80.9		
33	15.30	3.38	11.92	77.9		
35	17.50	4.30	13.20	75.4	semi gel	
37	19.20	4.88	14.32	74.6		
38	18.76	4.78	13.98	74.5		
39	20.62	5.18	15.44	74.9		
40	24.30	6.85	17.45	71.8		
42	25.63	7.27	18.36	71.6		
44	28.15	8.30	19.85	70.5		
45	28.84	9.06	19.78	68.5		
47	29.60	9.71	19.89	67.2		
48	31.80	10.20	21.60	67.9		
49	32.20	10.69	21.51	66.8		
51	32.04	11.25	20.79	64.9	firm & rubbery	yellow w/ green
52	33.30	11.63	21.67	65.1		
55	32.12	11.99	20.13	62.7		
57	31.50	12.32	19.18	60.9		
59	31.47	12.75	18.72	59.5		
60	30.56	13.42	17.14	56.1		
Ripe seed (average)	14.92	13.27	1.65	11.0	glassy & brittle	yellow

Seed age: days after anthesis

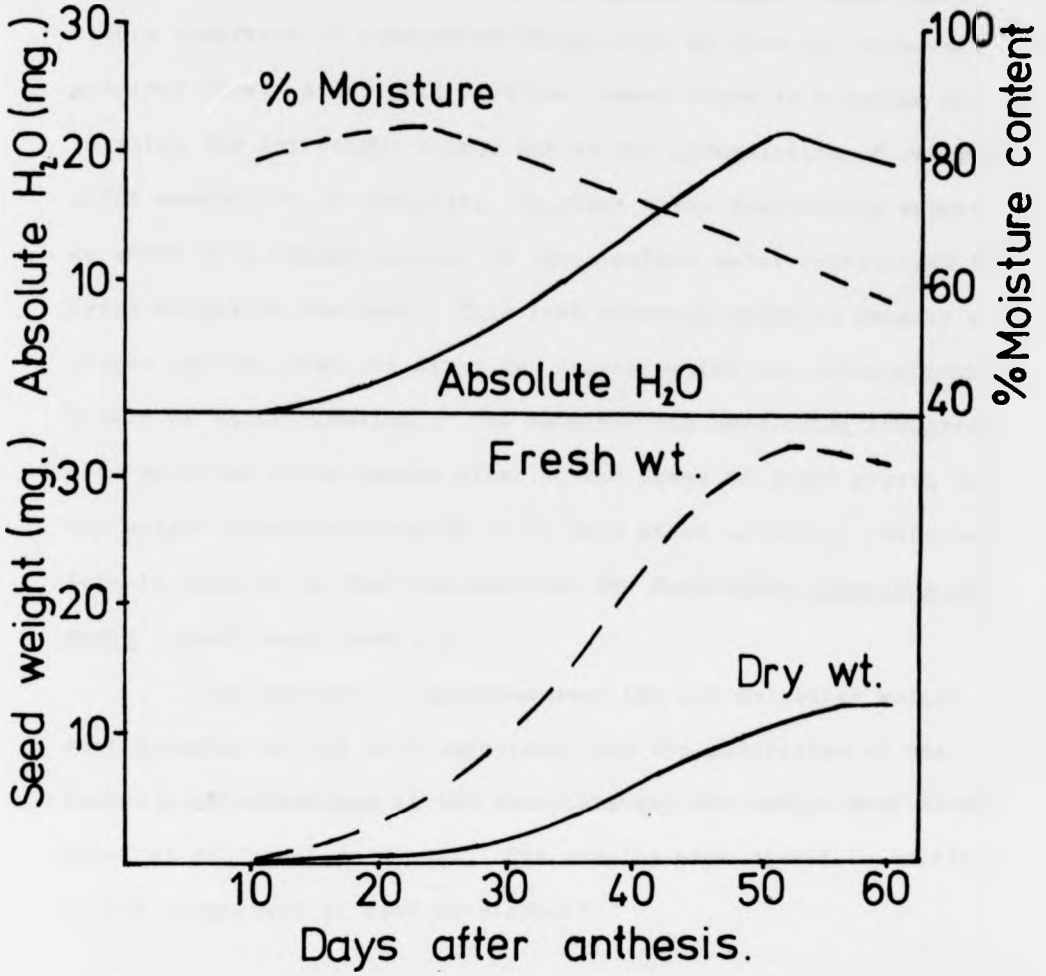


Fig. 1 Basic growth parameters during seed development.

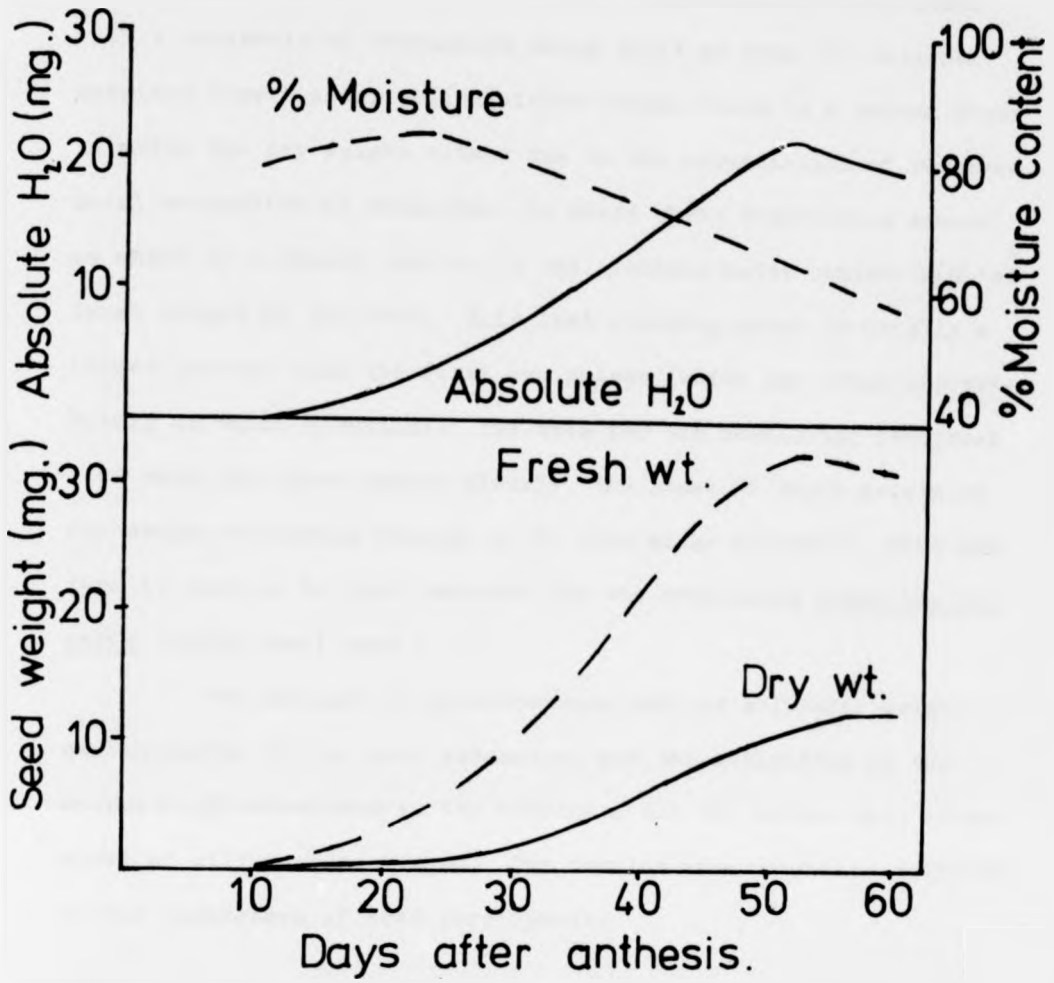


Fig. 1 Basic growth parameters during seed development.

three phases(40). In phase one rapid cell division takes place with a reservoir of precursors being built up from the maternal nutrient flow. After cell division ceases there is a second phase in which the dry weight climbs due to the accumulation of reserves, until maturation is complete. In phase three desiccation ensues as shown by a steady decline in the absolute water content and the fresh weight of the seed. This last ripening phase is usually a longer process than the first two phases (which are often approximately of equal duration). The data for the developing fenugreek seed show the three phases clearly, the phase of rapid growth in dry weight occurring from 30 to 55 days after anthesis. This pattern is similar to that recorded for the developing Phaseolus vulgaris (dwarf bean) seed (8).

The amounts of galactomannan and low molecular weight carbohydrates in the seed endosperm, and the activities of the enzyme α-galactosidase in the endosperm and the embryo were determined at different seed ages. The results were viewed in relation to the parameters of seed development.

In vivo studies

The quantities of galactomannan and the reserve tetrasaccharide stachyose in the endosperm at different stages of seed maturity are given in Fig. 2. Galactomannan deposition begins after the seed has reached 30 days of maturation. A slow increase is seen from 30-37 days after anthesis and subsequently the deposition increases markedly and is approximately linear until completion at 55 days after anthesis, when the seed contains 2.5 mg of polysaccharide. The polysaccharide is therefore deposited throughout phase two of seed development. A slight decrease in the amount of galactomannan is noticed in seeds taken from the third, ripening phase or at the fully ripe end-point. This was noticed by Reid

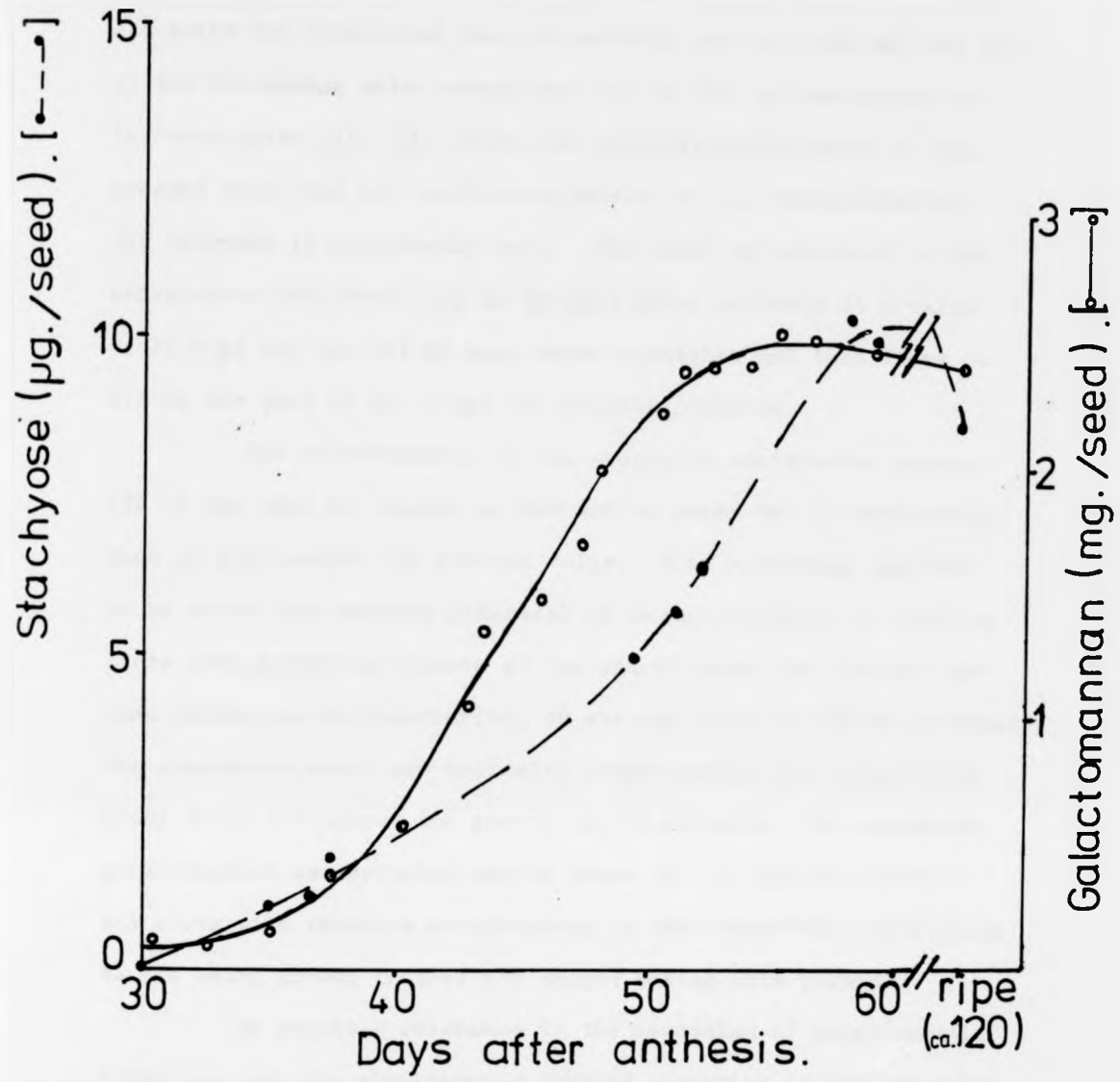


Fig. 2 The levels of galactomannan and stachyose in the endosperm, from the onset of deposition.

and Meier who considered that it probably was not real and was due to the decreasing water extractability of the polysaccharide as the seed dries out (129. Since the analysis method used in this present study did not involve extraction of the polysaccharide, the decrease is undoubtedly real. The level of stachyose in the endosperm climbs from zero at 30 days after anthesis to a value of 10.5 µg per seed at 60 days after anthesis, and then drops to 8.6 µg per seed at the stage of complete ripeness.

The galactomannan in the endosperm contributes approx. 19% of the seed dry weight at the end of phase two of maturation, when it has reached its maximum value. This percentage appears to be around the maximum potential of fenugreek seed, as ripening seeds from greenhouse plants at the end of phase two yielded the same percentage polysaccharide, on average equal to 19.7%, although the greenhouse seeds are typically larger seeds than growth-room grown seeds throughout the growth period (129,128). The endosperm galactomannan is deposited during phase two of seed maturation, and along with reserves accumulating in the cotyledons contributes to the rapid growth in seed dry weight during this period.

Of possible relevance to the mechanism of galactomannan formation, are the simultaneous changes occurring in the low molecular weight carbohydrates of the endosperm during and on either side of the period of galactomannan deposition. This analysis which was carried out semi-quantitatively by paper chromatography is recorded in Table 2. No free (non-phosphorylated) mannose or galactose was detected on any of the chromatograms of the endosperm extracts. At the beginning of phase two (i.e. reserve accumulation stage) of development the endosperm contains mainly sucrose, raffinose

Table 2 Composition and amount of the low molecular weight carbohydrates in fenugreek endosperm taken at different maturity stages

Seed age (days after anthesis)	Relative amounts of				
	Fructose	Glucose	Sucrose	Raffinose*	Stachyose
29	1	2	4	4	
31	tr.	2	4	4	tr.
35	tr.	2	4	4	1
39	tr.	2	3	4	2
41	tr.	2	3	3	2
43	tr.	1	3	3	2
46	tr.	1	3	3	3
50	tr.	1	3	3	3
53	tr.	1	3	3	3
57	tr.	1	3	3	4
60	tr.	1	3	2	4
64	tr.	tr.	2	2	4
85	tr.	tr.	1	1	4
104	tr.	tr.	1	1	4
Ripe seed (approx. 120 onwards)	tr.	tr.	1	1	4

According to the colour reaction with $AgNO_3$/NaOH on chromatograms; tr. = trace, 1 = weak, 2 = medium, 3 = strong, 4 = very strong.

* Myo- inositol also present in this fraction, as the two components are not separated in solvent (B).

and lesser amounts of glucose and fructose. During the next thirty days of development, from 30-60 days after anthesis, the sucrose and raffinose levels dwindle whilst stachyose is produced gradually until at the end of phase two it is the largest component of the low molecular weight carbohydrates. The stachyose is present in the fully ripe seed (from approx. 120 days onwards) in considerable quantities in relation to the other soluble components, but the quantity of stachyose stored in the endosperm is less by a factor of approx. 240 than that of the cell wall galactomannan (see Fig. 2). These findings are in agreement with an earlier investigation of galactomannan and low molecular weight carbohydrates in forming fenugreek seeds, in less well defined seed maturity stages (129).

The biosynthesis of stachyose in Phaseolus vulgaris has been investigated and a soluble enzyme isolated which is capable of transferring D-galactose from galactinol (1-O-α-D-galactopyranosyl-myo-inositol) to raffinose giving rise to stachyose and myo-inositol. It has been suggested that this is the final reaction on the pathway to stachyose(162). Galactinol has been shown to be a constituent of the bean seed and is formed, prior to stachyose, from UDP-D-galactose (specific for this sugar nucleotide) and myo-inositol. There is a possibility that galactinol might be involved in the transfer of galactose residues to both stachyose and the fenugreek galactomannan, as they both have galactose linked α(1- 6) and are synthesised at the same time in the seed endosperm. The possible involvement of galactinol in the transformation of UDP-(U-^{14}C) galactose by fenugreek endosperm homogenates will be discussed later (see page 73).

An interesting feature of the ripe fenugreek seed which has been previously observed(133)is that the mature embryo possesses high α-galactosidase activity, whereas the endosperm does not.

High α-galactosidase appears in the endosperm only after approximately 24 hours germination (133)and coincides with the onset of galactomannan mobilisation(128). The development and increase of an embryo α-galactosidase from 30 days after anthesis to a high level at the end of maturation (phase two) at approx. 60 days, is recorded in Fig. 3. During seed ripening (phase three) the activity declines to a level in the fully ripe seed of three-quarters of this peak level. This level of approx. 6×10^{-2} enzyme units (1 unit is equivalent to 1 μmole of p-nitrophenol released from the substrate per minute) in the ripe seed embryo agrees almost exactly with the value recorded for the embryo of the resting, ripe fenugreek seed before germination by Reid and Meier(133). The endosperm on the other hand has only a very small but quite detectable α- galactosidase activity throughout phase two of seed development. The possibility that this very low α-galactosidase activity in the endosperm preparation might have been caused by traces of seed coat adhering to the endosperm tissue was considered and rejected on the grounds that if the seed coats were not removed, the enzyme activity recorded reached a level only 2-3 times that of the endosperm tissue on its own. This weak endosperm α-galactosidase activity may play a role in germination and in particular the hydrolysis of the raffinose oligosaccharides, particularly stachyose, during germination. The oligosaccharides have been observed to be metabolised in the earliest stages of germination and broken down before the level of α-galactosidase increases dramatically, in the endosperm, at 20-24 hours germination(133)and galactomannan hydrolysis commences.

This very weak α-galactosidase level appearing during the galactomannan deposition period may relate to the low level of

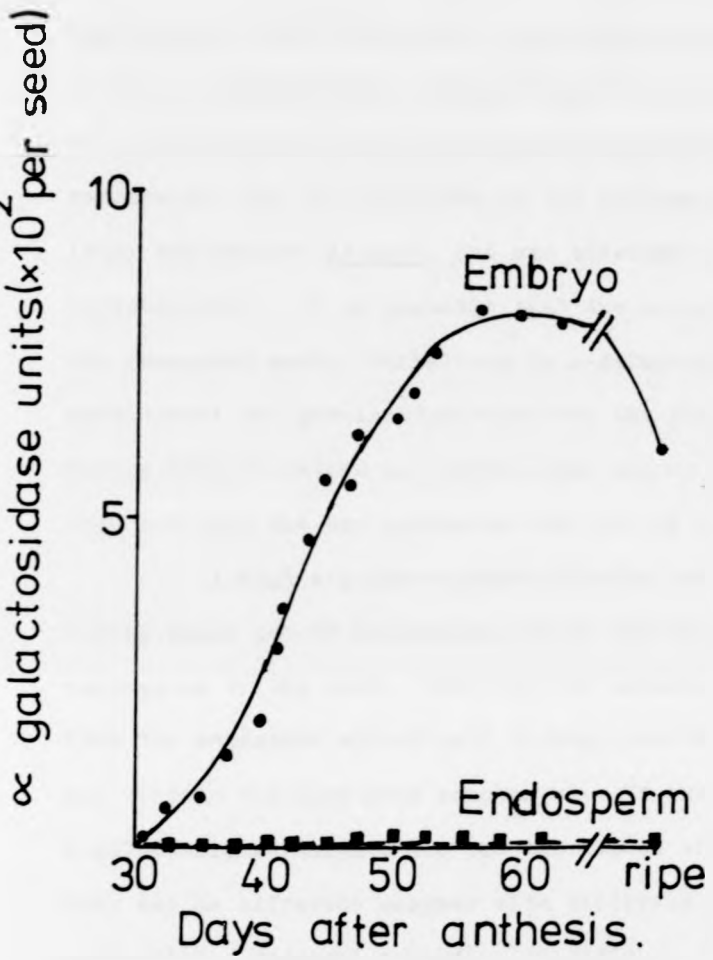


Fig. 3 α-Galactosidase activities in the endosperm and embryo during the period of galactomannan deposition.

activity encountered in the endosperm of the mature, ripe seeds of carob or Ceratonia siliqua (146). The weak enzyme activity in the ripe carob seeds could not be suppressed by inhibitors of transcription and translation and these inhibitors did not prevent degradation of the raffinose oligosaccharides in the endosperm. The very large proportion of the germinating seed's α-galactosidase responsible for the breakdown of the endosperm galactomannan was later synthesised de novo, and was affected by actinomycin D and cycloheximide. It is probable that the situation is similar in the fenugreek seed. Variations in α-galactosidase activity during development and germination reinforce the viewpoint that events during seed formation and germination cannot be analysed in isolation but that the two processes are part of an integrated whole.

A high α-galactosidase activity level in the endosperm during phase two of maturation, as in the embryo, might be disadvantageous to the seed. Yet both the endosperm and embryo originate from the megaspore mother cell through meiotic and mitotic divisions and contain the same gene complement. If the embryo and endosperm α-galactosidase enzymes are the expression of the same gene (but they may be different enzymes with different specificities) then regulation - repression and/or inhibition - of α-galactosidase in the endosperm must be enforced during maturation. With the onset of appropriate factors during germination release from regulation occurs. This might come about from the endosperm's proximity to the vascular flow from the parent plant, which could contain the necessary inhibitors as part of the plant hormones present therein (40). Such inhibitors could accumulate in the endosperm cells during the maturation process.

After 55 days of development the fenugreek seed has reached the end of its growth, with the major carbohydrate reserve being the endosperm, cell wall galactomannan. The accumulation of this storage polysaccharide is very marked and the cell walls of the endosperm become enlarged to the point of filling the cells, as seen in Fig. 4 (Plate 1)(132). The polysaccharide nature of the secondary cell wall material was corroborated after specific PAS carbohydrate staining of the sections, which gave the characteristic deep red colouration in the endosperm cell wall. The aleurone cell layer in the endosperm remains practically unfilled and stays living throughout maturation, ready to respond to the appropriate factors during germination and secrete the galactomannan hydrolytic enzymes (133).

"Semi in vivo" monosaccharide incorporation

Attempts were made to demonstrate incorporation of sugar residues into galactomannan by applying labelled monosaccharides to intact endosperm tissue halves. This technique has been called "semi in vivo" incorporation in this thesis to differentiate it from a purely in vivo or in vitro operation. It clearly lies between these two extremes as the tissue is intact and incubated in conditions applying in the seed (temperature, lack of light). Yet the seed's integrity as a whole has been broken, after disengaging the endosperm tissue from the overlying and underlying tissues.

Monosaccharides were applied, rather than sugar nucleotides as the latter would almost certainly not have been transported intact. Uptake was effected by floating endosperm halves on the radioactive solution under conditions which allowed oxygen to freely pass into the tissue whilst the underlying radioactive monosaccharide solution was imbibed. Levels of incorporation of radioactivity into

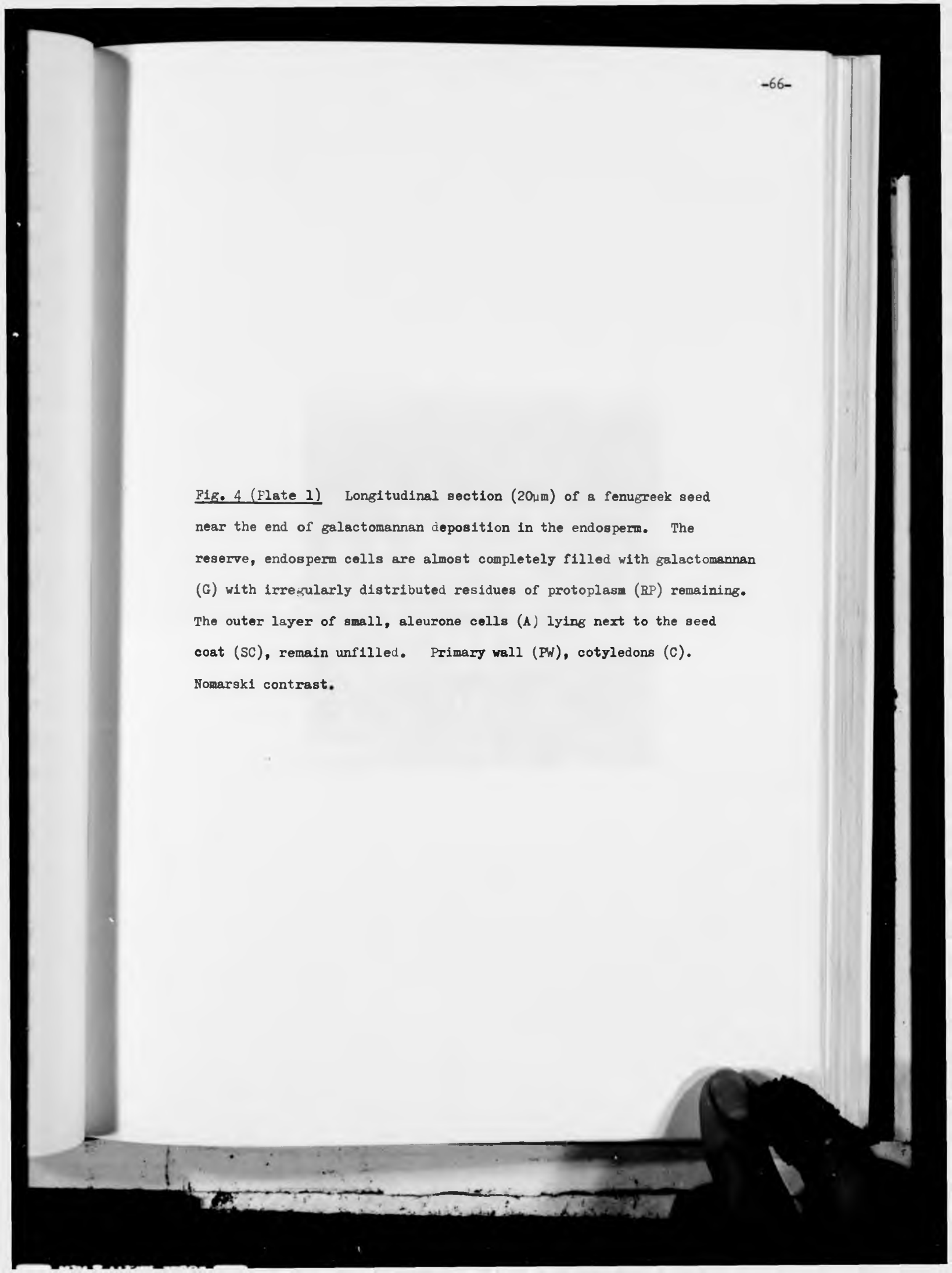
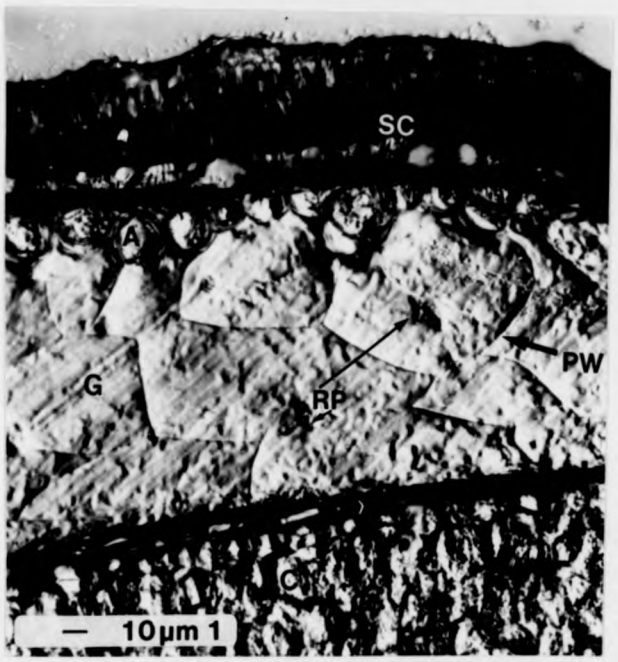


Fig. 4 (Plate 1) Longitudinal section (20µm) of a fenugreek seed near the end of galactomannan deposition in the endosperm. The reserve, endosperm cells are almost completely filled with galactomannan (G) with irregularly distributed residues of protoplasm (RP) remaining. The outer layer of small, aleurone cells (A) lying next to the seed coat (SC), remain unfilled. Primary wall (PW), cotyledons (C). Nomarski contrast.


SC
A
G
RP
PW
C
— 10µm 1

the endosperm galactomannan of endosperm taken from seeds atvarious maturity stages over the whole period of galactomannan deposition *in vivo*, are recorded for D-(U-^{14}C) galactose in Fig. 5 and for D-(U-^{14}C) mannose in Fig. 6. The pattern of incorporation would seem to follow the same format for the two sugars over the maturation phase, except that the maximum rate of incorporation for galactose appears slightly sooner than for mannose. For both sugars there appears a slow rise in incorporation from 30-44 days after anthesis, followed by a sudden sharp rise at 44 days which falls away after 46 days for galactose and after 48 days for mannose. These peaks correspond with the middle of the fast phase of galactomannan deposition *in vivo* (see Fig. 2, page 58).

For each precursor monosaccharide the radioactivity incorporated into the galactomannan was not exclusively in the corresponding sugar residue. With mannose some activity resided in the galactose component of the galactomannan and vice versa. During the maturation phase the mannose:galactose ratio of the incorporated radioactivity from mannose increased and decreased after the peak of incorporation had been passed. In the case of the substrate galactose solution some mannose residues of the galactomannan became labelled but the galactose residues always accounted for the greater part of the radioactivity within the polysaccharides.

Analysis of the low molecular weight sugars extracted from the tissue after the monosaccharide incubations showed clearly that for both radioactive precursor sugars the major components incorporating radioactivity were sucrose and stachyose (throughout the maturation phase). These two oligosaccharides always accounted for at least 90% of the radioactivity in the low molecular weight sugar fraction. With the galactose solution the stachyose oligosaccharide was more highly labelled than sucrose at the end of

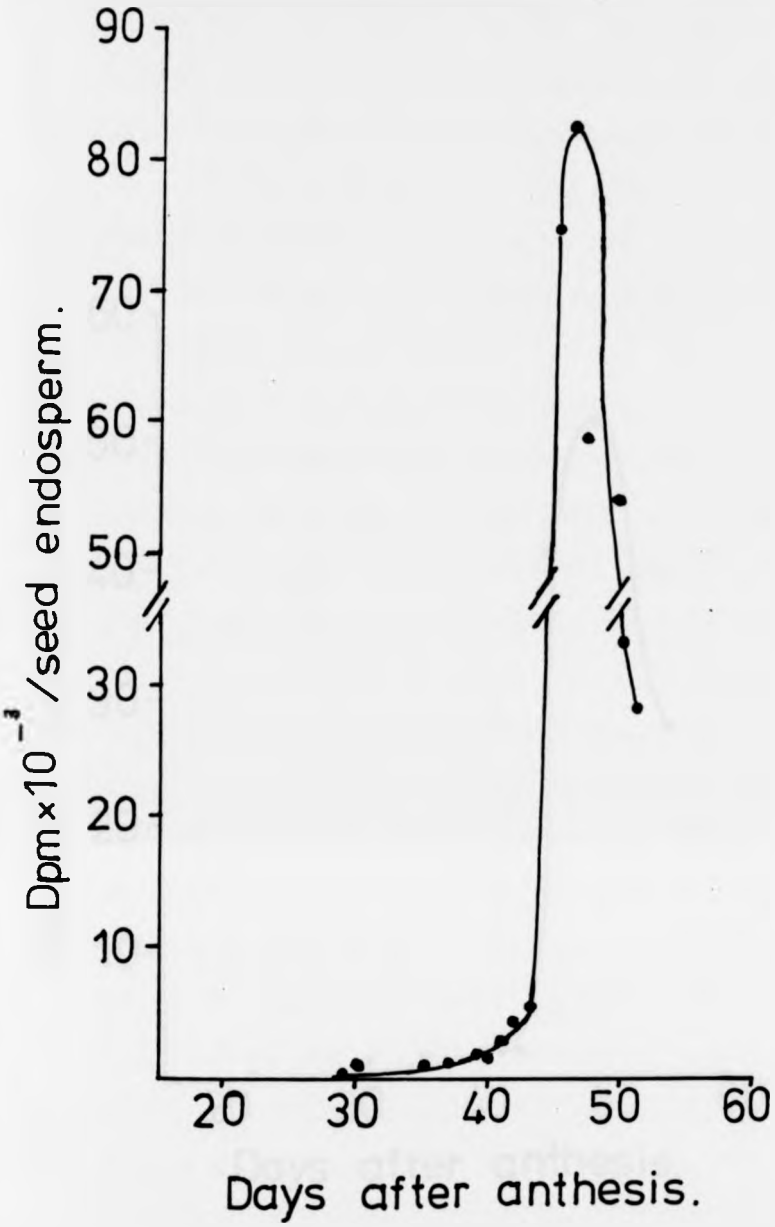


Fig. 5 "Semi in vivo" (U-^{14}C)D - Galactose incorporation into the galactomannan of intact endosperm halves.

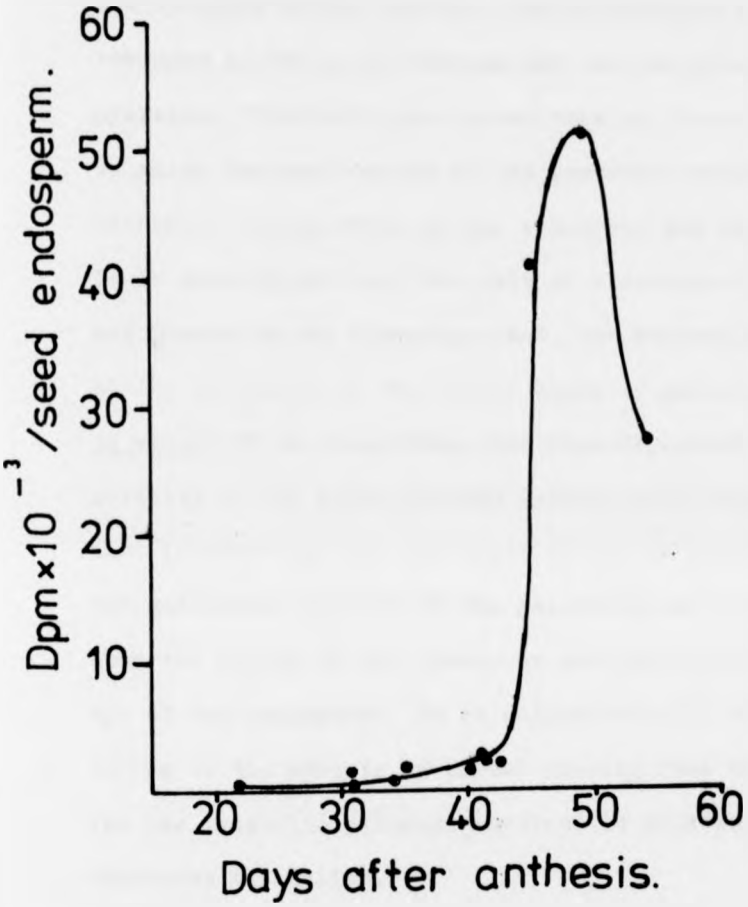


Fig. 6 "Semi in vivo" (U-^{14}C)D- Mannose incorporation into the galactomannan of intact endosperm halves.

maturation, 59% compared to 33%, respectively, whereas with the mannose solution the sucrose was more highly labelled than the stachyose at the end of maturation, 73% compared to 19%, respectively.

The results of the "semi in vivo" incorporation experiments are amenable only to qualitative interpretation. No data was obtained on the specific radioactivities of monosaccharide residues in the galactomannan and the low molecular weight carbohydrates. Metabolic pool sizes were not determined, and the extent to which the application of the precursor solutions perturbed the metabolic steady state in the endosperm was unknown. Nevertheless it is significant that the rate of incorporation of both D-galactose and D-mannose was time-dependent, the maximum rates being observed at the mid-point of the linear phase of galactomannan deposition in vivo. It is compatible with time-dependent variation of the activity of the galactomannan synthesising enzyme system. It is also interesting that the distribution of label between the mannose and galactose residues of the galactomannan is dependent not only upon the nature of the precursor monosaccharide but also upon the age of the endosperm. It is suggestive of a time-dependent modulation of the amounts of carbon passing from the labelled precursor via the metabolic pathways involved in galactomannan, sucrose and stachyose metabolism.

Studies in vitro, using cell free enzyme preparations from developing endosperm tissue.

The stage of seed development at which the galactomannan was laid down in the endosperm having been established, as well as the stage of development at which maximum incorporation of sugar was observed, it was possible to select endosperm tissue of an

appropriate stage of development for *in vitro* assays. An enzyme activity capable of transfering mannosyl units from GDP-(U-^{14}C) mannose to a soluble polysaccharide product was detected. The *in vitro* product was identified as a galactomannan by novel approaches to several precipitation techniques. This enzymic activity has been called by a trivial name throughout, galactomannan synthetase, and is more properly assigned the name GDP-mannose: galactomannan mannosyltransferase. Certain characteristics of the galactomannan synthetase were studied, and its subcellular distribution and activity at different stages of maturation observed. Less extensive investigation was also carried out on an enzyme capable of transfering galactosyl units from UDP-(U-^{14}C) galactose to the galactomannan.

(a) Preparation and assay of galactomannan synthetase activities

Seeds at varying stages of maturity, throughout phase two of maturation and beyond, encompassing the galactomannan deposition period, were taken and the endosperms removed, homogenised and incubated along with GDP-(U-^{14}C) mannose or UDP-(U-^{14}C) galactose.

Homogenisation and incubation conditions were found which gave very high percentage transfer (up to 60%) of radioactivity from GDP-(U-^{14}C) mannose to a soluble product which could be precipitated with 70% methanol along with carrier fenugreek galactomannan. The standard incubation mixture for the assay of the mannosyltransferase activity contained: Tris-HCL buffer pH 8.9 (see page 84); dithiothreitol (dTT, "Cleland's reagent) to stabilise protein sulphydryl group; Mg^{2+} ions (see page 85; and 94, 18, 48 , 35); UDP-galactose; and "primer" fenugreek galactomannan.

On differential centrifugation of the total homogenate the mannosyltransferase activity was distributed between a gross particulate or

"cell wall" fraction, a "particulate" fraction or 100,000g pellet and a "soluble" or post 100,000g fraction (see page 91). The homogenisation procedure adopted as standard was a brief grinding in a mortar in the presence of sand and polyvinyl polypyrrolidone (PVP, to complex phenols and quinones). This procedure gave a maximum yield of enzyme in the particulate fraction, and has also been found to give an optimum yield of intact organelle species from Pisum sativum (122). More severe homogenisation either brought about by a longer period in the hand mortar or by using a powered Potter homogeniser resulted in considerable denaturation of the particulate synthetase enzyme.

UDP- galactose galactosyltransferase activity was also detected in homogenates prepared as for the assay of the mannosyltransferase, and incubated in the presence of labelled UDP-galactose and unlabelled GDP-mannose. The levels of incorporation into high melocular weight material which were achieved however, were very low (1.5-2.0% of the incorporation from GDP(U-^{14}C) mannose, on a molar basis), and this activity was not pursued.

This very low galactosyltransferase activity may have been caused by the denaturation of this enzyme distinct from the mannosyltransferase, or a lack of a specific independent cofactor. It was suspected, however that a further contributing factor to the low level of incorporporation into the galactomannan co-precipitate might be the presence of other enzymes in the homogenate capable of transferring galactosyl units from UDP-galactose to methanol soluble products. A homogenate was incubated in the standard manner and the 70% MeOH soluble material was subjected to paper chromatography in solvent (B). Radioactivity other than that in the residual UDP-galactose was detected on the chromatogram, and

85% of it [some 14% transfer from UDP-(U-^{14}C) galactose) resided in the stachyose region of the chromatogram. Solvent (B) is incapable of resolving stachyose and its immediate metabolic precursor galactinol (see page 61). The stachyose region was therefore eluted and rechromatographed in solvent (C) (see Table 3). Although much of the stachyose had obviously been inadvertently hydrolysed during evaporation of the acidic solvent (B) (hence the peak of radioactivity in the melibiose and galactose regions), there is a small peak of radioactivity which corresponds to galactinol. It is clear that galactinol is being formed at the same time as stachyose in this endosperm cell free system and in fact may act *in vivo* as the precursor for galactose units in stachyose as found in the dwarf bean seed (162).

(b) Identification of the synthetase in vitro product

A vital question which must be answered before any direct relevance of the *in vitro* investigation to the *in vivo* operation of the polysaccharide synthetase is claimed, is whether the product of the reaction is in fact the naturally occurring polysaccharide. Tests to identify the *in vitro* product of the "galactomannan synthetase" (particulate mannosyltransferase) from fenugreek endosperm were undertaken.

The material precipitated by 70% MeOH from the GDP-(U-^{14}C) mannose incubation mixture was washed with 70% MeOH, hydrolysed and subjected to paper chromatography in solvent (A). On development of a paper with development reagent (1), mannose and galactose were found in approximately equal amounts. When a further paper was cut into strips and these were counted, radioactivity was found only in the mannose region. No radioactivity

Table 3 Chromatographic separation of "stachyose" component[+] from the low molecular weight carbohydrates after incubation of an endosperm homogenate with UDP-[U-^{14}C] galactose

Migration distance (cm)	Radioactivity (dpm)	Standard mobilities [*]
0 - 1.0	0	
1.0 - 2.5	0	
2.5 - 4.0	0	
4.0 - 5.5	0	Stachyose
5.5 - 7.0	0	
7.0 - 8.5	0	
8.5 -10.0	111	Galactinol
10.0 -11.5	0	
11.5 -13.0	6	(Raffinose)
13.0 -15.0	3,402	
15.0 -17.0	3,792	Myo-inositol (Melibiose)
17.0 -19.0	337	
19.0 -21.0	0	
21.0 -23.0	0	
23.0 -25.0	12	
25.0 -27.0	180	
27.0 -29.0	189	Galactose (Glucose)
29.0 -32.0	168	
32.0 -35.0	0	
35.0 -38.0	0	

+ After chromatography in solvent (B) of the soluble carbohydrate fraction (70% MeOH soluble) from the synthetase incubation, the "stachyose" region was eluted and re-chromatographed in solvent (C).

* Un-bracketed standards were run on the same paper as the "stachyose" fraction. Bracketed standard mobilities are shown as a guide, from published work (148).

was detected in the galactose region and no activity was associated with the area of the paper which would have been occupied by glucose or any other monosaccharide.

To determine whether the mannose was indeed incorporated into galactomannan a number of tests were carried out designed to differentiate between galactomannan and other mannose-containing plant polysaccharides. These were then applied to the in vitro product. The only other plant polysaccharides known to contain mannose are the mannans, the glucomannans and the galactoglucomannans. The first two are normally insoluble in water and would in any case on the basis of their linear structures (see page 3) be easily separable from a galactomannan. Galactoglucommans are water soluble polysaccharides with structures similar to that of galactomannan except that glucose is present in the main chain and the percentage of galactose in them is smaller than in most galactomannans (see page 6). Methods were therefore sought which were capable of separating artificial mixtures of fenugreek galactomannan and a soluble galactoglucomannan from spruce wood (92; mannose: glucose: galactose equal to 5.5:1.8:1.0). It should, of course, be borne in mind that no mannan, glucomannan or galactoglucomannan has ever been isolated from the fenugreek seed.

High voltage electrophoresis in borate buffers (46 ,172) was considered as a means of characterising the in vitro product but this was abandoned when no discrete separation of fenugreek galactomannan and spruce galactoglucomannan could be achieved. Attempts to identify the product by enzymic stripping of galactose side chains to leave an insoluble mannan had also to be abandoned. A purified α-galactosidase from germinating fenugreek seed (127) removed only a small proportion of the side chains

from the fenugreek galactomannan, leaving the residue water-soluble. This confirms other observations (34) that α-galactosidases cannot hydrolyse all the galactosyl residues from a galactomannan unless accompanied by other activities capable of hydrolysing the mannosyl linkages of the main chain (see page 11).

A novel and much more successful approach to the problem of identifying the in vitro product was to develop selective galactomannan precipitants.

The first precipitation test applied to the synthetase in vitro product was gellation with borate ions. Borate has been shown to complex with the galactomannan from carob (1930) (63) and later was shown to react similarly with several seed galactomannans (1949) (3). It has been shown that other polysaccharide molecules do not give this specific reaction e.g. pectic acid, mannan, gum arabic (37). The sensitivity of galactomannan solutions to borate ions has been attributed to the cis- glycol groups on carbon-2 and carbon-3 of the mannose residues (104). The specificity of this test in relation to fenugreek galactomannan and the spruce galactoglucomannan was observed, when each polysaccharide was treated alone and then a mixture of both was tested. Fenugreek galactomannan produced a gel, whilst spruce galactoglucomannan did not react, and an equal mixture of the two polysaccharides formed a gel which when decomplexed was shown by paper chromatography to be galactomannan and the supernatant solution contained only galactoglucomannan. With the introduction of glucose, having trans- glycol grouping at carbon-2 and carbon-3, in one-quarter of the main chain residues, then sufficient cross linking with borate ions is not achieved by

the galactoglucomannan. The result of testing the synthetase *in vitro* product with borate ions is shown in Table 4. All the labelled product normally recovered by precipitation with 70% MeOH was recovered as the borate gel. No further labelled material could be precipitated from the borate soluble fraction after treatment with methanol. This test indicates that the mannose residues from GDP mannose are incorporated into a polysaccharide similar in structure to the native galactomannan, and not a soluble galactoglucomannan.

Table 4 Identification of the *in vitro* particulate galactomannan synthetase product.

Test	Product isolated	*Incorporation level (dpm) 1	2	3	4
Borate gellation:	Standard 70% MeOH	1192	1026		
	Borate precipitate (S)	1190	1182		
Lectin precipitation:	Standard 70% MeOH	98656	90248	59058	73210
	Lectin precipitate	+89150	+84285	+24167	++71725
	70% MeOH precipitate from lectin soluble	10000	15080	31924	zero
Metal ion(Mn^{2+}) precipitation:	Standard 70% MeOH	12708			
	Metal ion precipitate	12672			

* Radioactivity incorporated into the designated fractions following a standard incubation. Different enzyme preparations were used for each test and each experiment (1,2,3 and 4).

(S) = Sonicated. Sonication of the compacted gel before counting was essential, otherwise counting was not reproducible.

\+ Lectin equivalent to added primer.

++ Excess lectin.

Ricinus communis lectin (RCA - II, castor bean haemagglutinin - molecular weight of 120,000) has been shown to bind galactomannans. Equilibrium dialysis data showed that the lectin contained two identical and independent binding sites per molecule, and hapten inhibition studies showed that galactopyranosyl glycosides are favoured in binding to this protein (169). Also worth noting from this report was that more carob seed galactomannan (mannose:galactose = 4:1) was required to cause maximal precipitation of a standard amount of lectin than fenugreek galactomannan (mannose:galactose = 1.1). It was considered that the lectin precipitation of seed galactomannans was due primarily to the side chain galactopyranosyl units.

Using the system employed for precipitation of fenugreek galactomannan by Van Wauwe et al, enough lectin was added to precipitate all of the original primer galactomannan in the synthetase *in vitro* assay. The precipitate was radioactive and contained nearly all the activity which would have been isolated in the standard manner by 70% MeOH (see 1,2 and 3 in Table 4). However a proportion of labelled high molecular weight material remained in solution and was precipitable with methanol, after removal of the lectin precipitate. When two consecutive lectin incubations of the *in vitro* assay system were undertaken and the precipitates pooled then it was found that all the polymeric labelled product was present in the galactomannan-lectin complex. No further precipitate was obtained with methanol. This combined activity was similar to the activity from the standard isolation procedure (experiment 4, Table 4). Presumably in the first three lectin precipitation experiments there was not enough lectin to precipitate all the

galactomannan present. The excess galactomannan was almost certainly present in the enzyme preparations, and this can be inferred from the observed non-essentiality of primer galactomannan in the synthetase assay (see page 87). That all the *in vitro* product of the particulate synthetase can be precipitated with castor bean lectin shows that the labelled product is a high molecular weight soluble molecule with galactosyl residues which interact with the bivalent lectin causing aggregation.

The precipitation of galactomannan with transition metal ions to form a gel-like product with industrial applications as a gumming agent, has been carried out (26) but the specificity of the reactions have not been reported. Treatment of fenugreek galactomannan, spruce galactoglucomannan or a mixture of both showed that with Cr^{3+}, Mn^{2+} and Fe^{3+} ions only the galactomannan formed insoluble complexes (see Fig 7(a), (b) and (c)). The galactomannan was completely precipitated, whereas the galactoglucomannan remained completely soluble. When the polysaccharide mixture was treated the decomplexed precipitates (gel-like for Cr^{3+} and Fe^{3+} ions) were shown by hydrolysis and paper chromatography to be galactomannan. The soluble polysaccharide recovered by methanol precipitation proved to be the galactoglucomannan. Even with a more concentrated solution of galactoglucomannan (1.25% instead of 1%) together with the galactomannan, no galactoglucomannan polysaccharide was precipitated. Precipitation of the synthetase *in vitro* product with Mn^{2+} ions showed that all the activity present in a standard 70% MeOH isolated product was also precipitable with Mn^{2+} (Table 4). This test also points to the synthetase *in vitro* product being of the same molecular structure as the native galactomannan.

Fig. 7 a. Hydrolysates of the decomplexed metal ion precipitates of fenugreek galactomannan alone or a mixture of fenugreek galactomannan and spruce galactoglucomannan.

G-M: standard fenugreek galactomannan hydrolysate.

ggm: " spruce galactoglucomannan hydrolysate.

M+G: " mannose and galactose.

1-3: galactomannan solution only.

4: mixture of galactomannan solution and galactoglucomannan solution A.

5-7: mixture of galactomannan solution and galactoglucomannan solution B.

1,4,5: treated with $Cr(NO_3)3$

2,6: " " $Mn(CH_3COO)_2$

3,7: " " $Fe(NO_3)_3$

All solutions are 1% (w/v), except galactoglucomannan solution B which is 1.25% (w/v).

Fig. 7 a.

Fig. 7 b. Hydrolysates of the isolated, soluble polysaccharide after metal ion precipitation of a mixture of fenugreek galactomannan and spruce galactoglucomannan.

G-M: standard fenugreek galactomannan hydrolysate.

ggm: " spruce galactoglucomannan hydrolysate.

M+G: " mannose and galactose.

8: mixture of galactomannan solution and galactoglucomannan solution A.

9-11: mixture of galactomannan solution and galactoglucomannan solution B.

8,9: treated with $Cr(NO_3)_3$

10: " " $Mn(CH_3COO)_2$

11: " " $Fe(NO_3)_3$

All solutions are 1% (w/v), except galactoglucomannan solution B which is 1.25% (w/v).

Fig. 7 b.

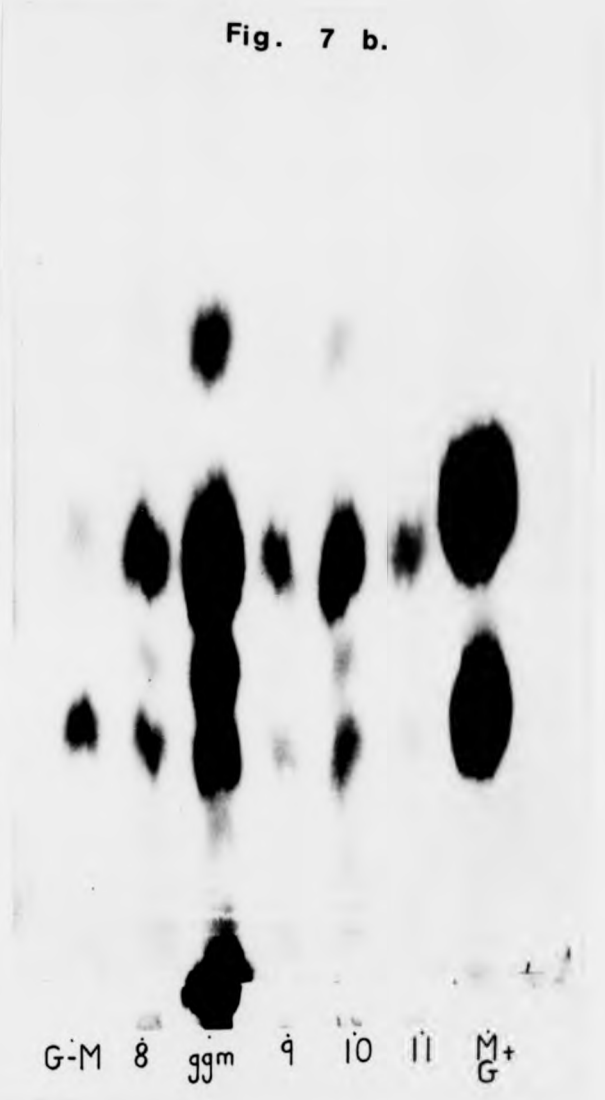


Fig. 7 c. Hydrolysates of the isolated, soluble polysaccharide after metal ion precipitation of spruce galactoglucomannan.

ggm: standard spruce galactoglucomannan hydrolysate.

12-14: galactoglucomannan solution A.

15-17: " " B.

12,15: treated with $Cr(NO_3)_3$

13,16: " " $Mn(CH_3COO)_2$

14,17: " " $Fe(NO_3)_3$

All solutions are 1% (w/v),

except galactoglucomannan solution B which is

1.25% (w/v).

Fig. 7 c.

The solubility characteristics of the *in vitro* product of the particulate synthetase show that it is water soluble and also high molecular weight. The accumulated evidence of the selective precipitation techniques provide more information and show that the synthetase *in vitro* product is the same as the naturally occurring galactomannan. In this system, mannosyl residues are transferred from GDP-mannose to galactomannan.

(c) Characteristics of the galactomannan synthetase (GDP mannose: galactomannan mannosyltransferase)

Several different factors were tested to see what the particulate synthetase enzyme's *in vitro* characteristics were. The effects of the following on enzymic activity was investigated: pH; cations; dialysis; attempted solubilisation; and the influence of substrate concentration. The possibility that the reaction might involve a lipid intermediate was also briefly tested. The experiments are detailed and discussed individually below with a general summary following.

pH optimum. The effect of varying the pH of the incubation medium, from pH 5.8 to pH 9.8, upon galactomannan synthetase activity is shown in Fig. 8. The optimum pH value for the reaction was pH 8.9, with half optimal activity at approximately pH 7.9 and pH 9.1. A degree of stimulation of the galactomannan synthetase is produced by the Tris-HCl buffer system itself relative to the two other buffers used. This can be seen at pH 7.5 and pH 8.9 where the range of the Tris-HCl buffer system overlaps with the ranges covered by the two other buffer systems. An alkaline pH optimum is not unusual for plant polysaccharide synthetases (see for example 69, 150) and stimulation by Tris-HCl buffer has been observed for plant β-glucan synthetase activities (118a). The quite high pH optimum might also indicate that the enzyme is perhaps isolated from the general cytoplasm in the cell whose pH is generally held to be around 7.0 (see for example 102, 57a).

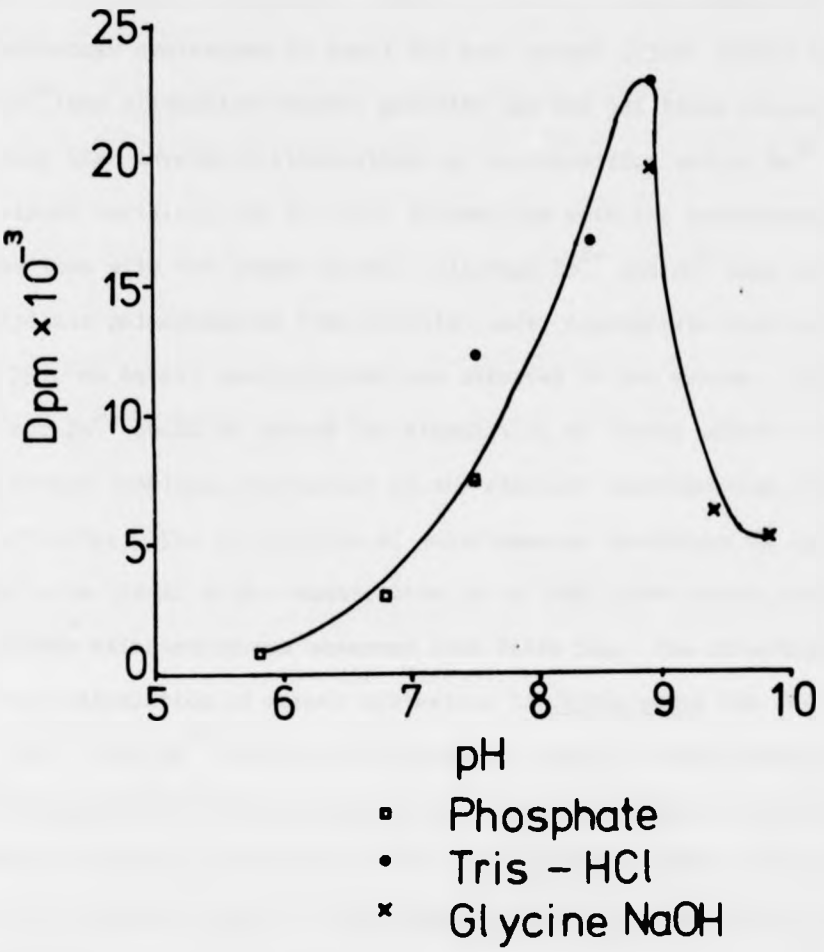


Fig. 8 Effect of pH on the transfer of ^{14}C mannose from GDP-(U-^{14}C) mannose to galactomannan in vitro using the particulate enzyme. (See Materials and Methods).

Cations. The effect of various cations (all at 10mM in the assay) upon galactomannan synthetase activity was studied and the results are given in Table 5A. Both Mg^{2+} and Ca^{2+} ions stimulated the galactomannan synthetase to about the same extent (23-24 times) but Mn^{2+} and Co^{2+} ions stimulated enzymic activity 348 and 524 times respectively. The very high levels of stimulation by the transition metals Mn^{2+} and Co^{2+} are almost certainly due to their interaction with the galactomannan product rather than with the enzyme alone. Although Co^{2+} and Mn^{2+} ions will precipitate galactomannan from solution under appropriate conditions (see page 79), no actual precipitation was observed in the assays. Neither Fe^{2+} nor Zn^{2+} could be tested for stimulation of enzyme activity because they formed insoluble hydroxides at the standard concentration (10mM) in the pH 8.9 buffer. The stimulation of galactomannan synthetase by Mg^{2+} ions was shown to be linear with concentration up to 10mM, above which concentration no further stimulation was observed (see Table 5B). The investigation of the cationic stimulation of mannan synthetase in Orchis morio has shown that Co^{2+}, Mn^{2+}, and Mg^{2+} in descending order of magnitude have a positive, stimulatory effect on this enzyme (48). The stimulation of galactomannan synthetase by Mn^{2+} and Co^{2+} was very much higher and almost certainly indicates a large element of ion-product interaction, like that observed with galactomannan preparations.

Table 5 Effect of cations on the particulate synthetase enzyme, and the optimum concentration of Mg^{2+} ions.

A. Cation	Relative incorporation level	B. Mg^{2+} conc (mM)	Incorporation activity (dpm)
None	1	0	71
Mg^{2+}	23	1	620
Mn^{2+}	348	3	831
Cu^{2+}	9	5	1127
Ca^{2+}	24	7	1385
Co^{2+}	524	10	1518
K^{+}	2	20	1525

Dialysis. A more complete understanding of the relevance of cations and other cofactors to the synthetase activity was gained from an investigation of the effect of dialysis on the particulate enzyme. When dialysis of the resuspended particulate enzyme was carried out at room temperature in dialysis membrane tubing against running tap water (16 hours) then distilled water (corrected to pH 8.9, 4 hours), it was found that all activity had been lost in the preparation and could not be recovered by addition of metal ions or other cofactors. When dialysis was effected rapidly using a micro-pressure dialyser/concentrator at 4^{o} (approx. 5 hours) against 0.05M Tris-HCl buffer (pH 8.9) then the preparation again lost activity but it could be reinstated (see table 6). It was found that the dialysed

Table 6. Effect of dialysis+ on the particulate synthetase enzyme

Test	Radioactivity (dpm)	Relative activity (%)
Particulate enzyme (s)	101,065	100.0
Dialysed enzyme alone	471	0.5
Dialysed + UDP-galactose	585	0.6
Dialysed + primer	624	0.6
Dialysed + Mg^{2+}	104,724	103.0
Dialysed + UDP-galactose, primer, and Mg^{2+}	84,915	84.0
Dialysed + diffusate only	759	0.8

+ Dialysis was effected against 0.05M Tris HCl buffer (pH 8.9) in an Amicon micro pressure dialyser/ concentrator at 4^{o}C.

(S) Standard incubation of particulate enzyme source used for dialysis.

enzyme on its own was almost incapable of transferring sugar residues to form the product, yet when Mg^{2+} ions were added back to the assay system the enzyme was fully active. The addition of galactomannan primer or UDP-galactose on their own to the assay did not activate the dialysed particulate enzyme. When these two components were present along with Mg^{2+} ions the activity of the enzyme was somewhat decreased over the enzyme plus Mg^{2+} ions alone. It is clear from this experiment that a divalent cation (Mg^{2+}) is an absolute requirement for mannosyltransferase activity. Furthermore sufficient endogenous primer is present in the enzyme preparation to allow transfer of sugar residues. This was confirmed by the observation that when a dialysed enzyme preparation was reactivated by Mg^{2+} ions, the product of the primer-free incubation was precipitable by castor bean lectin. The dialysis experiment shows furthermore that the inclusion of UDP-galactose in the standard assay mixture was unnecessary.

Attempted solubilisation. Several attempts at solubilisation of the particulate synthetase enzyme proved to be unsuccessful in that the enzyme was denatured. The use of the detergent, Triton X-100, and the bile salt, deoxycholate, at the 0.5% and 0.1% levels (final concentrations, treatment at $4^{\circ}C$ throughout) both gave a solubilised protein which was very low in activity relative to the original particulate enzyme. The solubilised activity was less than 4% of the original particulate activity after both the 0.5% and 0.1% treatments. The residual particulate enzymes after treatment and separation of the solubilised and particulate enzymes also proved to be denatured (10% activity remaining after 0.1% treatment and 2% activity after 0.5% treatment in each case).

When the resuspended particulate enzyme was treated once with digitonin, 0.8% final concentration, approx. 17% of the activity was solubilised with the residual particulate enzyme retaining 50% of the original particulate activity. [A similar solubilisation schedule by digitonin of "cellulose synthetase" from mung beans by Liu and Hassid (94) with two consecutive treatments of the enzyme, produced a solubilised synthetase representing 104% of the activity of the residual particulate synthetase. If a second, repeated solubilisation procedure produced the same percentage solubilisation in galactomannan synthetase as the first, then approx. 103% activity of the soluble enzyme over the residual particulate synthetase would result.] The digitonin schedule with its protection of most of the particulate synthetase during treatment and maintenance of an active soluble enzyme, seemed a feasible way of solubilising sufficient enzyme to effect further purification. Treatment of the digitonin-solubilised galactomannan synthetase with successive ammonium sulphate concentrations resulted in the loss of all enzymic activity and no further purifications were attempted.

Substrate concentration. The effect of altering substrate concentration upon the velocity of the reaction is shown in Fig. 9. The graph of concentration against velocity (Fig. 9A) appears hyperbolic up to 77μM UDP-(U-^{14}C) mannose (normal Michaelis-Menten kinetics) and inhibition at higher concentrations is apparent. A Lineweaver-Burk plot of these results (Fig. 9B) is linear for the first four concentrations and the K_m is obtained about 33μM. This is around the same value (55-62μM) obtained for the mannose-polymerising enzyme from Phaseolus aureus studied by Heller and Villemez (68). A similar type of enzyme inhibition at higher substrate concentrations has been observed for another particulate transferase, from

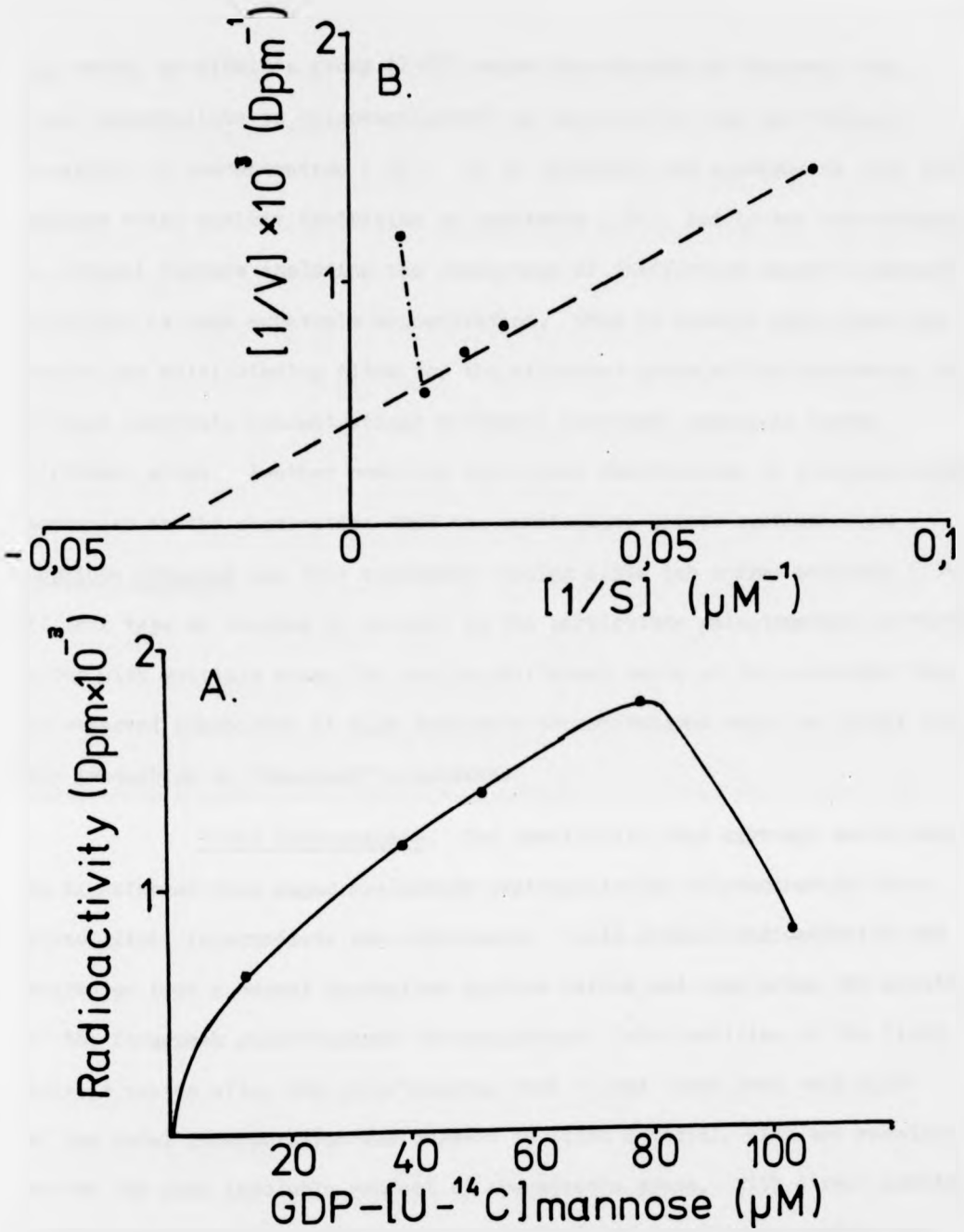


Fig. 9 A. Effect of substrate concentration upon particulate synthetase activity. B. Double reciprocal plot of velocity and substrate concentration.

pig aorta, by Elbein's group (1977) where the transfer of mannose, via a lipid intermediate to oligosaccharides of glycoprotein was particularly sensitive to concentration (25). It is certainly not uncommon to find other enzymes which exhibit inhibition by substrate (39), and it has been attributed to several factors including the production of ineffective enzyme-substrate complexes at high substrate concentration. This is brought about when the enzyme has multi-binding sites for the different parts of the substrate, and at high substrate concentrations different substrate molecules occupy different sites. Another possible additional complication of substrate-binding mechanism is the observation that the particulate chitin synthase from Coprinus cinereus has four substrate binding sites per enzyme molecule (36). If this type of feature is present in the particulate galactomannan synthetase along with multiple sites for binding different parts of the substrate then an observed inhibition at high substrate concentrations would be likely from the production of "dead-end" complexes.

Lipid intermediate. The possibility that glycosyl units might be transferred from sugar nucleotide residues to the polysaccharide via a glyco -lipid intermediate was considered. Lipid soluble radioactivity was extracted from a normal incubation mixture before and also after the addition of the fenugreek galactomannan co-precipitant. With addition of the lipid solvent system after the galactomannan then it was found that only 0.7% of the total incorporation was present in lipid material, with the remainder as the 70% meOH insoluble product of the aqueous phase. With direct addition of the lipid solvent system then only 2.8% of the total incorporation was present in lipid material, again with the remainder as the 70% MeOH insoluble product of the aqueous phase. From these results it would seem that no appreciable pool of lipid intermediate could be involved in the galactomannan

synthetase reaction. Using a similar lipid isolation procedure for the mannan synthetase of orchid tubers it was found that almost equal amounts of radioactivity were incorporated into lipid material and into the polysaccharide (48). A lipid intermediate, possibly a polyprenol, was postulated for this system.

From the above experiments several points relating to the enzyme's characteristics are clear. The particulate galactomannan synthetase has an optimum pH of 8.9 and has an absolute requirement for a divalent cation. The stimulation of enzymic activity is optimal at 10mM for Mg^{2+} ions. The observed, very large incorporation values in the presence of Mn^{2+} or Co^{2+} ions are most likely to be caused by product interaction, from their known ability to complex galactomannans. From a dialysed preparation it is obvious that endogenous galactomannan is present in the enzyme preparation. An active soluble enzyme can be produced by the action of digitonin on the particulate enzyme, with some loss of overall activity. No evidence was obtained for a lipid intermediate being involved in the transfer of sugar residues from the sugar nucleotide to the galactomannan. The possibility of multi-binding sites for distinct parts of the substrate seems likely from the observed inhibition of enzymic activity at high substrate concentrations.

The temporal and subcellular distribution of galactomannan synthetase.

(a) Temporal distribution

Total galactomannan synthetase in endosperm homogenates from fenugreek seeds was assayed at all stages of development. Results of this investigation are presented in Fig.10B, and show that the enzyme is present only during the phase of endosperm galactomannan deposition (from 30-55 days after anthesis). The activity peaks at 37 days after anthesis and again at

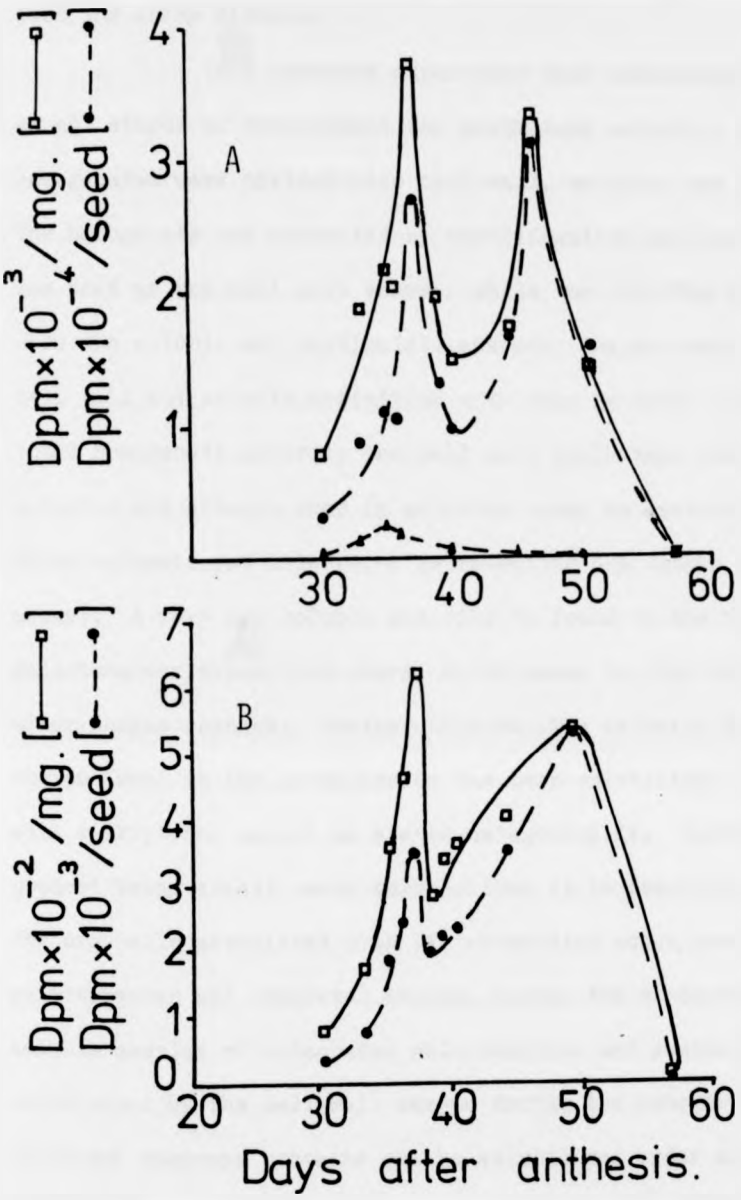


Fig. 10 A. Development of cell wall (——) and soluble (- - -) galactomannan synthetase within the endosperm tissue, during seed development. B. Levels of galactomannan synthetase in total endosperm homogenates, during seed development.

46 days after anthesis. The major peak represents 60% uptake of radioactivity from the assay mixture.

In a separate experiment seed endosperms were again assayed at all stages of development for synthetase activity, but this time the homogenates were divided into cell wall, soluble, and particulate enzymes. The homogenate was separated by centrifugation and the washed 1,000g pellet was used as the cell wall enzyme, while the 100,000g supernatant and pellet were the soluble and particulate enzymes, respectively. The variation of the cell wall and soluble activities with time is shown in Fig. 10A. Like the total homogenate activity the cell wall synthetase peaks at 37 days after anthesis and after a drop in activity rises to another peak around 49 days after anthesis (at this point representing 32% uptake of radioactivity in the assay). A very low soluble activity is found in the homogenates during the galactomannan deposition phase, which seems to rise in parallel with the other enzyme changes. Whether this soluble activity is truly an enzyme source found in the cytoplasm or has been solubilised by organelle or cell wall disruption, cannot be stated categorically. With the galactomannan product being itself water soluble then it is feasible that a proportion of the organelle associated with its production could have lost newly synthesised galactomannan and complexed enzyme, during the homogenisation step. Also a soluble complex of integrated galactomannan and enzyme may have been contributed by the cell wall enzyme during the homogenisation. In certain instances membrane proteins can be solubilised under dialysis conditions (116) and therefore not all membrane associated proteins are held strongly to the membrane in question. This very low soluble enzyme activity may indeed be an artefact of the homogenisation procedure.

The activity levels of the particulate galactomannan synthetase

during endosperm development are given in Fig.11. The peak of activity around 35 days after anthesis corresponds with the beginning of the fast phase of galactomannan deposition *in vivo*. This activity represents 41% uptake of radioactivity from the assay. By 40 days after anthesis the activity has reached as low a level as encountered at the beginning of phase two, 30 days after anthesis, and by 50 days after anthesis no discernible level of activity remains in the particulate fraction.

The highly active particulate galactomannan synthetase appears at the beginning of galactomannan accumulation in the endosperm, and this is a feature of the particulate β-glucan synthetase of developing cotton fibres which has an active peak just at the onset of secondary wall synthesis and falls off well before the end of deposition (35). The appearance two days later of a peak of galactomannan synthetase in the cell wall would suggest that the particulate synthetase, as the subcellular origin of galactomannan synthetase, moves to the growing cell wall along with preformed galactomannan (see also101). Near the end of the *in vivo* galactomannan deposition phase, from 45-55 days after anthesis, virtually all the endosperm cell's galactomannan synthetase activity is associated with the cell wall. Knowing from previous observations (101) that during deposition galactomannan containing ER vesicles in the protoplasm transfer their contents into the growing cell wall across the plasmalemma, then the particulate glactomannan synthetase would seem to be active *in vivo* in synthesising galactomannan (also see page 111). That biosynthesis does not only take place in the protoplasm is obvious from the cell wall activities during phase two. That the *in vitro* assay system has in fact been measuring an *in vivo* activity would seem to be the case from the period of maturation covered

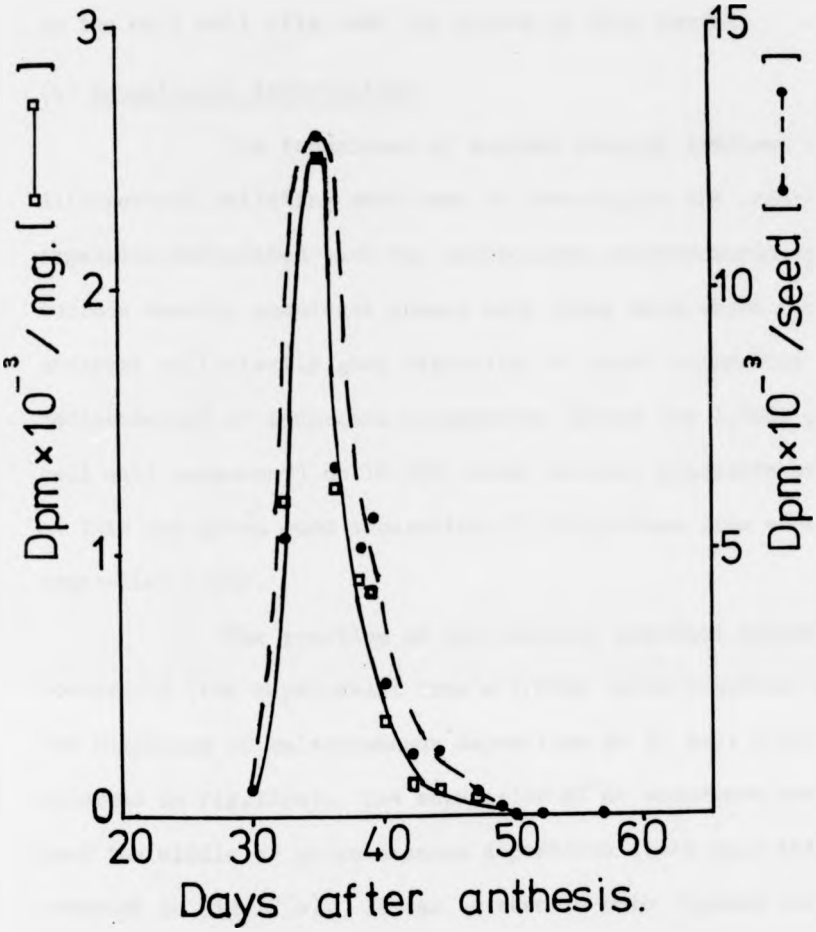


Fig. 11 Development of particulate galactomannan synthetase within the endosperm tissue, during seed development.

by the galactomannan synthetase, and from the apparent transference of activity from the protoplasm in the early part of galactomannan deposition to the cell wall site near the middle of this period.

(b) Subcellular distribution

The techniques of sucrose density gradient centrifugation and differential pelleting were used to investigate the nature of the protoplasmic organelle associated with the particulate galactomannan synthetase. The sucrose density gradients chosen were those with which previous authors achieved sufficiently good separation of plant organelles. The velocity sedimentation of endosperm homogenates (minus the 1,000g pellet i.e. the cell wall component) on 10-35% linear sucrose gradients was initially chosen as this has given good separation of dictyosomes from other cellular organelles (122).

The profiles of the velocity gradient separation of an endosperm homogenate (the supernatant from a 1,000g centrifugation) of seeds just after the beginning of galactomannan deposition at 36 days after anthesis, is recorded in Fig.12(a). The separation of an endosperm homogenate of seeds past the middle of galactomannan deposition at 46 days after anthesis is recorded in Fig.12(b). It can be seen in both figures that the major peak of galactomannan synthetase in the gradient is associated with a low density fraction at the top of the gradient, at 1.06g/ml, with no other appreciable synthetase component in the gradient. The corresponding velocity separation of β-glucan synthetase in Pisum sativum (122) showed that golgi-associated activity resided at a density of about 1.09g/ml, well into the sucrose gradient. Analysis of a velocity sedimentation gradient under exactly the same conditions as in Fig.12 showed that the only portion of the endosperm homogenate in the gradient containing any NADH-cytochrome C reductase

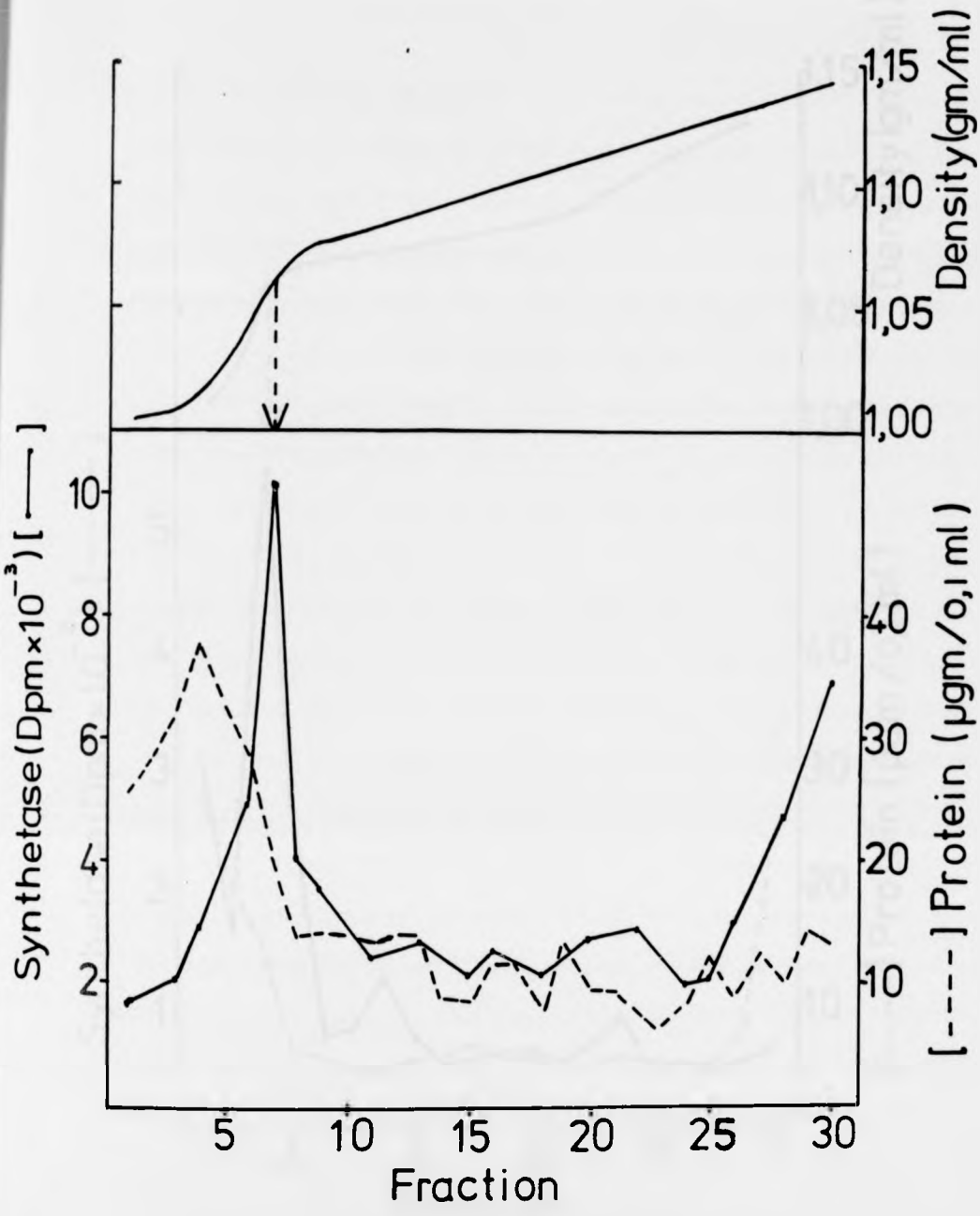


Fig. 12 (a) Velocity sedimentation behaviour of 1,000g supernatent from an endosperm homogenate, in a 15 - 35% linear sucrose gradient. Arrow shows the 15% sucrose fraction (1.0620 gm/ml) i.e. top of gradient. (See Materials and Methods for analysis of all sucrose gradients.)

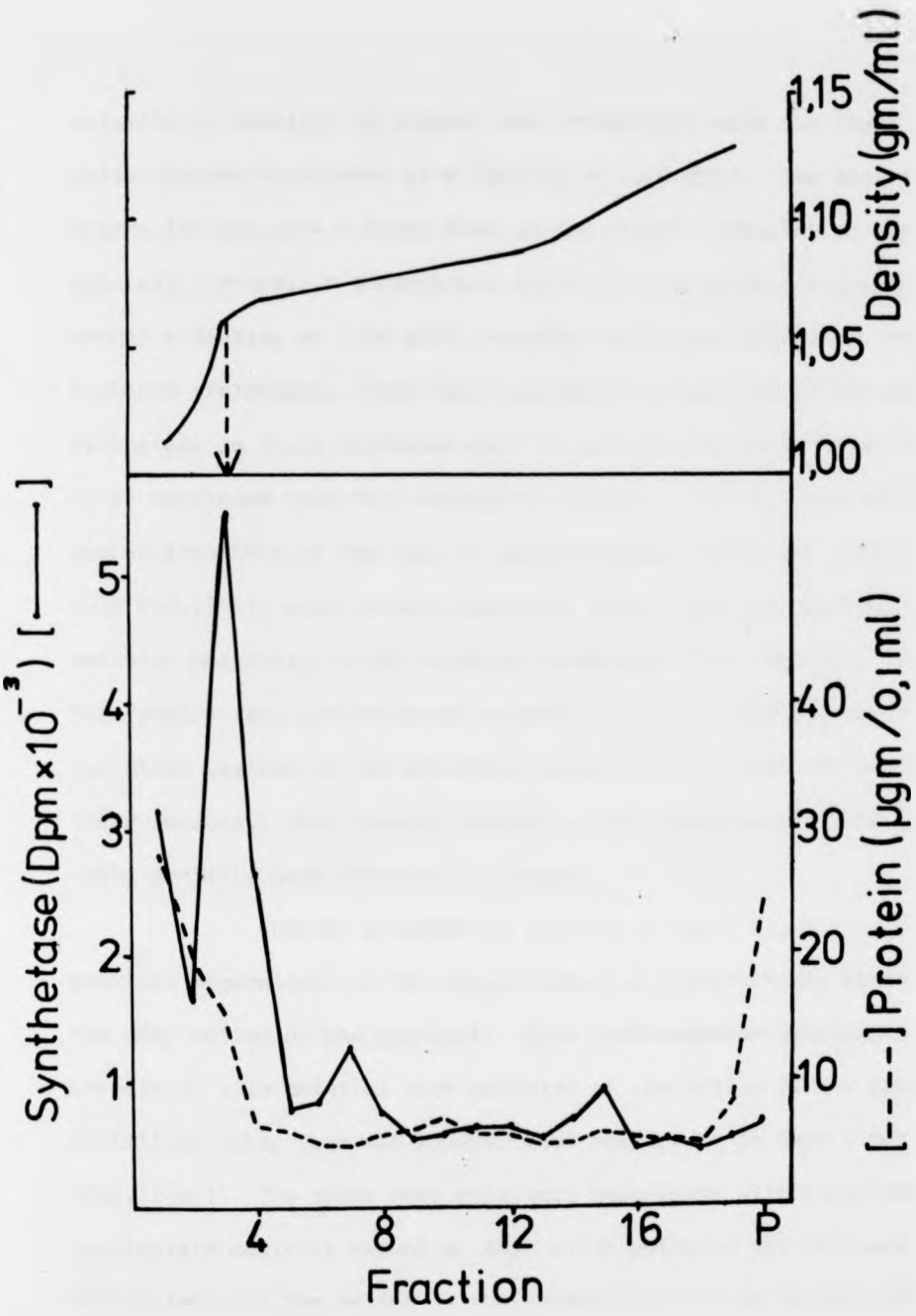


Fig. 12 (b) Repeat of velocity sedimentation presented in Figure 12 (a). Arrow again showing the 15% sucrose fraction.

activity (a specific ER enzyme) was co-incident with the light peak of galactomannan synthetase at a density of 1.06 g/ml. The dictyosome marker enzyme IDP-ase gave a broad peak at the soluble fraction on top of the gradient (probably a phosphatase activity) and another significant peak around a density of 1.08 g/ml, similar to the separation of pea golgi β-glucan synthetase. From the relatively low density of the galactomannan synthetase in these gradients and its association with the ER marker enzyme it is concluded that this organelle species is endoplasmic reticulum. The pooled fractions at the peak of galactomannan synthetase (Fractions 6, 7 and 8 in Fig.12(a)) were further examined, after fixation and staining as pelleted material, in the electron microscope (see page 109, and Fig.18-21). This pooled fraction contained significantly more absorbance at 260mm than the other regions of the gradient, more than 2.5 times the absorbance of other regions. This feature indicates that these possible ER membranes would probably have attached ribosomes.

Another interesting feature of these velocity sedimentation gradient separations is the appearance of a significantly active fraction at the very bottom of the gradient. This galactomannan synthetase activity was associated with material that pelleted at the bottom of the gradient centrifuge tube, from the younger seed samples at 36 days after anthesis (Fig.12(a)). The older seed endosperm homogenate with relatively low particulate activity around 46 days after anthesis did not have any activity associated with the pellet on the centrifuge tube walls (Fig.12(b)). The endosperm cell at the early phase of galactomannan deposition, around 30-35 days after anthesis, is seen to contain numerous ER vesicles filled with galactomannan-like material (101). The particulate enzyme which has its high activity peak at 35 days after anthesis is therefore associated with

quantities of galactomannan and a protein-galactomannan bound complex could explain why some of the galactomannan synthetase has such a high apparent density. A similar pattern emerged for chitin synthetase in Mucor rouxii (149). When the enzyme was pre-incubated with unlabelled UDP-GluNAc and then separated on a sucrose density gradient, a proportion of enzymic activity was associated with a very high density fraction at the bottom of the gradient (the majority of activity residing above the gradient). When the chitin synthetase was pre-incubated with ^{14}C labelled UDP-GluNAc and separation was effected on a parallel gradient then a band of radioactivity collected at the bottom of the gradient, and was associated with a visible band of fibrous material. Thus the dense chitin microfibrils had carried a portion of the chitin synthetase down to the bottom of the gradient, and was not any non-specific adsorption since unincubated enzyme did not adsorb to chitin. The same type of persistent enzyme-product bound complexes are also seen in the case of particulate glycogen synthetase (86).

The existence of a persistent tightly associated enzyme-product complex would certainly provide an explanation of the behaviour of the particulate synthetase during a differential pelleting schedule (Table 7).

Table 7 Particulate synthetase distribution after differential pelleting of endosperm homogenate+

10^{-3} x g	% of particulate synthetase Test 1 (38 days old)	Test 2 (47 days old)
10	54	58
25	14	15
40	12	6
75	12	12
100	8	9

+ Homogenisation carried out in the presence of the normal isolation buffer plus 0.4M sucrose. The 1,000g supernatant was taken for analysis at the higher g values.

It can be seen that the majority of the particulate synthetase, from 54-58%, can be sedimented at 10,000g a typical "mitochondrial" g value. Bearing in mind that the major galactomannan synthetase particulate component is of a low density from the sucrose density gradient analysis, one would expect that a typical ER associated enzyme would sediment from a post 40,000 g up to 100,000 g centrifugation. This relatively low g, active pelletable material would seem to be the result of co-sedimentation of endogenous galactomannan and enzyme as a complex.

To see whether this differential centrifugation regime might itself irreversibly bind enzyme and galactomannan then the material that sedimented between 1,000g and 10,000g was resuspended and placed on a velocity sedimentation gradient similar to those in Fig.12. The enzymic profile of this separation is given in Fig.13. The characteristic light synthetase (density of 1.06g/ml) peak is not present and a heavier peak at around a density of 1.11g/ml is observed. This would point to the combination during the initial 10,000g centrifugation being strong, with the production of this heavier synthetase particle. This preparation exhibits a high galactomannan synthetase activity level associated with the pelleted material which has moved completely through the gradient, and the increase in this activity as well as the appearance of the new heavier synthetase on the gradient can well be explained by the production during the centrifugation of enzyme-product bound complexes.

This feature was further investigated when the reverse of the previous experiment was carried out. In effect a normal velocity gradient centrifugation of the endosperm homogenate was produced and the light galactomannan synthetase peak was pooled and then subjected to a centrifugation at 10,000g. When this was done it was found that 70% of the recoverable activity was in the supernatant and 30% in the sediment. It would seem

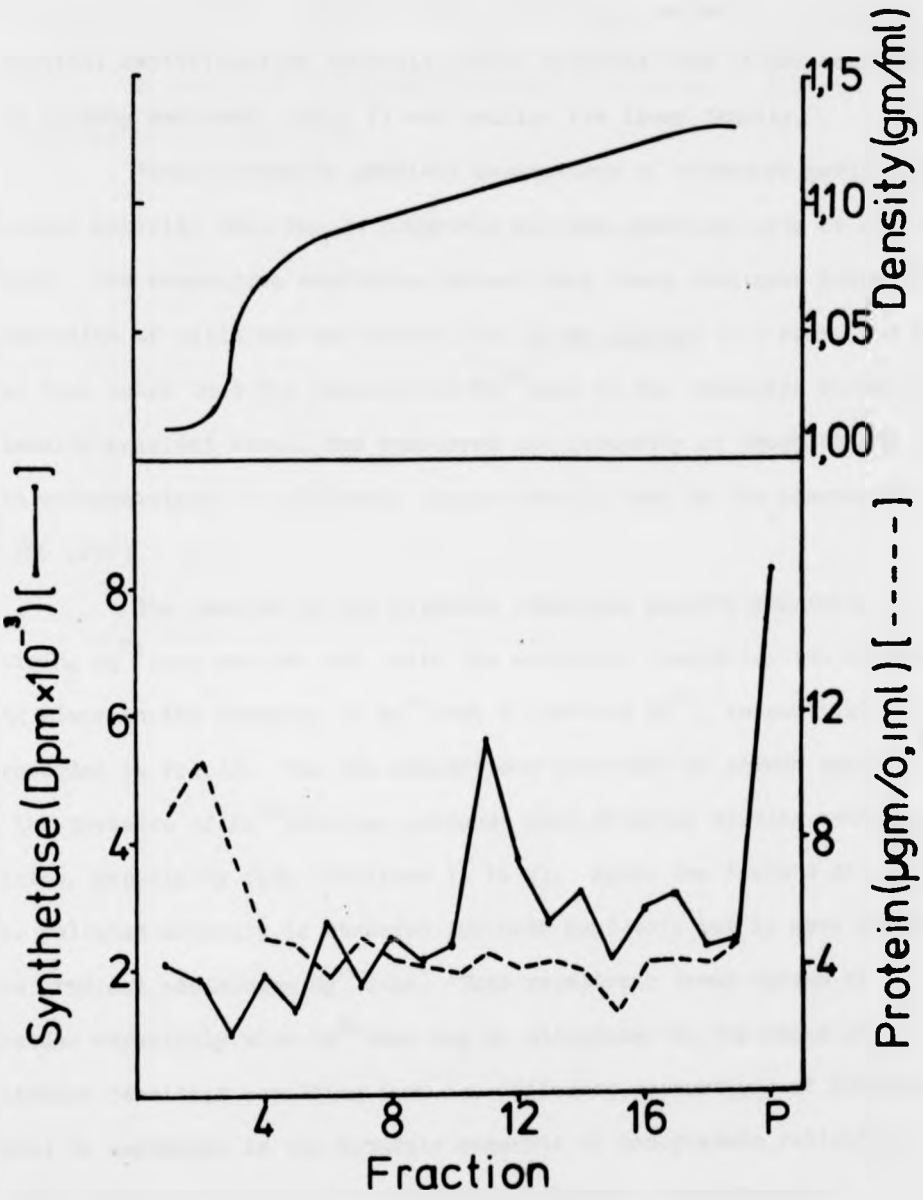


Fig. 13 Velocity sedimentation behaviour of a 10,000g pellet resuspended and placed on a 15 - 35% linear sucrose gradient.

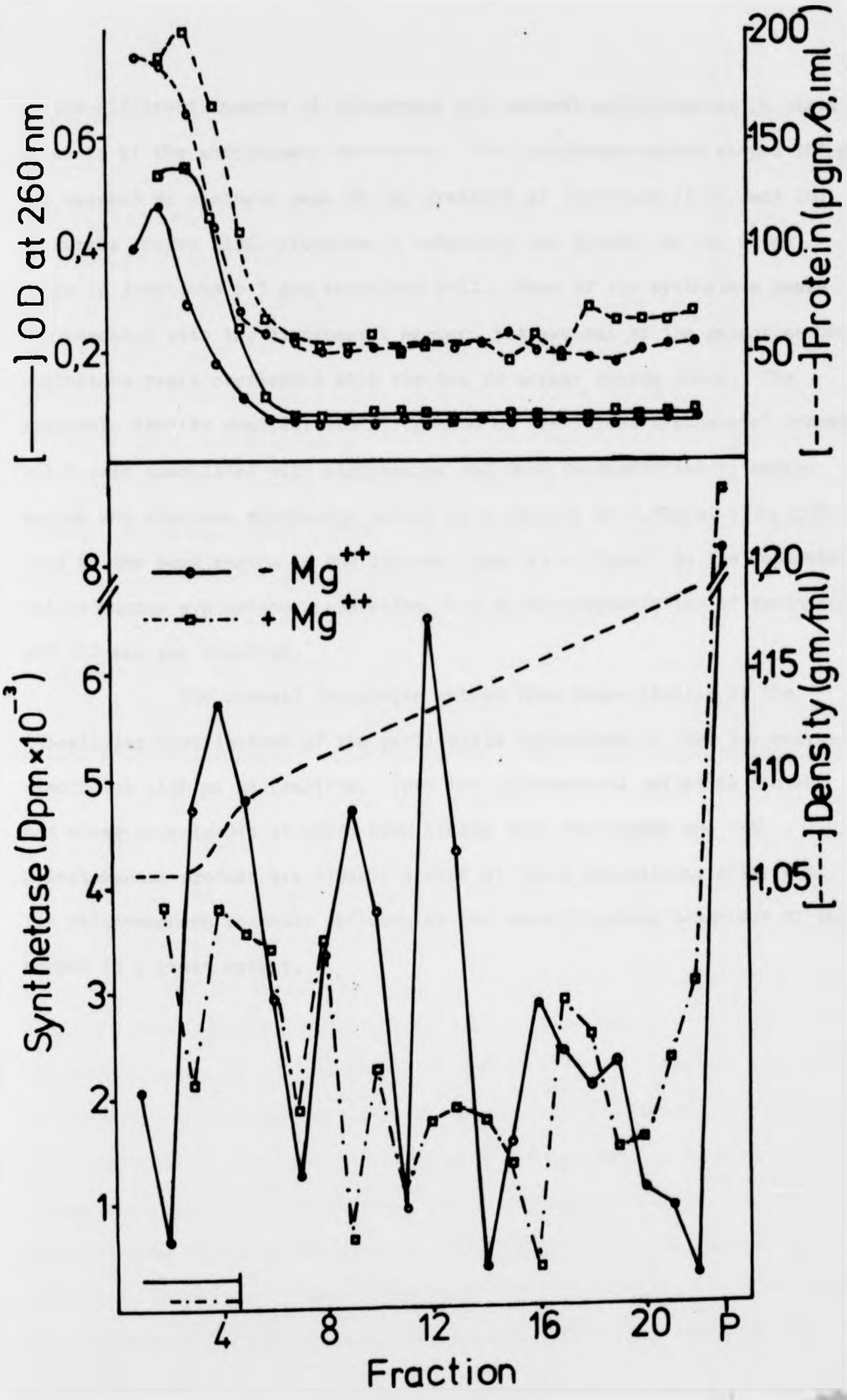
Fig. 14 (overleaf) Isopycnic distribution of 1,000g supernatants on 20 - 50% linear sucrose gradients. One galactomannan synthetase sample was extracted in, and placed on a gradient containing 5 mM Mg^{++}. The other sample was extracted in buffer without Mg^{++} and placed on a gradient containing 0.1 mM Mg^{++}. The bars appearing along the abscissa denote the fractions that represent the original samples. Both gradients had identical density profiles.

likely, therefore, that the gradient separation of the total particulate activity has separated most of the possible complexing galactomannan from most of the particulate synthetase enzyme. A significant activity still resided with the pellet from the sucrose gradient. The light galactomannan synthetase is no longer, after the above procedure, susceptible to the differential centrifugation schedule (which produced from 54-58% activity in the 10,000g sediment, Table 7) and retains its lower density.

Finally density gradient separations of endosperm particulate synthetase activity were run on isopycnic sucrose gradients with or without Mg^{2+} ions. The separation conditions chosen were those that gave isopycnic sedimentation of cellulose synthetase from Pisum sativum in 2 hours (151). It has been noted that the presence of Mg^{2+} ions in the isolation buffer and in the density gradient itself has conserved the integrity of rough ER and leads to its sedimentation at relatively higher density than in the absence of Mg^{2+} (96 ,150).

The results of two separate identical density gradients one containing Mg^{2+} ions and one not, with the endosperm homogenisation schedule taking place in the presence of Mg^{2+} ions or without Mg^{2+}, respectively, are recorded in Fig.14. The two separations have much in common except that the presence of Mg^{2+} ions has produced more slightly heavier synthetase particles, especially from fractions 12 to 21. Again the feature of a very dense, pelleted activity is observed for both gradients but is more marked in the gradient containing Mg^{2+} ions. This relatively broad spread of synthetase especially with Mg^{2+} ions may be attributed to the range of equilibrium densities resulting from the different proportions of ribosomes attached to membranes in the separate segments of endoplasmic reticulum. A similar broad distribution of cotton fibre β-glucan synthetase was observed after isopycnic sedimentation with activity present in several fractions (35). These broadly spread component activities of the galactomannan particulate synthetase may also have been brought about

Fig. 14 (overleaf) Isopycnic distribution of 1,000g supernatants on 20 - 50% linear sucrose gradients. One galactomannan synthetase sample was extracted in, and placed on a gradient containing 5 mM Mg^{++}. The other sample was extracted in buffer without Mg^{++} and placed on a gradient containing 0.1 mM Mg^{++}. The bars appearing along the abscissa denote the fractions that represent the original samples. Both gradients had identical density profiles.


OD at 260 nm
0,6
0,4
0,2
Protein (µgm/0,1ml)
200
150
100
50
- Mg++
+ Mg++
Synthetase (Dpm×10⁻³)
8
6
5
4
3
2
1
Density (gm/ml)
1,20
1,15
1,10
1,05
4
8
12
16
20
P
Fraction

by the different amounts of endogenous and nascent galactomannan in certain segments of the endoplasmic reticulum. The dictyosome marker enzyme IDP-ase was present as a single peak on the gradient at fractions 13-15, and the ER marker enzyme NADH-cytochrome c reductase was present as two close peaks in fractions 5-7 and fractions 8-11. None of the synthetase peaks corresponded with the dictyosomal marker, but several of the galactomannan synthetase peaks overlapped with the two ER marker enzyme peaks. The isopycnic density gradient centrifugation of "cellulose synthetase" preparations which were associated with dictyosomes and were characterised by marker enzyme and electron microscopy banded at a density of 1.15g/ml (122, 150). This is the same region as the IDP-ase (peak at 1.15g/ml) in the separated galactomannan synthetase preparation, but no co-sedimentation of synthetase and IDP-ase was observed.

The overall impression gained from these studies of the subcellular distribution of the particulate synthetase is that the enzyme is associated with an ER fraction. From the differential pelleting routine and other experiments it would seem likely that the enzyme and the galactomannan product are closely linked at their subcellular site, with the galactomannan probably influencing the centrifugation behaviour of the enzyme to a great extent.

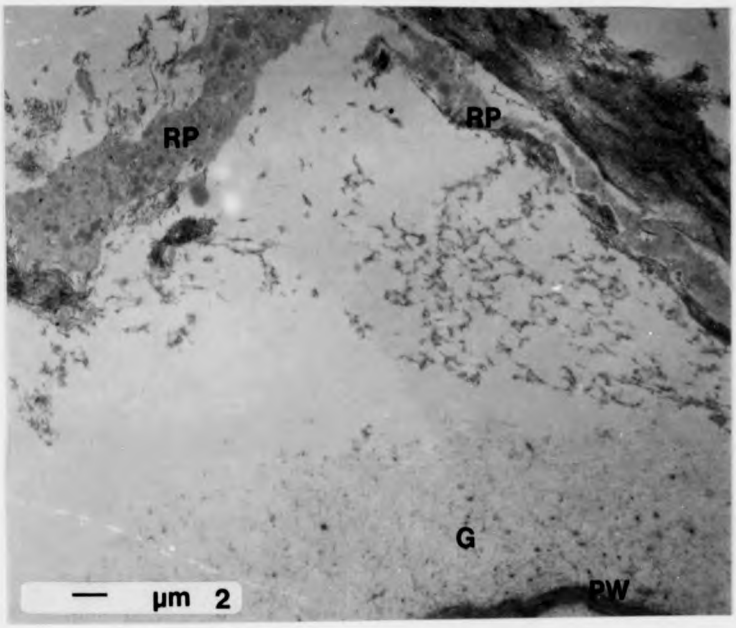
Microscopic investigation of endosperm tissue and an isolated organelle fraction

Endosperm tissue: The general pattern of galactomannan deposition in the developing fenugreek seed endosperm has been recorded by Reid and Meier (132, 10). Galactomannan deposition begins in the cells nearest to the cotyledons and proceeds outwards filling the rest of the endosperm cells except for a single cell layer adjacent to the seed coat. Rough endoplasmic reticulum is involved in the synthesis of galactomannan within the protoplasm and its subsequent transfer to the cell wall (see page 11). Light and electron microscope examination of the tissue used in the present biochemical study confirmed the earlier observations.

The previously presented Figure 4 (plate 1) (page 66) is a light micrograph of fenugreek seed at the end of galactomannan deposition (entering phase three) and shows the reserve cells of the endosperm completely filled with galactomannan. The following three figures are electron micrographs of endosperm cells during the galactomannan deposition stage of seed development. Figure 15 (plate 2) and 16 (plate 3) show endosperm cells near the beginning and near the end of galactomannan deposition, respectively. Material similar to the wall galactomannan is seen in the regions of protoplasm near the growing secondary cell wall in both figures. The younger endosperm (Fig.15 (plate 2)) has a rather loose cell wall arrangement in comparison to the more mature seed endosperm (Fig.16 (plate 3)). In a further examination of endosperm tissue, not only were there numerous cytoplasmic vesicles with galactomannan-like material in evidence, but also distinct bridges across the plasmalemma, with the same densely staining material being transported from the cytoplasm to the external space, were observed (Fig.17 (plate 4)). Such continuity between protoplasmic vesicles, and their contents, and the cell wall shows that the galactomannan is being formed to some extent in the

Fig. 15 (Plate 2). Endosperm cell near the beginning of galactomannan deposition. The galactomannan (G) adjacent to the primary wall (PW) is present as a fine network. Present within the residual protoplasm (RP) are pockets of similarly-staining material to the galactomannan of the secondary wall thickenings. Section contrasted with uranyl acetate - lead citrate.

Fig. 16 (Plate 3). Endosperm cell near the middle of galactomannan deposition. The galactomannan (G) of the wall is a more compact feature than in Fig. 15. The residues of protoplasm (RP) again contain pockets of similarly-staining material to the galactomannan. Section contrasted with uranyl acetate - lead citrate.


RP
RP
G
PW
μm 2

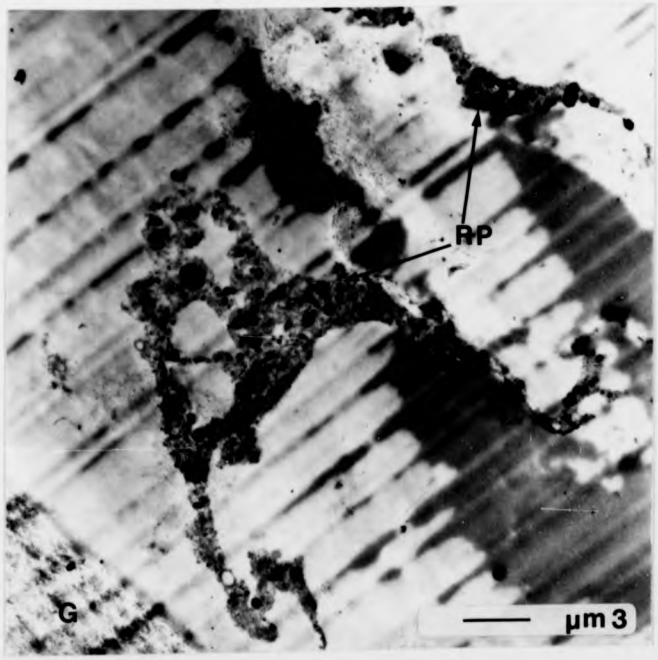
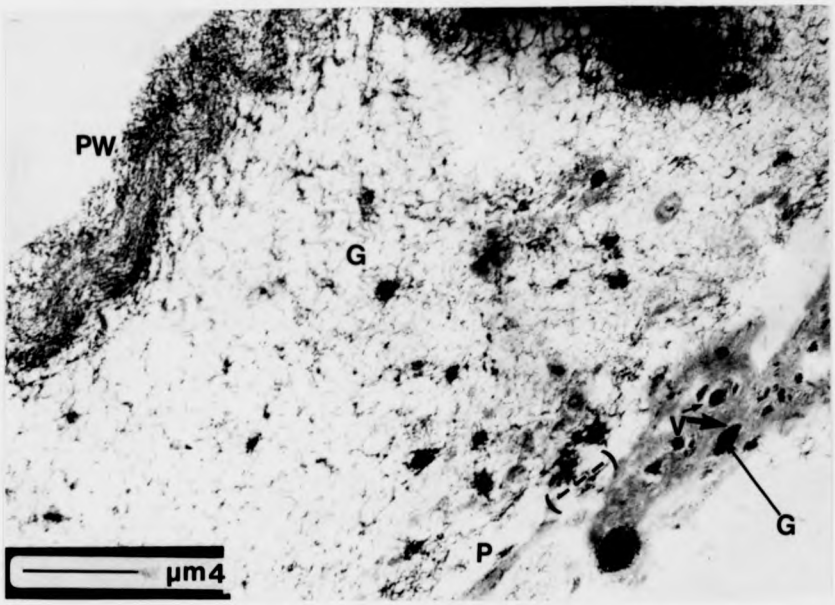

RP
G
μm 3

Fig. 17 (Plate 4) Endosperm cell during galactomannan secretion in the secondary cell wall. The galactomannan (G) is built up between the primary wall (PW) and the plasmalemma (P). Similarly - staining material is present in vesicles (V) contained within the adjacent cytoplasm. A connection (---) can be seen from the cytoplasm to the external space with galactomannan like material being transported. Section contrasted with uranyl acetate - lead citrate.


PW
G
V
G
P
µm4

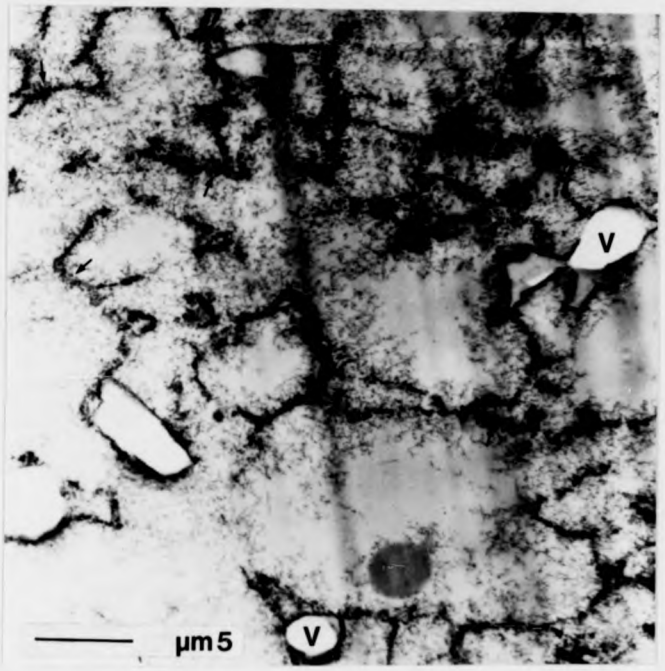
protoplasm and then being expelled into the growing wall as has been observed by Meier and Reid (1977) (101). The pockets of material in the protoplasm are similar to the galactomannan of the cell wall in general and to features within the wall. The secondary wall appears homogenous at lower magnification, yet areas of deeply staining material can be seen in Fig.17 in an almost vesicular formation. The thin, compact primary cell wall appears wavy probably due to contraction during the dehydration phase of tissue preparation. That the protoplasmic material held in the membrane vesicles was galactomannan in transit to the cell wall seems definite, but the origin of these vesicles was not observed.

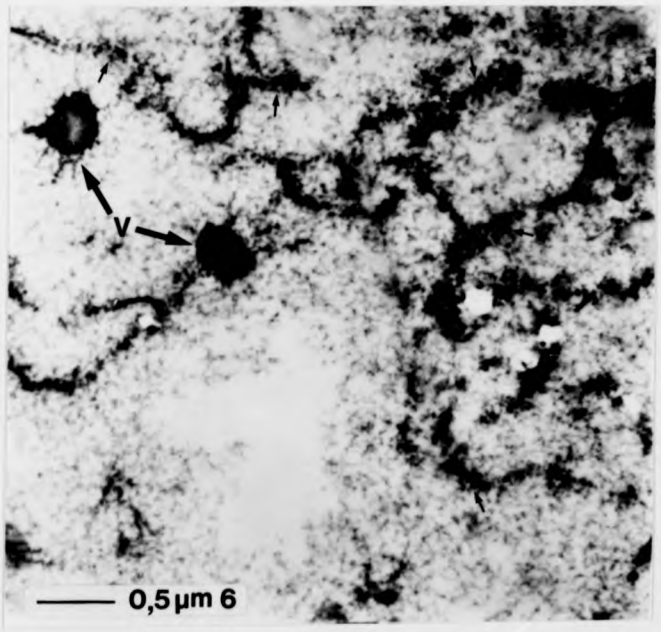
Isolated organelle fraction: A pooled sample of a galactomannan synthetase-containing organelle from a sucrose density gradient was pelleted and then prepared for the electron microscope in situ (see page 46). The material of the pellet was stained either by the standard uranyl acetate-lead citrate regime or by subjecting sections to the Thiéry reaction (PATAg) (164). This last procedure, which specifically stains polysaccharides, is based upon the formation of aldehyde functions from the polysaccharide glycol groups by periodic acid. These are then complexed with thiocarbohydrazide whose thio-groups are revealed by deposition of silver grains at their site.

On examination by the typical membrane staining procedure, uranyl acetate-lead citrate, the major features of the pellet were membrane segments showing a rough ER like appearance and vesicles which either appeared empty (see Fig.18 (Plate 5)) or were stained inside (see Fig.19 (Plate 6)). The membrane segments were similar to those from a peak of enzyme involved in lecithin synthesis in castor bean, taken from a density gradient and identified as rough ER (96). The observation of the ribosome attachment to the membranes involved in galactomannan synthesis provides an explanation for the relatively high absorbance at 260 nm this gradient

Fig. 18 (Plate 5). Particulate fraction isolated from the peak galactomannan synthetase region of a sucrose density gradient. Regions of membrane indicative of rough endoplasmic reticulum (small arrows) and ER vesicles (V). Section contrasted with uranyl acetate - lead citrate.

Fig. 19 (Plate 6). Preparation as in Fig. 18. Regions of membrane indicative of rough endoplasmic reticulum (small arrows) and ER vesicles with heavily stained interiors (V). Section contrasted with uranyl acetate - lead citrate.


V
V
µm 5

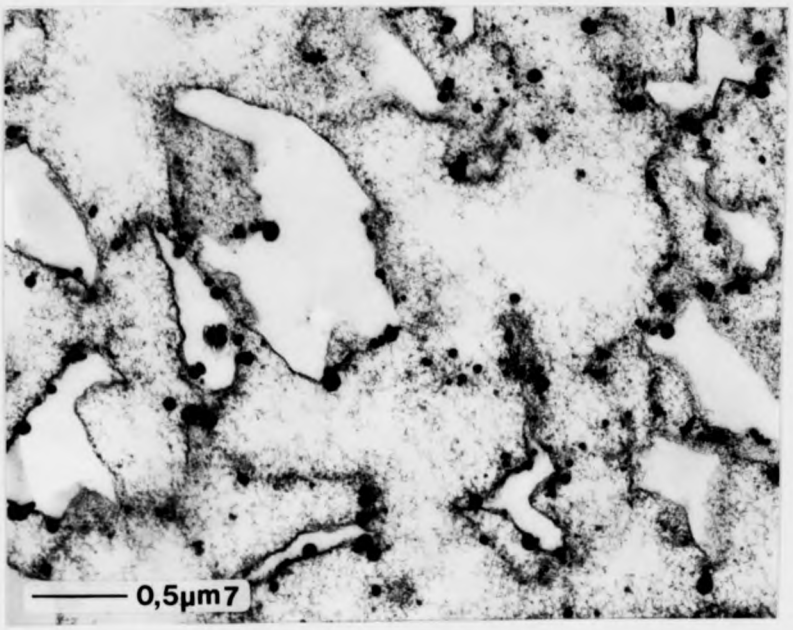

V
0,5 µm 6

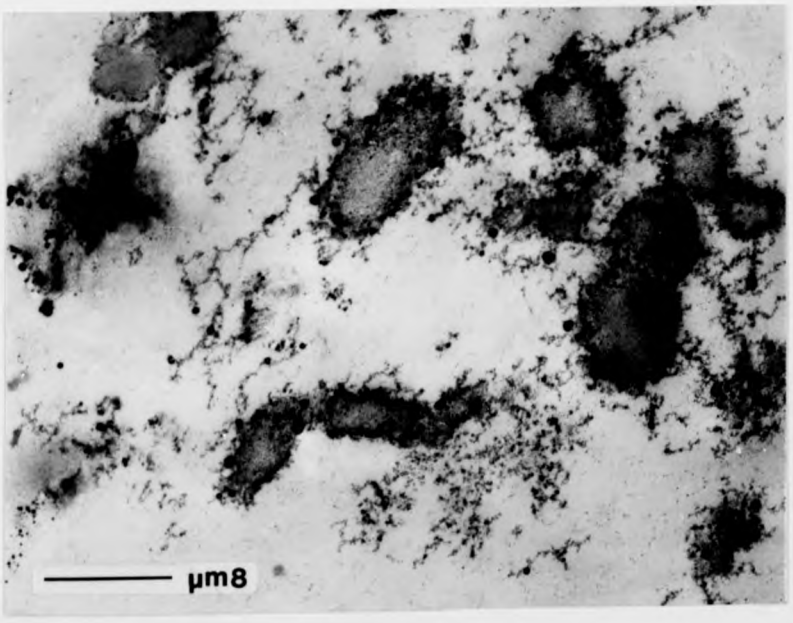
fraction had over the rest of the gradient (see page 99). The appearance of vesicles and membrane segments was again observed after staining by the Thiéry reaction. A section from the middle of the pellet can be seen in Fig.20 (Plate 7) and a section from the bottom of the pellet in Fig.21 (Plate 8). The appearance of the background is of a fibrous material and from the distribution of silver grains on this material in both figures, it could be galactomannan associated with the membranous material. In the middle of the pellet the vesicles have grains deposited at their periphery (Fig.20), whereas in the more dense bottom material the grains appear not only at the periphery of vesicles but also within the vesicles themselves (Fig.21). Sections taken as blanks in the staining procedure (no periodic acid step) showed no silver grains and were only contrasted by virtue of the osmium fixation step.

The indication from this specific polysaccharide ultrahistochemical reaction is that the more dense parts of the pelleted membranes in fact contain more polysaccharide within the vesicular material than those at the middle of the pellet which have no interior staining. The vesicles were of similar size in both regions of the pellet (0.7 µm and 0.6 µm diameter value in Fig.20 and Fig.21, respectively). The overall information that can be gathered from this examination of endosperm tissue and the subcellular organelle responsible for the biosynthesis of galactomannan, is not incompatible with ER membranes and vesicles being involved in the formation of polysaccharide in the protoplasm and its transport to the cell wall.

Fig. 20 (Plate 7). Particulate material isolated from the peak galactomannan synthetase region of a sucrose density gradient. Section taken from the middle of the pelleted material and contrasted by the Thiéry reaction. Silver grains are mainly at the periphery of vesicles, and distributed over the fibrous background material.

Fig. 21 (Plate 8). Preparation as in Fig. 20. Section taken from the bottom of the pelleted material and contrasted by the Thiéry reaction. Silver grains are accumulated inside the vesicles, at their periphery and also some grains are associated with the fibrous background material.


0,5µm7


µm8

Section 2: Chemotaxonomic Study

The first part of this section deals with the main structural features of the total polysaccharide in the endosperm of *Laburnum anagyroides* Medicus. In conjunction with a chemotaxonomic survey of the tribe *Genisteae* (*Leguminosae*) the results showed not only that the galactomannan from this tribe conforms to the established structural pattern, but also that the mannose:galactose ratio of individual member galactomannans appears to have taxonomic significance.

Structural characterisation of Laburnum anagyroides galactomannan

Endosperms of laburnum seed were manually isolated, freed of low molecular weight carbohydrates and subjected directly to hydrolysis, light scattering, methylation analysis and periodate oxidation. Although it is generally accepted that the galactomannans are present only in the endosperms of the leguminous seed, no structural studies have hitherto been carried out in which the seed endosperm has been used as starting material. Polysaccharide fractions have always been obtained by extraction of comminuted seeds, usually with hot water followed by a fractionation of the extract, commonly by precipitation of the galactomannan as its copper complex (34). The laburnum endosperm residue material after re-hydration and freeze-drying regained its original shape but had the texture and appearance of cotton wool. The protein content of the endosperm material was less than 2%, 0.9% by the method of Lowry (97) and 1.8% by the method of Waddell (171).

On complete acid hydrolysis the endosperm material gave an almost quantitative yield of mannose and galactose in the ratio 2.15:1.00. Only trace amounts of other monosaccharides were detected by paper chromatography and by GLC of the alditol acetates.

(a) Light scattering

Light scattering intensities were measured for aqueous solutions of the laburnum endosperm material. This data is presented in Table 8 which shows an almost linear relationship between concentration (c) and the values for C/I_θ.

Table 8 Light scattering data

Concentration (c) (10^4 x g/ml)	Scattering intensity, I_θ (galvanometer units)	$I_\theta{}^{\alpha}$ *	$C/I_\theta \times 10^5$
4.3	33.0	23.5	1.839
10.7	51.0	41.5	2.583
13.8	49.3	39.8	3.470
19.0	51.0	41.5	4.578

* Scattering intensity relative to the solvent i.e. water (I_θ = 9.5)

Extrapolation of the straight line relationship to zero concentration results in an intercept value on the C/I_θ axis of 0.825×10^{-5}. Refractive index measurements were made for these same polysaccharide solutions and, using the refractive index increment (4.13×10^{-2}) in the standard weight average molecular weight formula (see page 49; 160), a value of approximately 69,500 was obtained. This value compares to one of the few reported molecular weights for a galactomannan, that of Caesalpinia pulcherima where a M_w of 60,000 was indicated by ultracentrifugation data (167).

(b) Methylation analysis

The endosperm material was methylated once with dimethyl sulphate and sodium hydroxide, and twice with sodium hydride, dimethyl sulphoxide and methyl iodide. The product had no IR absorption which could be

attributed to free hydroxyl groups. On formolysis followed by hydrolysis the following methylated sugars were detected: 2,3,4,6 tetra -O-methyl galactose, 2,3,4,6 tetra-O-methyl mannose, 2,3,6 tri-O-methyl mannose and 2,3 di-O-methyl mannose. The methyl ethers were identified by paper and thin-layer chromatography and quantitative estimations were carried out by GLC of their alditol acetates, using standard reference sugars. The quantitative results are presented in Table 9.

Table 9 GLC separation of the alditol acetate derivatives of the partially methylated sugars of Laburnum anagyroides extract.

Derivative	Relative retention time	% Molar composition
2,3,4,6-tetra-O-methyl-D-Man	0.80	1.2
2,3,4,6-tetra-O-methyl-D-Gal	1.00	31.0
2,3,6-tri-O-methyl-D-Man	1.80	36.6
2,3-di-O-methyl-D-Man	3.94	31.2

The identifications were confirmed by GLC of the methylated methyl glycosides released on methanolysis of the permethylated endosperm polysaccharides. Trace amounts of other methylated derivatives were detected by GLC but were not identified.

The results of the methylation analysis are exactly those which would be expected if the endosperm material from the laburnum seed were composed almost entirely of a galactomannan with the established leguminous seed type of structure, and a mannose:galactose ratio of 2.2:1.0. Using the proportion of 2,3,4,6 tetra-O-methyl mannose detected, and assuming that the terminal non-reducing mannose residue is never galactose-substituted, then a DP_n of 82 is indicated.

(c) Periodate oxidation

Periodate oxidation of the endosperm material was complete after 22 days (at 4°C) when values of 1.33 moles of periodate consumed with the release of 0.32 moles of formic acid per mole of anhydrohexose were recorded (see Table 10).

Table 10 Periodate oxidation analysis

Time (days)	Periodate consumed per anhydrohexose unit (mole/mole)	Formate released per anhydrohexose unit (mole/mole)
1	0.53	0.19
2	0.81	0.22
3	0.83	0.24
4	0.87	0.27
15	1.10	0.31
22	1.33	0.32

No galactose or mannose could be detected by paper chromatographic or GLC analysis of the periodate oxidised polysaccharide. The polysaccharide structure revealed by methylation of the endosperm material would be expected to consume 1.31 moles of periodate with the release of 0.31 moles of formic acid per mole of anyhydrohexose, and to contain no periodate-resistant monosaccharide residues.

It is clear that the high molecular weight material in Laburnum anagyroides endosperm consists almost entirely of a galactomannan polysaccharide of the established "leguminous seed type." It is concluded that the galactomannans of other seeds of the Leguminosae-Genisteae probably all conform to the established structural pattern.

Aspects of the galactomannans from Genisteae species

Seeds of all available species within the tribe Genisteae were examined for galactomannan content and composition. The hot water soluble

galactomannans extracted and purified via their insoluble copper complexes were analysed after hydrolysis by GLC of their alditol acetates. In Table 11 are listed the sub-tribes and genera of the Genisteae according to Engler (102), and the analysis results.

Table 11 Composition of galactomannans isolated from the seeds of some species in the tribe Genisteae

Subtribe and species	Man:Gal ratio	Subtribe and species	Man:Gal ratio
Lipariinae		Crotalariinae	
Priestleya hirsuta D.C.	2.2	Crotalaria retusa L.	3.2
Bossiaenae		Crotalaria capensis Jacq.	2.8
Hovea Longifolia R.Br.	1.8	Crotalaria crassipes Hook.	3.0
Genistinae		Crotalaria incana L.	2.9
Spartium junceum L.	2.2	Crotalaria sagittalis L.	2.9
Genista ovata W.K.	2.0	Crotalaria nitens H.B.K.	2.9
Genista tinctoria L.	2.1	Crotalaria striata D.C.	2.3
Genista monosperma Lam.	2.1	Crotalaria dissitiflora Benth.	2.7
Petteria ramentacea (Sieb.)Presl.	2.1	Argyrolobium linnaeanum Walp.	1.9
Laburnum alpinum (Mill.)Presl.	2.1	Aspalathus macrantha Harv.	1.8
Laburnum anagyroides Medicus	2.2	Lupinus angustifolius L.) Lupinus arboreus Sims Lupinus luteus L. Lupinus albus L. — no galactomannan (no endosperm)	
Ulex europaeus L.	1.9		
Cytisus hirsutus L.	2.3		
Cytisus supinus L.	2.0		
Sarothamnus scoparius (L) Wimmer ex Koch	2.3		

It is clear that the data set produced from 13 of the 17 genera of the tribe Genisteae does not have the same degree of uniformity of galactomannan presence and composition which was encountered in the Trifolieae (130). The tribe Genisteae, however, does not exhibit the same taxonomic self-consistency as does the tribe Trifolieae; it represents,

rather, an area of leguminous taxonomy where there is considerable doubt and disagreement between authorities as to the placing of species within genera and genera within tribes (compare for example 77, 102 and see 13). Despite the absence of a constant mannose:galactose ratio throughout the tribe it is clear that galactomannan analysis in the Genisteae does sub-divide the tribe into groups which reflect classical taxonomic divisions. All the species within the sub-tribe Genistinae yielded galactomannans with mannose:galactose ratios in the range 2.3 to 1.9 (mean, 2.1; S.D. 0.13). Within the sub-tribe Crotalariinae there were three distinct sub-groups: the genus Crotalaria; the genus Lupinus; and Argylobium and Aspalathus. The Crotalaria species examined all contained galactomannans whose mannose:galactose ratios fell within the range 3.2 to 2.3 (mean, 2.8; S.D. 0.24.). This range is distinct from but considerably wider than that spanned by the Genistinae, which is perhaps a reflection of the relative homogeneities of the two groups. None of the Lupinus species examined yielded galactomannans, which is not surprising as the genus is known to have endosperm-free seeds. Although only one species from each of the two genera Aspalathus and Argylobium was examined they both yielded galactomannans whose mannose:galactose ratios were closely similar and outside the ranges covered by the Genistinae and Crotalaria. Another feature of this analysis was that the galactomannan fraction from the Laburnum anagyroides seed exhibited the same mannose: galactose ratio of 2.2 as the total endosperm polysaccharide preparation from laburnum. This shows that the galactomannan extracted from laburnum is representative of the galactomannan in the endosperm, a fact which should be true for other seeds of Genisteae.

The presence or absence of an endosperm in the seed is a morphological feature which in itself might be considered in the first stage taximetry of leguminous species. When it is present, however, the leguminous endosperm invariably contains a galactomannan whose mannose: galactose ratio appears to be a useful taxonomic feature. Galactomannan analysis could considerably expand the usefulness of the seed endosperm in traditionally undecided taxonomic areas such as the Genisteae.

General Summary

Section 1: The biosynthesis of fenugreek galactomannan

Galactomannan formation was shown to commence at the start of phase two of fenugreek seed maturation. This period of rapid increase in dry weight in the seed started approximately 30 days after anthesis and lasted for 25 days. Galactomannan amounted to nearly 20% of the seed dry weight at the end of phase two, and along with reserves accumulating in the cotyledons made a major contribution to the rapid growth in seed weight during maturation. The spectrum of low molecular weight carbohydrate in the endosperm changed markedly during this same period. The seed endosperm at first contained mainly sucrose and raffinose, but no stachyose, and became chiefly composed of stachyose from the end of phase two onward. Thus two simultaneously produced carbohydrate components with the same galactosyl residue linkage ($\alpha 1 \rightarrow 6$) were present in the ripe seed endosperm. In vitro work showed that the immediate precursor of stachyose, galactinol, was being produced at the same time as stachyose in the endosperm cell free system.

The fenugreek endosperm was shown to produce from the beginning of phase two a very low detectable level of α-galactosidase, which was sustained in the ripe seed. The fenugreek α-galactosidase activity may be responsible for the early breakdown of stachyose, which is metabolised in the earliest stages of germination (133), in the same manner as previously shown to occur in the germinating carob seed (146). A much higher level of α-galactosidase was reached in the embryo coincident with the low level in the endosperm.

Incorporation of radioactive precursor monosaccharides by endosperm halves showed that the maximum rates of incorporation into galactomannan occurred at the middle of galactomannan deposition *in vivo*. From analysis of the incorporation into the low molecular weight sugars and of the endosperm galactomannan over the period of cell wall reserve accumulation, it would seem that there was a modulation of the flow of carbon with regard to the metabolic pathways involved in galactomannan, sucrose and stachyose metabolism, which was dependent upon the age of the seed endosperm.

In vitro analysis of the endosperm polysaccharide biosynthesis showed that endosperm homogenates contained a particulate enzyme capable of transferring mannose residues from GDP-mannose to a high molecular weight galactomannan molecule. The identity of the product was established by using selective precipitants. This mannosyltransferase enzyme (galactomannan synthetase), in common with many polysaccharide synthetase systems, required the presence of a divalent cation for activity. The particulate galactomannan synthetase was also shown to have the following characteristics; an alkaline pH optimum; retention of activity after digitonin solubilisation; and no appreciable lipid intermediate involved in the galactomannan synthesis. A K_m of 33μM was evaluated from substrate concentration data at concentrations of GDP mannose below 77μM, because above this value enzyme inhibition was observed.

The activity of the particulate galactomannan synthetase was highest at the start of the rapid phase in galactomannan accumulation *in vivo*, and dwindled to a low level before the end of galactomannan deposition. Cell wall preparations also contained a galactomannan synthetase activity which rose to a high level a few days after the peak of the

particulate enzyme in the endosperm. The *in vitro* particulate galactomannan synthetase activity would seem to be an active *in vivo* enzyme, from the observation that galactomannan-containing endoplasmic reticulum vesicles in the protoplasm are transferred to the growing cell wall (101). The transferrence of galactomannan synthetase from the protoplasm in the early part of galactomannan deposition to the cell wall site near the middle of deposition again pointed to the involvement of this *in vitro* system *in vivo*.

Density gradient centrifugation of the particulate galactomannan synthetase showed that the enzyme was associated with an endoplasmic reticulum fraction. The endoplasmic reticulum origin of the enzyme was compatible with observations made in the electron microscope using the active galactomannan synthetase material from a density gradient. Another feature of the particulate galactomannan synthetase that could be deduced from its centrifugation behaviour in a differential pelletting scheme and in density gradients, was that the galactomannan product and the enzyme are likely to be closely linked at their subcellular site. Electron micrographs of endosperm tissue during galactomannan accumulation showed vesicles in the protoplasm containing galactomannan-like material, which was seen to be transported across the plasmalemma into the growing cell wall.

Section 2: Chemotaxonomic studies

The proposal that the mannose:galactose ratio of galactomannans can be useful as a chemotaxonomic feature (130) was substantiated by the galactomannan analysis of the tribe Genisteae. It was apparent from the overall chemotaxonomic study that the extracted galactomannan from

the seed exhibited the same mannose:galactose ratio as the total galactomannan in the endosperm. The Genisteae tribe galactomannans probably all conform to the leguminous seed structural type, in the same manner as the Laburnum anagyroides galactomannan.

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