STUDIES WITH PLANT α-GALACTOSIDASES

A Thesis submitted by

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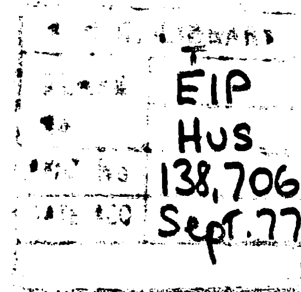

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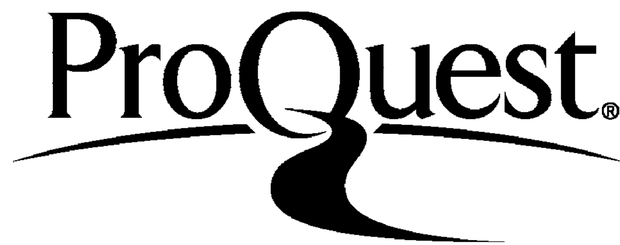

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To my Mother

'Begin at the beginning,' the King said, gravely, 'and go on till you come to the end; then stop.'

Lewis Carroll
(Alice in Wonderland, Ch. 12)

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ABSTRACT

The two molecular forms, $_{I}$ and $_{II}$, of α-galactosidase from immature, mature (resting) and germinated *Vicia faba* seeds have been studied. The enzymes have been purified by a multistage procedure and, in particular, the effect these stages have upon the relative isoenzyme levels has been investigated.

Upon purification in McIlvaine buffer the relative isoenzyme pattern for mature seeds was seen to change from the low molecular weight enzyme $_{II}$ being the major form in the crude extract, through the two molecular forms being almost equal after the citric acid precipitation and ammonium sulphate fractionation, to the high molecular weight enzyme $_{I}$ being the major form after the final purification stage of dialysis. A parallel purification carried out in Acetate buffer introduced a time lag between the change in isoenzyme levels found upon acid precipitation in the McIlvaine system. Purification of extracts of whole (testas intact) immature seeds in McIlvaine buffer showed enzyme $_{II}$ to be the dominant form throughout all the purification stages, while beans devoid of testas produced extracts where enzyme $_{I}$ was the greater. Drying the green immature beans in the

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presence of testas gave the normal mature bean isoenzyme patterns, while drying in the absence of testas, or where the testa has been replaced by a non-physiological covering produced extracts where enzyme $_{I}$ remained the major isoenzyme throughout all stages of purification. Enzyme $_{II}$ was the more abundant isoenzyme in the crude McIlvaine buffer extracts from germinated seeds, with small amounts of enzyme $_{I}$ appearing upon purification.

A preparation of α-galactosidase $_{II}$ when incubated with a 30 - 65% ammonium sulphate fraction from green bean testa was observed to convert in vitro to an enzyme with very similar physical properties to enzyme $_{I}$ from mature seeds. Some factors affecting this conversion have been investigated and it appears that the active component in the green testa is proteinaceous.

The physiological role of α-galactosidases in seeds has been considered.

Attempts were made to modify galactomannans by hydrolysis with α-galactosidases, so as to produce galactose-depleted products which would form stronger gels with κ-carrageenan than would the parent galactomannans. *Vicia faba* enzymes failed to produce any significant hydrolysis whereas coffee and clover enzymes increased the mannose/galactose

ratio but also appeared to hydrolyse the mannan backbone so that gel formation was inhibited. This was believed to be due to the presence of contaminating ß-mannanase. Unsuccessful attempts were made to remove this activity from clover enzyme by several purification stages culminating in affinity chromatography.

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ABBREVIATIONS

The abbreviations and symbols used in this thesis are as suggested by the Biochemical Journal[328], except for the following:-

BSA	bovine serum albumin
$\overline{DP}$	degree of polymerisation
E.C.	enzyme commission
Man/Gal, M/G	mannose/galactose ratio
PNPG	p-nitrophenol galactoside
Vmax	maximum velocity

I N T R O D U C T I O N

A. GENERAL CONSIDERATIONS

α-Galactosidases (α-D-galactoside galactohydrolases) were first reported in 1895 by Bau[1] and Fischer and Lindner[2] who isolated enzyme preparations (melibiases) from bottom yeast which hydrolysed the disaccharide melibiose. The name was later changed from melibiase to α-galactosidase by Weidenhagen[3,4,5]. α-Galactosidases have now been isolated from approximately sixty different sources mostly from seeds and fungi, as well as from some animal preparations. The enzyme exists in several forms which vary in molecular weight and ionic characteristics.

α-Galactosidases are generally known to hydrolyse the α-D-galactosidic linkages of substrates possessing non-reducing terminal galactose residues. Carbohydrates with such terminal α-D-galactosyl residues are widely distributed in nature. In particular, oligo- and poly-saccharides containing these units are common reserve metabolites in plants. The occurrence and structure of these carbohydrates have been reviewed by French[6], Courtois and Percheron[7] and Whistler and Smart[8]. (See also p.30)

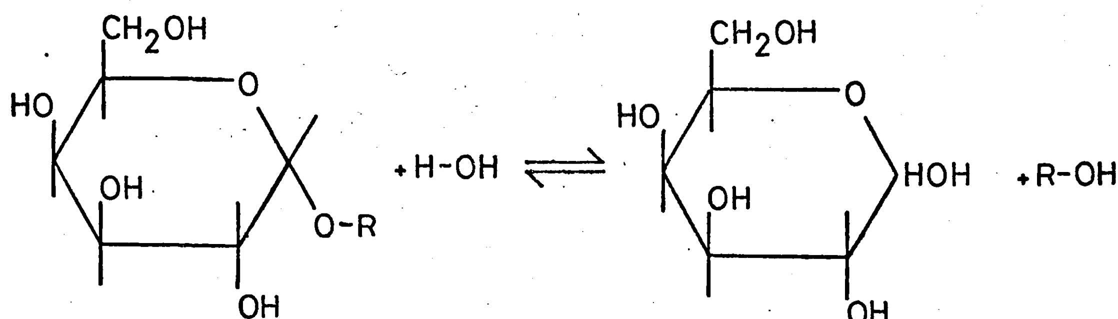
The galactose containing polysaccharides, the galactomannans, are found in the seeds of many leguminous plants, and some,

such as guar, locust bean, gleditsia, fenugreek and tara are used in industry. These gums, which may account for up to 35% of the weight of the endosperm, are used in textile (as fabric dressings) and paper (as "wet-end" additives) production. In the Food Industry they are employed as thickening agents. One of the most important properties of galactomannans is their ability to act synergistically with other polysaccharides (e.g. agarose, carrageenan) to form or modify gel structure.

The main purpose of the research was to examine: (1), the multiple forms of α-D-galactosidase occurring in the leguminous seed, Vicia faba, at various physiological stages. The interconversion of the forms was also investigated in relation to their physiological significance; (2), the modification of galactomannans from guar and locust bean, using purified α-galactosidase preparations, in the hope that such studies would lead to an understanding of their synergistic behaviour in gel formation.

B. α-D-GALACTOSIDASES (E.C. 3.2.1.22.).

α-D-Galactosidases catalyse the following reversible reaction:-



α-D-Galactopyranoside D-Galactose

With most enzymes, R may represent alkyl, aryl or monoglycosyl or polyglycosyl residues.

Most enzymes are not absolutely specific for the glycon residue and will hydrolyse the structurally related α-D-fucopuranosides and β-L-arabinopyranosides[9]. Water can be replaced by a number of organic galactose acceptors, including monosaccharides, and in this way simple galactosides and oligosaccharides (de novo synthesis) have been synthesised. The equilibrium for α-galactosidase-catalysed reactions, as with other glycosidases[10], normally favours hydrolysis.

1. Location.

α-D-Galactosidases have been detected in all the major groups of organisms, particularly in microorganisms and plants. A recent reveiw[11] discusses the occurrence and cellular location of these enzymes. In plants, they have been isolated from numerous seed species, but only in a few cases have highly purified and apparently homogenous preparations been obtained. These include α-D-galactosidase from Prunus amygdalus[12,13], Vicia sativa[14] and Medicago sativa[15]. An homogeneous preparation has been separated from Diplococcus pneumonias[16,17]. Suzuki et al[18] obtained the first crystalline preparation of α-D-galactosidase from the fungus Mortierella vinacea. The enzyme has also been detected in other plant tissues including leaves of Spinacia oleracea[19], phloem tissue of Populus tremuloides[20], in Ficus sp.[21] and in the exudates of cultured orchid tubers[22].

Tissue and cellular location of α-galactosidases using histochemistry and sub-cellular fractionation has been reported for the rat[23,24,25] and various other animals[11,26,27,28,29,30]. However, such studies on plant tissues are limited. Spinach leaves contain the enzyme in the chloroplast, mitochondrial and microsomal fractions[19]. In fenugreek seeds activity has been found in both cotyledon

and endosperm tissue[31]. In most organisms the enzyme occurs in a soluble form, but a particulate enzyme has been reported to occur in pig renal tissue[26].

2. Physical Properties.

a. Multimolecular Forms. Recent reports from several sources have shown that in some organisms two or more forms of the enzyme may exist. The occurrence of different molecular forms of an enzyme derived from the same organism which show essentially similar catalytic activities is common. These forms or isoenzymes may be located in the same or different tissues of the organism or parts of the cell[32,33,34]. The presence of multiple forms of an enzyme may be caused by a variety of factors and these have been reviewed by the IUPAC-IUB Commission[35]. (See Table 1)

Petek *et al*[36,37,38] were the first to report the existence of different forms of α-galactosidase. They obtained two activities from the seeds of both *Coffea sp.* and *Plantago ovata* by fractionation on alumina columns using graded pH elution. These enzymes had very similar kinetic properties. The two forms were not apparent when extracts of coffee seed were examined by Sephadex G-100 gel filtration[39].

TABLE 1.

Multiple Forms of Enzymes[35].

GROUP	REASON FOR MULTIPLICITY	EXAMPLE
1	Genetically independent proteins	Malate dehydrogenase in mitochondria and cytosol
2	Heteropolymers (hybrids) of two or more polypeptide chains, non-covalently bound	Lactate dehydrogenase
3	Genetic variants (allelic)	Human glucose 6-phosphate dehydrogenase
4	Protein conjugated with other groups	Phosphorylase a and b
5	Proteins derived from one polypeptide chain	The family of chymotryprins arising from chymotrypsinogin
6	Polymer of a single subunit	Glutamate dehydrogenase of molecular weight 1,000,000 and 250,000
7	Conformationally different forms	All allosteric modifications of enzymes

Dey and Pridham separated two forms with different molecular weights from ungerminated *Vicia faba* seeds[40,41,42]. These, enzyme $_{I}$ and enzyme $_{II}$, unlike those from *Coffea sp.* and *Plantago ovata* differed markedly in their kinetic properties. On carboxymethyl cellulose, using stepwise pH elution, the lower molecular weight enzyme ($_{II}$) was further resolved into two active fractions, enzymes $_{II}1$ and $_{II}2$, in equal amounts, but the specific activity of $_{II}1$ was three times higher than that of $_{II}2$. Chromatography of the original, high molecular weight enzyme ($_{I}$) on a CM-Cellulose column gave rise to two protein peaks, but only one, which was eluted at pH 4.5, possessed α-galactosidase activity. In addition to *Vicia faba* α-galactosidase isoenzymes differing in molecular size have been found in dry seeds of a number of other plant species[43]. The fungi, *Mortierella vinacea*[18] and *Aspergillus niger*[44], also each possess three forms of α-galactosidase. The separation in the case of *Mortierella vinacea* was accomplished by DEAE-Sephadex column chromatography: One form was crystallised. The three active peaks of α-galactosidase separated on CM-Cellulose in the case of *Aspergillus niger* gave only one enzymically active peak when examined by chromatography on Sephadex G-200, Biogel P200, DEAE-Sephadex or DEAE-Cellulose. The existence of three forms was, however, confirmed by isoelectric focusing.

Some evidence for the interconversion of multimolecular

forms of α-galactosidase _in vitro_ has been obtained for enzymes from _Vicia faba_[39]. Here an enzyme preparation from a batch of broad beans (brown, resting) which appeared to be mature (but were in fact observed to be abnormal in that they contained only the low molecular weight enzyme $_{II}$) was purified by pH precipitation and acetone and ammonium sulphate fractionations, and then stored at pH 5.5 and 4°C. Over a period of days the specific activity of the solution increased rapidly, and examination by Sephadex gel filtration revealed that the activity of enzyme $_{II}$ was decreasing at the expense of a higher molecular weight enzyme with an elution profile identical to that of enzyme $_{I}$. The apparent interconversion did not occur if the preparation of enzyme $_{II}$ had previously been passed through Sephadex G-100. Further experiments suggested that the two forms of enzyme $_{I}$ (original, and from $_{II}$ by conversion) were closely related with respect to the kinetic properties and amino acid composition.

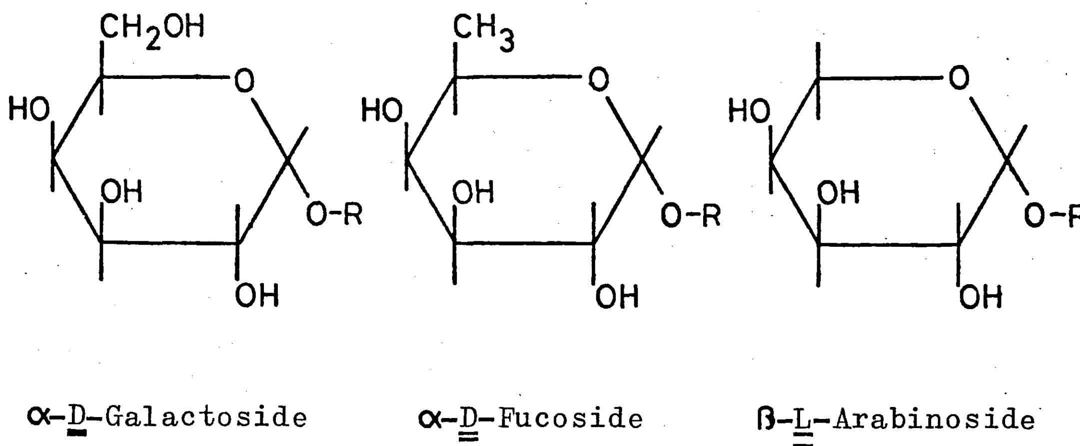
b. _Molecular Weights_. Apparent molecular weights have been determined for a number of α-galactosidases from various organisms, and values obtained vary from 20,000 to about 200,000. In most cases these have been estimated by gel filtration techniques[45]. Many dry seeds[43] contain two forms of the enzyme, one having a substantially higher molecular weight (2-6 times) than the other. The high

molecular weight α-galactosidase ($_I$) from _Vicia faba_ is believed to have a sub-unit structure and when passed through a Sephadex G-100 column in the presence of 6M urea is dissociated into six inactive fractions[41]. Some dormant seeds appear to contain enzyme of only one molecular size[43], however the number of forms of α-galactosidase may be related to the physiological state of the seed.

3. Specificity.

a. Hydrolase Activity. In general, a change of configuration of hydrogen and hydroxyl groups on any single carbon atom of a glycoside substrate may be sufficient to reduce the rate of hydrolysis or completely inhibit the hydrolytic action of the corresponding glycosidase. Absolute specificity for ring size and the configuration at the anomeric carbon atom of the glycoside, in particular, is normal. A great deal of work has been done on the specificity of a number of glycosidases (E.C. 3.2.1.20-26) and this has been extensively reviewed by Helferich[46], Pigman[47], Gottschalk[48] and Wallenfels[49]. In the case of α-galactosidases the ring structure of the glycone must normally be pyranoid and the configuration of -H and -OH on carbon atoms 2,3 and 4 must be the same as that of

D-galactose. Changes at C-6 of the glycosyl moiety of the substrate are normally tolerated by α-galactosidase. Hence, β-L-arabinosides(18,41,50,51,52,53) and p-nitrophenyl-α-D-fucosides(9) are normally hydrolysed by the enzyme. However, α-galactosidase from Streptococcus bovis(54), Epidinius ecaudatum(17), Diplococcus pneumonas(16) and Calvatia cyanthiformis(55) cannot use β-L-arabinosides as substrates. The quantitative evaluation of glycon specificity has been carried out with α-galactosidases from sweet almond(51) and Vicia faba(9). The affinity (1/km) of the enzymes for the substrates seems to depend largely on the structural changes in the glycon moiety and follows the order: α-D-galactoside > α-D-fucoside > β-L-arabinoside.



α-D-Galactoside α-D-Fucoside β-L-Arabinoside

This suggests that one of the specific points of attachment of the substrate to the enzyme might be through the primary alcohol group of the galactose structure.

Naturally occurring α-D-galactosides known to be hydrolysed by α-galactosidases include umbelliferose, raffinose, planteose, stachyose, verbascose, lychnose and isolychnose[11]. A large number of synthetic galactosides are also hydrolysed[11]. In general substrates with aryl aglycones are hydrolysed more rapidly than alkyl derivatives or disaccharides. For galactose-containing oligosaccharides, such as melibiose and manninotriose, reduction of the terminal reducing group (producing melibiitol and manninotriitol, respectively) decreases the rate of enzymic hydrolysis[54,56,57]. Oxidation of the reducing group, as in the case of the conversion of melibiose to melibionic acid, does not appear to affect the rate of hydrolysis[58]. In an homologous series of α-D-galactosides, the rate of hydrolysis seems to be reduced by an increase in the chain length[9,14,16,26,29,54,55,57,59,60,61], but in two microbial enzymes the reverse is reported to occur[54,62].

In addition to hydrolysing terminal galactosyl residues, almond α-galactosidase is also capable of splitting the internal galactosidic linkage of stachyose, forming galactobiose and sucrose[63,64]. On the other hand, coffee α-galactosidase can only cleave stachyose[65,66] and tetra-O-D-galactosyl sucrose[59] in a stepwise fashion starting from the non-reducing end. α-Galactosidases from Streptococcus bovis[54,57] and Epidinium ecaudatum[56]

acting on verbascose and verbascotetraose behave in a similar manner.

Many α-galactosidases also hydrolyse α-D-galactose linkages in polysaccharides. These include α-D-galactosyl residues in blood group B substances[62,67] and in galactomannans[18,68,69]. (See p. 41)

Differing reports on the rate of hydrolysis of galactomannans relative to the rates of hydrolysis of other substrates such as raffinose and stachyose have been presented. α-Galactosidase from germinated fenugreek seeds[60] is capable of hydrolysing oligosaccharides, and it will also partially hydrolyse galactomannan from fenugreek, (Man/Gal 1.2:1) liberating galactose. This enzyme also possesses galactosyl transferase activity. α-Galactosidase preparations from other sources do not appear to be so general in their activity. An α-galactosidase from honey locust seeds (Gleditschia sp.) will liberate galactose more easily from its own natural galactomannan substrate (Man/Gal 3.2:1) than from clover galactomannan which has a higher galactose content (Man/Gal 1.6:1), so much so, that on prolonged incubation with honey locust galactomannan an insoluble mannan product is produced. In 1964 Hui and Neukom reported[71] that despite lengthy incubations of α-galactosidase from green coffee beans with galactomannan

from guar (Man/Gal 1.54:1) and locust bean gum (Man/Gal 3.75:1), not all available galactose could be split off. Only about 20% of the galactose units in guaran could be hydrolysed, whereas with locust bean gum, about 70% was cleaved. This agreed with similar, earlier findings by Courtois _et al_[72]. Hui could offer no explanation for the production of this apparent "enzyme resistant" hydrolysis product. Agrawal and Bahl with enzyme preparations from both _Aspergillus niger_[73] and _Phaseolus vulgaris_[69] also obtained a 30-40% removal of total galactose from locust bean and guar galactomannans.

In contrast, an α-galactosidase isolated from the seeds of guar by Su Rae Lee[74], and purified by column chromatography on DEAE-Cellulose and DEAE-Sephadex, was more able to hydrolyse aryl-α-D-galactosides and guaran than oligosaccharides or alkyl-α-D-galactosides. He also claimed that the D-galactose residues in guaran were completely removed on the grounds that the hydrolysis product was an insoluble mannan.

Courtois and Le Dizet[68] investigating galactomannan hydrolysis by coffee bean α-galactosidase also confirmed that there were variations in hydrolysability according to the source of galactomannan substrate. Galactomannan from _Gleditsia ferox_ (Man/Gal 3.73:1) was reduced to a water insoluble material containing 24 residues of mannose

for each residue of galactose, but those of white clover (Man/Gal 1.6:1), genista (Man/Gal 4.14:1) and locust bean (Man/Gal 3.75:1) were only slightly hydrolysed.

Further, a purified α-galactosidase from germinating _Vicia sativa_ seeds, reported by Courtois _et al_[14], did not liberate galactose from galactomannans from various leguminous seeds. However, Bahl and Agrawal[73] found that an α-galactosidase purified from a commercial _Aspergillus niger_ enzyme was capable of hydrolysing both small molecular weight substrates and galactomannans. The crystalline α-galactosidase from _Mortierella vinacea_[18] was not able to liberate D-galactose from the galactomannan of guaran (Man/Gal 1.54:1).

b. _Transgalactosylation Reactions_. Apart from hydrolytic activities glycosidases are also known to catalyse transgalactosylation reactions[75,76]. These reactions of α-galactosidases have been studied extensively with respect to the effect of various parameters such as galactosyl donor and acceptor specificity, acceptor concentration, pH, temperature and the source of the enzyme[37,49,60,77]. Hydrolysis and transgalactosylation are essentially similar reactions. In hydrolysis glycosyl residues are transferred to water, and in transgalactosylation they are transferred to other hydroxylated compounds such as simple alcohols,

carbohydrates or phenols. The results obtained so far show that both hydrolytic and glycosyl transfer reactions are catalysed by the same enzyme, and also that they occur on the same active site of the enzyme molecule. In general, most enzymes have a low transfer/hydrolysis ratio, but preparations from Phaseolus vulgaris[78] and wheat[79] have high ratios. In the case of an enzyme isolated from Phaseolus vulgaris by Tanner and Kandler[78] transgalactosyl--ation from galactinol (O-α-D-galactopyranosyl-(1→1)-D-myo-inositol) to raffinose (with the formation of stachyose) occurs more readily than hydrolysis. The specificity of transgalactosylation catalysed by α-galactosidases from various sources has been reviewed by Dey and Pridham[11]. Phosphorylation, reduction or oxidation of either C-1 or C-6 of D-glucose or of D-galactose have been shown by Li and Shetlar[17] to destroy the acceptor properties of these hexoses using a Pneumococcal sp. α-galactosidase. They have also reported that 2-deoxy-D-galactose but not 2-deoxy-D-glucose served as acceptor for transgalactosylation with the same enzyme. Methylation of the C-3 hydroxyl of D-glucose also destroyed the acceptor properties of this hexose.

c. De Novo Synthesis. Galactosidases are also known to synthesis oligosaccharides when incubated with high concentrations of monosaccharides[80,81] (de novo synthesis)

and this procedure has been used for the synthesis of several glucose and galactose derivatives[82,83,84]. Clancy and Whelan reported, for example, that when a yeast of α-galactosidase was incubated in a solution of 17% D-galactose, 7.5% of the sugar was polymerised, and about 60% of this amount appeared as 6-O-α-D-galacto--pyranosyl-D-galactose[85,86]. Other products also formed in small amounts were α-1,3-, α-1,4- and α-1,5-D-galactobioses. Clancy and Whelan[86] also produced 3-O- and 6-O-α-D-galactosyl-2-acetamido-2-deoxy-D-glucose by allowing D-galactose and 2-acetamido-2-deoxy-D-glucose to react in the presence of the same yeast enzyme.

4. Kinetic Properties.

Some α-galactosidases are inhibited by 'sulphydryl agents'[16,87,88], while others are not[9,18,19,56,89]. Heavy metal ions such as Ag^{+}, Hg^{++} and Cu^{++} cause inactivation[9,74]. In the case of α-galctosidase enzyme $_{I}$ from *Vicia faba* Ag^{+} inhibition was found to be competitive[9]. Dey and Pridham further showed that Hg^{++} inhibition is weaker than that of Ag^{+} at pH 4.0, and that the former ion reacted noncompetitively. This discounts the possibility that Hg^{++} reacts with the carboxyl or histidyl residues at the active site of the enzyme. On the other hand,

inhibition by Hg^{++} at pH 6.0 was much greater and competitive, and hence in the latter case the reaction may have occurred with the histidine at the active site. Other inhibitors include D-galactose[9,18,44,56,87] and related structures such as, D-fucose[9], L-arabinose[9,18,44,89], myo-inositol[90] and D-galactal[44]. High concentrations of the substrate, p-nitrophenyl-α-D-galactoside inhibits Vicia faba α-galactosidases, whereas other galactose containing oligosaccharides, such as raffinose, are ineffective[41,51]. Sweet almond and Vicia faba enzymes are inhibited by photo-oxidation in the presence of methylene blue, suggesting that histidine residues are important for activity[89]. Little information is available regarding the mechanism of action of α-galactosidases[9,89,91] because of insufficient knowledge of the chemistry and kinetics of the enzymes from most sources. However, a "two step" mechanism has been postulated for the action of sweet almond α-galactosidase[89].

The pH optima of α-galactosidases vary to a considerable extent. In several cases the enzymes show two peaks (e.g. Fenugreek enzyme at pH 3.2 and 4.6[60] and Vicia faba, enzyme $_{I}$ at pH 3.0-3.5 and 6.0-6.5, and enzyme $_{II}$ at pH 2.5-3.5 and 5.0-5.5) but in general most α-galactosidases exhibit single broad optima[11]. From the effect of pH on the Km and Vmax of α-galactosidases from sweet almond and Vicia faba, the possible participation of a carboxyl

and a histidine imidazolium group in the enzyme-catalysed hydrolysis of substrates has been suggested[9].

In general, purified α-galactosidases are very stable if kept at low temperatures (2-4°)[13,16,28,29,30,44]. The enzyme from Vicia faba, however, was completely inactivated at temperatures below 0°[11]. At high temperatures α-galactosidases are relatively stable: several enzymes can be heated to 50-60° for 30 minutes, and lose only 50% of their original activity[13,41,44].

C. NATURALLY OCCURRING GALACTOSE DERIVATIVES AND THEIR METABOLISM.

1. Oligosaccharides.

In plants, galactose-containing oligo- and poly-saccharides and galactosylglycerides are ubiquitous[92,93], and these are commonly accompanied in the tissues by α-galactosidase. On maturation of many seeds there is an apparent increase in α-galactosidase activity with the concomitant synthesis of galactosyl sucrose derivatives[94,95,96,97]. Upon germination, α-galactosidase is undoubtedly involved in the hydrolysis of these oligosaccharides, which serve

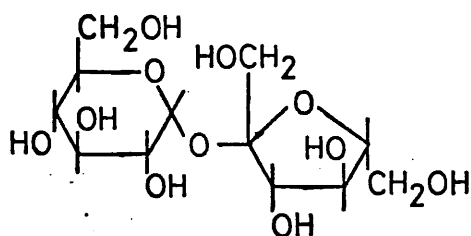
as a soluble and a readily metabolizable energy reserve[97]. In order that galactosyl sucrose derivatives may accumulate during seed maturation, there must be some mechanism that prevents the interaction of enzyme and substrate. This could perhaps be effected by compartmentalization or by an endogenous inhibitor. Vicia faba seeds contain two α-galactosidases[9] and it was originally reported that one of these (low molecular weight) was present in green immature seeds, and that during maturation this appeared to be converted into another high molecular weight form which had a higher specific activity on natural substrates. Thus it was believed that a maximum rate of breakdown of the reserve oligosaccharides could occur at seed germination.

The raffinose family of oligosaccharides (See Table 2) is the most widely occurring group of galactosyl sucrose derivatives in plants[98,99,100,101]. The lower members of the series, raffinose and stachyose, have been reported to occur in many plant organs, such as roots, seeds, shoots and underground tubers[100,102,103,104,105] in amounts usually equal to or greater than that of sucrose: Stachyose is commonly present at higher levels than raffinose. An isomer of raffinose with the trivial name planteose (Table 2) is found in several plant species, commonly in association with stachyose and raffinose. In

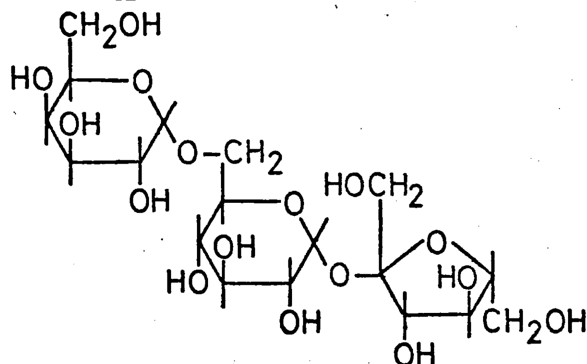
TABLE 2.

The Raffinose Family of Oligosaccahrides[6].

Sucrose (O-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside)



Raffinose (O-α-D-galactopyranosyl-(1→6)-O-α-D-glucopyranosyl-
-(1→2)-β-D-fructofuranoside)



Stachyose (O-α-D-galactopyranosyl-(1→6)-O-α-D-galactopyranosyl-
-(1→6)-O-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside)

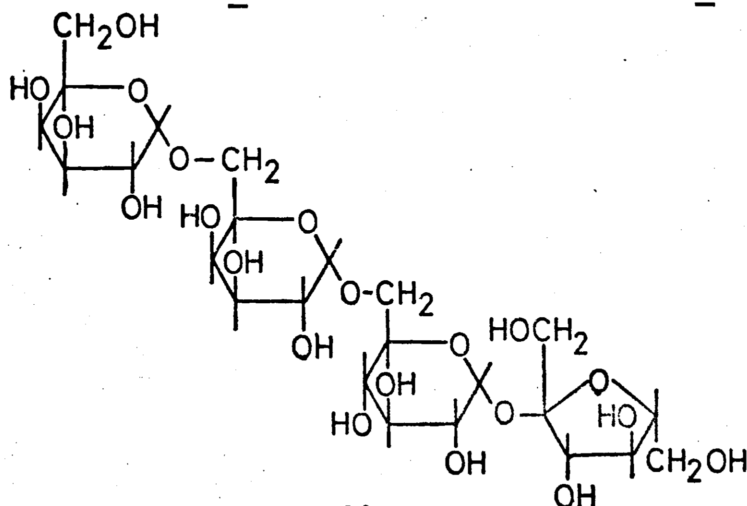
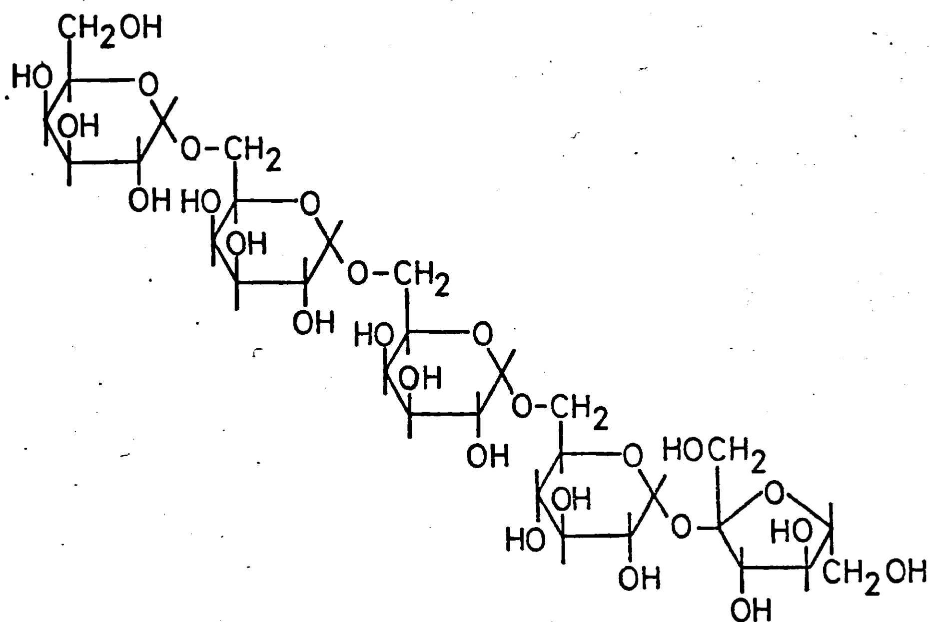


TABLE 2.-Continued.

Verbascose (O-α-D-galactopyranosyl-(1→6)-O-α-D-galactopyranosyl--(1→6)-O-α-D-galactopyranosyl-(1→6)-O-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside)



Planteose (O-α-D-~~gluc~~galactopyranosyl-(1→6)-O-β-D-fructofuranosyl--(2→1)-α-D-glucopyranoside)

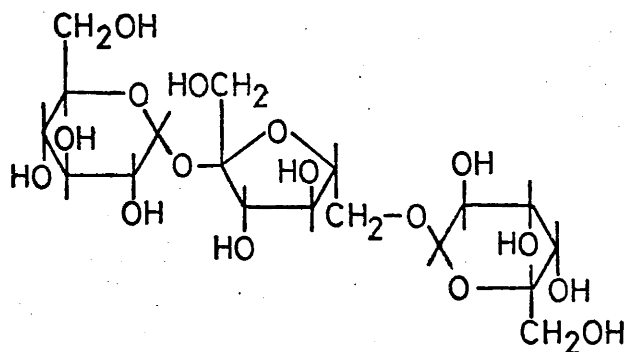
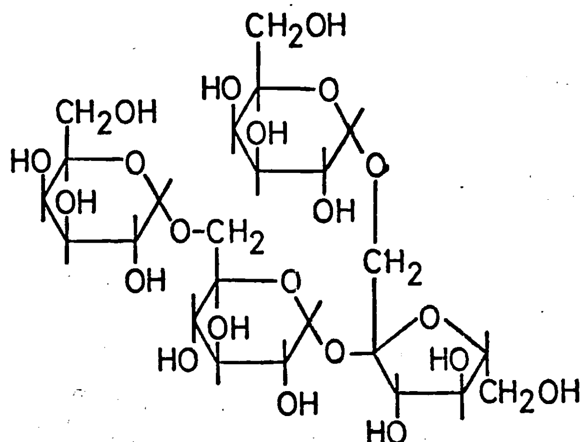


TABLE 2.-Continued.

Lychnose (O-α-D-galactopyranosyl-(1→6)-O-α-D-glucopyranosyl-(1→2)-O-β-D-fructofuranosyl-(1→1)-α-D-galactopyranoside)



Family derived from Lychnose

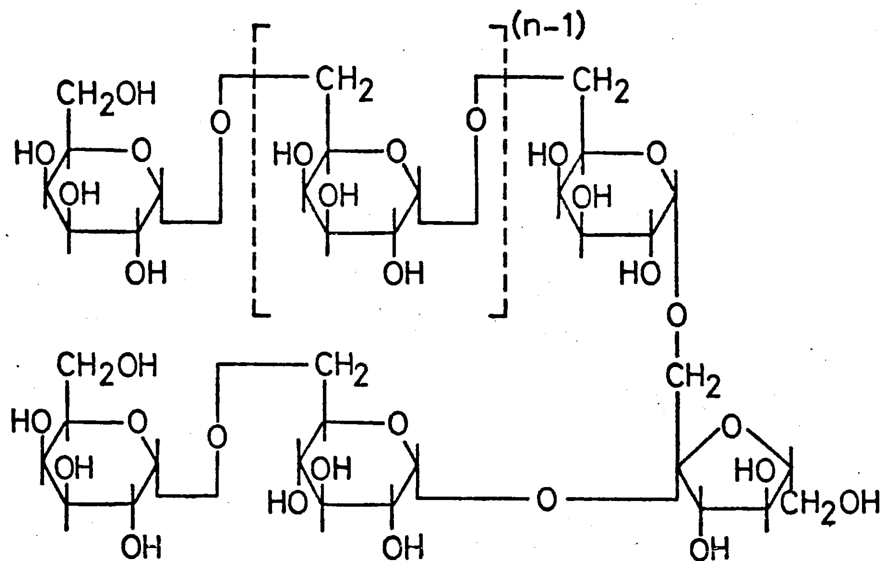
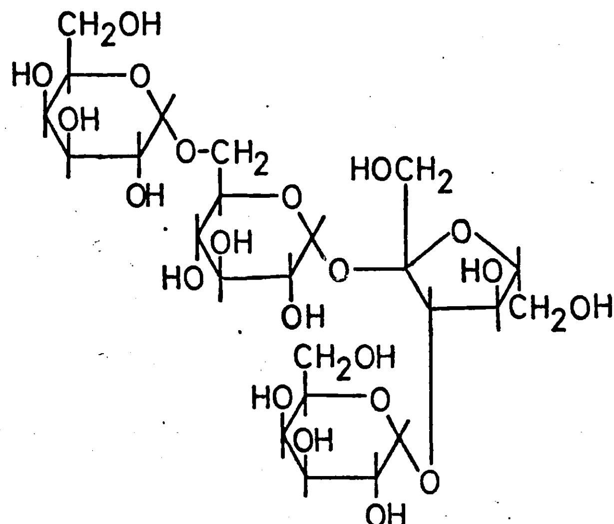
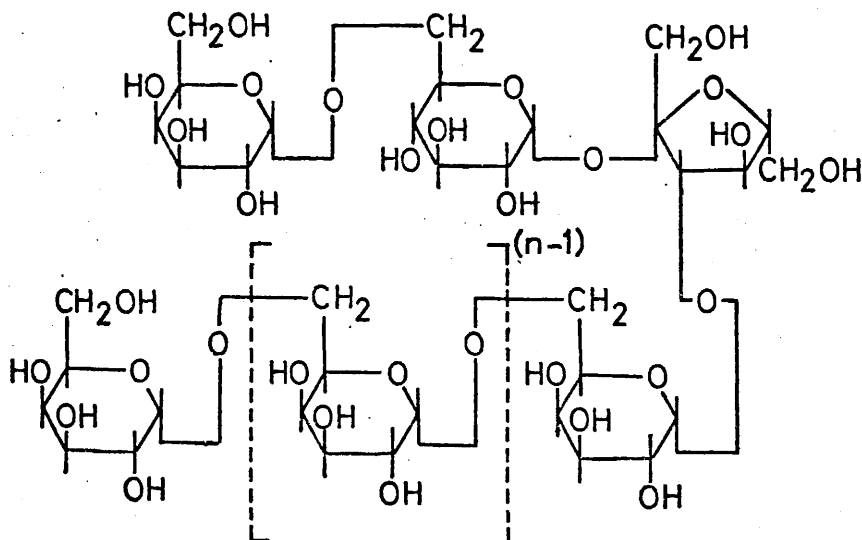


TABLE 2.-Continued.

Isolychnose (O-α-D-galactopyranosyl-(1→6)-O-α-D-glucopyranosyl-(1→2)-O-β-D-fructofuranosyl-(3→1)-α-D-galactopyranoside)

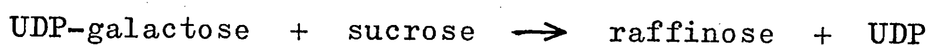


Family derived from Isolychnose



Plantago sp. this trisaccharide is present in the roots but not the seeds[7]. Other oligosaccharides related to the raffinose family that occur naturally include lychnose and isolychnose which have been isolated from roots of various plant species: in spring isolychnose appears to be the predominant oligosaccharide, while in autumn it is lychnose[106,107]. They are usually accompanied by stachyose. Higher homologues with increasing numbers of galactose residues are also known. The higher members of the raffinose family of oligosaccharides in general, however, have only been isolated from seeds and roots. In leguminous seeds it has been reported that the raffinose family of oligosaccharides constitute the soluble reserve carbohydrates during dormancy[95,108,109]. In Vicia faba[110] raffinose and stachyose were found to accumulate during seed maturation.

The possible biosynthetic pathways for the formation of raffinose in several plant species has been examined by a number of workers some[110,111,112] of whom have concluded that the galactose is derived directly from UDP-galactose with sucrose acting as an acceptor.



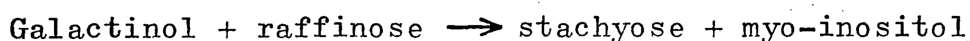
UDP-Galactose has been detected in many plant tissues, including *Vicia faba* seeds[110], and may originate from UDP-glucose by the action of UDP-glucose 4'-epimerase[113], or from galactose via galactose 1-phosphate which involves galactokinase and UDP-galactose pyrophosphorylase. Duperon[114] has shown that ^{14}C-labelled glucose rapidly gives rise to labelled sucrose as well as raffinose in developing *Phaseolus vulgaris* seeds.

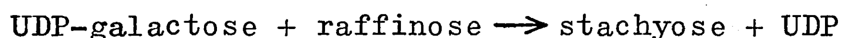
Raffinose has also been synthesised *in vitro* by allowing high concentrations of sucrose and melibiose (or *p*-nitrophenyl α-D-galactoside) to react in the presence of α-galactosidase [115,116,117,118,119]. The physiological importance of such a reaction is in doubt. The thermodynamics of the process are unfavourable although it is conceivable that high concentrations of sucrose could be present in the cell to overcome the problem. There are, however, no known endogenous galactose donors for such a reaction.

Tanner and Kandler[120,121] believe that galactose is not transferred directly from UDP-galactose to sucrose to form raffinose. They originally isolated a specific transferase with poor hydrolytic properties from the ripening seeds of *Phaseolus vulgaris*. This enzyme catalyses, *in vitro*, the transfer of a galactosyl moiety from galactinol (O-α-D-galactopyranosyl-(1→1)-D-myoinositol) to raffinose

with the production of stachyose. The author suggests that this reaction can occur in vivo and that galactinol is regenerated from the free myo-inositol by the transfer of a galactosyl residue from UDP-galactose.

GDP-galactose + myo-inositol → galactinol + UDP


Galactinol + raffinose → stachyose + myo-inositol


UDP-galactose + raffinose → stachyose + UDP

In subsequent work Sensor and Kandler[122] showed that galactinol functions as a galactosyl donor in several plants containing raffinose and stachyose and that raffinose could be formed from galactinol and sucrose.

When plants were subjected to photosynthesis in the atmosphere of $^{14}CO_2$ for 30 - 60 minutes, label first appeared in galactinol and this was followed by introduction of label into raffinose and stachyose. A similar observation was reported by Shiroya[123] who showed that when $^{14}CO_2$ was fed to needles of *Pinus strobus*, sucrose and raffinose in the phloem became labelled after 6 hr. A study of the kinetics of the labelling in the leaves of *Lamium macalatum*

after feeding $^{14}CO_2$[122] revealed that the galactinol pool was saturated with ^{14}C more rapidly than the oligomers of the raffinose series, and under conditions of continued photosynthesis, label accumulated in raffinose and stachyose and decreased in galactinol. During the process, label accumulated initially in the galactosyl portion of the galactinol and then in the galactosyl residue of raffinose and stachyose. This shows that in this case raffinose and stachyose are produced by the specific transfer of a galactose moiety from galactinol to sucrose and raffinose respectively. It is likely that by a repetition of this process the whole series of raffinose oligosaccharides is produced[124]. In the case of stachyose, the highest specific activity during a short period of exposure to $^{14}CO_2$, was found in the terminal galactosyl residue[125]. A study of this biosynthetic process in the ripening seeds of Phaseolus vulgaris confirms the work with Laminium sp. leaves, and shows that the synthesis of raffinose and stachyose takes place in the seeds and not in the pod[126]. During ripening of Phaseolus vulgaris seeds, the pool of inositol and sucrose, which accumulates during the early stages of seed development, disappears as galactinol is formed. Later, the level of galactinol decreases as galactinol-raffinose-galactosyl transferase[127,128] activity increases and stachyose is formed[126].

The formation of stachyose *in vitro* was demonstrated by Shiroya[123] who incubated raffinose with a cotton seed extract which contained α-galactosidase. Again, it is not known whether the reaction has any physiological significance.

No informaticn is available regarding the synthesis of planteose or the lychnose or isolychnose series of oligosaccharides.

Gould and Greenshields who studied the changes[95] in levels of galactose-containing oligosaccharides in ripening and germinating seeds of *Phaseolus vulgaris* demonstrated that upon germination these oligosaccharides disappeared leaving mainly sucrose in the cotyledons, and sucrose, glucose and fructose in the embryo. No free galactose was detected in any part of the germinating seeds. Shiroya[123] similarly reported that raffinose and stachyose from resting cotton seeds disappeared rapidly and completely in the early stages of germination. Shiroya also observed that the activity of α-galactosidase in resting, soaked cotton seeds was much higher than that of invertase, and that raffinose and stachyose were hydrolysed by the former enzyme giving rise to sucrose and galactose. The failure to detect free galactose in tissues where active hydrolysis of galactosylsucrose derivatives is occurring suggests that

galactose utilisation is very efficient, and that the rate is as at least as high as the rate of hydrolysis of galactose from the oligosaccharide by α-galactosidase. There is no evidence that invertase plays any role *in vivo* in the initial degradation of galactosylsucrose derivatives.

2. Galactomannans.

One of the major galactose-containing reserves in leguminous seeds is galactomannans[129,130,131]. These neutral polysaccharides commonly encountered in the endosperm of some plant seeds are one of the two main groups of galactomannan polysaccharides. The other group being derived from microbial sources, in particular the yeasts and other fungi.

The seed galactomannans occur in the endosperms of seeds of a wide range of species of the *Leguminoseae*[132,133,134]. Galactomannan has been identified in about seventy species of this family, and except for *Gymnocladus dioica*[135], in which the mucilage is extracted from the inner side of the seed coat, and *Glycine max.*[130], in which it occurs in the hulls, the sole source of the galactomannan has been the endosperm. The endosperm is the translucent white region of the seed which lies between the outer husk and

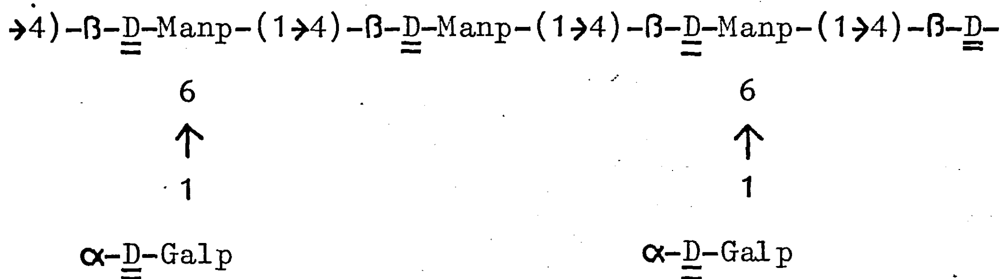
the innermost seed-germ, and which functions as a food reserve for the germinating embryo. The general chemistry of the galactomannans has been reviewed on several occasions by Whistler[8] and others[129,136].

The galactomannans from the seeds of leguminous plants vary in the proportions of the two constituent sugars (and the variance is characteristic of the species), but they belong to the same structural type in which chains of (1→4)-linked β-D-mannopyranose residues carry at intervals single unit side chains of α-D-galactopyranose residues attached by (1→6) linkages. These polysaccharides, notably those from guar and from the carob tree (locust bean gum) are widely cultivated in Mediterranean countries and find commercial applications as sizes in the Textile industry, as beater additives in paper making, and as thickening agents in the Food industry.

Guaran, the galactomannan from guar seeds, was the first polysaccharide of this type for which the main structural features were established[137,138,139,140]. Hydrolysis of guaran gave D-galactose and D-mannose in the ratio of 1 : 2.

The following structure for the polysaccharide is based on the isolation of 2,3,4,6-tetra-O-methyl-D-galactose,

2,3,6-tri-O-methyl-D-mannose and 2,3-di-O-methyl-D-mannose in equimolar proportions on hydrolysis of the methylated polysaccharide[141,142], together with the characterisation of 4-O-β-D-mannopyranosyl-D-mannose, the polymerhomologous trisaccharide, 6-O-β-D-mannopyranosyl-D-mannose and O-α-D-galctopyranosyl-(1→6)-O-β-mannopyranosyl-(1→4)-D-mannose as products of partial depolymerisation with acid or by enzyme preparations.



Guar Galactomannan

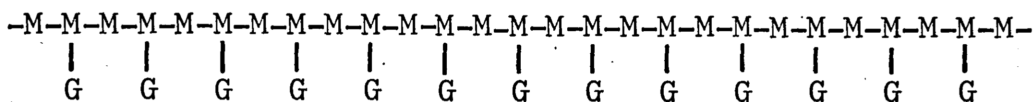
The above structure is only an average representation of the polysaccharide based on chemical investigation. However, this average representation is supported by infra-red spectrophotometry, optical rotation studies and X-ray crystallography[129].

For some galactomannans there are reports of small contributions of linkage types other than those accounted for in the above representation. These variations include the presence of small quantities of β-(1→3)[143,144,145] and β-(1→2)[146] linkages in the mannan backbone and the occurrence of galactose as non-terminal groups, linked through position 1 of the new galactose to position 6[147,148] of the original. Also, in the galactomannan from Cassia absus[147], it has been reported that some of the galactose is linked to carbon -2 of mannose. It has also been shown that kentucky coffee bean galactomannan contains significant quantities of terminal mannopyranosyl units[135] as single side chain stubs.

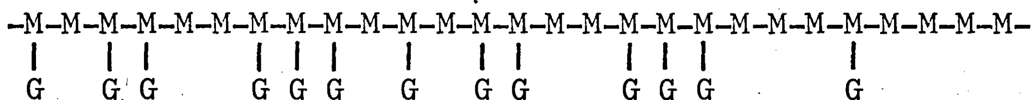
In seed galactomannans the distribution of the galactose along the mannan backbone has been the subject of a number of conflicting reports. Problems discussed have been whether the distribution is specific for any one species, and whether within a species it is totally random, or whether the galactose takes up an ordered "block" like distribution. A knowledge of the distribution of the α-galactosyl stubs along the main chain is necessary before a correlation can be made between structure and properties of the galactomannans.

Formulas 1-3 indicate the three possible extremes of

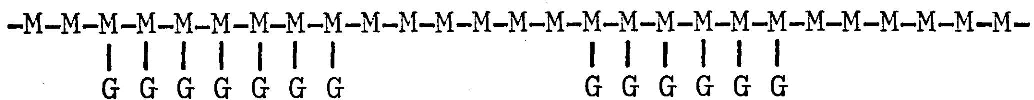
structures where a polysaccharide has -D-mannose (M) : -D-galactose (G) in a ratio of 2 : 1.



(1)



(2)



(3)

Structure (1) represents a regular arrangement of side chains; (2), a random distribution of side chains, and (3), a structure in which the side chains occur in blocks. Intermediates between these structures are of course possible.

Rees *et al*[149] are supporters of the 'block structure' on the grounds of the behaviour of the galactomannans in mixed gels. He cites both guar and locust bean gum. On the other hand, however, another group of workers[150] state that the distribution of the galactose along the mannan chain in both guar and locust bean gum is totally random. A more recent paper by Baker and Whistler[151] who have made chemical derivatives of galactomannans states that the side chain units of guar are alternately disposed along the D-mannan backbone, whereas those of locust bean gum are disposed in uniform blocks. These findings are in accordance with the mixed gel interaction experiments of Rees[149].

Reid and Meier[152] claim that the ratio of galactose to mannose in the galactomannans of leguminous seeds reflect their botanical classification, and they suggest that this may be an aid to the actual classification of this group.

In contrast to the comparative conformity of common structure of the galactomannans from plant sources, galactomannans from microorganisms have structures which are quite different. A variety of linkages have been detected[153,154,155,156], and in addition the galactose is frequently present in the furanoid form[153,154,157]. These polysaccharides are produced, for example, by dermatophytes[156], which are

responsible for a number of skin diseases. Here, the galactomannans may even be causative agents[158]. The nuclear magnetic spectra of the yeast mannans and galactomannans have been used to aid the taxonomic classification of yeasts[155,159,160,161]. Microbial galactomannans have recently been reviewed[153,154].

The biosynthesis of seed galactomannans has attracted relatively little attention. The earliest reports[162,163] proposed that the galactose residues were attached randomly to the main chain of mannose residues. However, when sucrose and raffinose were detected in the seeds of Medicago sativa Hough et al[164] suggested that one plausible pathway for the synthesis, and also the degradation, of the galactomannan might be transglycosylation of α-D-galactopyranosyl units, in the first case from raffinose to a mannose polymer, and in the second from galactomannan to sucrose. It was not until 1970 when the topic was reopened by two different laboratories that further information came to light.

Sioufi, Percheron and Courtois[165] demonstrated that in Trigonella foenum graecum (fenugreek) seedlings sugar nucleotides were essential factors in the metabolic pathways leading to the synthesis of the cell wall polysaccharides. The work of Reid and Meier[166] on the other hand attempted

to determine at what stage of seed development the galactomannan was synthesised, and if the mannose/galactose ratio varied during its formation. They also examined seed extracts for possible low molecular weight carbohydrates which might be used as galactomannan precursors[166]. With fenugreek they were able to remove seeds at different stages of ripening from a single plant and to investigate the galactomannan structure. They found no change in the chemical composition (Mannose/Galactose ratio) of the hot water extracted polysaccharide from young or old seeds. Regarding possible precursors of the polysaccharide, no free mannose was present in the tissues, nor were mannose-containing oligosaccharides detected. A trace of free galactose was found and raffinose, galactinol (O-α-D-galactopyranosyl-(1→1)-D-myo-inositol) and stachyose were also identified. Two further galactose-containing compounds were tentatively identified as verbascose and digalactosylmyoinositol. Sucrose, myo-inositol, glucose, fructose and trace amounts of other carbohydrates were also present. The young seeds contained small amounts of low molecular weight carbohydrates consisting predominately of sucrose, myo-inositol, galactinol, glucose and possibly raffinose. With increasing seed maturity the amount of low molecular weight carbohydrates in the seed increased, and the composition of the mixture changed markedly; the proportion of stachyose increasing, and then remaining at a constant

level, and the sucrose, galactinol and hexoses decreasing. Reid and Meier by cataloguing the levels of galactomannans in developing seeds claimed that the galactomannan began to be laid down at an early stage of development, and continued to be formed until the seeds reached their full size and began to become yellow. They futher claimed that the polysaccharide appeared to be synthesised by a specific mechanism involving simultaneous deposition of both galactosyl and mannosyl residues. This is contrary to the earlier suggestions[162,163,164]. Further, stachyose, also a reserve carbohydrate in these seeds, is formed simutaneously with the galactomannans, and because galactinol is thought to have a place in the biosynthesis of the former[121], it was suggested that galactinol might also play a part in galactomannan formation.

Courtois and co-workers have also shown[70,72,167] that there is no change in the proportion of galactose to mannose residues during the formation of polysaccharide in the seeds of Gleditsia ferox and Gleditsia triacanthos.

In 1889 Tschirch[168] first reported that galactomannans were rapidly metabolised during seed germination. This was followed in 1894 by Schulze publishing similar results for a number of other galactomannan-containing seeds. Much more recently, the biochemical degradation of the

galactomannan of fenugreek seeds has been the subject of an extensive investigation by Reid and Meier[169]. Their original report was founded upon light and electron microscopy and chemical analysis of the seeds at different stages of germination. They found that in the earliest stages of germination the raffinose family of oligosaccharides were metabolised both in the endosperm and in the cotyledons of the seed, but that there was no change in the appearance, amount or composition of the main reserve galactomannan in the endosperm. About 18 hr. after the emergence of the radical the endosperm galactomannan began to be mobilised. Then, over a period of hours the polysaccharide was completely degraded, and the breakdown products, mainly galactose and mannose were absorbed by the cotyledons in which the level of the sucrose increased and starch was formed. It was noticed that this mobilisation of the galactomannan was accompanied by the formation in the endosperm of a dissolution zone the form of which implied that the aleurone layer was involved in the degradation process.

The above hypothesis was then further tested[170,171] and Reid and Meier were able to state that degradation was independent of the embryo, and that the enzymes required for it were synthesised by the aleurone layer during the first stages of germination, and were secreted

into the reserve tissue. Not only in fenugreek, but also in crimson clover[171] and lucerne[171] the reserve endosperm galactomannans were observed to be broken down to free galactose and mannose when dry, isolated endosperms (devoid of embryo) were incubated under germination conditions. Breakdown could be prevented by inhibition of protein synthesis or of oxidative phosphorylation in the aleurone layer. Reid and Meier[171] reported that resting aleurone cells contained *inter alia* a large number of ribosomes more or less regularly distributed in the ground plasma. At the onset of germination, before galactomannan breakdown began, polysomes were formed and seen, at least partly, to become associated with vesicles and flat cisternae both probably newly formed and derived from the endoplasmic reticulum. Concurrently with galactomannan breakdown in the reserve cells, wall erosion occurred in the aleurone layer; the contents of the aleurone grains disappeared, and the rough vesicles and cisternae proliferated. Later, a large central vacuole was formed which incorporated smaller vacuoles emerging from the cytoplasm, and at the same time the rough endoplasmic reticulum vesicles and cisternae became highly distended. From such evidence they concluded that the cells of the aleurone layer were responsible for the synthesis and secretion into the storage cells of enzymes necessary for galactomannan degradation.

They were unable to associate galactomannan depletion with any significant accumulation of released galactose and mannose in whole seeds. The large accumulation of these sugars only occurred in incubated, isolated endosperms. Reid and Meier were only able to suggest that the products from such hydrolysis were taken up by the cotyledons and/or embryos where they were further metabolised.

Contrary to these findings by Reid and Meier[171] who were unable to detect any significant change in the galactose content of fenugreek galactomannan as germination proceeded, Courtois and Le Dizet[148] have suggested that during galactomannan hydrolysis in Gleditsia ferox there is an initial removal of most of the galactose residues from the polymer, followed by cleavage of the mannan chain giving manno-oligosaccharides. A similar mechanism of hydrolysis was reported for the galactomannan of fenugreek seeds where the percentage galactose in the polysaccharide was reported to decrease from 49% to 12% on germination[165].

The enzymes most likely to be involved in galactomannan breakdown are:-

a. α-D-Galactosidase

b. β-D-Mannanase

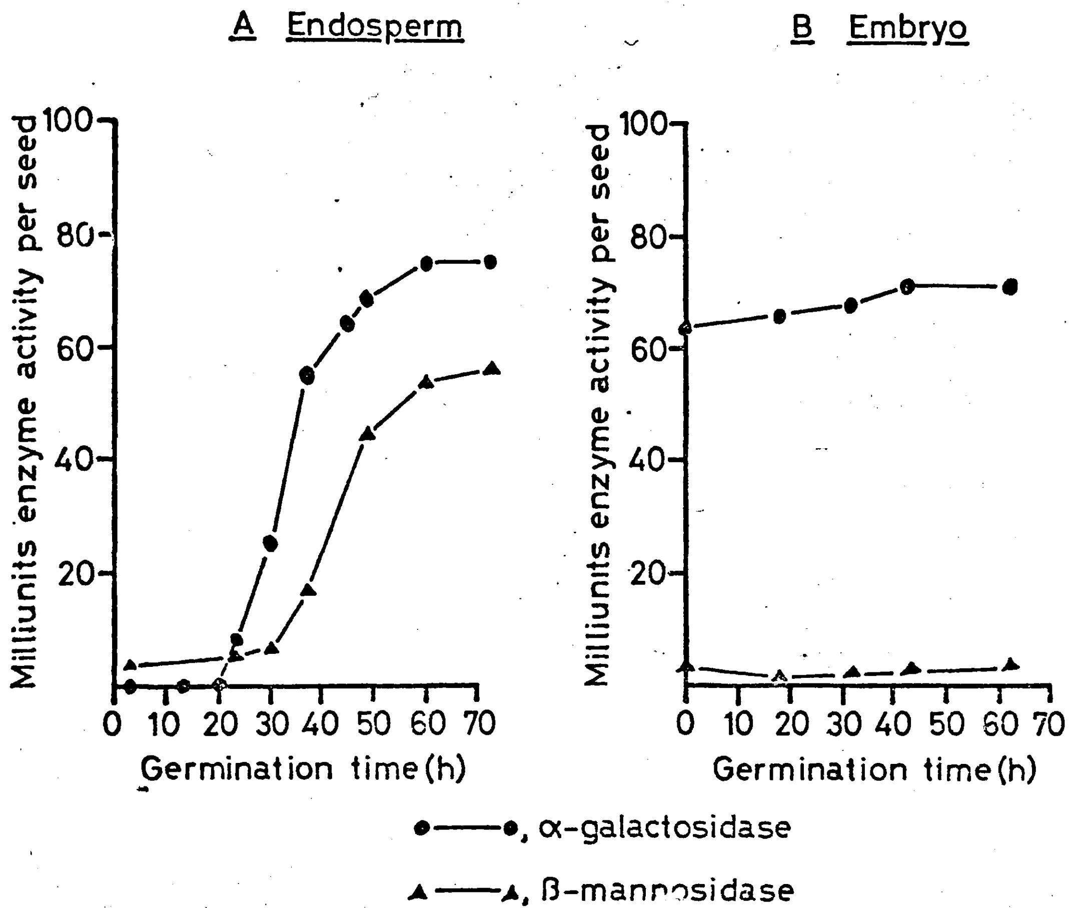
c. β-D-Mannosidase

d. Oligo-D-mannosyl β-(1→4)-phosphorylase

a. α-D-Galactosidase. The properties of these enzymes have been considered in detail in Section B. (See page 15)

Increases in α-galactosidase activity concurrent with galactomannan hydrolysis have been found in seeds of fenugreek[165,172], guar[74], Gleditsia sp.[167] and some other species[11,172]. Fenugreek seeds[31] have α-galactosidase activity in the endosperm and in the embryos. Activity in the embryos is quite high in dry seeds and remains constant on imbibition, whereas the endosperm activity is undetectable in ungerminated seeds, but increases markedly after imbibition. (See Graph 1) Increases in endosperm α-galactosidase activity were believed to be associated with galactomannan hydrolysis. This was later confirmed by the work of McCleary and Matheson[172] on α-galactosidases extracted from the embryos and endosperms of lucerne, guar, locust bean and soybean. In contrast, the α-galactosidases from the embryo which could be separated from the β-D-mannanase

GRAPH 1.



Per seed activities of α-galactosidase and β-mannosidase in endosperms and in embryos of germinating fenugreek seeds. (Dry, punctured seeds were set out to germinate at time zero; they were fully swollen after 5 hr.)[31].

activity found only in the endosperm, appeared to be primarily involved in galactosylsucrose oligosaccharide hydrolysis.

b. β-D-Mannanase. (E.C. 3.2.1.25.). β-D-Mannanases are endo hydrolytic enzymes capable of hydrolysing the (1→4)-β-D-mannopyranosyl linkages of D-mannans, D-gluco-D-mannans and D-galacto-D-mannans.

β-D-Mannanases have been reported in fungal and microbial sources[173,174,175,176,177,178,179,180,181], plant material[182,183] and in the digestive juice of Crustacea[184].

Bourquelot and Herissey (1899)[185,186] were the first to study the appearance of β-mannanases in plants and their role in the hydrolysis of various mannan-type polysaccharides. They observed the appearance of an enzyme activity capable of converting galactomannan to galactose and mannose on germination of the seeds of locust bean, fenugreek, lucerne and clover. Since then a preparation from Bacillus subtilis [181], endo-specific only for the β-D-(1→4) mannosidic linkages, has been isolated in a crystalline state.

Most β-mannanases have optimal pH values for activity between pH 4-6[74,177,181,182,187,188,189,190], however,

some like those of Sporocytophaga sp. and variuus rumen bacteria exhibit maximal activity at pH 7[191,192]. Reese and Shibata[193] found that the activity 'peaks' of several fungal preparations ranged from pH 2-8. Most followed the same pattern between 6 and 8, but below pH 4.5 the enzymes were divided into two groups, one of which was relatively inactive at pH 3.5, and the other had quite high activity even at pH 3.0 . These enzymes, in general, were stable between pH 4 and 8.

Microbial β-mannanases[177,181,187,193] are usually most active at temperatures between 50-60°, with inactivation above these values. Plant β-mannanases[189,190] are inactivated near 50° and show maximal hydrolysis rates at temperatures of 35-40°. Bacillus subtilis [181] β-mannanase is believed to be stabilised at high temperatures by Ca^{2+}. It has an optimal temperature for activity of 60° in the absence of E.D.T.A., but if this is present the temperature optima drops to 40°.

Since galactomannans are heteropolysaccharides and mannans are insoluble, the significance of Michaelis constants determined with these substrates is limited. Km values obtained for plant and microbial β-mannanases on these substrates range from 0.071 to 0.59% carbohydrate[74,181, 189,193]. The two guar β-mannanases isolated by Lee[74]

gave values of 0.083 and 0.27% with ivory nut mannan.

Molecular sizes of β-mannanases have been determined almost exclusively by gel filtration and values of 26,000 and 74,000 for two guar β-mannanases[74] and 22,000 for _Bacillus subtilis_ β-mannanase[181] have been obtained. From sedimentation equilibrium studies, a Mw value of 42,000 was found for the β-mannanase in the 'Cellulase 36' preparation obtained from _Aspergillus sp._[177].

The major role of β-mannanase in plants appears to be the hydrolysis of galactomannans and mannan reserves. As with α-galactosidase there is an increase in activity paralleling galactomannan depletion. This has been monitored in germinating seeds of fenugreek[165], guar[74] and other species[185].

Studies on the properties of plant β-mannanases have been hampered by the difficulty in obtaining these enzymes free of interfering glycosidases. Beaugiraud and Percheron[194] had only a limited success in obtaining a β-mannanase free of α-galactosidase from germinated fenugreek seeds. They reported that continuous flow electrophoresis gave α-galactosidase minus an assayable amount of β-mannanase, but not the reverse. β-Mannanase was obtained free of α-galactosidase from fenugreek by fractional precipitation

with ammonium sulphate, but in low yield. Lee[74] using column chromatography on DEAE-Cellulose and gel filtration on Sephadex G-100 isolated three forms of β-mannanase from germinated guar seeds. One of these was obtained almost free of α-galactosidase, but the other two contained quite high levels of this activity.

β-Mannanases, free of interfering glycosidase activities, hydrolyse mannans almost exclusively to mannobiose and mannotriose.

Ahlgren, Eriksson and Vesterberg[175,195] attempted among other things to characterise by gel filtration, isoelectric focusing and zone electrophoresis, β-mannanase and to compare it with β-mannosidase, the related exo-enzyme. In studies with these enzymes from Aspergillus sp., Stereum sanguinolentum and other sources they found that the extracellular endo-enzymes were smaller in molecular size than the corresponding exo-enzymes.

Beaugiraud and Percheron[183] using a β-mannanase separated from germinated fenugreek seeds studied the hydrolysis products obtained from mannan, oligosaccharides and reduced oligomannoside substrates. They reported that the enzyme preparation would hydrolyse both mannopentaose and mannotetraose to mannobiose and mannotriose, with the

production of only traces of free mannose. To explain the products obtained by hydrolysis of mannotetraose, it was suggested that some of the mannobiose units produced combined with mannotetraose by a transglycosylation reaction to form mannohexaose, which was then split into two mannotriose units. The rate of hydrolysis of manno-oligosaccharides also markedly increased with increasing $\overline{DP}$ up to 5. Thus the pentasaccharide was hydrolysed much more readily than the tetrasaccharide. As a result of these experiments they considered their enzyme to be an endo-β-D-(1→4)-mannanase.

A β-mannanase purified from a commercial preparation of Aspergillus sp. enzyme[177] was found to be active against guar, breaking it down only to mannobiose and mannotriose. Similarly, Courtois and Le Dizet[186] reported that a commercial preparation of fungal hemicellulase depolymerised galactomannans with a Galactose/Mannose ratio of 1 : 4 . Mannobiose and mannotriose were released, together with a non-dialysable product in which nearly all the mannose units were substituted with galactose residues. The β-mannanase from this preparation did not liberate mannose or galactose from galactomannan, but behaved like an endohydrolase.

A β-mannanase from Rhizopus niveus[196], purified by DEAE

Sephadex column chromatography and Sephadex G-100 gel filtration, hydrolysed green coffee bean galactomannan mainly to di-, tri- and tetra-saccharides containing galactose and mannose. β-Mannanases from Bacillus subtilis, Alfalfa and Leucaena sp.[197] also act in a similar way on galactomannans and hydrolyse β-mannosyl linkages where the mannose residue does not carry a galactose residue. After the endo-attack, a non-dialysable residue remains, in which almost all the mannoses are substituted with galactose. The β-mannanase extracted from Sporocytophaga myxococcoides [192] was also characterised as an endo-polysaccharide. It had no activity against oligomannosides when there were four or less residues.

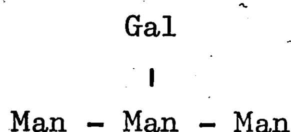
A β-mannanase purified from the extracts of Aspergillus niger[187] when incubated with galactomannan from soybean hull, guaran and coffee bean gave manno-oligosaccharide products, again suggesting that the activity was of an endo-nature.

The β-mannan hydrolysing enzyme from Bacillus subtilis[181], which was isolated in a crystalline state, was found to attack only β-D-(1→4)-mannosidic linkages in the main chain of galactomannan of soybean seed coat, guar gum, coffee bean or glucomannan of Konjac. Attack on the coffee bean polysaccharide was 'endo-wise' to form mannobiose,

mannotriose and mannotetraose. Attack patterns on several mannohomo-oligomers from coffee indicated that the enzyme preferentially attacked the β-D-(1→4)-mannosidic linkages that were present in the position three to four mannose residues from the non-reducing end of the mannose chain. Contrary to this detailed account of a systematic, stepwise attack, konjac β-mannanase[189] is reported to hydrolyse mannan randomly.

In conclusion, it would appear that, whereas preparations of β-mannanase free of β-mannosidase yield mainly mannobiose and mannotriose, if β-mannosidase is present, mannose is produced[198]. It can, therefore, be seen that the degree of hydrolysis of galactomannans with preparations of β-mannanase, free of α-galactosidase, is controlled by the galactose content of the polysaccharide. Reese and Shibata[193] stated that two contiguous unsubstituted mannosyl units are required for β-mannanase hydrolysis of galactomannans. They found that a fungal β-mannanase, substantially free of interfering glycosidase activities, hydrolysed locust bean gum to a much greater extent than guar. On hydrolysis of guar gum there remained a high $\overline{DP}$ product which precipitated with alcohol and had a Mannose/ Galactose ratio of 1 : 2. So it was suggested that one unsubstituted mannose unit between galactosyl branches does not allow sufficient space for the approach of the enzyme

to the hydrolysis site. The isolation of a tetrasaccharide containing galactose, from the locust bean gum hydrolysate, for which the probable structure:



was suggested, was given as further evidence for this theory. A galactose-containing tetrasaccharide, as well as a galactose-containing pentasaccharide were also reported by Reese and Shibata as products of hydrolysis of coffee bean galactomannan by Bacillus subtilis β-mannanase. In contrast, Courtois and Le Dizet[186,197] have suggested that only one unsubstituted mannosyl unit is required for hydrolysis.

c. β-D-Mannosidase. (E.C. 3.2.1.25.). β-D-Mannosidases are necessary for the complete hydrolysis of the β-D-manno-oligosaccharides released by β-mannanase to mannose. These former enzymes are exo-hydrolases which remove single β-(1→4)-linked mannose residues from the non-reducing end of manno-oligomers[198], manno-polymers[74] and mannose-containing glycopeptides.

β-D-Mannosidases have been extracted from various seeds, including those of fenugreek[31,194], red clover[199] and guar[74]. Activity has also been detected in pineapple bromelain[200] where it completely released the terminal D-mannose from the Man-NAG-NAG-Asn fragment obtained from serum albumin. Indirect evidence for the presence of β-mannosidase in other legumes has been suggested by the fact that monosaccharides are released when extracts of the corresponding seeds are incubated with galactomannan. Somme[201] taking aqueous extracts of *Trifolium repens*, *T. pratense*, *Medicago sativa*, *Anthyllis vulneraria* and *Lotus corniculatus* was able to demonstrate the production of galactose, glucose and fructose by auto-digestion. No mannose could be detected, however, and the extracts would not hydrolyse β-phenyl-D-mannoside or mannobiose. From these results she concluded that β-mannosidase does not participate in the utilization of galactomannan reserves. She then went on to suggest that phosphorolysis and isomerisation were possible alternative mechanisms for the mobilisation of this food reserve. This suggestion was followed up by a later paper[202] which reported the presence of oligo-D-mannosyl β-(1→4)-phosphorylase in fenugreek.

Reid and Meier[31] working on seeds of fenugreek determined the activity of β-mannosidase in extracts of embryos and endosperms at different stages of germination. Endosperm

homogenates contained little or no activity in the early stages of germination before the reserve galactomannan began to be mobilised. The onset of galactomannan breakdown coincided with the appearance of α-galactosidase and β-mannosidase activities, which increased throughout the period of galactomannan degradation and then remained constant. (See Graph 1). A similar rise in activities occurred during galactomannan breakdown in dry, isolated endosperms incubated under germination conditions. The increases in the activities of both enzymes could be suppressed by the metabolic inhibitors cycloheximide (2×10^{-5} M), abscisic acid (10^{-5} M), 5-fluorouracil (10^{-5} M) and actinomycin D (100ug/ml) which also inhibited galactomannan breakdown. Embryo homogenates contained low levels of β-mannosidase at all stages of germination. They concluded that the galactomannan of fenugreek is broken down by a series of hydrolases secreted by the aleurone layer of the endosperm, and these included a β-mannosidase.

β-Mannosidase activity has also been detected in some micro-organisms[203,204,205] and in a whole range of animal tissues including marine gastropods[206,207], the liver of <u>Turbo cortunus</u>[208], human blood platelets[209] and human epidermis[210].

Purification and kinetic studies on plant β-mannosidases

have been hindered by the apparent limited distribution and the low levels of activity of this enzyme. Recently, attempts have been made to obtain β-mannosidase free of α-mannosidase activity for use in structural studies on glycopeptides. Using gel filtration, a β-mannosidase preparation, containing only 4% α-mannosidase activity, was obtained from <u>Turbo cornutus</u>[206]. Relatively pure preparations have been obtained from the viscera of the snail <u>Achatina fulica</u>[211,212] and from hen oviduct[213]. The snail enzyme was purified 100-fold, and contained no α-mannosidase activity. β-Mannosidase from hen oviduct was purified 10,000 times and was free of α- and β-glucosidase, α- and β-fucosidase, α- and β-galactosidase and α-mannosidase activities. Reese and Shibata[193] detected β-mannosidase activity in a number of fungal species and obtained this enzyme free of α-galactosidase and β-mannanase in culture filtrates of <u>Penicillium sp</u>. The β-mannosidase from <u>Rhizopus niveus</u> was obtained free of β-mannanase by ion exchange chromatography and gel filtration[204].

The optimal pH values for activity of β-mannosidases are between pH 3.5 - 5.0[74,200,204,209,211,213]. The stability range is generally from pH 3 - 6, although an enzyme from guar[74] is stable at pH 8. Temperatures giving maximal activities of these enzymes vary from 37 - 55°[204,209,211] and the enzymes are unstable above 55°[200,211].

The affinities of various β-mannosidases with a number of substrates have been determined. Km values for the substrate p-nitrophenyl-β-mannoside are of the order of 5mM[209,212,213]. The enzymes have less affinity for the possible natural substrates, ivory nut mannan[74] and glycopeptides[213] (Km = 16mM).

β-Mannosidases are actively inhibited by D-manno-1,5-lactone, but not by D-manno-1,4-lactone. Levy et al [207] found that the 1,4-lactone was more than 500 times as active as the 1,5-lactone on a β-mannosidase from limpet. Similar results have been obtained from a number of microbial sources[193] and from hen oviduct[213]. The heavy metal ions Hg^{2+} and Pb^{2+} at concentrations of 1-2mM gave almost complete inhibition of β-mannosidase activity[74,209,213], whereas Ag^{2+} and organomercurials had only a slight effect[209].

Relatively little is known of the hydrolysis mechanism or physical or chemical properties of β-mannosidase. Molecular weights have been estimated by gel filtration and values of 100,000 and more have been obtained for the enzymes from hen oviduct[213] and from guar[74].

There have been a few papers commenting on the comparative rates of hydrolysis of various substrates. Reese and Shibata[193] claim that, in general, mannotriose is

hydrolysed more rapidly than mannobiose, and that reduction of mannotriose to mannotriitol gives no decrease in the initial rate. Mannobiitol is hydrolysed much more slowly than mannobiose itself. Further, a β-mannosidase, free of β-mannanase activity, prepared from Koji culture of _Rhizopus niveus_[204] had a substrate specificity as follows:-

mannotetraose = mannotriose > mannobiose > mannopentaose > mannohexaose

An enzyme from guar, described as an exo-β-(1→4)-mannanase reduced ivory nut mannan almost completely to mannose[74].

d. _Oligo-D-Mannosyl β-(1→4)-Phosphorylase_. The enzyme β-D-mannanase reduces β-D-(1→4)-mannan mostly to mannobiose and mannotriose with the release of only traces of D-mannose. Similar products, plus galactose, are produced by the action of β-D-mannanase and α-D-galactosidase on galactomannans. For the quantitative conversion of mannan to mannose a β-D-mannosidase would be needed to hydrolyse the products of mannanase action. The existence of a β-mannosidase is in dispute. In 1970 Somme[201] first reported that she was unable to detect β-mannosidase activity in seeds of clover, lucerne, _Anthyllis vulneraria_ or _Lotus corniculatus_ and she suggested that the mobilization of the mannose in

galactomannan on seed germination might not depend upon the presence of this enzyme, but on a mannan phosphorylase. At the time an enzyme catalysing this reaction had not been detected. It had been noted by Reid and Meier[171] that mannose and galactose accumulated in isolated fenugreek endosperms that had been incubated under germination conditions. They suggested that a β-D-mannosidase was functioning under these conditions, but they were only able to detect low levels of this enzyme. However, they reported an increase in β-mannosidase activity on seed germination, paralleling galactomannan breakdown. In the case of the coconut it has been reported[214] that β-D-mannosidase activity remains at a constant level.

In contrast to the Reid and Meier paper[31] and in support of Somme[201] Foglietti and Percheron[202] published a report, again unsubstantiated, on the existence of oligo-D-mannosyl β-(1→4)-phosphorylase in germinated fenugreek seeds. They found that crude homogenates of the germinated seeds were capable of catalysing the reversible phosphorolysis of β-D-(1→4)-linked manno-oligosaccharides ($\overline{DP} > 2$) to β-D-mannose 1-phosphate. The Reid Meier[31] paper disagreed with these conclusions on two grounds. Firstly, that they had observed galactomannan to be broken down to free galactose and mannose in fenugreek endosperms. Secondly, that the enzymes responsible for

the degradation of the polysaccharide were extracellular [170,171] and that no extracellular phosphorylases had been previously reported.

3. Glycolipids.

Photosynthetic tissues of plants contain galactoglycerides [215,216,217,218,219,220,221,222]. The total galactolipid content of the chloroplast is related to the chlorophyll content of the leaf tissue [219]. Rosenburg and co-workers [223,224,225] demonstrated that in the cells of _Euglena_ _sp_. the appearance and disappearance of chlorophyll is accompanied by the simultaneous appearance and disappearance of galactosyl diglycerides in an approximately fixed ratio. The authors suggested that the galactolipids may have a possible function in the localization of the porphyrin structure of the chlorophyll by forming a stable 'lock and key' fit between the phytol chains of the chlorophyll molecules and the fatty acyl chains of the galactolipids. Bauber and Park [226], in studying the Hill Reaction of isolated spinach chloroplasts noted that a crude _Phaseolus aureus_ preparation, containing galactolipases and galacto-sidases, changed the physiological activity of the organelles, and in this connection it is of interest to note that α-galactosidase has been detected in spinach [19] and

sugar cane chloroplasts[11]. Helmsing[227] believes that galactolipids play an important role in the photosynthetic apparatus of plants. Sastry and Kates[228] and Helmsing[229] suggested that *in vivo* digalactosyl glycerides are hydrolysed to monogalactosyl glycerides and galactose by the action of α-galactosidase, and that the latter are then broken down to glycerides and galactose by the action of β-galactosidase. Several plant species have been tested for the presence of galactolipases, specific enzymes for the hydrolysis of galactolipids. These have been found in spinach leaves[229] and runner bean leaves[227] and they catalyse the following reactions:-

Digalactosyl diglyceride ⟶ digalactosyl monoglyceride
+ fatty acid

Digalactosyl monoglyceride ⟶ digalactosyl glyceride
+ fatty acid

Hydrolysis of the digalactosyl glycerides are then carried out by α- and β-galactosidases.

The biosynthetic pathway to galactolipids in plants is not yet clear. Ferrary and Benson[230] observed a rapid

incorporation of ^{14}C into monogalactosyl diglyceride and a slow entry into digalactosyl diglyceride during the growth of Chorella pyrenoidoas in $^{14}CO_2$. They concluded that the digalactosyl diglyceride was synthesised by the galactosylation of monogalactosyl diglyceride and that both galactose transfers involved UDP-galactose, although in the first the linkage formed is β, and in the second case α. Neufeld and Hall[231] have demonstrated that spinach chloroplasts catalyse the transfer of galactose from UDP-galactose to an uncharacterised endogenous acceptor with the appearance of mono-, di-, and tri- and possibly tetra-galactosyl diglycerides.

4. Utilization of Galactose and Mannose.

Galactomannan depletion is not associated with any significant accumulation of released galactose and mannose. On germination of fenugreek, Daoud[232] was unable to detect either of these two sugars. Glucose, fructose and sucrose were the only mono- and di-saccharides isolated. Much more recently, Reid and Meier[171], with more sensitive techniques were able to detect a very slight increase in galactose and mannose in whole seeds on germination. However, a large accumulation of these sugars occurred in incubated endosperms[169,171]. They suggested that the

products of galactomannan hydrolysis are taken up by the cotyledons/embryos where they were further metabolised. A similar, slight accumulation of galactose and mannose, as well as manno-oligosaccharides, has been reported in <u>Gleditsia ferox</u>[148] and <u>Gleditsia triacanthos</u>[167] seeds.

The formation of transitory starch granules in the cotyledons of fenugreek was first observed by Nadelman in 1889, and has recently been confirmed by Reid[169] who has also calculated that the amount of starch formed in these seeds is approximately equal to 1/5 of the original amount of galactomannan: he believes that the latter polysaccharide is a precursor of starch in fenugreek.

The galactose and mannose released on hydrolysis of galactomannan and the galactose from the raffinose family of oligosaccharides during seed germination is undoubtedly rapidly metabolised. Each of these monosaccharides can be used by plants in at least two ways. Firstly, as a source of energy via glycolysis, and secondly, in the formation of structural polysaccharides. (See Figure 1) In either case the first step would be phosphorylation, catalysed by a kinase, to yield mannose 6- Phosphate and galactose 1-phosphate. Hexokinase, a rather non-specific phosphorylating enzyme, may be involved in

FIGURE 1.

The Utilization of Galactose and Mannose by Plants.

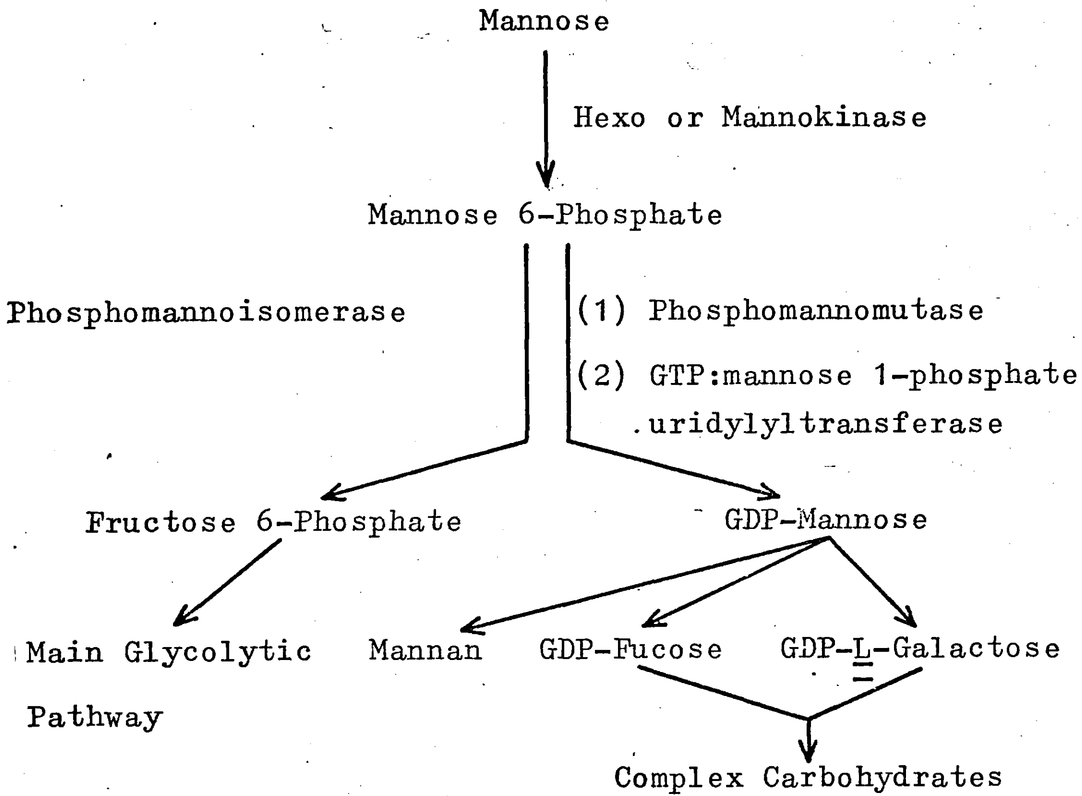


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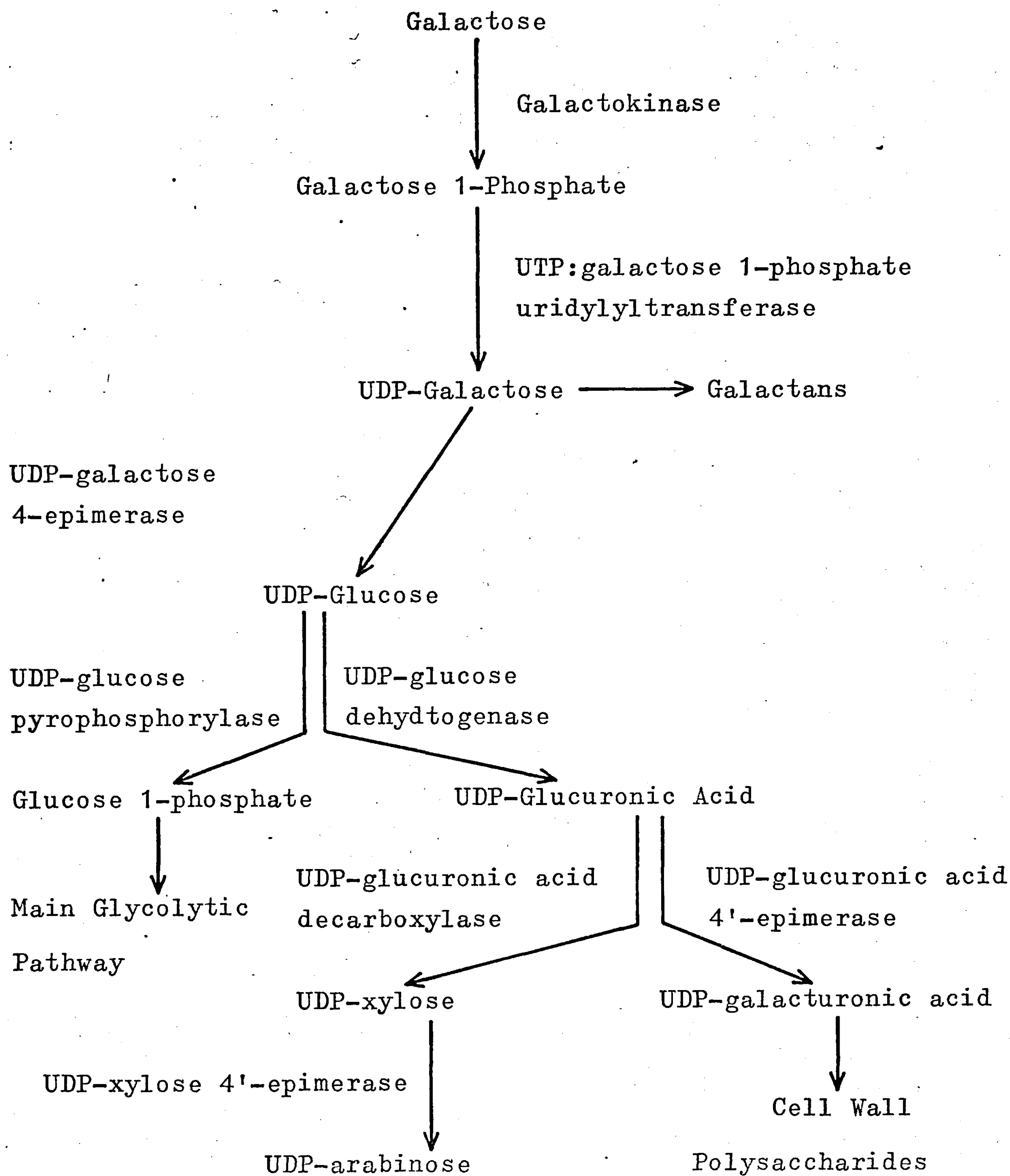
FIGURE 1.-Continued.



the formation of mannose 6-phosphate in plants. However, Saltman[233] separated a kinase from wheat germ that could phosphorylate mannose at about 68% the rate of glucose. More specific mannokinases have been obtained from microbial sources including Leucocstoc mesenteroides[234] and Escherichia coli[235]. Crude enzyme preparations from plants capable of phosphorylating galactose have been obtained from Canna sp.leaves[236] and wheat[236], Phaseolus aureus[237] and Vicia faba[238] seedlings.

Mannose 6-phosphate can either be incorporated into the main glycolytic pathway through fructose 6-phosphate[239] or be converted to GDP-mannose by the sequential action of a mutase followed by a pyrophosphorylase[240,241,242]. GDP-Mannose is converted to mannan, for example, in corn root tips[243], and is also the precursor of a number of other related nucleoside diphosphate sugars in algae including GDP-L-fucose[244,245,246] and GDP-L-galactose[247]. These sugar nucleotides are substrates in the synthesis of a range of complex polysaccharides. Phosphomanno-isomerase (E.C. 5.3.1.8.) is the enzyme involved in the conversion of D-mannose 6-phosphate to D-fructose 6-phosphate. This enzyme has not been reported to occur in plant tissue, although it is present in a number of animal and microbial species. The enzyme was first demonstrated in rabbit muscle in 1950[248], and since then a highly

purified preparation from the yeast Sacharomyces cerevisiae [249] has been reported.

Galactose metabolism involves an initial phosphorylation at C-1. A galactokinase has been identified in fenugreek seeds[250]. The enzyme activity is located in the cotyledons and in the seedlings, but the endosperm is devoid of activity. The optimum pH of the kinase is in the range of 7.3 - 7.6, and there is a dependence on Mg^{2+} concentration. The galactose 1-phosphate can then be converted to UDP-galactose by the enzyme UTP-galactose 1-phosphate-uridylyltransferase[113,251,252,253]. UDP-Galactose can then be either channelled into glycolysis[254] and UDP derivatives of glucuronic acid, galacturonic acid and pentoses via UDP-glucose, or serve as an immediate precursor of galactans [255,256].

D. GALACTOMANNANS IN INDUSTRY.

The structure of the galactomannans has already been discussed in this thesis. (See Page 41) Table 3 lists some of the galactomannans found in the Leguminoseae.

Galactomannans of major commercial importance are those

TABLE 3.

Galactomannans of Leguminoseae.

Source	Yield of Gum (%)	D-Mannose: D-Galactose Ratio	Refs
Cassia absus		3.00	146,257,258
Cassia fistula L.	27	3.0	259,260
Ceratonia siliqua L. (Locust bean gum)	38	3.75	132
Caesalpinia pulcherima L.	31	3.0	143,260
Caesalpinia spinosa	24	2.7	132
Gleditsia triacanthos L. (Honey locust)	15.2	3.2	147
Gymnocladus dioica (Kentucky coffee bean)	15	2.71	132
Genista raetam	5	4.14	261
Cyamopsis tetragonoloba (Guar gum)	35	1.54	132
Medicago sativa (Alfalfa, lucerne)	18.6	1.56	132
Trifolium repens	1.6	1.3	262
Trigonella corniculata		1.17	152
Trigonella foenum-graecum (Fenugreek)		1.2	263

from carob[264] (locust bean gum) and guar[265]. Locust bean gum which was originally native to Southern Europe and the Near East, and has been known and cultivated for many hundreds and possibly thousands of years, and has more recently been transported to other parts of the world, including[266] Australia and the U.S.A. The carob pod has a long history of use as both animal and human food stuffs[267], although as the latter, more use of them was made in times of desperate need, than as a welcome staple diet. The synonym "St. John's Bread" reveals the Biblical associations of the carob, as "the locusts" (Matthew 3:4) eaten by John the Baptist in the Wilderness are believed to have been locust bean pods. One of the problems with the carob tree is the very long time it takes to mature. It fruits only after five years, and is not fully grown for another twenty[266]. The carob tree is of considerable economic importance to Cyprus, where it is cultivated on very poor soil[268].

In World War II, the supply of locust beans from Mediterranean countries to the U.S.A. was greatly reduced, and a search was initiated for a suitable replacement[132]: these searches were continued into the 1960's[133,259,269,270]. The plant that eventually emerged as a rival to the locust bean was guar (<u>Cyamopsis tetragonolobus</u>)[271]. Like the locust bean, guar seeds yield a high level of

galactomannan from the endosperm (locust bean, 48% ; guar bean, 42%), and from both sources, the galactomannan constitutes about 80% of the soluble seed polysaccharides[272].

The guar plant is native to North-west India and Pakistan where it is of considerable economic importance[273]. It was introduced into the U.S.A. at the beginning of this century (1903) and became a commercial commodity[274] in 1940. Unlike the locust bean tree, which grows to an average height of 8m., the guar plant is only about 0.6m. high and is grown as an annual crop[274]. Like the carob pod, the guar seeds have been used as both human and animal food, although it is most certainly not a complete nutrient in itself[275].

The unique thickening properties of locust bean gum and guar gum have found extensive application in Industry. The initial incentive for finding a substitute for locust bean gum came from the paper industry where gums are mainly used as 'wet end' additives (i.e. the gum is added to the pulp suspension before the sheet is formed thereby increasing the wet strength of the paper)[264,265,276,277], but industries now using either or both of these gums include foods, cosmetics and pharmaceuticals. The patent literature pertaining to the industrial exploitation of guar gum from 1948 to 1962 has been comprehensively

covered by Saxena[273].

The chemical and physical properties of the galactomannans are reflected in their industrial applications. In this way their ability to imbibe water to form a thick, mucilaginous paste which is impervious to further water, is utilised by the mining and related industries both as a method for waterproofing explosives[278,279,280] and as a plugging composition for leaking wells[281,282,283,284].

The slow hydration of guar gum makes it a suitable base for delayed release drugs[285], although it also has a direct medicinal use as a laxative. Galactomannans have also been used as flocculents, for example, in the purification of ores.

Apart from guar and locust bean gums, the galactomannans have hardly been exploited at all, although tara (*Caesalpinia spinosa*)[286] and fenugreek (*Trigonella foenum-graecum*)[286] gums show some promise in the food[287] and *Gleditsia triacanthos* in the cosmetics industries[288].

The very high viscosity obtainable with locust bean gum and guar gum solutions has been put to good use in the food industry where both gums are in great demand as

thickeners for soups, deserts, sauces etc., and have been put to this use for the last forty years. Although they do not gel themselves, galactomannans will promote gelling when mixed with other gelling polysaccharides (carrageenan and agar), and it is upon this basis that they have been added, very much on a trial and error principle, to this whole range of processed foods. Studies by Baker et al[289] and Deuel et al[290] included investigations of the effect of locust bean gum on the mechanical properties of carrageenan and agar-agar gels, and these showed that even small additions of locust bean gum made the gels much firmer and less fragile. The early work indicated that it is mainly the hot water-soluble fraction of locust bean gum that is responsible for the strengthening of agar gels. This fraction of locust bean gum has a higher mannose/galactose ratio than other polysaccharides present in the gum[71].

The industrial importance of these interactions is that locust bean gum and guar gum are much less expensive than carrageenan and agar (the gelable polysaccharides). Thus, gels of a specific strength can be made with galactomannans using less carrageenan and agar than would otherwise be required, at a significant saving in cost. In addition, the resulting gels, although of the required gel strength, have different textural properties

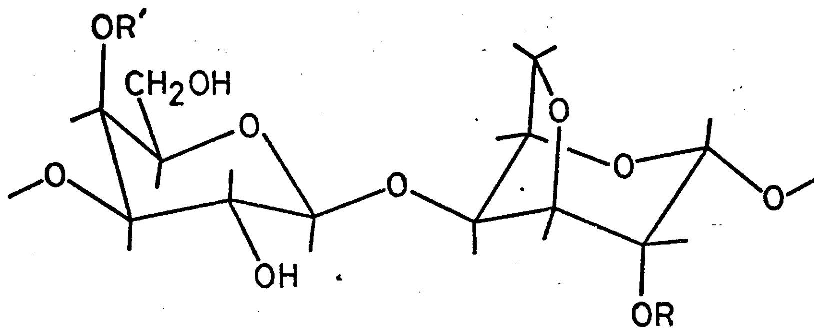
from the pure carrageenan and agarose gels, and these may be preferred in some applications.

The fact that non-gelling polysaccharides can increase the firmness of gels indicates that they take part in forming the gel framework. Rees and co-workers have put forward a model to explain this effect[149,291].

Carrageenans are a group of gel-forming sulphated polysaccharides found in marine algae of the class Rhodophycaceae and they have considerable economic importance as food additives. Rees[292] states that this whole group has a common structural theme of a repeating disaccahride unit masked by derivatisation of some of the residues in the sequence. Some representatives of the group definitely have sophisticated helical conformations, both in the solid state and in solution and model building experiments indicate that this could be a common feature of the whole group. The carrageenans are composed of D-galactose units linked alternately α-(1→3) and β-(1→4), and typically, have certain units along the chain derivatized by sulphation or 3,6-anhydride formation. The various carrageenans are assigned Greek letter prefixes, and the differences in their structure involve the nature of the derivatization. See Figure 2. The different types of carrageenan occur naturally. Thus, ideally ι-carrageenan

FIGURE 2.

Structure of Carrageenan.



a) $R = R' = SO_3^-$ ι-Carrageenan

b) $R = H$; $R' = SO_3^-$ κ-Carrageenan

c) $R = H$; $R' = 50\%\ SO_3^-$
50% H Furcellaran

bears a sulphate group on each sugar residue, κ-carrageenan a sulphate group on each D-galactose residue, and furcellaran a sulphate group on about 50% of the D-galactose residues. As the sulphate content of the carrageenan decreases so the concentration required for gelation decreases. Thus, ι-carrageenan gels at a concentration of about 2.5% (W/V), κ-carrageenan at 1.5% (W/V) and furcellaran at 0.4% (W/V). The most interesting of these molecules from the standpoint of conformation are κ- and ι-carrageenans. X-Ray diffraction studies on orientated fibres of these polysaccharides show that both are double helices[293] with three disaccharide residues per turn of the single helix and pitches of 24.6 (κ) and 26.0 (ι). In ι-carrageenan the second chain is displaced exactly half a pitch from the first. Rees and his group further showed that an appreciable fraction of this conformation is retained in solution, and that the gel-forming ability of the polysaccharide can be interpreted on this basis. The conformation can be changed in a controlled fashion by adding or taking away masking groups from the hydroxyl residues: it is by this means that the parent algae can regulate cell wall structure.

Agar, the classical gel-forming polysaccharide, is not sulphated, but it has many structural features in common with the carrageenans. Although there appears to be no definite information on its conformation, Rees[294] also

believes that agar is helical, and if this is so then presumably the helical structure could also be broken by the masking groups.

Rees and Scott[295] have pointed out that homopolymers of the pyranose forms of glucose, galactose, mannose, xylose and arabinose have conformations that are very restricted by steric factors alone and that they can all be classified into one of four characteristic shapes:-

Type A. Extended and ribbon-like

Type B. Helical and flexible

Type C. Rigid and crumpled

Type D. Very flexible and extended

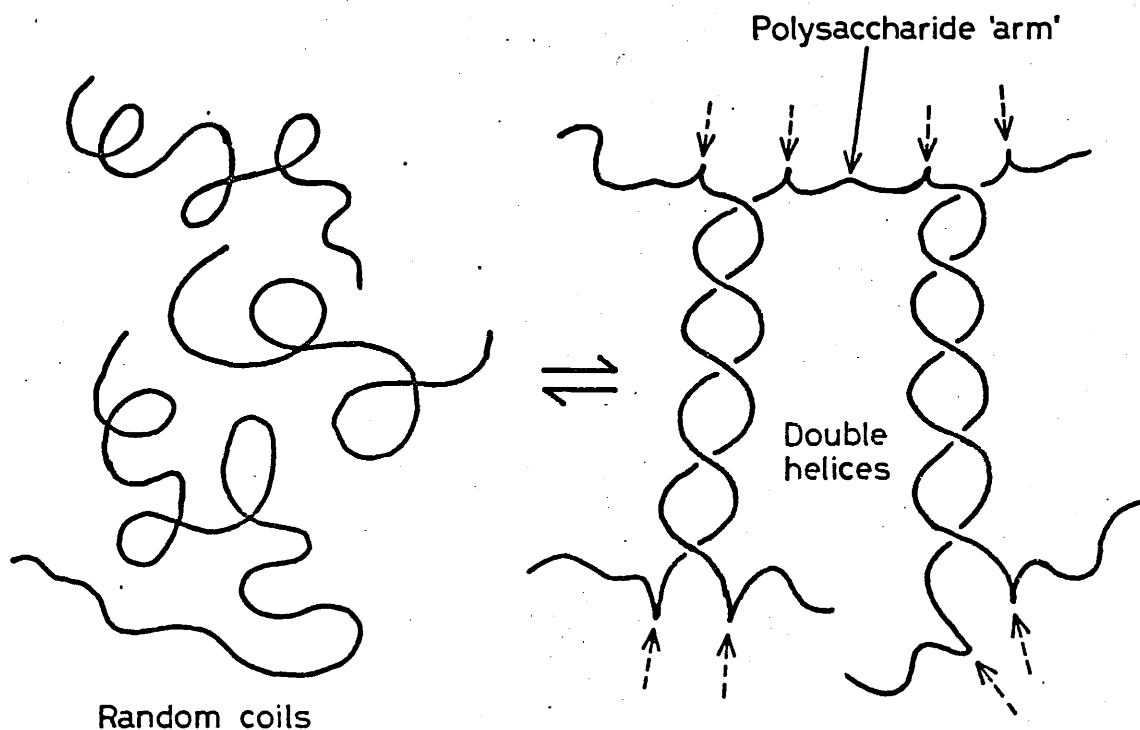
Most matrix materials, including the carrageenans, are in group B. It has long been recognised that a gel-forming substance must possess two structural characteristics: it must be a long molecule and the molecules must associate at certain points referred to as 'junction zones'. Association of the molecules by connection at the junction

zones then creates a network that can entrap solvent and form a gel. (See Figure 3.) Rees and co-workers[293] suggested that κ- and ι-carrageenans produced gels by forming double helices. The double helices form a complex network unlike, for example, the structure of DNA. Thus, one glycan chain can form a double helix with a number of other chains. Some of the monosaccharide units in the chain which exist as derivatives form kinks in the regular helical structure and these determine the position of the junction zones along the chain. (See Figure 3.) The kinks have been chemically identified[292,296] and they appear to occur when a 3,6 anhydro D-galactose unit in the disaccharide repeating sequence is replaced by D-galactose 6-sulphate: the kink results because the two galactose derivatives exist in two different chair conformations.

The theory of Rees[293] that carrageenans have double helical structures is supported by X-ray diffraction studies with the polysaccharides in the solid state. It is believed that the coiled conformation is retained, at least to a degree, when the polymers pass into solution, and this is supported by observations of changes in optical rotation during the processes of gelation and melting[292]. One prediction from this model is that by cleaving the polysaccharide chain at the kink points would produce 'simple' double helices unattached to other

FIGURE 3.

Model for the Molecular Basis of Gel Formation by the Carrageenans and Other Matrix Polysaccharides of Similar Conformational Type. (Rees[149]).



As a warm solution of the polysaccharide is cooled, those sections of the chain that have unbroken stretches of the repeating disaccharide unit combine to form double helices. This regular structure is broken up by kinks (indicated by arrows) in the chain caused by replacement of 3,6 anhydro galactose by galactose 6-sulphate. The whole then forms a network that entraps solvent to form the gel structure.

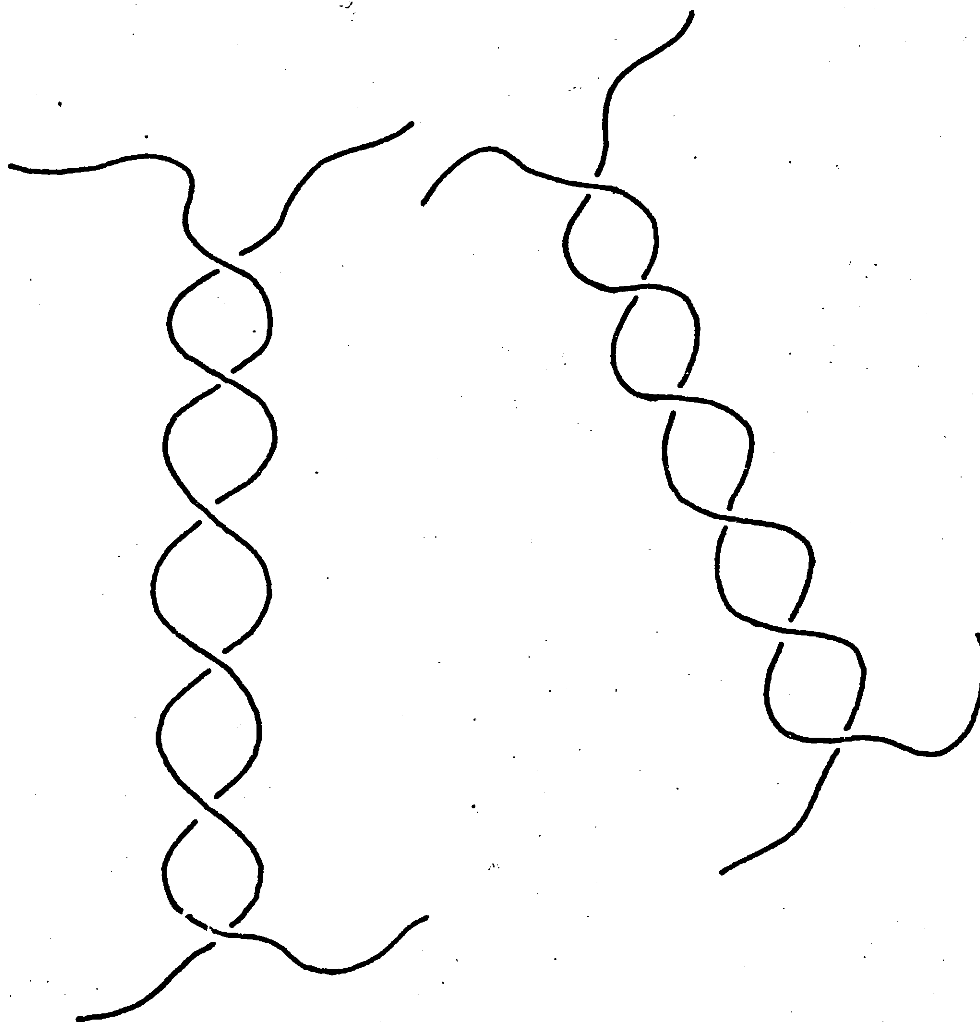
neighbouring helical systems (Figure 4.) and the polysaccharide 'arms' would then not be available to entrap solvent in the gel. Rees has succeeded in testing this prediction. κ-Carrageenan and agarose can be dekinked by means of the Smith Degradation, and in both cases the dekinked (or segmented) material behaves as predicted. It will not gel, but measurements of optical rotation indicate the formation of double helices at low temperatures and these revert to random coils as the temperature is raised[297].

A further prediction is that if a non-gelling polysaccharide could be found that would bond with the segmented helical material, then mixtures of the two should gel, even though neither gels alone under the same conditions. The added polysaccharide, when it forms the association, would simply replace the 'arms' removed by the Smith Degradation. To verify this prediction Rees investigated the interaction of dekinked κ-carrageenan and segmented agarose with galactomannans[149].

The confirmed ability of certain galactomannans to form gel structures when mixed with other non-gelling polysaccharides (i.e. with segmented κ-carrageenan or segmented agarose) or with gelling polysaccharides at concentrations that would be too dilute for gelling

FIGURE 4.

A Solution of Dekinked Polysaccharide after Cooling in a Similar Fashion to that shown in Figure 3.



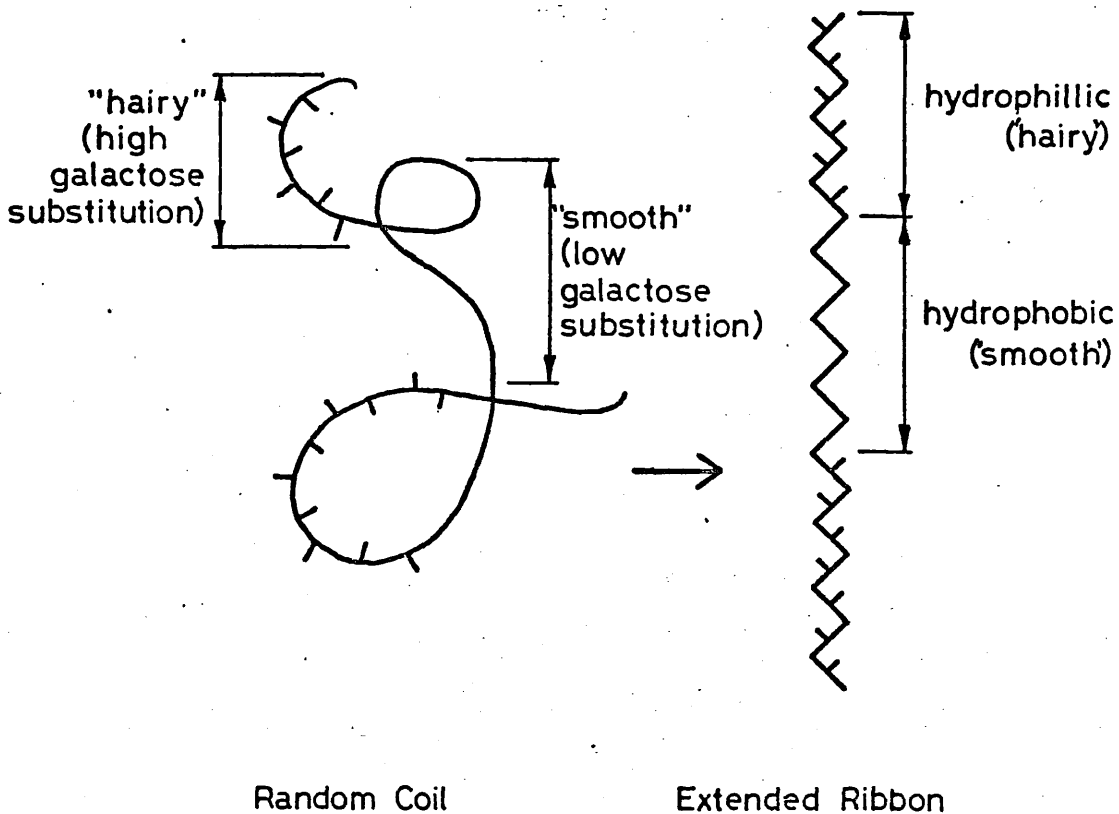
(native κ-carrageenan or native agarose) would imply that a network forms by interaction of unlike chains, perhaps by a mutual binding. Fibre diffraction evidence and indications from computer model building[295,298] for guar gum indicate that any ordered secondary structure of a galactomannan must involve the backbone in an extended ribbon-like form, which has alternate regions of high and low galactose substitution. Hence, if ever the random coil converts to an ordered form, for example, when binding to another chain, this can be represented as a molecular 'ribbon' that is alternately 'hairy' and 'smooth'. (Figure 5.)

The mixed guar galactomannan and κ-carrageenan gels show sharp melting and setting behaviour consistent with cooperative transitions and hence with networks that are cross-linked by ordered, non-covalent associations. The fact that galactomannans containing less galactose (and presumably more sparsely substituted regions) interact best with κ-carrageenan and furcellaran perhaps indicates that the galactose-poor regions are the most effective in any binding. Ordered binding, therefore, occurs between the ribbon-like, sparsely substituted regions of the galactomannans and the double helical portion of the carrageenan. (Figure 6.)

The interactions of these polysaccharides has also been

FIGURE 5.

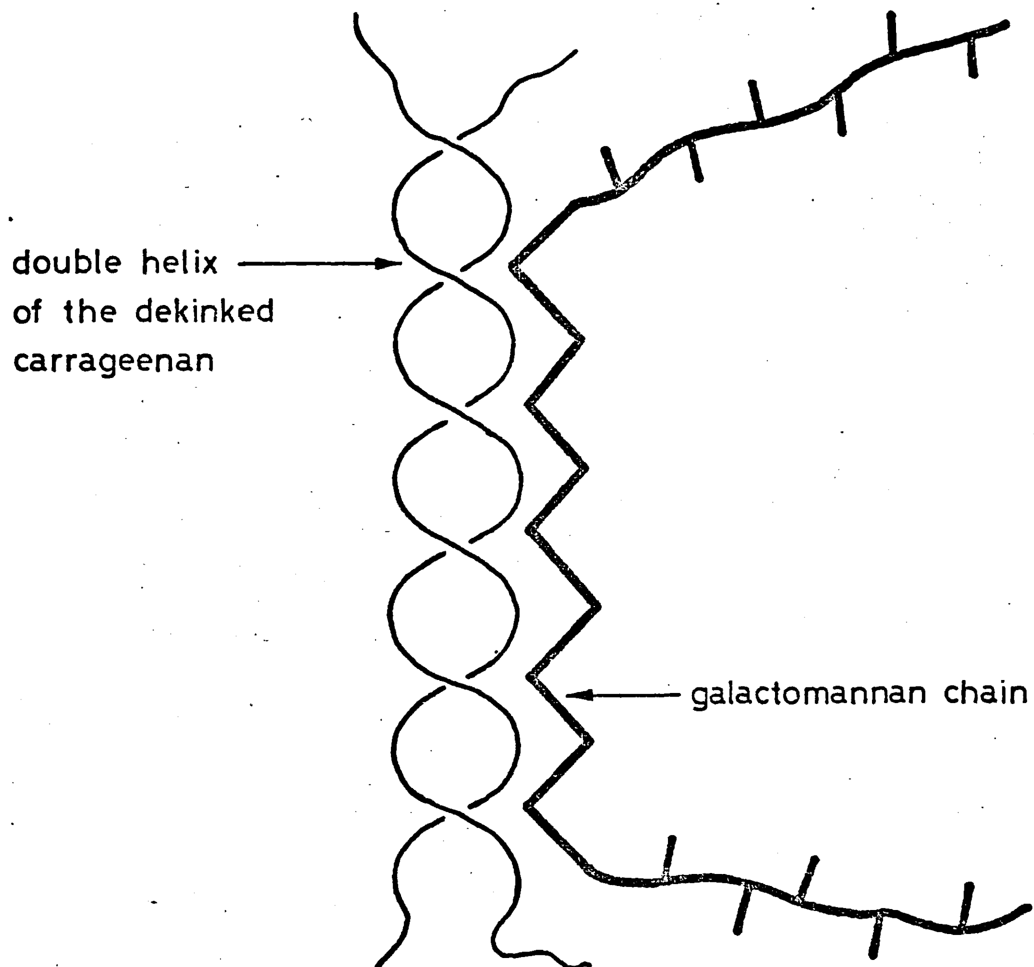
Guar Galactomannan in Random Coil and Extended Ribbon Conformations.



In the representation of the galactomannan, galactose residues are shown as protusions from the continuous mannan backbone. Although this figure shows only one smooth and two hairy sequences in the chain, a galactomannan may actually contain many such alternating sequences.

FIGURE 6.

Interaction Between Dekinked (segmented) Carrageenan and Galactomannan that accounts for Restoration of Gel-Forming Ability. (Rees[149]).



reviewed by Kirkwood[299].

If the above, proposed block structure for the galactomannans is accepted, one can imagine that synergism would be enhanced by:-

a), lack of isolated galactose stubs in the smooth regions, which would otherwise impair interaction;

b), optimum length of both 'hairy' and 'smooth' regions;

c), high molecular weight which aids cross-linking.

Modification of the galactomannans by enzymic methods would seem to be the most useful tool to test this hypothesis, and previous enzyme studies in this field appeared to support this approach. The work of Courtois *et al*[37] provided the main pointer for an investigation along these lines. The majority of the structural investigations with galactomannans have been carried out using coffee bean α-galactosidase. In general, release of D-galactose from galactomannans by this enzyme takes place readily at first, but then slows down until an enzyme-resistant product is formed. The initial rates of

release of D-galactose have proved to be indistinguishable for the galactomannans from Trifolium repens (Mannose/Galactose ratio 1.07 : 1), Genista scoparia (M / G ratio 1.6 : 1) and Gleditsia triacanthos (M / G ratio 3.82 : 1). This insensitivity with regard to the content of galactose was confirmed by Hui[300] for the galactomannans from Medicago sativa (M / G ratio 1.56 : 1) and fenugreek (M / G ratio 1.2 :1) and also Caesalpinia spinosa (M / G ratio 2.7 : 1). However, the results of prolonged action of α-galactosidase differ quite significantly for galactomannans from different plant species. Thus, the action of α-galactosidase on Trifolium repens galactomannan for six days raised the mannose/galactose ratio from 1.07 : 1 to 1.54 : 1; a further 20 days treatment with the enzyme raised the ratio to 1.72 : 1 [68]. However, similar treatment of Medicago sativa galactomannan raised[197] the ratio from 1.1 : 1 to 3.04 : 1. This large difference in the ratio of the product produced by long term enzymic treatment of galactomannans having an almost identical initial mannose/galactose ratio (and in which the galactose-mannose linkages are all α-(1→6)) presumably indicates that the arrangement of the D-galactosyl stubs along the D-mannan backbone must differ markedly.

A comparison of Gleditsia ferox galactomannan (Mannose/Galactose ratio 3.9 : 1) and locust bean gum (Mannose/

Galactose ratio 3.88 : 1) revealed an even more striking difference[68]. On treating <u>Gleditsia ferox</u> galactomannan with α-galactosidase, a precipitate appeared after 2 days; this was separated by centrifugation and washed in ethanol to remove free galactose. The mannose/galactose ratio of this degraded polysaccharide was found to be 13.8 : 1. It was then treated with the enzyme for a further 48 hr. to yield an insoluble material having a mannose/galactose ratio of 17 : 1. After a further treatment (48 hr.) with the enzyme an insoluble product with a ratio of 30 : 1 was produced. The ratio increased steadily with the number of successive treatments, but it was not possible to detach all the D-galactosyl groups from the galactomannan by this procedure. In contrast, treatment of locust bean gum with α-galactosidase for 5 days yielded only a small amount of precipitate that had a mannose/galactose ratio of 4.57 : 1. A further 5 days treatment with the enzyme gave an insoluble fraction with only a slightly increased ratio of 5.27 : 1. Several similar attempts by Courtois and co-workers[37] failed to raise the ratio of mannose/galactose significantly.

Dea <u>et al</u>[301], however, claim to have obtained a degradation product from a sample of locust bean gum with a mannose/galactose ratio of 19 : 1 using the same enzyme as Courtois. This result probably indicates batch

to batch variation in the fine structure of locust bean gum preparations, resulting from different extraction and purification procedures. However, the results obtained for <u>Gleditsia ferox</u> galactomannan are different from those for locust bean gum[37], and as they have similar mannose/galactose ratios, it again seems likely that they must have markedly different distributions of α-(1→6) linked galactosyl stubs along the mannan backbone. Dea and Morrison[291] extrapolated from the above findings and came to the conclusion that as relatively little galactose is released by α-galactosidase from locust bean gum, in comparison to <u>Gleditsia ferox</u> galactomannan, the former had fewer isolated galactosyl stubs interspersed in the 'smooth' regions. They further concluded that as the <u>Gleditsia</u> polysaccharide gives rise to a greater proportion of insoluble product than the locust bean gum, then the former has much shorter 'high density' D-galactose regions and hence much longer blocks of D-mannose residues which associate together causing the higher degree of insolubility[302].

MATERIALS AND METHODS

General.

Analar grades of reagents and only glass distilled or deionised water were used. All operations involving enzyme purification, unless specified, were carried out at 4°. *Vicia faba* seeds, variety Bunyard Exhibition (long pod), coffee and clover were used as a source of enzyme preparations.

Enzyme Assays.

α-Galactosidase.

Enzyme activities were determined by measuring only the initial rates of substrate hydrolysis. α-Galactosidase was assayed by addition of appropriately diluted enzyme solution (0.1ml) to a mixture of McIlvaine Buffer[303], pH 5.5 (0.6ml) and 2.0mM-PNPG (0.3ml) preheated to 30°. The temperature was maintained for 15min. and then the reaction was stopped with sodium carbonate (0.1M, 5.0ml), and the *p*-nitrophenol released was measured using a Unicam SP500 spectrophotometer at 405nm. The molar extinction coefficient of *p*-nitrophenol, under the assay conditions used is 1.53×10^{4} cm^{3}/mmole[9]. A unit of activity is defined as the amount of enzyme that hydrolyses 1µmole substrate per min. under

the condition specified. Specific activities are expressed as enzyme units per mg of protein.

β-Mannanase.

β-Mannanase was estimated by one of two methods:

i) Viscometric Assay for β-Mannanase.

An aliquot of carob galactomannan (15ml, 0.1% in acetate buffer; pH 5.0, 0.05M) was equilibrated to 25° in an Ubbelohde viscometer. To this, the enzyme preparation (0.1-1.0ml) was added and the viscosity determined immediately and at various times after addition.

ii) Incubation with Glucomannan.

To a solution of glucomannan (5.0ml; 0.25%,W/V) in acetate buffer (pH5.0, 0.05M) the enzyme preparation (0.1-1.0ml) was added and the resulting mixture was incubated for 1 hr. at 30°. Increase in reducing power was determined by the micro-cuprimetric method of Nelson. (See page 102.)

Determination of Proteins.

This was carried out by the method of Folin and Ciocalteu[304] as modified by Lowery et al[305]. The procedure was as follows:-

Reagent A: 2.0% Na_2CO_3 in 0.1N NaOH

Reagent B: 0.5% $CuSO_4.5H_2O$ in 1.0% Rochelle Salt

Reagent C: Reagent A (50ml) mixed with Reagent B (1ml)

Folin-Ciocalteu reagent (Obtained from BDH, Poole, Dorset, U.K.) Diluted 1:1 (V/V) with water to reduce the acid strength from 2N to 1N.

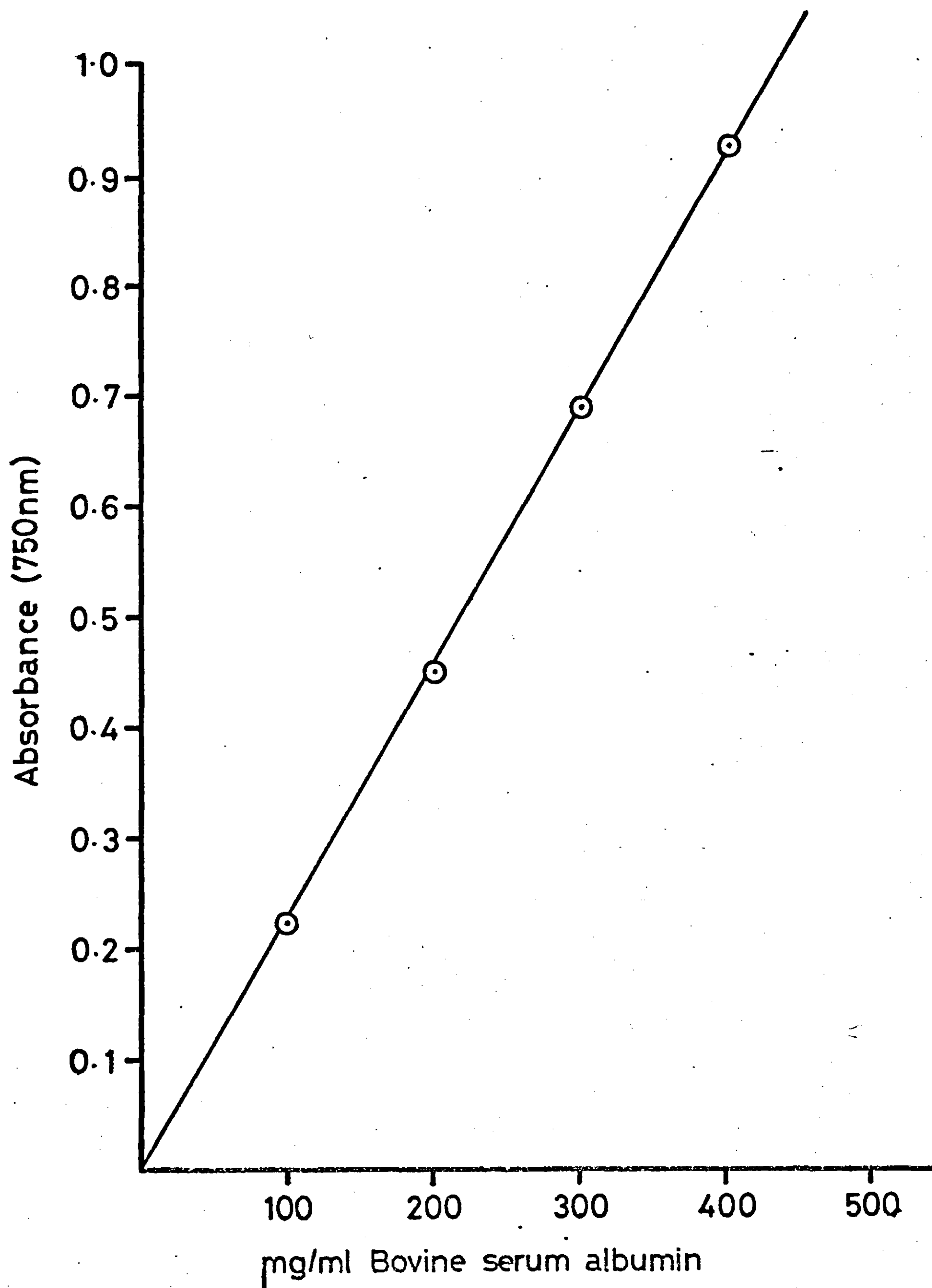
3M-Trichloroacetic acid (0.4ml) was added to the appropriately diluted enzyme solution (1.0ml). The precipitated protein was then collected by centrifugation at 5,000 rpm using an MSE bench centrifuge and the supernatant solution was discarded. The precipitate was then suspended in water (0.9ml) using a Rotamixer, 1N NaOH (0.1ml) was then added. After solubilization of the protein, Solution C (5ml) was added followed 10 min. later by the diluted Folin-Ciocalteu reagent (0.5ml). The solution was immediately mixed using a Rotamixer, and allowed to stand for 30min. The absorbance was then measured at 750nm. (Unicam SP 500). Crystalline Bovine Serum albumin was used to prepare a calibration curve. (Graph 2)

Estimation of Carbohydrates.

The total carbohydrate content in enzyme preparations was measured by the phenol/sulphuric acid method as

GRAPH 2.

Standard Curve for the Determination of Protein by the Method of Lowery *et al*[305].



described by Dubois _et al_[306]. The procedure was as follows:

The sample (2ml) containing about 5-30μg/ml of carbohydrate was measured into a boiling tube and 80% phenol (0.05ml) was added. Sulphuric acid (5ml) was allowed to flow rapidly on to the liquid surface so as to cause a maximum rise of temperature. The sample was then left to stand for 10 min., shaken, and then cooled to 25° and maintained at 25-35° for 10-20 min. The absorbance was then measured at 490nm (Unicam SP500) against a reagent blank. Glucose was used to prepare a standard calibration curve (Graph 3) and the results are expressed as glucose equivalents.

The Galactose Released from Galactomannans was estimated by two methods:

i) Reducing sugars were measured by the micro-cuprimetric method of Nelson[307] using arsenomolybdate reagent[308]. The copper reagent was prepared as follows:

Copper Reagent A: Na_2SO_4 (20.0g), $NaHCO_3$ (2.0g) and Rochelle Salt (2.0g) in water (100ml)

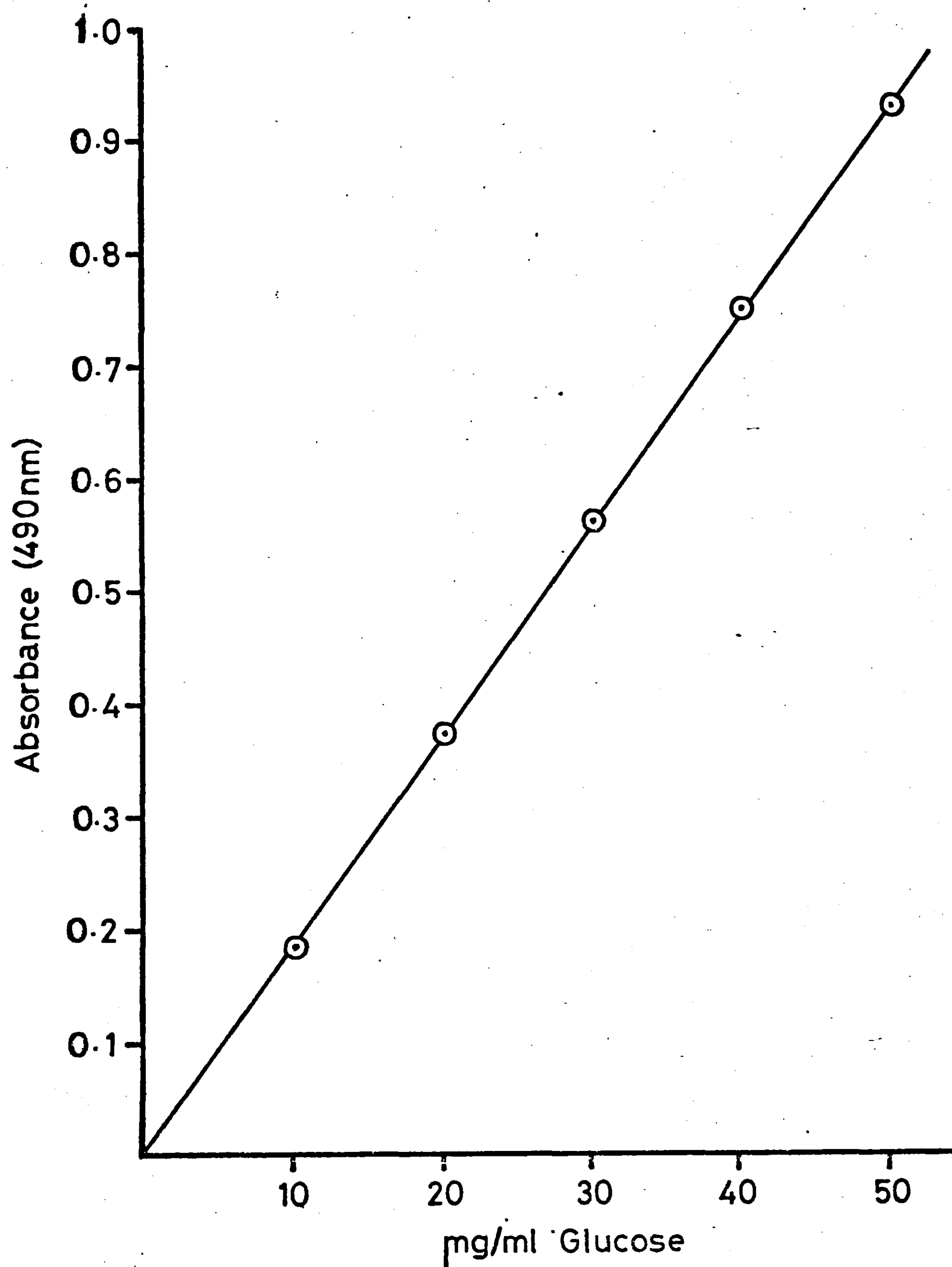
Copper Reagent B: 15% $CuSO_4.5H_2O$ containing 1-2drops of concentrated H_2SO_4 per 100ml.

25 volumes of A and 1 volume of B were mixed together.

The arsenomolybdate reagent was prepared as follows:

GRAPH 3.

Standard Curve for the Determination of Carbohydrate Content by the Method of Dubois *et al*[306].



Ammonium Molybdate (25g) was dissolved in distilled water (450ml) and concentrated H_2SO_4 (21ml) was added with stirring. $Na_2HAsO_4,7H_2O$ (3g) dissolved in water (25ml) was added with mixing, and the resulting solution placed in an incubator at 37° for 24 hr. (The reagent was stored in a glass stoppered, brown bottle)

The copper reagent (1ml) was added to each digest at the completion of the enzyme reaction. The mixture was then heated in a boiling water bath for 20 min., cooled and arsenomolybdate reagent (1.0 ml; diluted 1:2, V/V with 1.5N H_2SO_4) and water (3.0ml) then added. The resulting colour was measured at 560nm (Unicam SP 500). The amount of reducing sugar released was determined from a calibration curve prepared using D-galactose. (Graph 4)

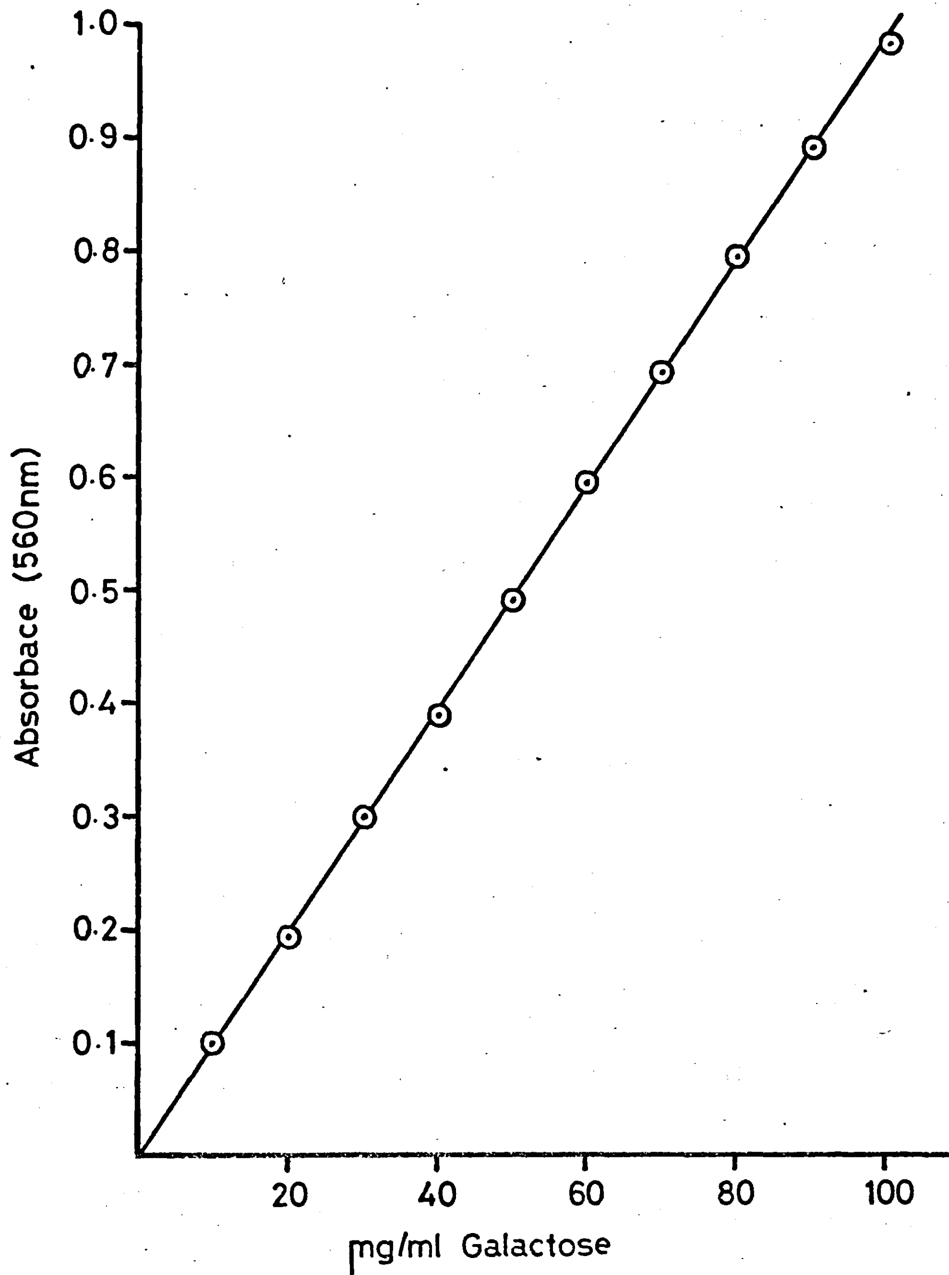
ii) Galactose was estimated enzymically. NAD and a suspension of galactose dehydrogenase was added to the sample in phosphate buffer (pH 7.5) in a cuvette. The dehydrogenation was allowed to proceed and the reduced NAD was estimated spectrophotometrically at 366,340 and 334nm. (Biochemica Test Combination-Boehringer)

Ammonium Sulphate Fractionation.

Powdered ammonium sulphate was added in *ca.* 0.5g

GRAPH 4.

Standard Curve for the Determination of Reducing Sugars by the Method of Nelson[307].



quantities with gentle and constant stirring at 4°
and pH 5.5. Percentage saturation was calculated from
the nomogram described in Methods in Enzymology[309,310].

Sephadex Gel Filtration.

Sephadex G-100 and G-200 columns (1,000 x 26 mm
internal diameter) were prepared by the method
described by Andrews[45]. The columns were eluted
(unless otherwise stated) with 0.1M-KCl-McIlvaine
buffer, pH 5.5, at a flow rate of 30ml per hr.,
and 3ml fractions were collected. The packing of
the column was checked and the void volume (Vo)
determined by using 0.2% blue dextran 2,000 (Pharmacia
Chemicals, Uppsala, Sweden.). Columns were water
jacketed, and were calibrated and run with the aid
of this refinement at room temperature. The recovery
of enzyme activities from Sephadex columns was of
the order of 90-95%. Enzyme fractions were concentrated
and dialysed using an ultrafiltration cell with a
PM 10 membrane. (Amicon Corporation, Lexington,
Massachusetts, U.S.A.) Sephadex G-50 columns (50mm x
20mm internal diameter, unjacketed) were prepared
and operated in a similar way. Being unjacketed,
however, they were run in the cold room (4°).

Concanavalin A Chromatography[311].

A 30-65% ammonium sulphate α-galactosidase preparation from Vicia faba was dialysed against 10mM sodium phosphate buffer, pH 6.8, containing 0.5M-NaCl. This dialysed material (10ml) was chromatographed on a column (30mm x 10mm) of Concanavalin A-Sepharose (Pharmacia (G.B.) Ltd.) which had been equilibrated in the same buffer. The column was eluted with the equilibration buffer at room temperature(19°) at a flow rate of 200ml/hr. until the E280 of the effluent became zero, when the material that had bound to the column was eluted with 0.5M-methyl α-D-mannopyranoside in the equilibration buffer (100ml). The fractions (2ml) were assayed for α-galactosidase activity.

Affinity Chromatography.- Purification of α-Galactosidases Using a Galactonate-benzidine Sepharose[21].

Cyanogen bromide activated Sepharose-4B (Pharmacia (G.B.) Ltd.) gel (10ml) was washed with deionised water (50ml) and suspended in water (20ml) and the pH was adjusted to 4.0. D-galactono-δ-lactone (1.00g) was added while stirring. To this mixture EDAC (1-ethyl-3(3-dimethylaminopropyl) carbodiimide-HCl) (100mg) was added and the pH allowed to rise to 5.7.

During the course of 24hr. gentle stirring (brought about by the slow use of a tumbling cell culture apparatus) the pH decreased to below 4.4. The pH was adjusted to 5.0 with 1N-NaOH and the gel allowed to stir overnight, by which time the pH was again below 4.0. The pH adjustment and stirring cycle was repeated twice more. The gel was then filtered and washed with water (200ml). The residue in water (18ml) was then allowed to stand overnight with acetic anhydride (2ml). The gel was again filtered, washed with water and suspended in a final volume of 20ml.

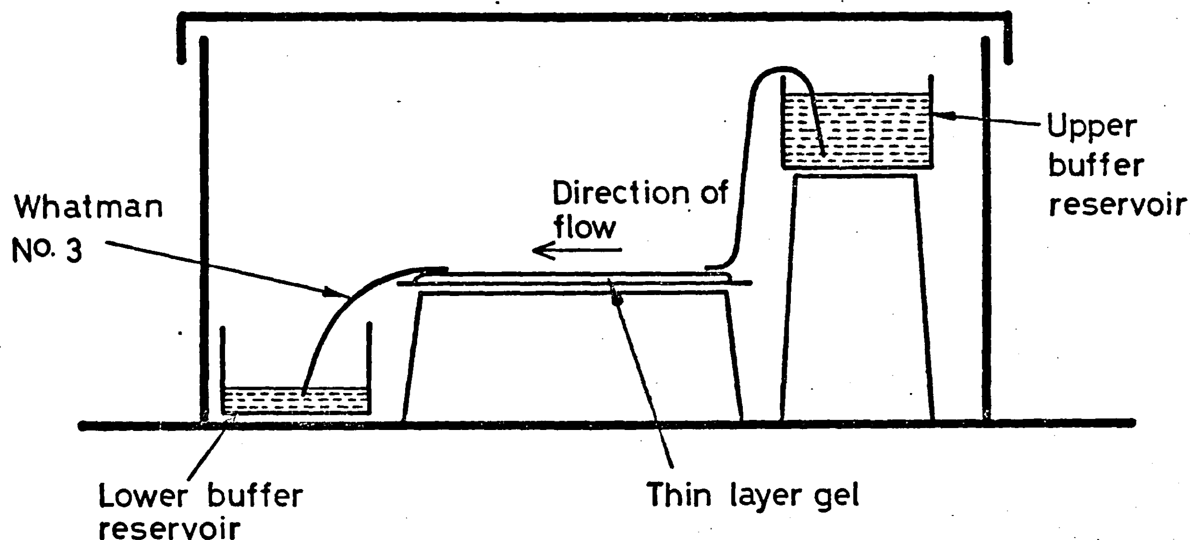
A slurry was poured into a water jacketed glass column and allowed to gravity settle to yield a 1 x 8cm bed volume. It was extensively washed with 0.01M phosphate buffer (pH7.0). A maximum of 100mg of protein could routinely be applied to a column of this volume. This was then followed by phosphate buffer (pH7.0) and fractions (3ml) collected until no further inactive protein was detected. At this time, 0.1M-borate buffer (pH 10) was used to elute the enzyme activities. A flow rate of 1-2ml per min. was maintained. Aliquots of each tube were assayed for α-galactosidase activity. Active fractions were dialysed against McIlvaine buffer (pH 5.5) and concentrated by Amicon untrafiltration. The figures

of the improved specific activity, after passage through an affinity column, of a typical dialysed ammonium sulphate (Stage iv.) preparation from Clover are given in the table below.

Source of α-Galactosidase Preparation	Specific Activity Prior to Affinity Column Chromatography (milliunits/mg Protein)	Specific Activity After Affinity Column Purification (milliunits/mg Protein)
Dialysed Ammonium Sulphate Preparation from Clover	584	87,327

Thin Layer Gel Chromatography.

Glass plates (200mm x 200mm) were spread with Superfine Sephadex G-100. These thin layer gels were equilibrated with McIlvaine buffer (pH 5.5, 0.1M) in a closed tank as shown over:-



After 24 hr. equilibration the enzyme preparation was applied as a spot (5µl) at the right hand side of the plate. A separate spot of blue dextran was applied as a marker. After 6 hr. (when the blue dextran had reached the end of the plate) the thin layer gel plate was removed and defined bands (marked on the under side of the glass plate) were scraped away into a centrifuge tube together with a small amount of buffer. Each band was assayed for enzyme activity. The α-galactosidase isoenzymes of *Vicia faba* were separated and quantitatively recovered.

Purification of α-Galactosidases.

The following steps were carried out in succession:-

Stage i. Extraction: Mature, brown, dry (referred to as resting) testa-free *Vicia faba* (Long pod, Bunyard Exhibition type) were finely powdered in a grinder and extracted for 30 min., three times with McIlvaine buffer, pH 5.5 (1kg/1,000ml buffer). The extract was then centrifuged at 10,000 x g for 30 min. and the clear supernatant solution used as a source of crude enzyme.

Stage ii. Variation of pH: The pH of the solution was lowered to 3.2 with 1.0M citric acid solution. Slow addition of the citric acid and efficient stirring was maintained for a further 30 min. after the addition of the last of the acid. The resulting inactive proteins were removed by centrifugation at 22,000 x g for 20 min., and the pH of the supernatant solution was re-adjusted to 5.5 with saturated disodium hydrogen orthophosphate solution.

Stage iii. Ammonium Sulphate Fractionation: The active enzyme solution was fractionated with ammonium sulphate using the procedure already described.

The precipitates obtained by saturation with ammonium sulphate to 30% were removed by centrifugation and discarded, and the concentration of ammonium sulphate in the supernatant solutions then increased to 65%; the active precipitate was centrifuged off, collected and disolved in McIlvaine buffer, pH 5.5.

Stage iv. Dialysis: The solution was then dialysed against McIlvaine buffer (pH 5.5, diluted 1 : 1 with deionised water, two changes of buffer) for 24 hr. to remove ammonium sulphate.

Stage v. Sephadex Gel Filtration: The dialysed enzyme solution was then passed through a G-100 Sephadex column. (See page 106.) Eluted enzyme fractions were then pooled separately, concentrated and dialysed against McIlvaine buffer, pH 5.5.

Stage vi. Recycling through Sephadex Column: The isolated dialysed enzyme was recycled twice through a Sephadex column and the various fractions obtained treated as in Stage v.

A summary of the complete purification procedure is given in Table 6. (See page 127.)

Synergistic Properties.

The synergistic interaction between agarose and the various galactomannans was followed by optical rotation measurements.

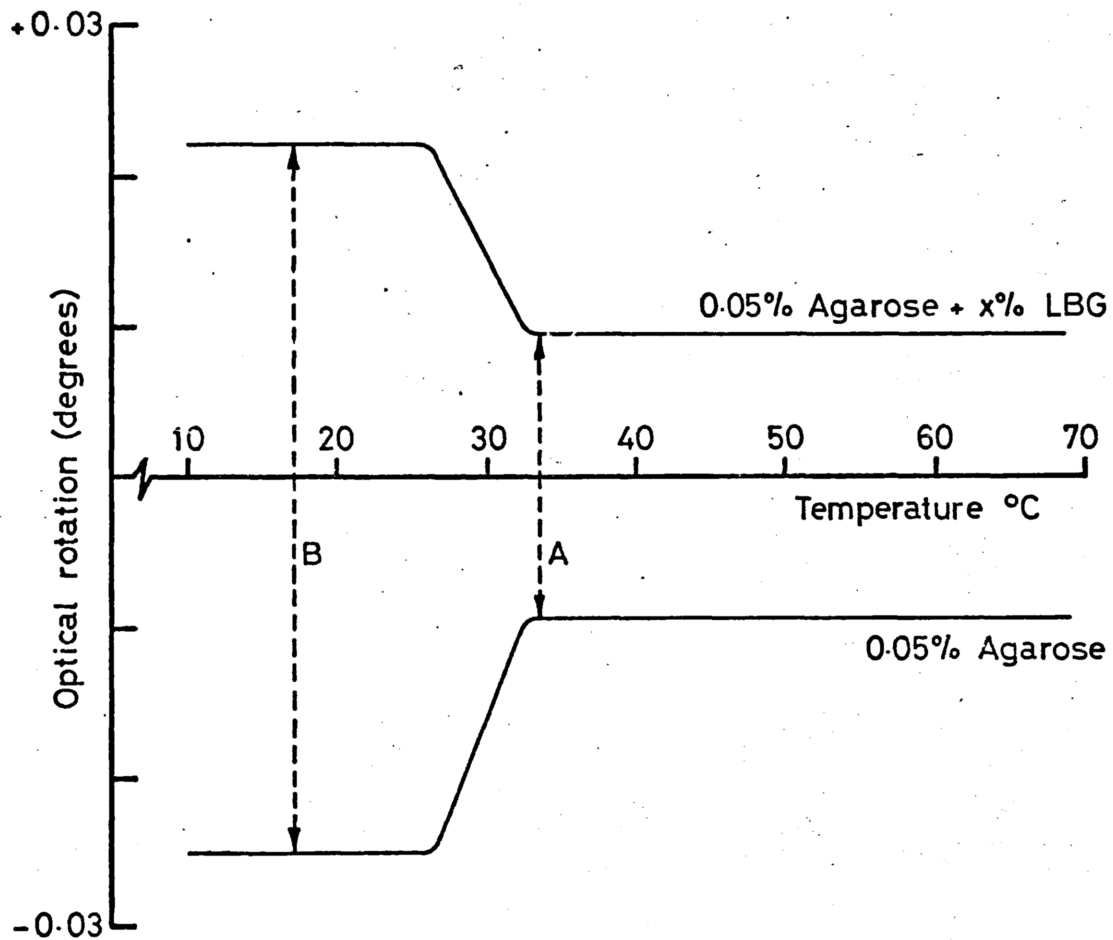
A 0.05% solution of agarose shows a sharp fall in optical rotation as it is cooled below 35°, but in the presence of, for example, locust bean gum a sharp rise is observed. (See Figure 7) The positive contribution to optical rotation of the galactomannan is measured by correcting the normal negative contribution of the agarose; from this the synergistic change in specific rotation $d[\alpha]$ due to the presence of the galactomannan can be calculated. The value of $d[\alpha]$ increases with increasing synergistic activity of the galactomannan under test, and was measured at two wavelengths. (546nm and 436nm).

Instrom Measurements.

To test the synergistic properties of the enzyme-treated galactomannans (guar and locust bean gums) mixed gels with κ-carrageenan were prepared in the following way:

FIGURE 7.

Variation With Temperature of the Optical Rotation of Agarose Solutions Containing Locust Bean Gum (LBG).



Change in d[α] in specific rotation due to LBG is equal to:

$$\frac{100\ (B - A)}{xp}$$

where p = path length

In the present study the concentration x of LBG was 0.3%

10cm cells were used (p = unity)

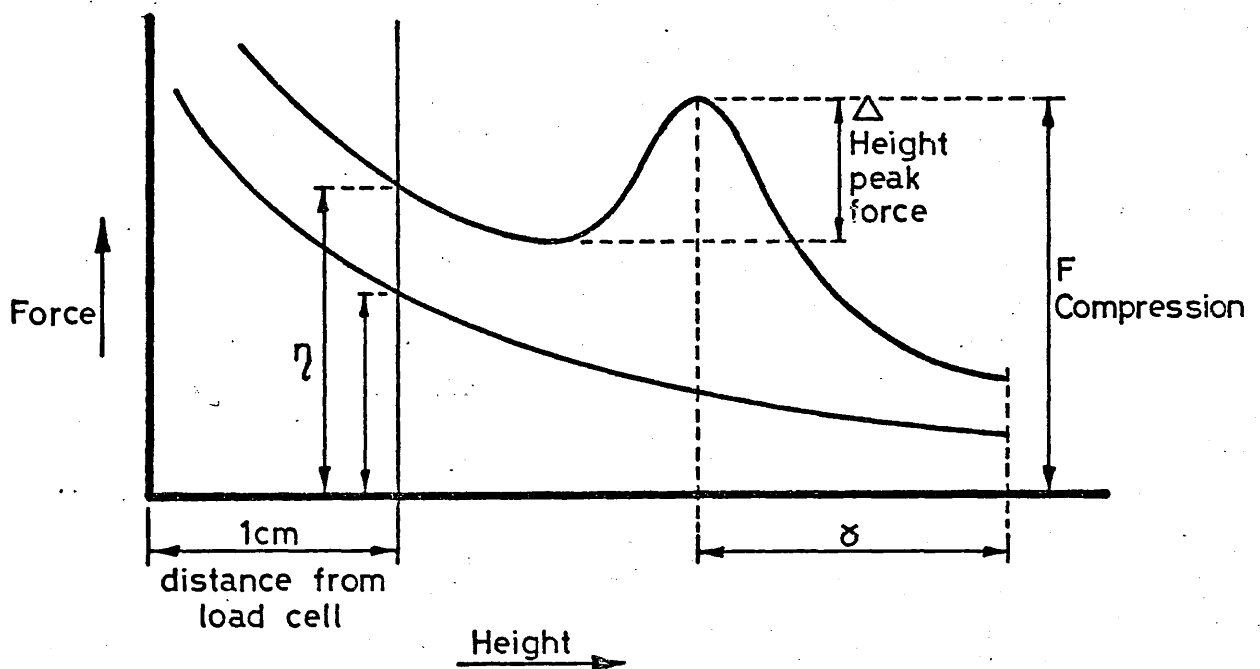
0.2% Galactomannan gum

1.5% κ-Carrageenan

10ml Water

The components were mixed cold and then treated at 90° for 30min. with stirring. They were then poured into moulds and stood at 4° for 24 hr.

The Instrom tensile strength tester produces a graph which can perhaps be interpretted as follows:



Δ gives a measure of brittleness

F gives a measure of firmness

η gives a measure of thickness

Polysaccharide Analysis.

Mannose/Galactose ratios of the galactomannans were carried out by gas chromatography of the mixture of mannitol and galactitol hexa-acetates that resulted from hydrolysis, reduction and acetylation. (alditol acetates.)

4N Trifluoroacetic acid (1ml) was added to the galactomannan (10mg). Standard inositol solution (5mg/ml, 1ml) was added. The mixture was heated at 121° in a pressure cooker (15lb) for 1 hr. The solvents were then removed by rotary evaporation.

The product from hydrolysis, still in the test tube, was then reduced in 1N-ammonium hydroxide (0.5ml) with sodium borohydride (10mg) for 1 hr. at room temperature. The reaction mixture was then treated with a few drops of glacial acetic acid to destroy residual sodium borohydride. The mixture was then taken to dryness by rotary evaporation. The remaining borate ions were removed as methyl borate by addition of methanol (1ml) to the residue and drying down by rotary evaporation. The cycle was repeated at least six times.

The alditols were then acetylated in acetic anhydride (2ml) at 121° in a pressure cooker for three hours. The solvent was then removed _in vacuo_ after the addition of a few drops of water. The residue was dissolved in chloroform and chromatographed.

The chromatography was carried out isothermally at 180° on a column of 10% ECNSS-M on J.J.'s "C" diatomic support (6 ft.). The polyacetates of galactitol and mannitol were separated. Inositol hexaacetate was used as an internal standard.

Intrinsic Viscosity Measurements.

Measurements of intrinsic viscosity are frequently used for characterisation, even though the method is not absolute, because accurate data can be obtained very simply and quickly.

All the measurements were carried out at 25° using an Ubbelohde suspended level viscometer. Dilutions of the gums (guar and locust bean) were made to give efflux times of between 100 and 300 secs., and four different concentrations of each gum were measured. The specific viscosity (η sp) was calculated from the formula:

$$\eta sp = \frac{t - to}{to}$$

where t = efflux time of solution

to = efflux time of solvent

The intrinsic viscosity (η) was then obtained by plotting ηsp/c against concentration of the solution and extrapolating the graph to zero concentration. The intrinsic viscosity was then used to calculate the molecular weight of the galactomannans using the Mark-Houwink equation:

$$\eta = KM^{\alpha} \qquad K = 6x10^{-4}$$

$$\alpha = 1.1$$

The results are given in Table 4.

Ultra Centrifugation.

The enzyme-modified samples of guar and locust bean gum were examined with a Sphinco Model E Analytical Ultra centrifuge with a R.I.T.C. rotor using the sedimentation equilibrium method. Runs were carried out at 20°. Weight, number and zeta averages for these samples are given in table 5.

TABLE 4.

Determination of the Molecular Weight of Galactomannans by Intrinsic Viscosity Measurements.

Sample	Concentration (g/100ml)	Efflux Time (secs.)	ηsp	η	Mz
Efflux time for water 89secs. - Unmodified Samples:					
Guar	0.075	238	1.67	1260	196,600
	0.050	172	0.933		
	0.025	124	0.343		
	0.0125	105	0.18		
Locust Bean Gum	0.10	225	1 53	880	141,600
	0.075	180	1.02		
	0.05	145	0.63		
	0.035	123	0.38		
Efflux time for water 78secs. - Modified Samples:					
Guar	0.2	127	0.63	320	23,000
	0.175	120	0.54		
	0.15	114	0.46		
	0.10	103	0.32		
Locust Bean Gum	0.2	108	0.38	310	22,700
	0.175	105	0.35		
	0.15	103	0.32		
	0.10	98	0.26		

TABLE 5.

Ultra Centrifugation Data For the Enzyme-Modified Samples of Guar and Locust Bean Gum.

Sample	Concentration (mg/ml)	Mn	Mw	Mz
Enzyme-Modified Guar	0.2	38,000	74,000	23,000
	0.6	36,000	38,000	33,000
Enzyme-Modified Locust Bean Gum	0.2	23,000	76,000	48,000
	0.6	14,000	18,000	40,000

RESULTS AND DISCUSSION

PART I

α-GALACTOSIDASE FROM VICIA FABA SEEDS

The occurrence and nature of the multiple forms of α-galactosidase in *Vicia faba* seeds have been discussed in the Introduction of this Thesis. (See page. 15.) This present investigation was started in order to study, further, the reported conversion of α-galactosidase $_{II}$ to $_{I}$[39] (i.e. the low molecular weight, low specific activity, to the high molecular weight, high specific activity form.). The first indication that this conversion occurred was obtained using a preparation of $_{II}$ from a batch of broad beans which seemed to be mature but in fact were abnormal as they contained only enzyme $_{II}$. On storage of this enzyme at 4°, after purification with ammonium sulphate, an increase in specific activity occurred and when a sample was applied to a Sephadex G-100 column, the appearance of enzyme $_{I}$ was noted; a steady increase in the formation of enzyme $_{I}$ was monitored over a storage period of 10 weeks. In the earlier work by Dey *et al*[39] it was reported that passage of enzyme $_{II}$ through a Sephadex G-100 column inhibited the conversion reaction. It has been suggested that enzyme $_{I}$ possesses a quaternary structure composed of units of α-galactosidase $_{II}$[10].

It was decided, therefore, to reinvestigate this phenomenon and, in particular, to examine factors likely to influence the formation of α-galactosidase I from II.

1. Purification and Changes in Isoenzyme Levels of α-Galactosidase I and II.

Early experiments were concerned with the preparation of purified forms of α-galactosidase which could subsequently be used to study the conversion phenomenon. Purification was effected by the following procedure:

i. Crude Extract.

Dried, mature beans (testas removed) were ground to a fine powder in a mechanical mill. The bean powder was stirred into McIlvaine buffer (pH 5.5) and allowed to soak for 1 hr. The cell debris was centrifuged off leaving the supernatant. (Crude enzyme extract)

ii. Citric Acid Precipitation.

The pH of the crude extract was lowered to 3.2 by the careful addition of 1M citric acid solution. The inactive protein thus precipitated was removed

by centrifugation. (This precipitation removes several contaminating glycosidase activities[41]). The pH of the solution was then readjusted back to 5.5 by addition of saturated disodium hydrogen orthophosphate solution.

iii. <u>Ammonium Sulphate Fraction</u>.

The acid-treated preparation was fractionated with ammonium sulphate and the 30-65% fraction which possessed most of the activity, was retained.

iv. <u>Dialysis</u>.

The ammonium sulphate fraction was finally dialysed for 48 hrs. against McIlvaine buffer (pH 5.5, diluted 1:1 with deionised water).

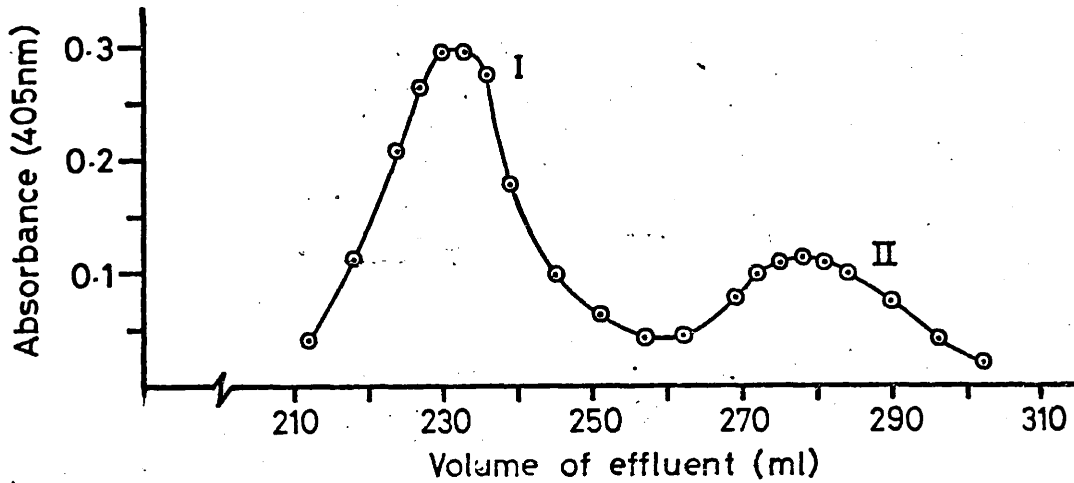
The dialysed preparation was then applied to a Sephadex G-100 column. This gel filtration resolved the α-galactosidase activity into two fractions, I and II[40] (See Graph 5i.). These were concentrated separately by using an Amicon ultra-filtration cell.

A typical purification is shown in Table 6. It was noted at this stage of the investigation that the total activity as well as the specific activity had increased significantly during the purification.

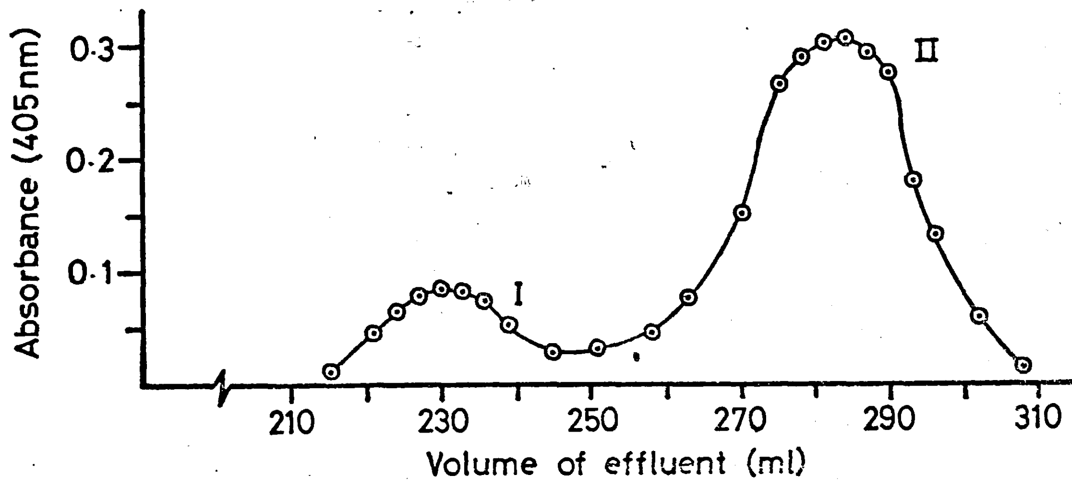
GRAPH 5.

Sephadex G-100 Eluent Patterns of:-

(i) Resting beans - applied after multi-stage purification[43].



(ii) Resting beans - crude extract applied direct to the column.



⊙—⊙ α-D-Galactosidase activity

Experimental conditions are given in the Experimental Section (Page 106)

TABLE 6.

Purification of α-D-Galactosidase from Mature *Vicia faba* Seeds (McIlvaine buffer System).

Fraction	Vol. ml	Enz. (milli units/ ml)	Tot. Act. (milliunits)	Prot. (mg/ ml)	Tot. Prot. (mg)	Spec. Act.	Purific. X	Recov. %
Crude	250	152	38,000	14.3	3,575	10.6	-	-
Acid ppt to 3.2 Citrate	250	408	102,000	10.0	3,500	40.8	3.8	268.4
$(NH_4)_2SO_4$ (30-65% saturated)	16.5	6,095	100,567	17.5	289	348.2	32.8	98.6
Dialysis	30	5,690	170,700	9.9	297	574.7	54.2	169.7
Sephadex G-100 Gel filtration and conc. I	18	8140	146,520	0.39	7.02	20,871	1,968	85.8
Sephadex G-100 Gel filtration and conc. II	18	6748	121,464	3.20	57.6	2,109	199	71.1

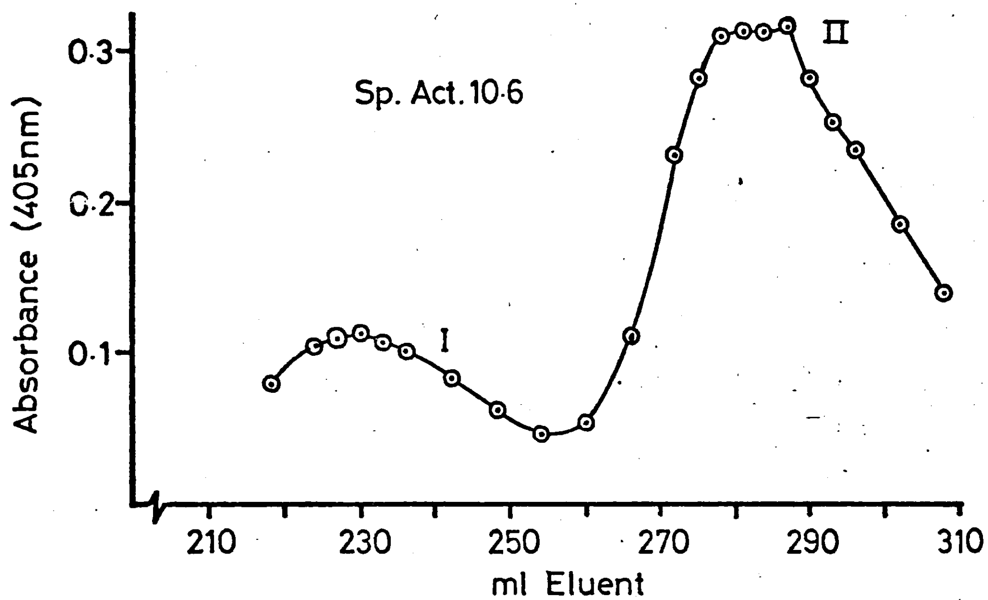
The final pattern obtained from the Sephadex gel resembled that obtained by other investigators working with _Vicia faba_[40] as well as other mature seeds[43].

The procedure described was found to take several days to complete, and thus possible methods that would expedite the purification were examined. These methods included passage of the crude enzyme preparation from mature beans through a Sephadex G-100 column before attempting other stages. The enzyme pattern obtained in this case, which was unexpected, is shown in Graph 5(ii) which differs from Graph 5(i) in that the proportion of α-galactosidase $_{II}$ is much higher. The pattern difference was then further studied by investigating the effect of increasing the tissue extraction time and by examining the fraction from each of the stages in the multistage purification (Table 6.) on a Sephadex G-100 column (Graph 6.). When the extraction time was increased from 1 hr. to 24 hr. at 4° and the enzyme pattern examined, it was clear that there had been some increase in α-galactosidase $_{I}$ (_cf_. Graph 6(i) and 7). It should be noted that the tissues were extracted for 24 hr. by other workers[312] who were interested in the isoenzyme levels at various stages of growth.

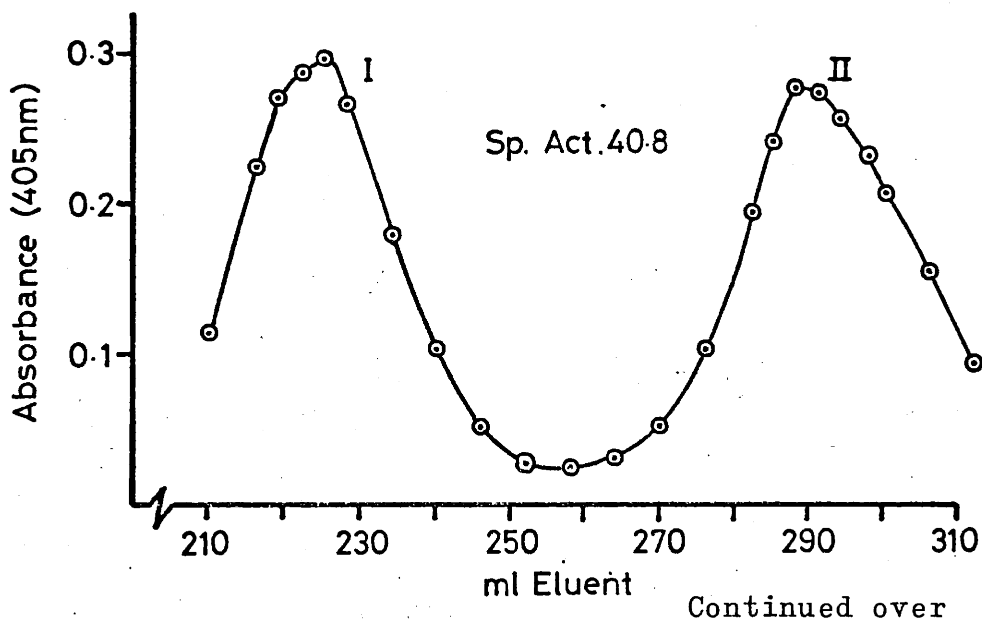
GRAPH 6.

Sephadex G-100 Eluent Patterns of the Purification Stages of α-Galactosidase:-

(i) Crude extract into Buffer



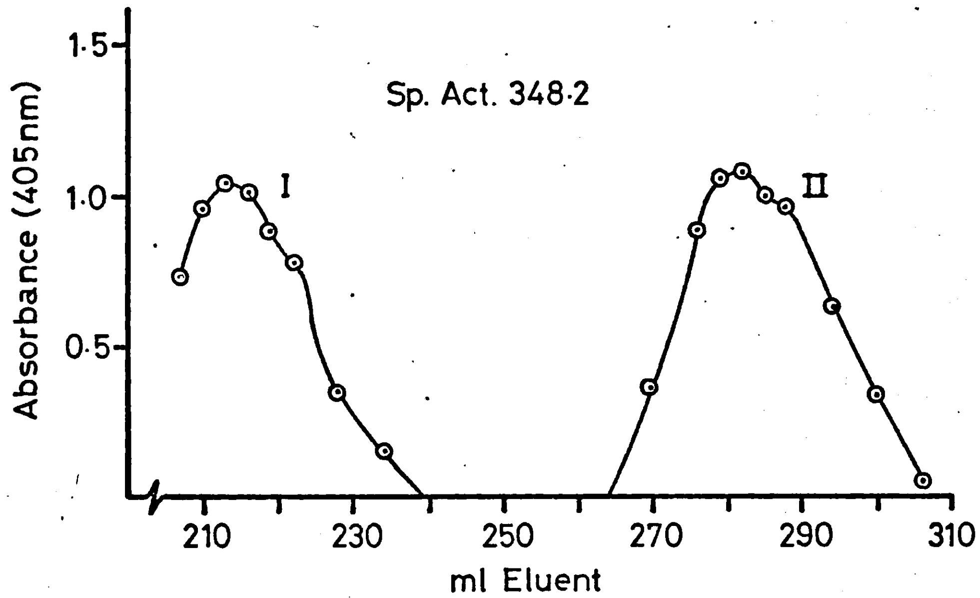
(ii) Citric acid Precipitation



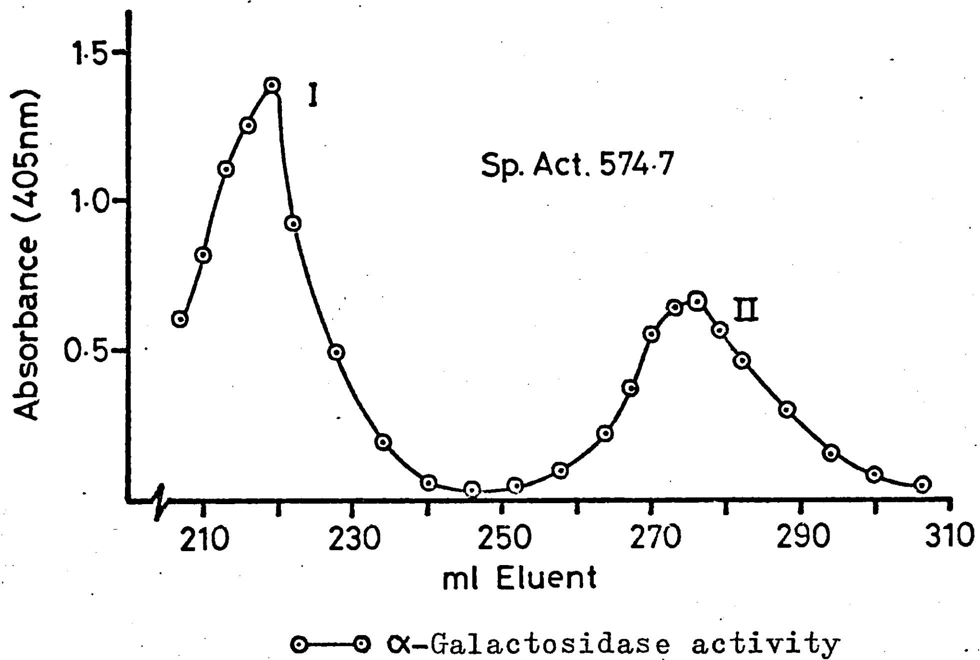
Continued over

<u>GRAPH 6.-Continued.</u>

(iii) Ammonium sulphate fractionation



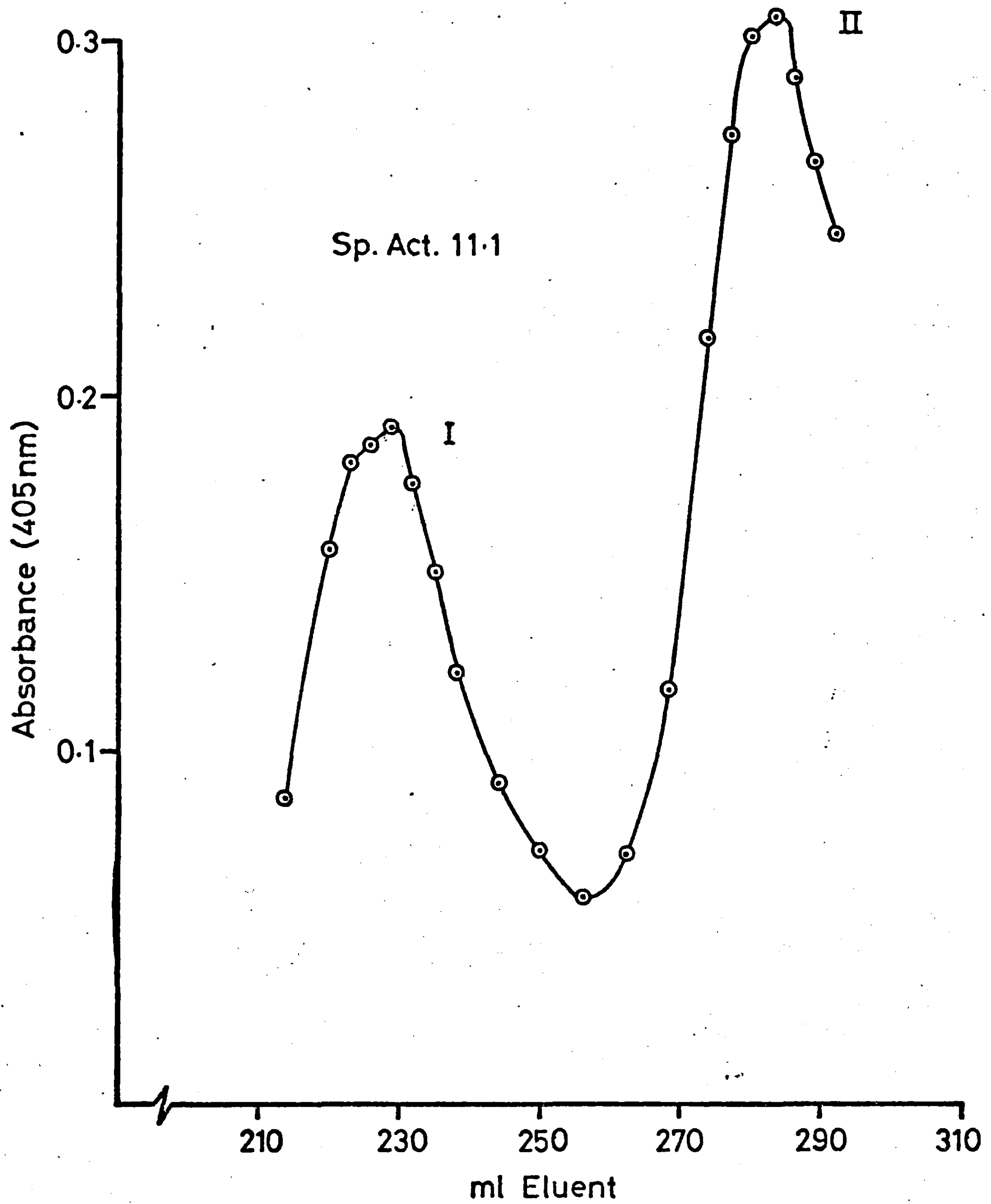
(iv) After Dialysis



⊙—⊙ α-Galactosidase activity

GRAPH 7.

Sephadex G-100 Eluent Pattern of a Crude Extract of Resting Beans (Extraction Time increased from 1hr to 24hrs).



α-Galactosidase activity.

In Graph 6 it can be seen that there are two points in the purification procedure where major changes in the isoenzyme levels also take place. These are during the Citric acid precipitation (Stage (ii)) and following dialysis (Stage (iv)).

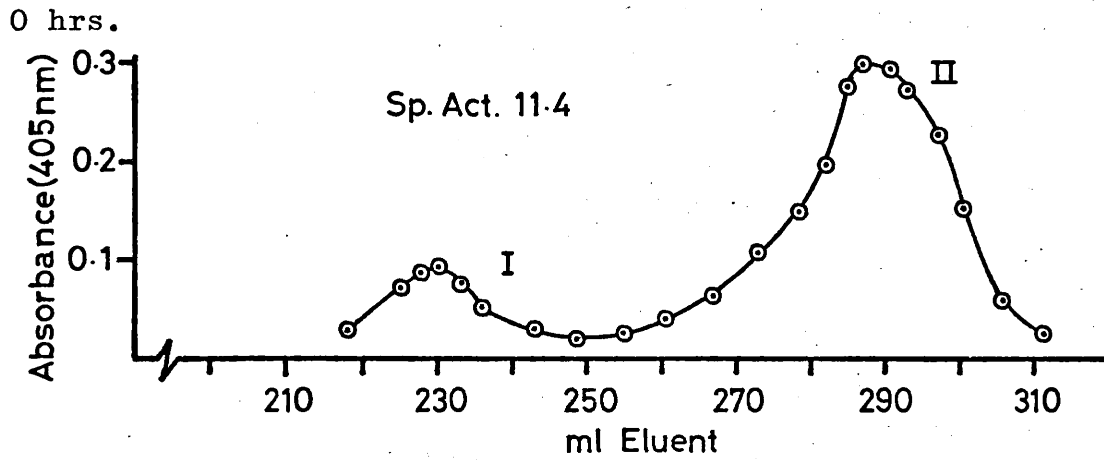
Citric acid precipitation increased both the specific activity of the preparation and the level of enzyme $_{I}$ relative to $_{II}$. It was observed that this could also be achieved by merely standing a crude extract (Stage (i)) at 4° for several days. (See Graph 8). Over a period of 60 hr. standing there was an increase in specific activity from 11.4 to 37.8 milliunits/mg protein, which was paralleled by the rise in total activity from 3,684 to 9,710 milliunits and an increase in enzyme $_{I}$. In the case of both citric acid treatment and storage a precipitate was produced; this had no activity, and when added back to a crude enzyme preparation or an ammonium sulphate fraction did not affect the rate of change of activity or the isoenzyme pattern.

It was shown that when the whole purification procedure was carried out in Acetate buffer (pH 5.5, 0.1M) (See Table 7) instead of McIlvaine (pH 5.5) that at the pH precipitation step (using acetic acid)

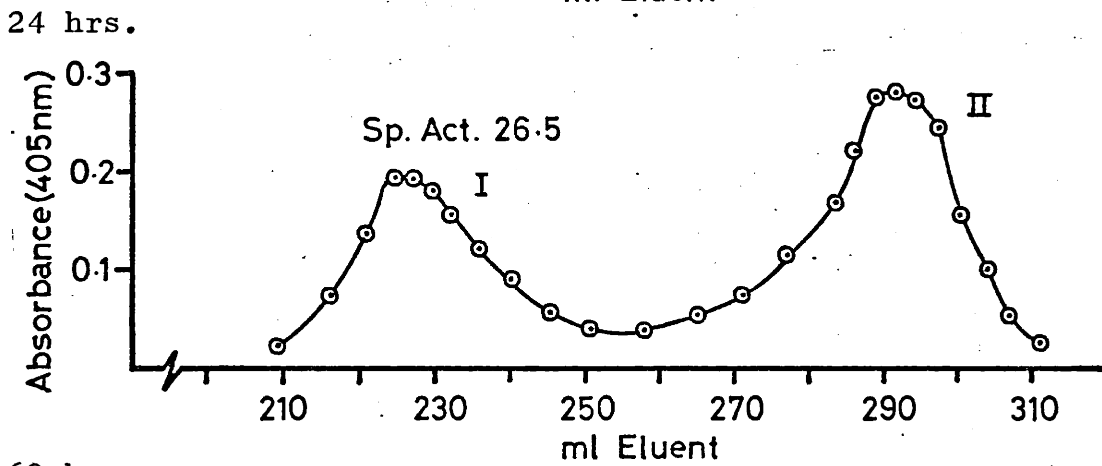
GRAPH 8.

Sephadex G-100 Eluent Patterns of a Crude Extract of Resting Beans after Storage at 4°.

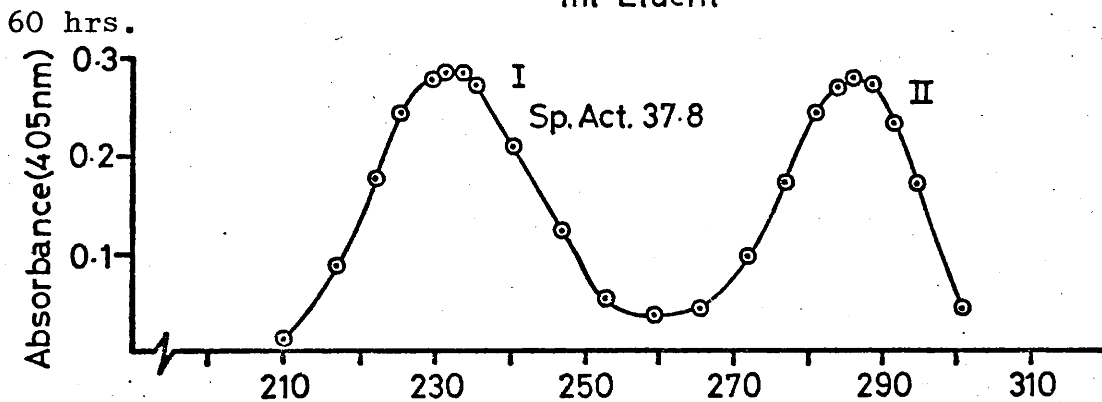
0 hrs.



24 hrs.



60 hrs.



⊙—⊙ α-Galactosidase activity. Sp. Act. = milliunits/mg protein.

TABLE 7.

Purification of α-D-Galactosidase from Mature *Vicia faba* Seeds (Acetate buffer System).

Fraction	Vol. ml	Enz. (milli units /ml	Tot. Act. (mil liun its)	Prot -ein (mg/ ml)	Tot. Prot. (mg)	Spec. Act.	Puri- fic. X	Recov. %
Crude	60	138	8,280	14.6	876	9.4	-	-
Acid ppt to 3.2 (acetic)	60	360	21,600	9.7	582	37.2	3.9	260.8
Storage (8hr., 4°).	58	352	20,416	9.4	545	37.4	3.9	94.5
$(NH_4)_2SO_4$ (30-65% saturated)	5	5,865	29,325	17.4	87	337.1	35.8	143.6
Dialysis	9	5,657	50,913	10.7	96	528.7	56.2	173.6

there was an increase in specific activity from 9.4 to 37.2 milliunits/mg protein, but no change in total activity, or immediate change in pattern (Graph 9). On standing at 4° for 8 hr. the acetic acid treated fraction yielded a flocculent precipitate which was removed by centrifugation, and when the resulting supernatant was applied to a Sephadex G-100 column the pattern was observed to have changed (Graph 9) and now resembled that obtained by the citric acid precipitation step (Graph 6(ii)). The specific activity of the preparation after the 8 hr. storage had not changed significantly.

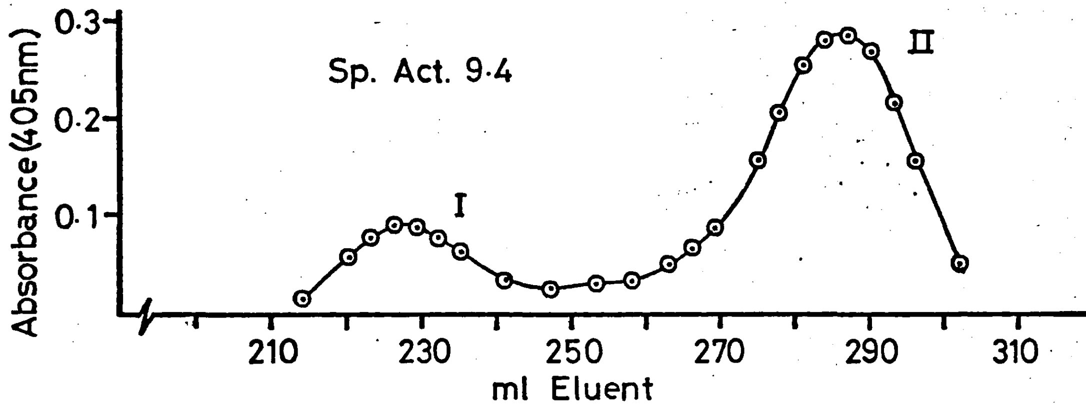
The remaining purification stages carried out in acetate buffer completely paralleled the McIlvaine buffer system.

The different α-galactosidase patterns resulting at stage (ii) after precipitating with citric acid and with acetic acid (in the presence of McIlvaine and acetate buffers, respectively) was further investigated keeping the buffer the same (i.e. McIlvaine) and then acidifying with HCl or acetic acid. Precipitates from both of these treatments were spun-off separately, and after readjustment of the pH of the supernatants to 5.5 with disodium hydrogen

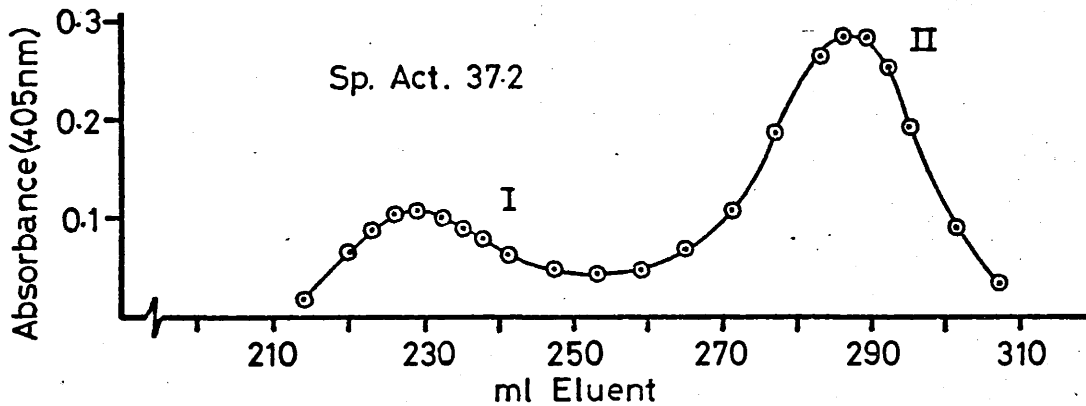
GRAPH 9.

Sephadex G-100 Elution Patterns of Resting Beans - Purification Carried out in Acetate Buffer.

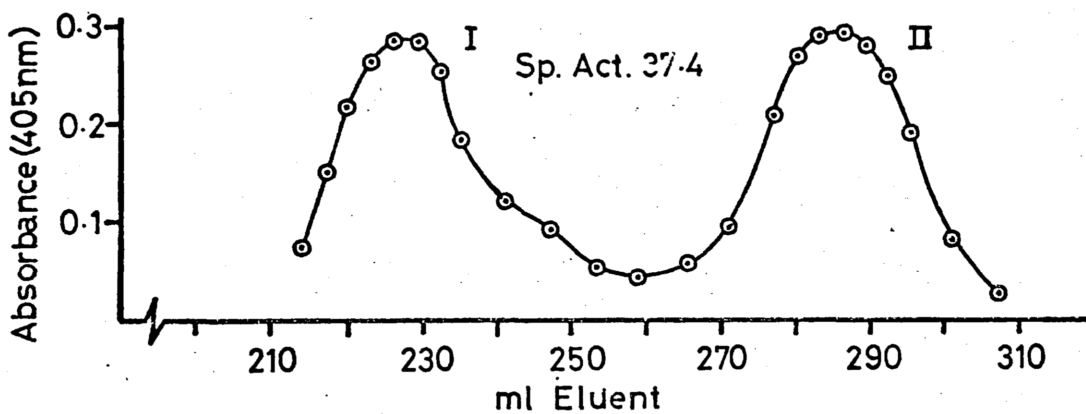
(i) Crude Extract



(ii) pH Precipitation (With 1M acetic acid)



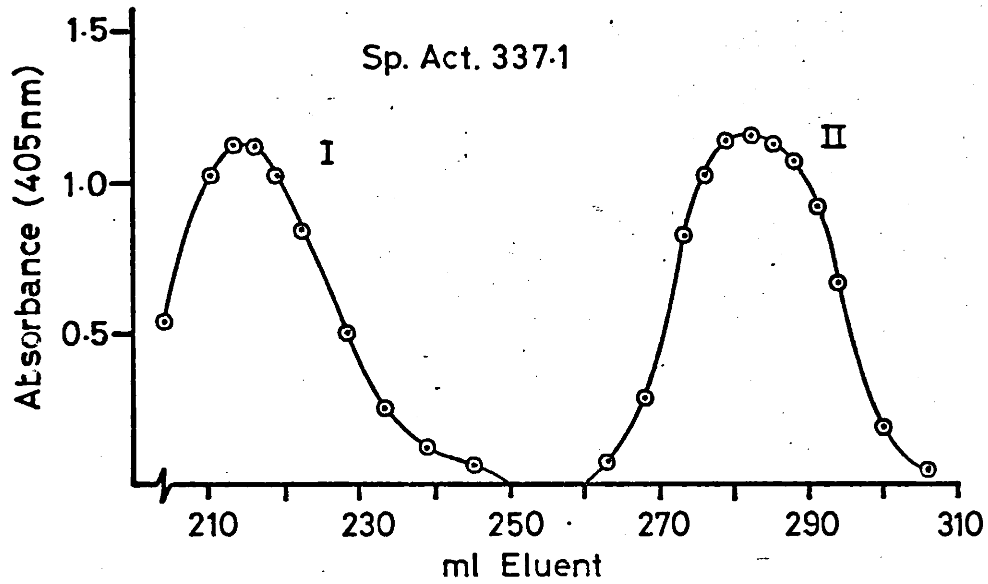
- 8 hr at 4°



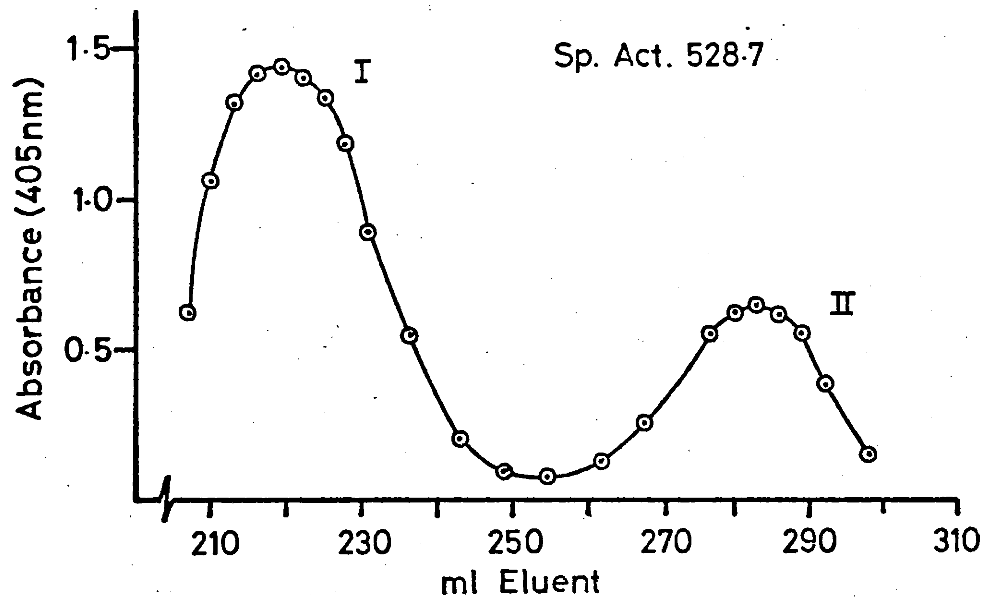
Continued over

GRAPH 9.-Continued.

(iii) Ammonium Sulphate (30 - 65% Fraction)



(iv) Dialysis

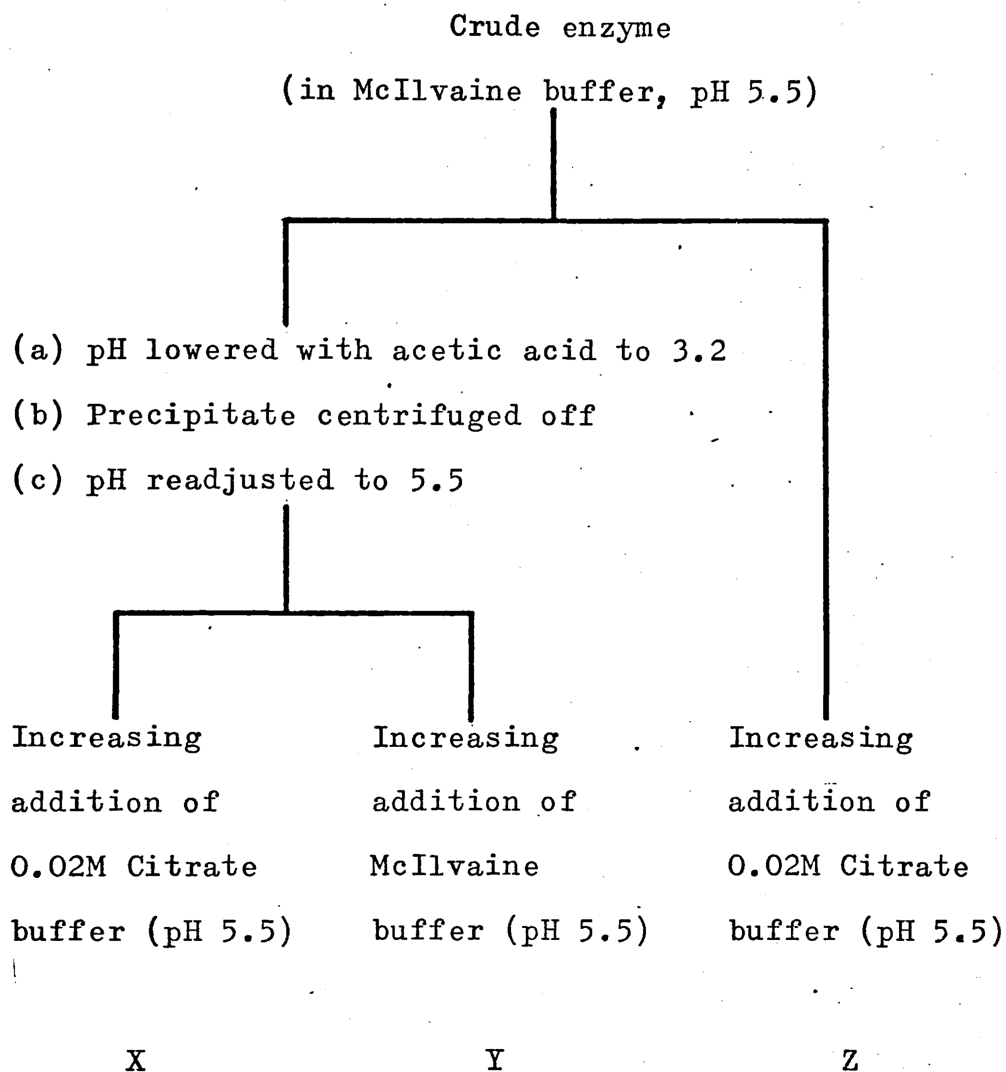


⊙—⊙ α-Galactosidase Activity.

phosphate they were examined by gel-filtration: no changes in isoenzyme pattern were observed in comparison with the crude preparation (Stage (i), Graph 6(i)). At this point it appeared that the concentration of the citrate ion was important in the acidification stage for the conversion of enzyme $_{II}$ to $_{I}$. Hence, experiments were designed to test the effect of citrate ion concentration on the system. In brief, increasing amounts of citrate were: (a) added to a crude enzyme in McIlvaine buffer keeping the pH constant at 5.5 (Experiment Z, page 139.); (b) added to a crude enzyme preparation in McIlvaine buffer that had received prior treatment with acetic acid (to pH 3.2) and then readjusted to pH 5.5 with buffer. Again the pH was kept constant during the citrate addition (Experiments X and Y, page 139.). The experiments are summarised in more detail in the scheme given overleaf.

The changes in specific activities, plus the Sephadex G-100 eluent patterns after the various treatments are shown in Graph 10.

All three experiments resulted in a steady increase in specific activity with increasing citrate concentration at constant pH, but it can be seen



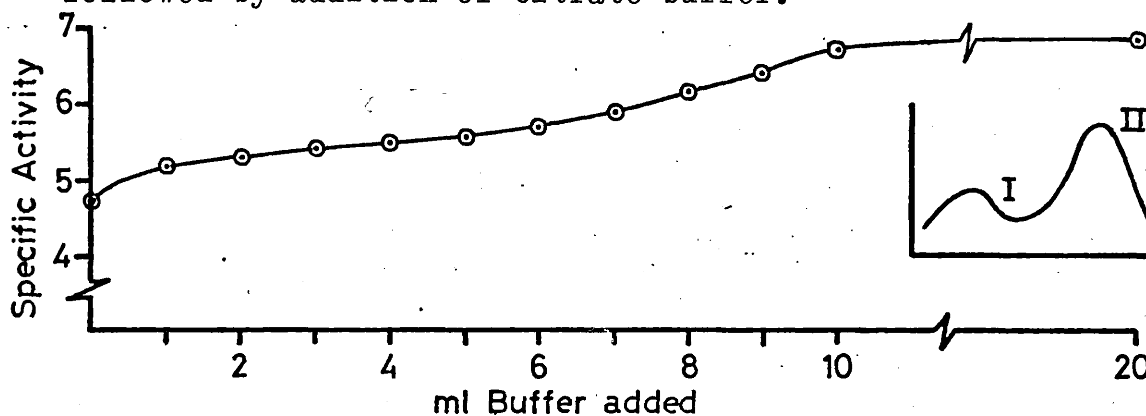
(Specific activity was checked after each addition and the isoenzyme pattern checked on Sephadex G-100 after the final addition.)

(in Graph 10) that a preliminary treatment of the crude enzyme with acetic acid to pH 3.2 (X and Y)

GRAPH 10.

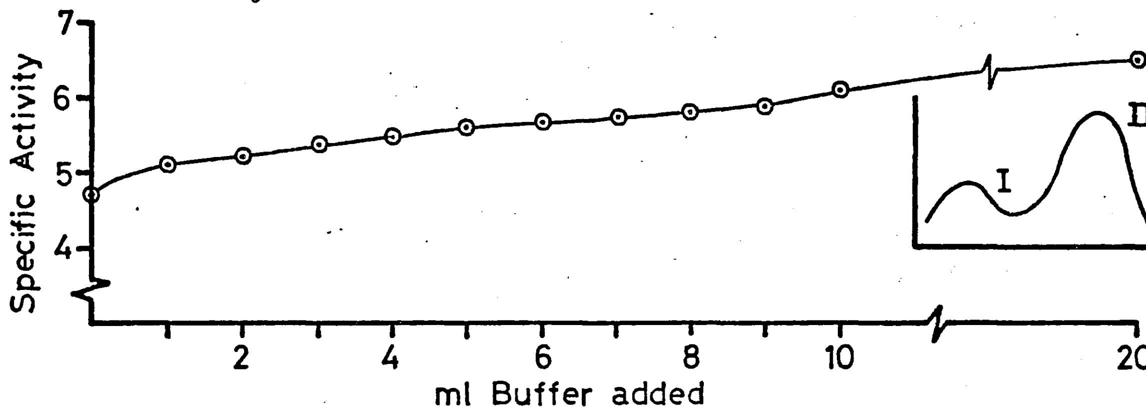
X Preliminary Treatment with Acetic acid to 3.2

followed by addition of Citrate buffer.



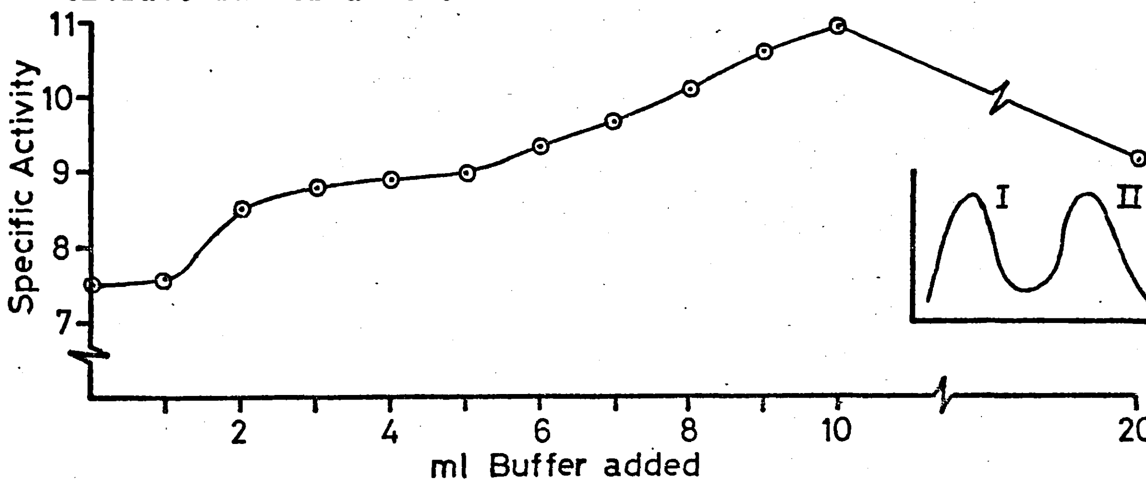
Y Preliminary Treatment with Acetic acid to 3.2

followed by Addition of McIlvaine buffer.



Z No Preliminary Treatment with Acetic acid:

Citrate buffer added.



(which in itself produced no pattern change (Graph 10)) prevented any change in the isoenzyme ratio on addition of citrate. The control experiment (Z) in which the crude enzyme had not received prior treatment with acetic acid, displayed the same initial increase in specific activity as the two acetic acid-treated samples when the citrate concentration was increased; but there was also a change in the G-100 gel filtration pattern with an apparent increase in enzyme $_{I}$. No precipitate resulted during the addition of citrate in Z and it should be noted that in this experiment, the specific activity decreased during the final addition of citrate which was not the case in X and Y.

Taking preparations of pure enzyme $_{I}$ and enzyme $_{II}$ (from Sephadex G-100 columns) in McIlvaine buffer (pH 5.5) and increasing the citrate concentration did not increase the specific activity of either enzyme and did not produce precipitates or other enzyme forms.

It appears, therefore, that the citrate ion activates both $_{I}$ and $_{II}$ in a crude α-galactosidase preparation and is the major factor determining whether $_{II}$ converts to $_{I}$. Increasing citrate ion concentration

in the absence of a pH change aids conversion in the case of the crude enzyme. When, however, the crude enzyme has received a preliminary low pH treatment with acetic acid (or HCl) but not citric acid, increasing citrate concentration no longer facilitates the conversion process. In other words increasing the acidity of the crude preparation in the absence of relatively large amounts of citrate can in some way prevent subsequent addition of citrate from changing the isoenzyme ratio.

The formation of a recognisable precipitate in any of these treatments is not a prerequisite for a change in the gel-filtration pattern. But, nevertheless, it was of interest to examine and compare the precipitate (A) produced by the action of acetic acid, which did not result in an immediate conversion, with the one that did (B) i.e. resulting from citric acid treatment. The third precipitate (C) which appeared after treating crude enzyme with acetic acid and then allowing the solution to stand was also investigated: the appearance of this precipitate coincided with conversion of enzyme $_{II}$ to $_{I}$.

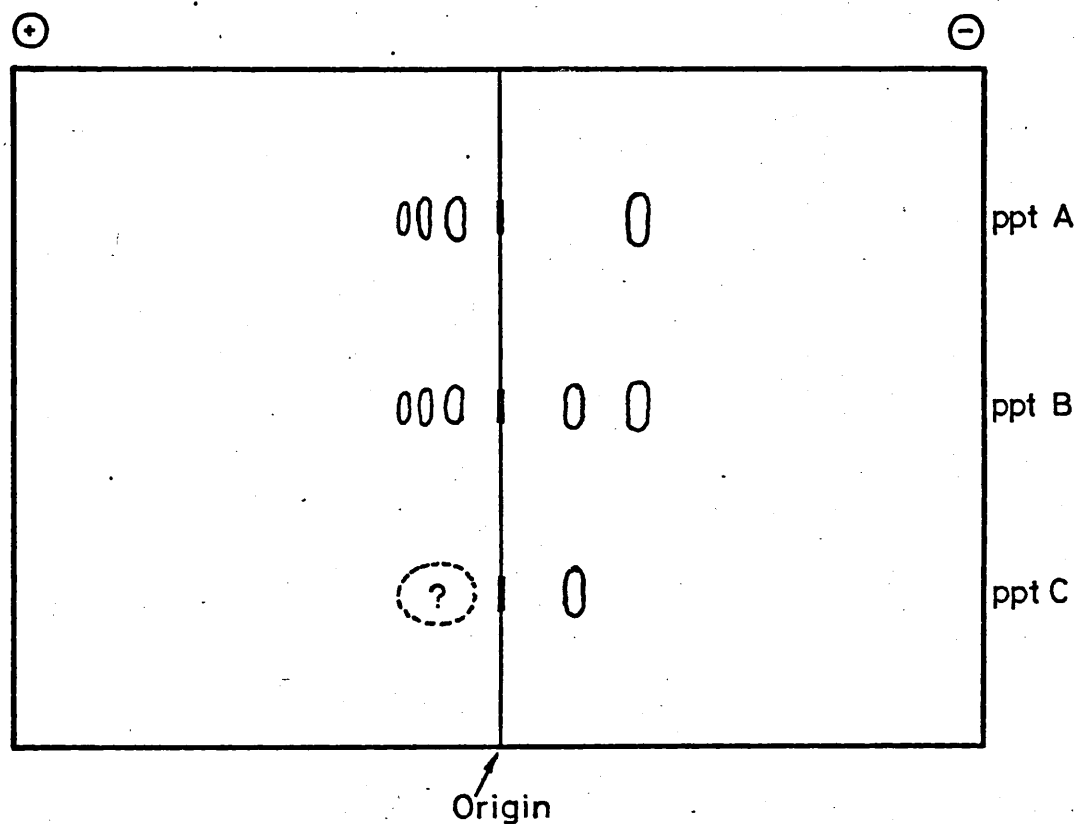
The three precipitates were subjected to cellulose acetate gel electrophoresis at pH 8.6 and protein

bands were visualised with Ponceau S (See Figure 8). All contained three relatively minor components (traces only in the case of C) which migrated towards the anode. Precipitate B gave rise to two relatively intense bands which were positively charged, whereas A gave a single band corresponding to the fast moving component in B. C, on the other hand, appeared to,contain a single, positively charged component corresponding to the slower moving protein in B. Examination of the precipitates by gel electrophoresis at pH 5.5 followed by treating the gel with 4-Methyl-umbellyferyl galactoside revealed no α-galactosidase activity. Precipitates A and B contained about 10% carbohydrate (measured as glucose) and C 2%.

It must be concluded that the major component in precipitate C is important for the $_{II}$ to $_{I}$ conversion reaction. It appears also in B i.e. where citric acid facilitates an enzyme pattern change, but not in A where acetic acid has no immediate effect on the components of the crude enzyme preparation. On leaving the acetic acid treated enzyme to stand the protein component in C is apparently removed from the system and this may trigger off the conversion reaction.

FIGURE 8.

Cellulose Acetate Electrophoresis (pH 8.6) of Protein Precipitates produced by lowering the pH by : addition of acetic acid (A); citric acid (B); leaving acetic acid-treated preparation to stand (C).



Constant voltage 250v., 3·5hr.
Barbital buffer (pH 8·6, 0·07M.)

* Located with Ponceau S.

One further possibility was that the change in isoenzyme levels at the pH lowering step might be associated with the removal of ions such as Ca^{2+} by citrate. The effect of E.D.T.A. on the pattern change was therefore investigated. A crude extract of mature _Vicia faba_ beans was made in the presence of E.D.T.A. (2.4mM) using the McIlvaine buffer system. A sample was applied to a Sephadex G-100 column, but the elution profile was identical to that obtained with a normal crude extract (e.g. Graph 6(i)).

Following the citric acid addition stage in the purification procedure (Table 6, Graph 6) there was only a very slow change (specific activity increase _ca_. 4 units/day) in isoenzyme pattern if the preparation was stored at 4°. The next fraction, resulting from the addition of 30 - 65% ammonium sulphate, on storing at 4° in ammonium sulphate solution also exhibited a change in pattern, the rate being somewhat greater (_ca_. 32 units/day). This difference between storage of the citric acid and ammonium sulphate fractions might be due to the higher protein concentration in the latter. Storage of both fractions gave rise to precipitates. In the former case it was extensive and examination by cellulose acetate gel electrophoresis revealed that it consisted of

a complex mixture of proteins possibly including component C (See Figure 8). The precipitate arising from the ammonium sulphate fraction was smaller and in this case contained C as a major component. When the ammonium sulphate fraction was dialysed against McIlvaine buffer, however, there was a very rapid change in specific activity (ca. 113 units/day) (although the protein concentration was decreasing) and a marked change in the gel filtration pattern. Here again there was a small precipitate containing C as a major component.

Dialysis, therefore, appeared to be speeding up a change which would have occurred naturally with time i.e. allowing the preparation to reach its natural equilibrium more rapidly.

The effect of increasing the total protein concentration on the rate of conversion of α-galactosidase $_{II}$ to $_{I}$ was also examined. Bovine serum albumin (final concentration 1%, W/V) in McIlvaine Buffer was, therefore, added to a 30 - 65% ammonium sulphate fraction of the enzyme which was then dialysed against buffer at 4°. The increase in specific activity with time was plotted and compared with a control to which no B.S.A. had been added. After 10 hr.

samples were applied to Sephadex G-100 columns. The resulting patterns are shown in Graph 11 together with the changes in specific activity. It is clear that B.S.A. increases the rate of isoenzyme conversion although the equilibrium, as shown by the specific activity eventually reaches the same level with and without B.S.A.

Glycoprotein Nature of α-Galactosidases.

Originally, α-galactosidase $_{I}$ resulting from resting seeds of *Vicia faba* was thought to be a glycoprotein containing 25% carbohydrate[41]. A later improved purification of the enzyme[10] resulted in a product containing only 8% carbohydrate, and a similar value was also obtained for enzyme $_{II}$. Enzyme $_{II}1$ and $_{II}2$, which were resolved from enzyme $_{II}$, were further shown to contain approximately 4% carbohydrate. The nature of this carbohydrate has now been investigated.

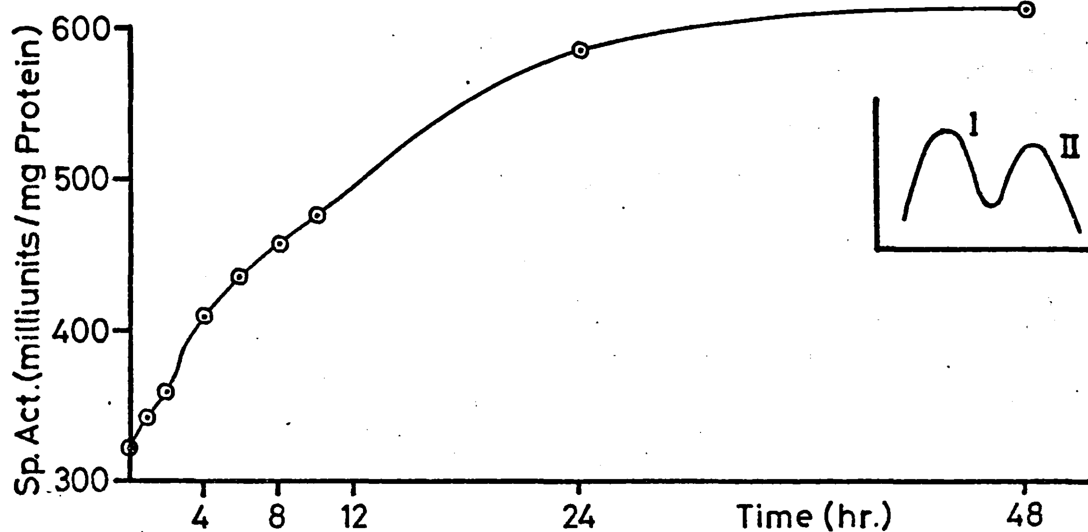
Using purified enzyme $_{I}$, containing 9% carbohydrate, measured as glucose, enzymic assays for the combined galactose, glucose, fructose and mannose, released by acid hydrolysis, were carried out[313]. (See Table 8.)

These results do not account for all the carbohydrate

GRAPH 11.

The Changes in Specific Activity (milliunits/mg protein) of Mature <u>Vicia faba</u> α-Galactosidases on Dialysis of: (a), the 30 - 65% ammonium sulphate fraction; (b), the 30 - 65% ammonium sulphate fraction plus bovine serum albumin (1% W/V).

(a) Ammonium Sulphate Fraction



(b) Ammonium Sulphate Fraction plus Bovine Serum Albumin.

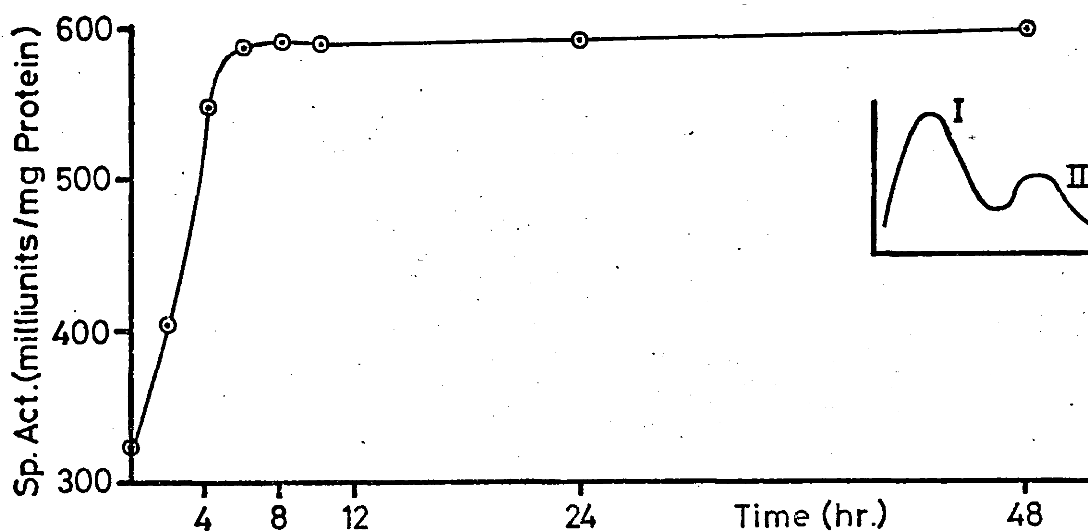


TABLE 8.

Determination of Combined Monosaccharides in α-Galactosidase $_{I}$ from Mature Vicia faba Seeds.

Monosaccharide	Amount (%, W/W)
Galactose	1.1
Glucose	1.4
Fructose	-
Mannose	1.4

in the enzyme sample and it is still not certain that it is covalently bonded to the protein, particualarly as the seeds are known to contain high concentrations of polysaccharides such as starch.

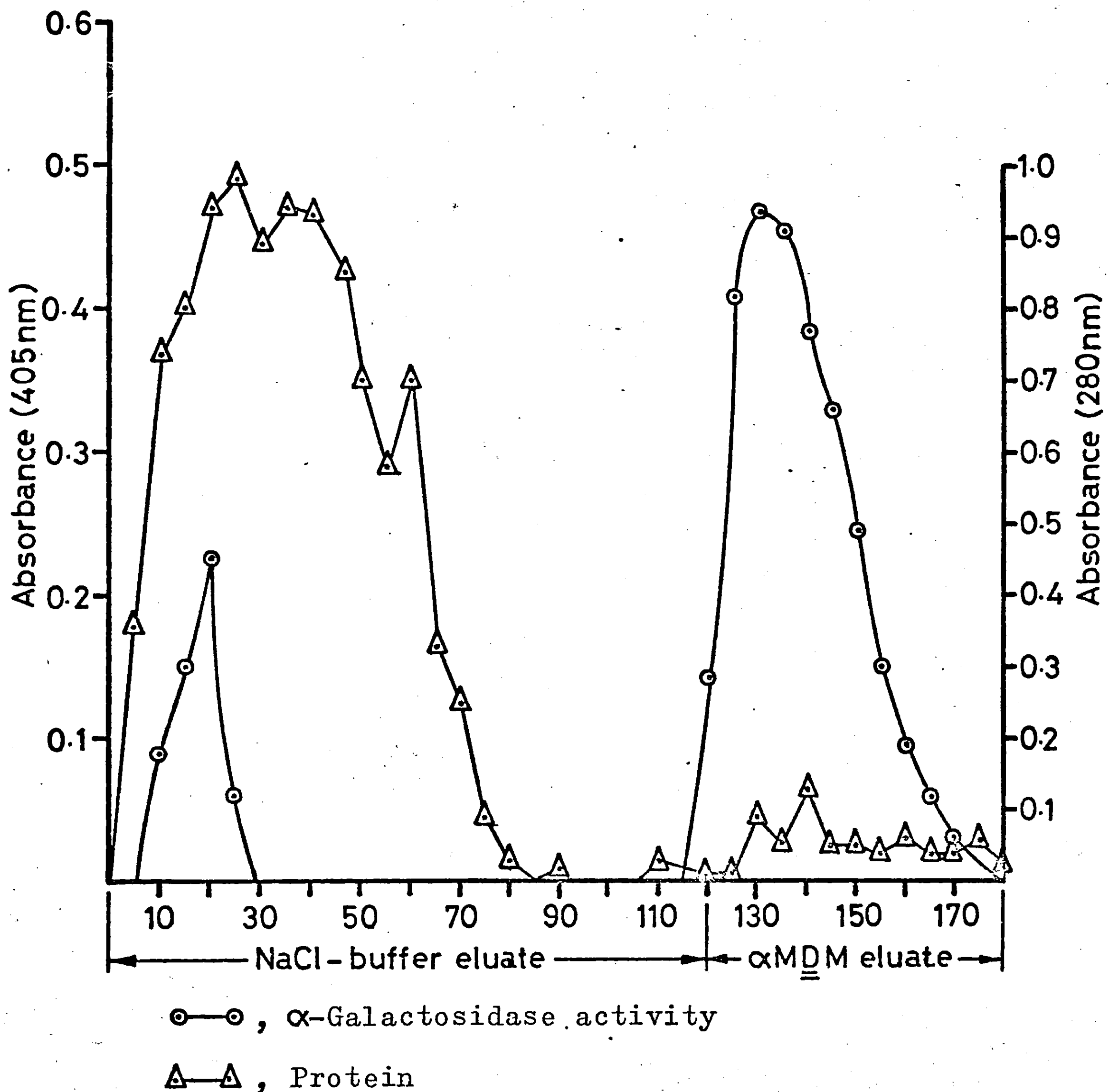
A further indication of the glycoprotein nature of the Vicia faba α-galactosidases was obtained, however, by passing a dialysed 30 - 65% ammonium sulphate fraction down a column of Concanavalin A-Sepharose[311]. The column was washed with potassium phosphate buffer (pH 7.0, 0.1M) containing 0.5M NaCl, and fractions collected and monitored for protein. When there was no further protein in the

eluate the enzyme was eluted with α-methyl-D-mannoside (0.5M) in the same buffer, and again fractions were collected. Each fraction was tested, after dialysis, for α-galactosidase activity. The elution profiles are shown in Graph 12. Approximately 16% of the α-galactosidase and the bulk of the protein was washed through the column with salt solution, but most appeared to be bound to the Concanavalin A-Sepharose and only eluted with the mannoside solution. Both the bound and unbound fractions were concentrated separately and applied to a Sephadex G-100 column. The elution profiles from this column are shown in Graph 13. The small differences in isoenzyme patterns were observed but were thought to be insignificant: approximately equal quantities of activities $_{I}$ and $_{II}$ were present. (the isoenzyme pattern of the enzyme preparation prior to application to the Concanavalin A-Sepharose column was as in Graph 6(iv)). Very similar results were obtained when the experiment was repeated using α-methyl-D-glucoside instead of the α-methyl-D-mannoside to elute the column.

The 16% of the total enzyme activity applied to the Concanavalin A-Sepharose which was washed through with the first 30ml of the NaCl buffer solution was not thought to arise from overloading of the

GRAPH 12.

Examination of a Dialysed 30 - 65% Ammonium Sulphate Fraction of Mature Vicia faba seed α-Galactosidase on a Concanavalin A-Sepharose Column. The column was first eluted with Potassium Phosphate buffer (pH 7.0, 0.1M) containing 0.5M NaCl, and then with α-Methyl-D-Mannoside (0.5M) in the same buffer.



⊙—⊙ , α-Galactosidase activity

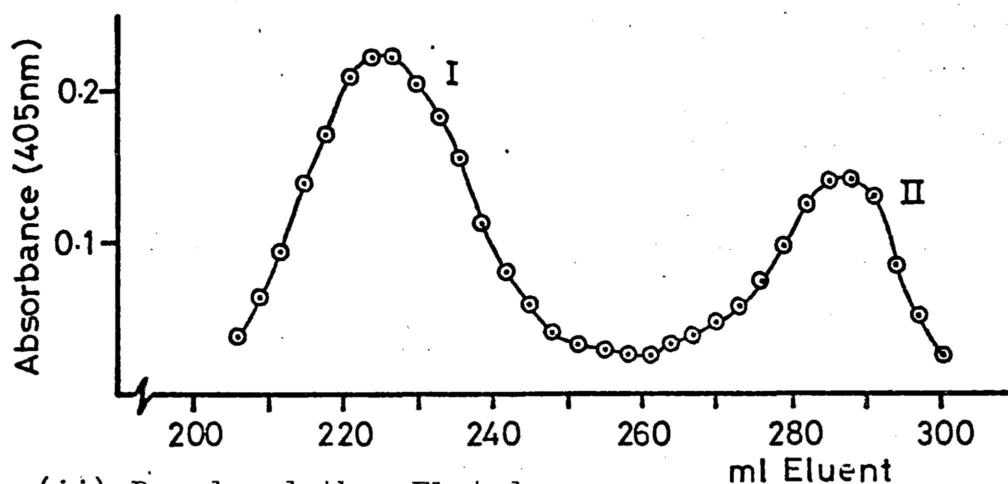
Δ—Δ , Protein

GRAPH 13.

Sephadex G-100 Elution Patterns of the two peaks of α-Galactosidase activity separated on the Concanavalin A-Sepharose column illustrated in Graph 12.

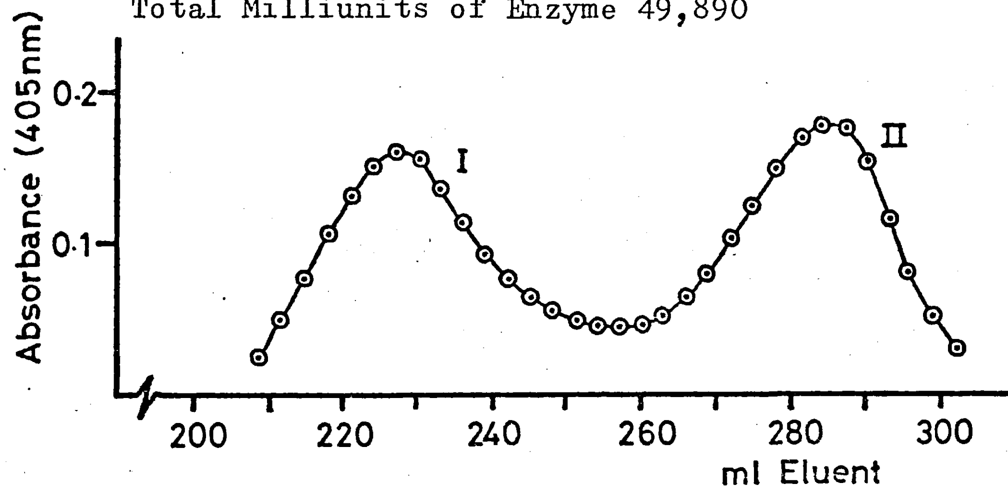
(i) Washed Off in First buffer

Total Milliunits of Enzyme 10,520



(ii) Bound and then Eluted

Total Milliunits of Enzyme 49,890



Total milliunits of enzyme activity applied to column 61,240

⊙—⊙, α-Galactosidase activity.

column. In another experiment much less of the α-galactosidase preparation (5,971 milliunits) was applied, and a similar percentage (14%) of the activity appeared in the first salt fraction. It must be concluded that this unbound enzyme was either not glycoprotein or a glycoprotein which for some reason did not bind to the lectin.

The specific binding properties of Concanavalin A have been extensively studied by Goldstein and co-workers[314,315]. The lectin binds glycans with terminal D-mannopyranose or D-glucopyranose units[315]. As binding does take place in the case of α-galactosidases $_{I}$ and $_{II}$ the glycoprotein nature of the enzymes is fairly certain and glucose or mannose residues (or both) are probably present as terminal groups. It is not certain, however, whether such evidence of glycoprotein structure is unequivocal. The possibility that the α-galactosidases are simple proteins physically associated with glycoproteins which in turn bond to Concanavalin A should be borne in mind.

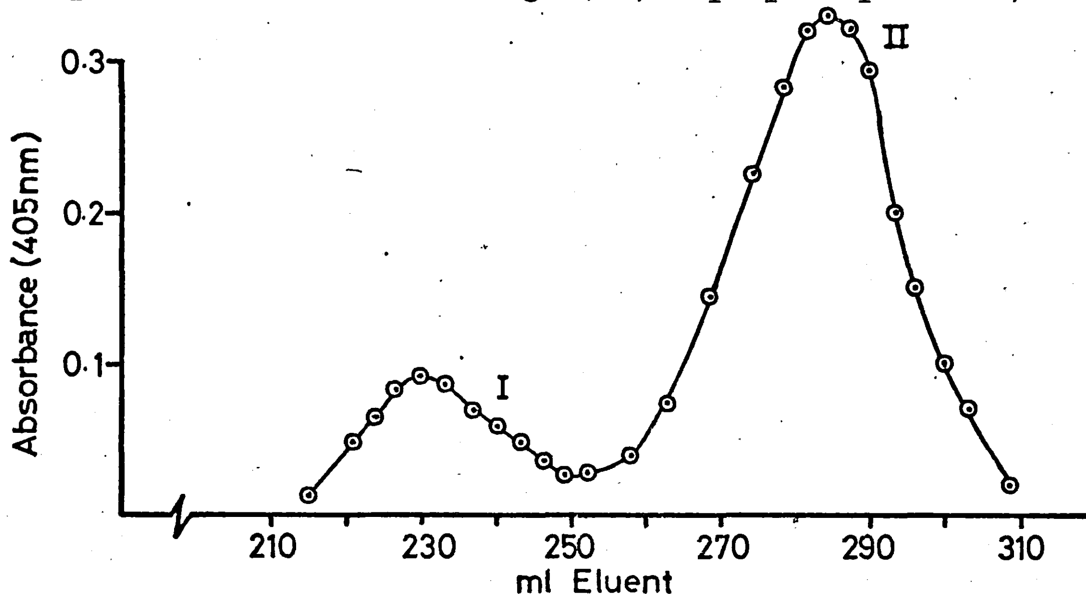
2. Changes in α-Galactosidase Isoenzyme Pattern during Seed Maturation.

In Section 1 the fact that the purification procedure altered the relative isoenzyme levels in the case of resting seed preparations was discussed. In view of these results it was decided to extend the study to find out if any of the purification procedures had any effect on extracts from immature beans. The previous work[10] on extracts of whole, maturing *Vicia faba* seeds had shown that fresh, young beans (1.9 - 2.9 cm in length) had low levels of enzyme $_{I}$ relative to enzyme $_{II}$. When these beans were allowed to dry out at room temperature the level of activity of enzyme $_{I}$ appeared to increase beyond that of enzyme $_{II}$. Graph 14 shows the Sephadex G-100 gel filtration patterns produced by Khaleque *et al* [312] before and after the seeds had dried. The upper profile (i) results from extracts of whole green beans which had been treated with citric acid and the pH lowered to 3.2 (Stage (ii) of the Purification Scheme), while the lower profile (ii) is of an extract from similar beans which had been allowed to dry out at room temperature for 14 days. This latter extract, however, had been taken through purification to Stage (iv), (dialysed ammonium sulphate fraction; See Page 125.) before being applied to the Sephadex column.

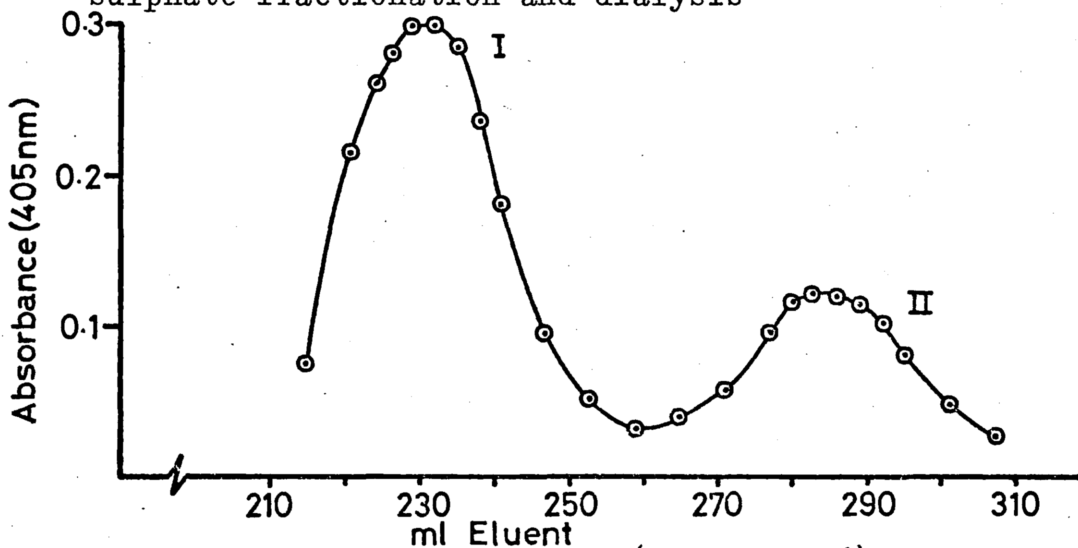
GRAPH 14.

Sephadex G-100 Elution Patterns of α-Galactosidases

(i) from green beans (Cotyledon, embryo and testa) freshly picked (Purification stage (ii) - pH precipitation)



(ii) from green beans (Cotyledon, embryo and testa) Dried 14 days at room temperature (Purification stage (iv) - ammonium sulphate fractionation and dialysis



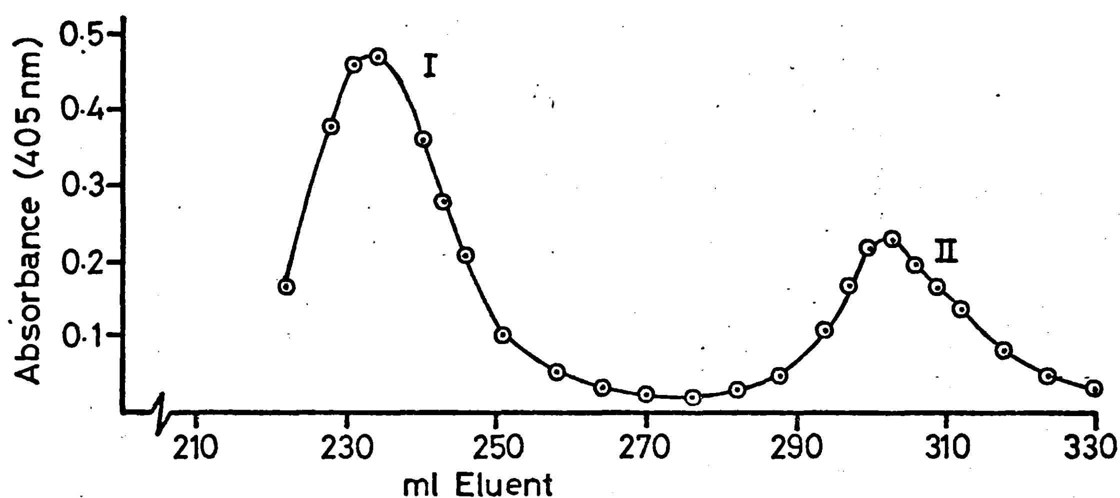
Taken from the work of A. Khaleque[10, 312,316]

In the present study a more rigorous screening of the immature bean extracts was carried out and the isoenzyme profile examined after each stage of purification. The results of this investigation are shown in Graph 15. The four profiles, corresponding to the four stages of purification all show enzyme $_I$ as the major peak, which is the opposite of what had previously been reported[316]. (See Graph 14(i)). The immature bean enzyme preparation, which gave the results illustrated in Graph 15, had been made by a method similar to that used for resting beans (See Page 124.) i.e. the testa had been removed, and the cotyledons and embryos then ground in a pestle and mortar with McIlvaine buffer. The extract had then been allowed to stand for 1hr, strained through muslin, and the cell debris centrifuged away. The possibility that the difference in results (cf. Graphs 14(i) and 15(ii)) might be connected with the testa was next considered. The experiments were thus repeated, but this time the crude enzyme preparation was made from the whole seed i.e. the testa was also included. The results are given in Graph 16 which show that the activity of enzyme $_{II}$ in this case is greater than that of Enzyme $_I$ at all stages of purification and, in particular, that the profile after pH precipitation was very similar to that obtained by Khaleque[316] (See Graph 14) in the previous work. Table 9 compares the specific activities after the

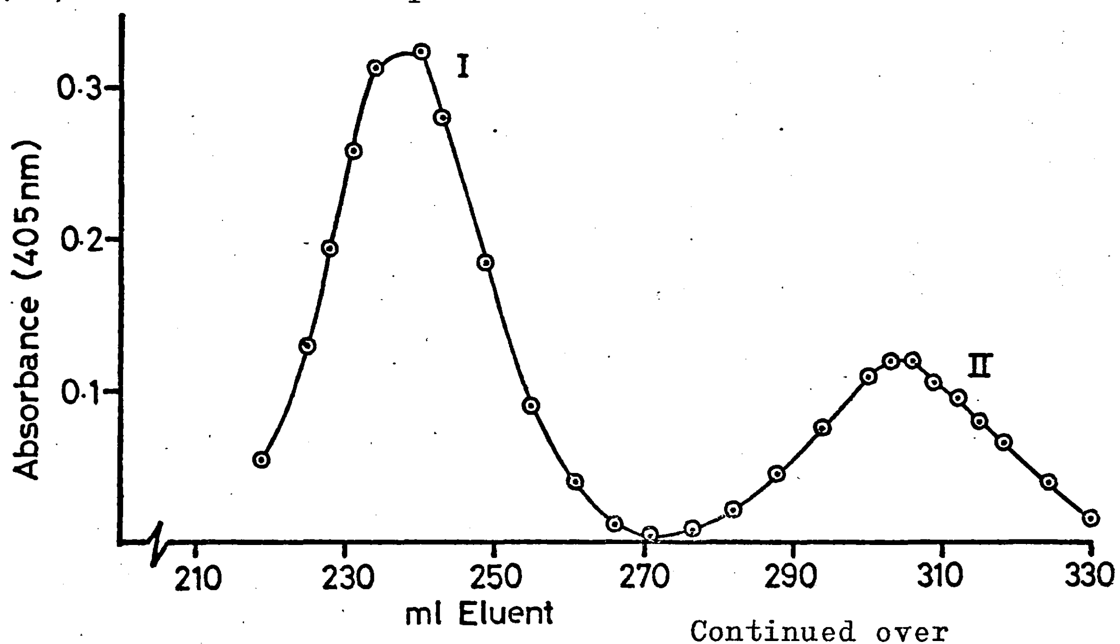
GRAPH 15.

Sephadex G-100 Gel Filtration Patterns obtained from Immature Beans (Purification in McIlvaine buffer).

(i) Crude exract into Buffer



(ii) Citric Acid Precipitation



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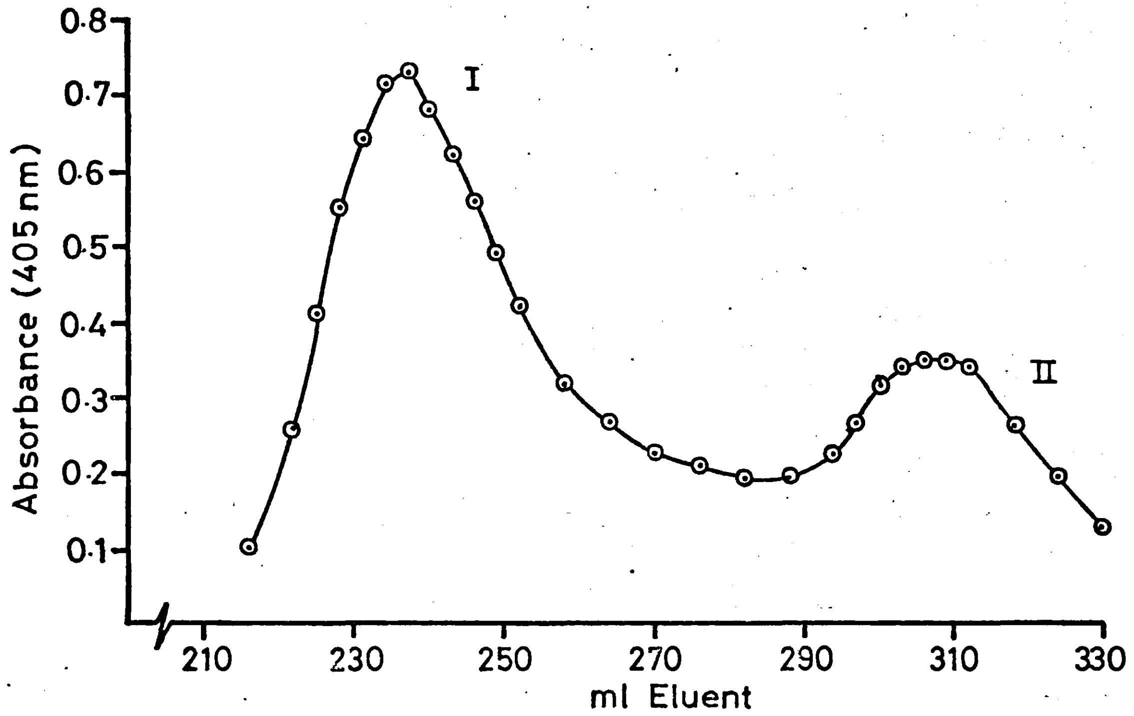
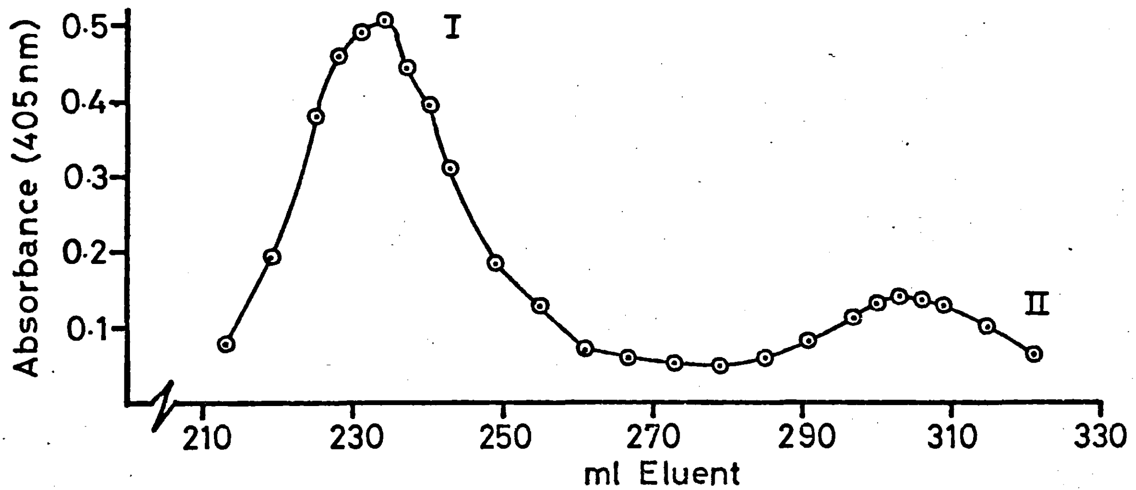
GRAPH 15.-Continued.

(iii) Ammonium Sulphate Fractionation



(iv) After Dialysis

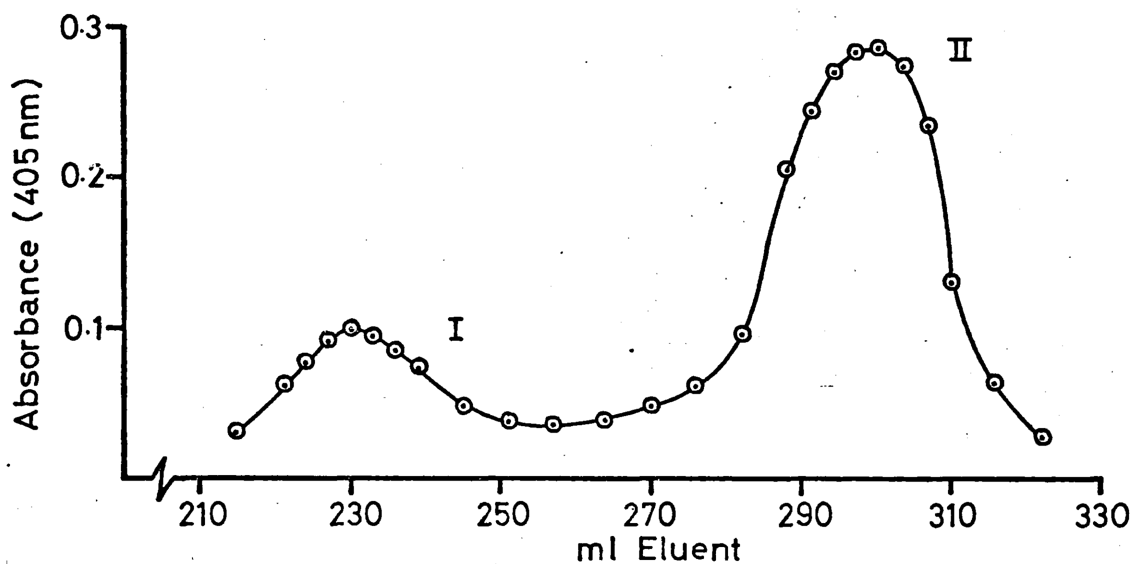


⊙—⊙ α-Galactosidase activity

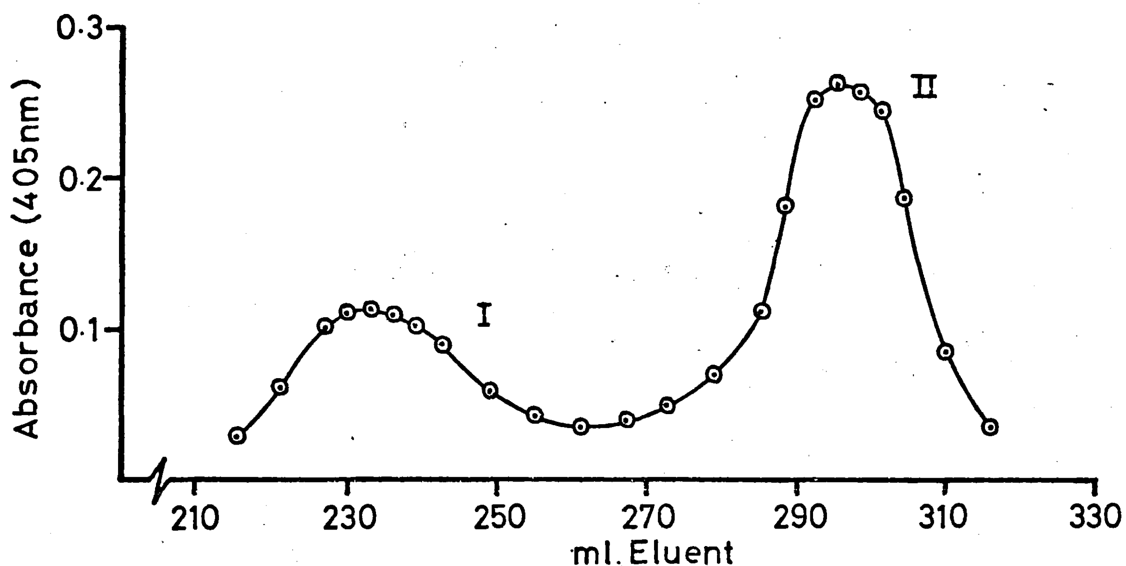
GRAPH 16.

Sephadex G-100 Elution Patterns of α-Galactosidases from Whole, Immature Beans (Cotyledon, embryo and testa) after four stages of Purification ((i) to (iv)).

(i) Crude Extract into Buffer



(ii) Citric acid Precipitation



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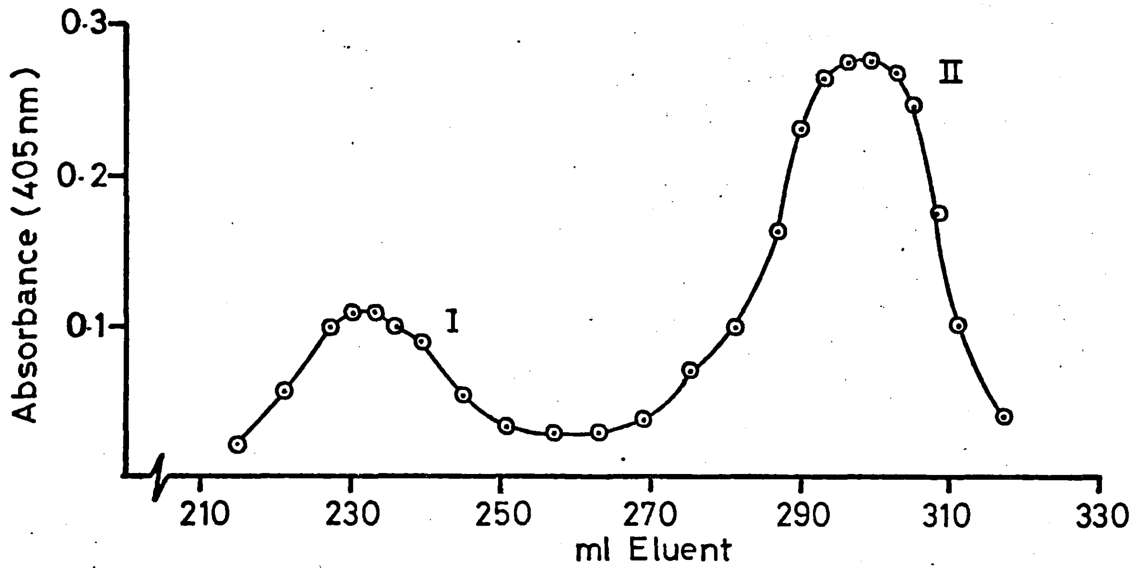
GRAPH 16.-Continued.

(iii) Ammonium Sulphate Fractionation



(iv) After Dialysis

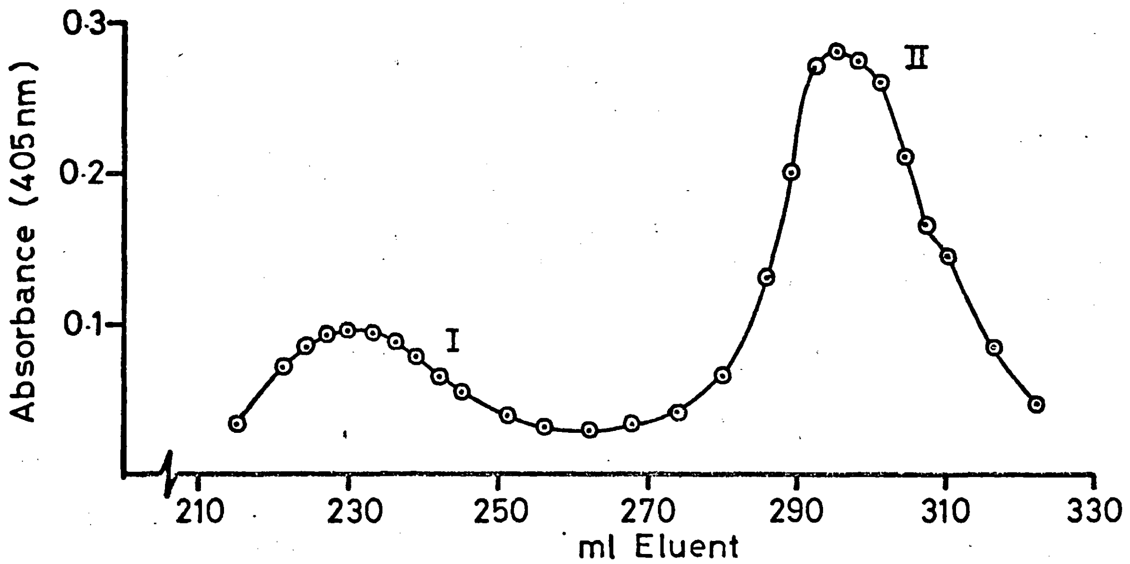


TABLE 9.

Specific Activities of α-Galactosidases from Extracts of Green Beans after Various Purification Stages.

Extract	Purification Stage 1	2	3	4
Cotyledon/ Embryo	9.1 (3,920)	9.8 (3,849)	28.7 (3,750)	33.4 (3,965)
Cotyledon/ Embryo/Testa	8.9 (3,726)	10.6 (3,752)	26.9 (3,810)	31.2 (3,826)

Bean size 20 - 25 mm

Total activities below, in parenthesis

different purification stages of enzymes from whole green beans with those from extracts of cotyledons and embryos only. It can be seen that there is no great difference between the two different types of preparation in terms of specific activities which also parallel the total activities. This cannot be explained: The specific activities of the enzyme fractions from the whole beans ought to have been somewhat lower than those from the cotyledon/ embryo fractions as the latter appeared to contain greater amounts of the high specific activity form $_{I}$. The testa contributed little to the whole seed extracts (See Page 173 .).

The previous experiments of Khaleque[316] who dried the immature beans at room temperature were again repeated but, drying was effected in two ways. One batch of beans was allowed to dry with their testas intact. At the end of the drying period (14 days) the sample was divided in half; one half was left with testas, whilst the testas were removed from the other half. Then both seed samples were subjected to the four stages of purification and each stage was examined on a Sephadex G-100 column. Graph 17 shows the resulting gel filtration patterns.

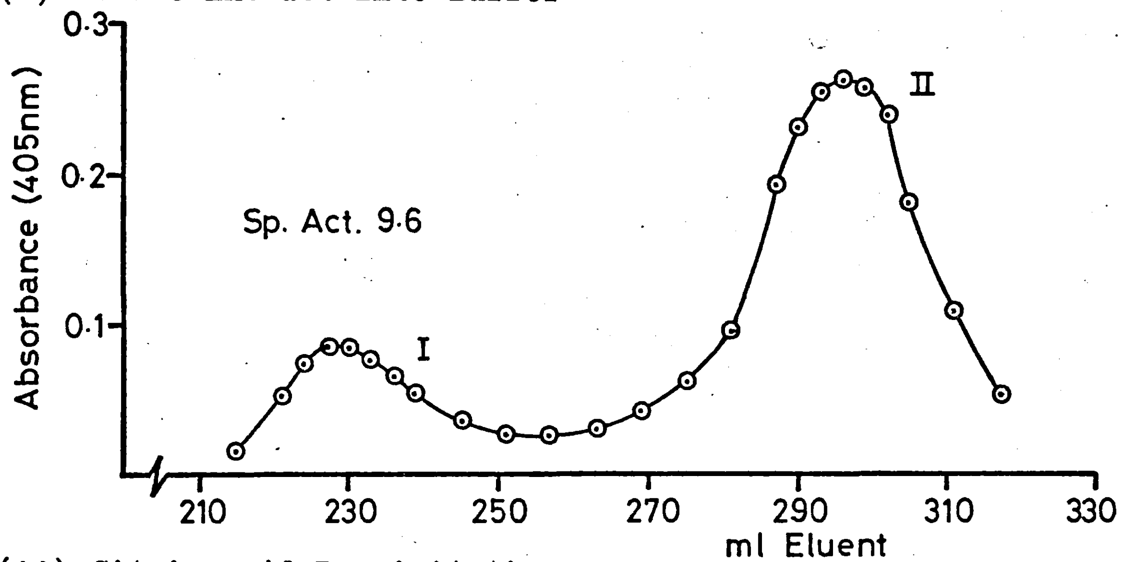
Testas were removed from a second batch of green beans and half of these were covered by a layer of cling film. Covered and non-covered seeds were then allowed to dry

GRAPH 17.

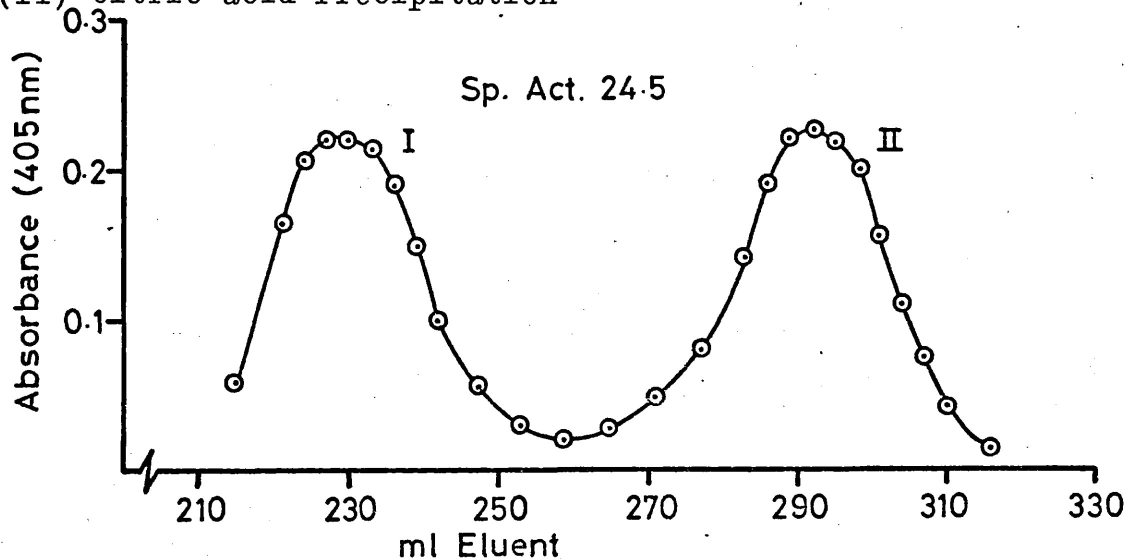
Sephadex G-100 Elution Patterns of α-Galactosidases from Intact Green Beans which have been allowed to dry (room temperature, 14 days). Seed samples were then subjected to purification; four stages ((i) to (iv)): (a), after removal of testa; (b), with testa intact.

(a) After removal of Testa

(i) Crude Extract into Buffer



(ii) Citric acid Precipitation



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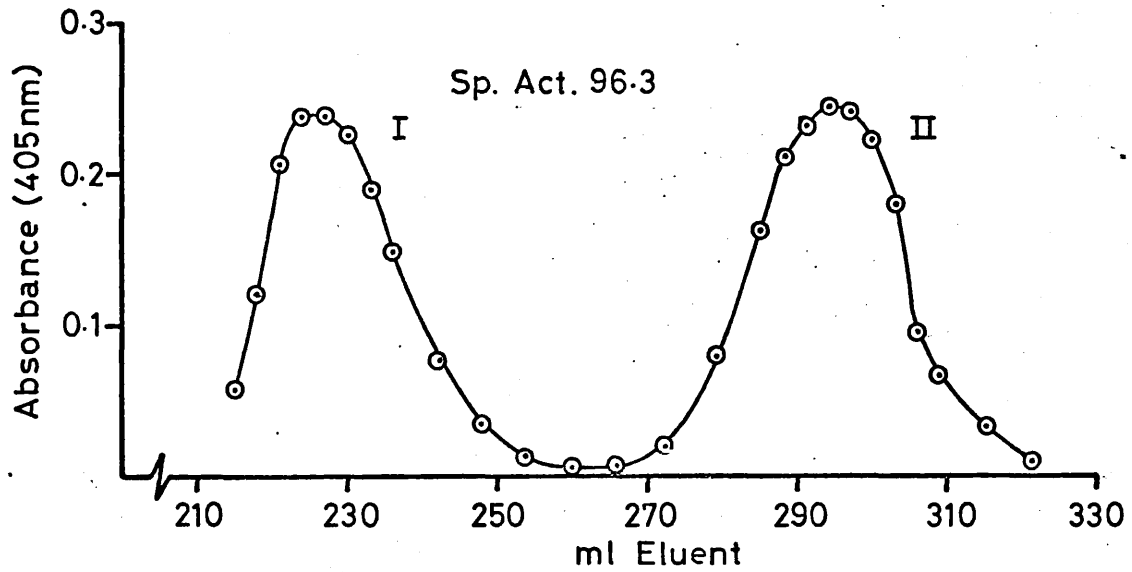
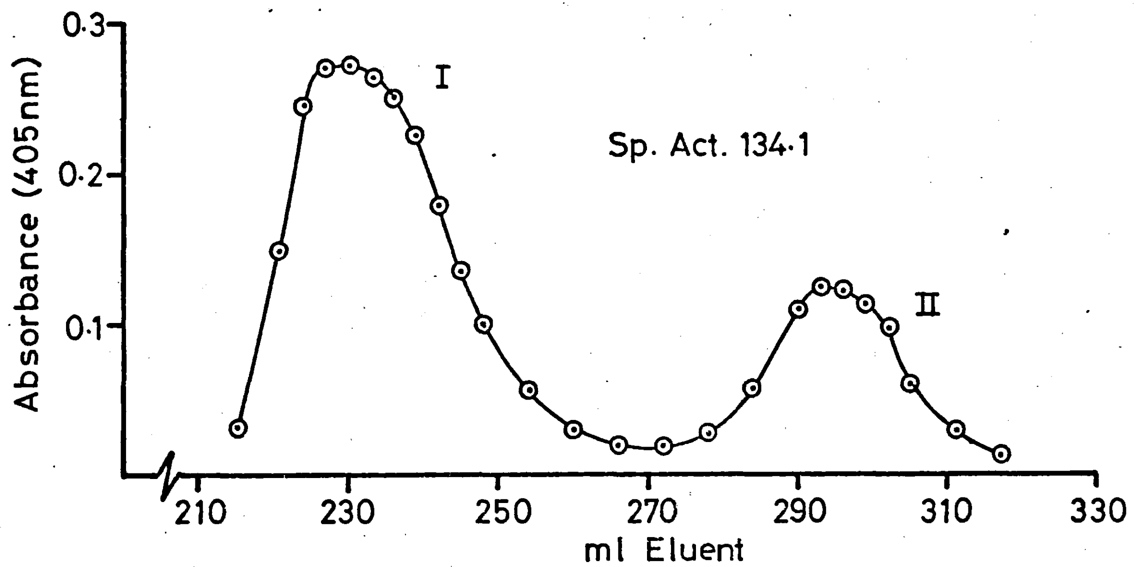
<u>GRAPH 17.-Continued.</u>

(iii) Ammonium Sulphate Fractionation



(iv) After Dialysis



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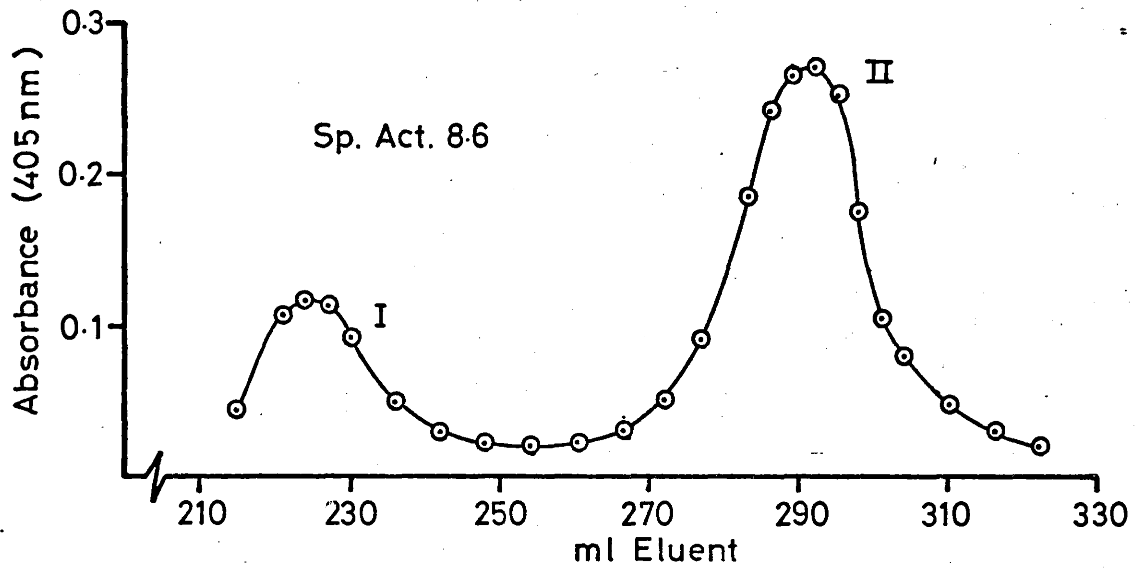
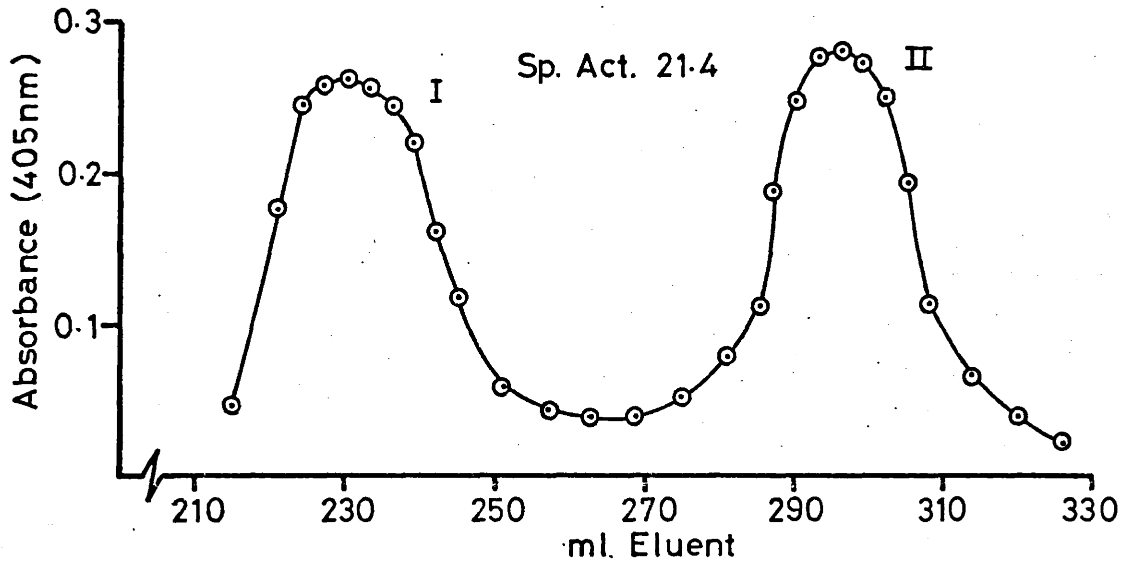
GRAPH 17.-Continued.

(b) With testa intact

(i) Crude Extract into Buffer



(ii) Citric acid Precipitation



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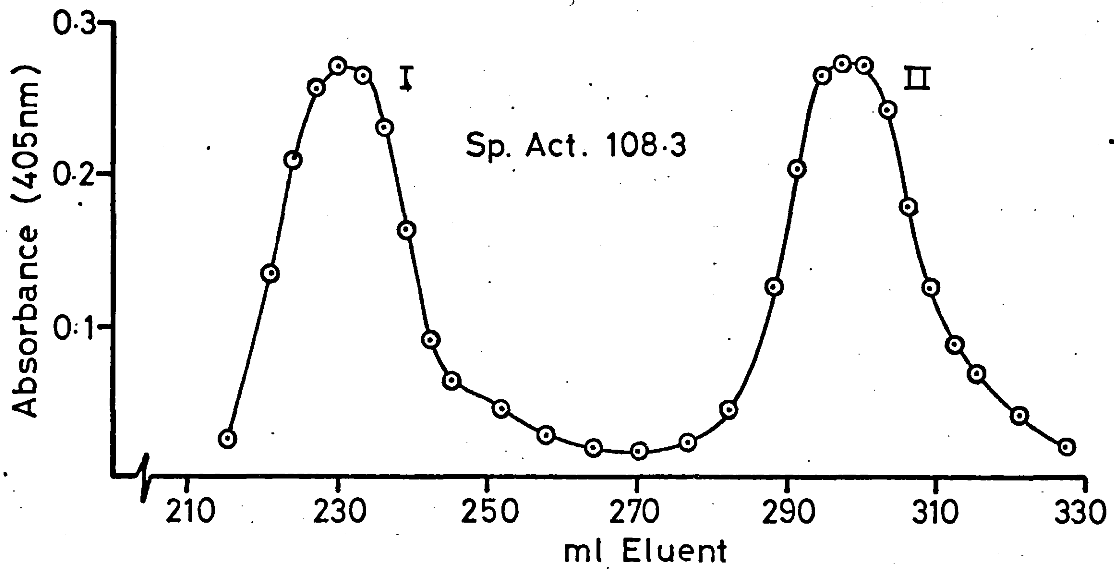
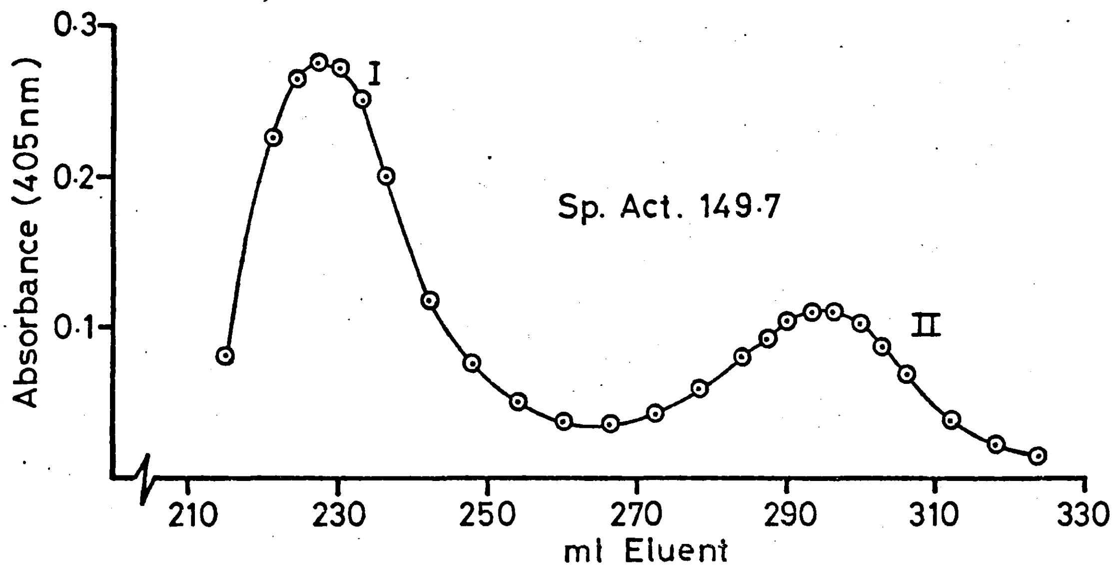
GRAPH 17.-Continued.

(iii) Ammonium Sulphate Fractionation



(iv) After Dialysis



(room temperature, 14 days), and the seeds were then examined for α-galactosidases at various stages of purification as before. The results are shown in Graph 18.

A comparison of Graphs 17 a and 17 b shows that by drying immature seeds with testas still in place the expected isoenzyme pattern in the crude extract is obtained with a low $_{I}/_{II}$ activity ratio. As purification proceeds this ratio increases as was shown with extracts from normal resting seeds (where conversion of α-galactosidase $_{II}$ to $_{I}$ was thought to occur, See Page 129.), and patterns are the same no matter whether initial seed extracts are made in the presence or absence of the testas.

In Graph 18 where immature seeds have been dried in the absence of testas the $_{I}/_{II}$ activity ratio is relatively high in the crude extracts and remains so during the purification stages. The cling film (Graph 18 b) does not appear to imitate the natural testa.

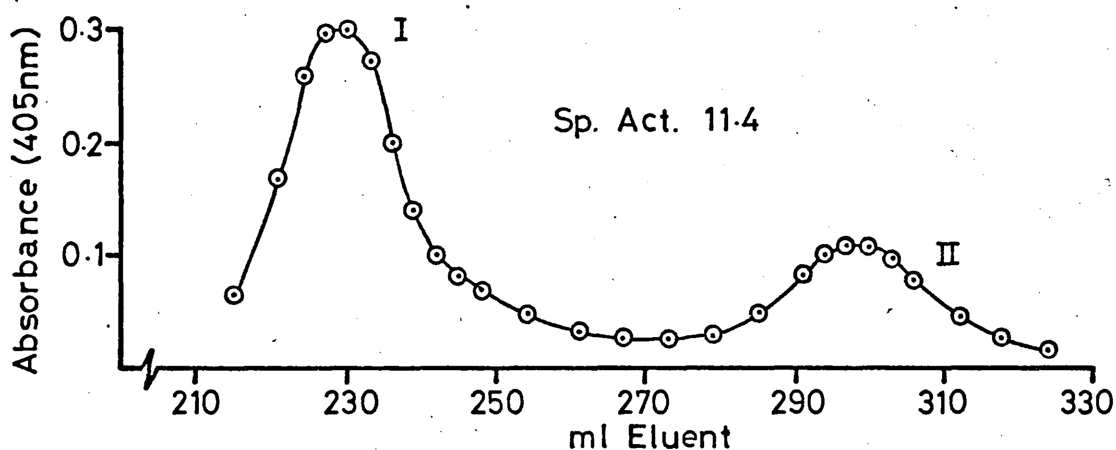
If the results illustrated in Graphs 17 and 18 are compared it must be concluded that the presence of the testa (i.e. under physiological conditions) during the drying of the green beans in some way prevents the conversion of α-galactosidase $_{II}$ to $_{I}$. Different rates of drying in the presence and absence of testa could be a factor

GRAPH 18.

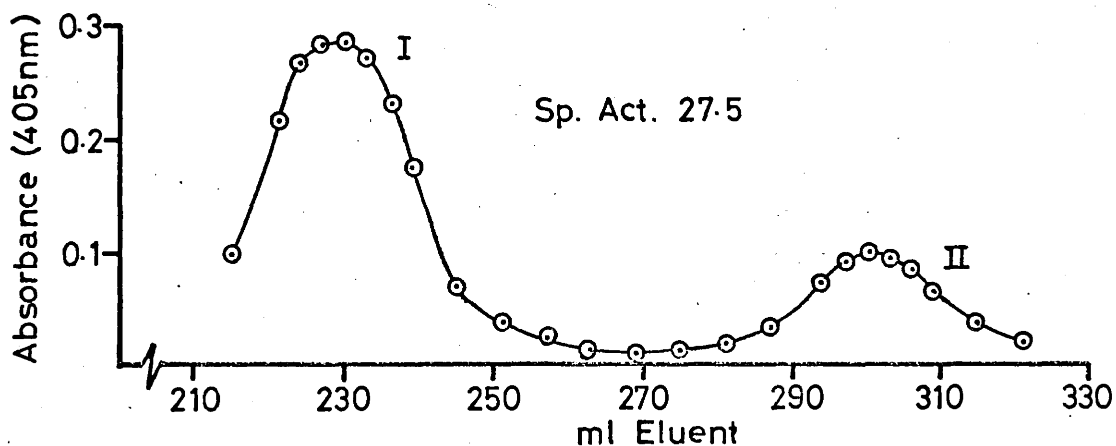
Sephadex G-100 Elution Patterns of α-Galactosidases from Intact Green Beans from which: (a), the testas had been removed; (b), the testas had been removed but replaced by a layer of cling film. Both (a) and (b) had then been allowed to dry (room temperature, 14 days) and seed samples were subjected to four purification stages ((i)-(iv)).

(a) Testas removed at start of drying

(i) Crude Extract into buffer



(ii) Citric acid Precipitation



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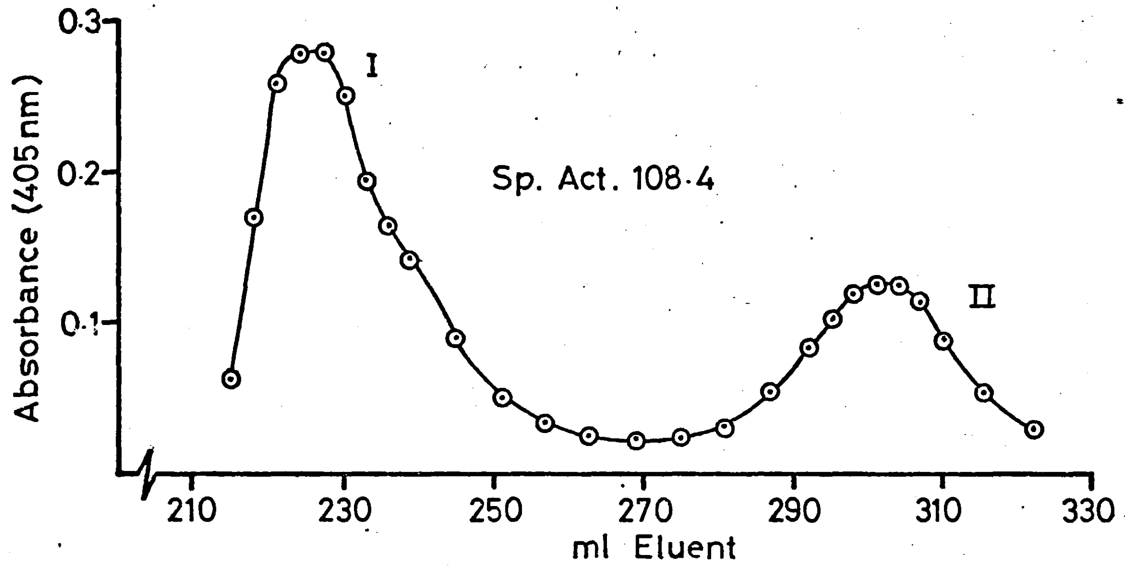
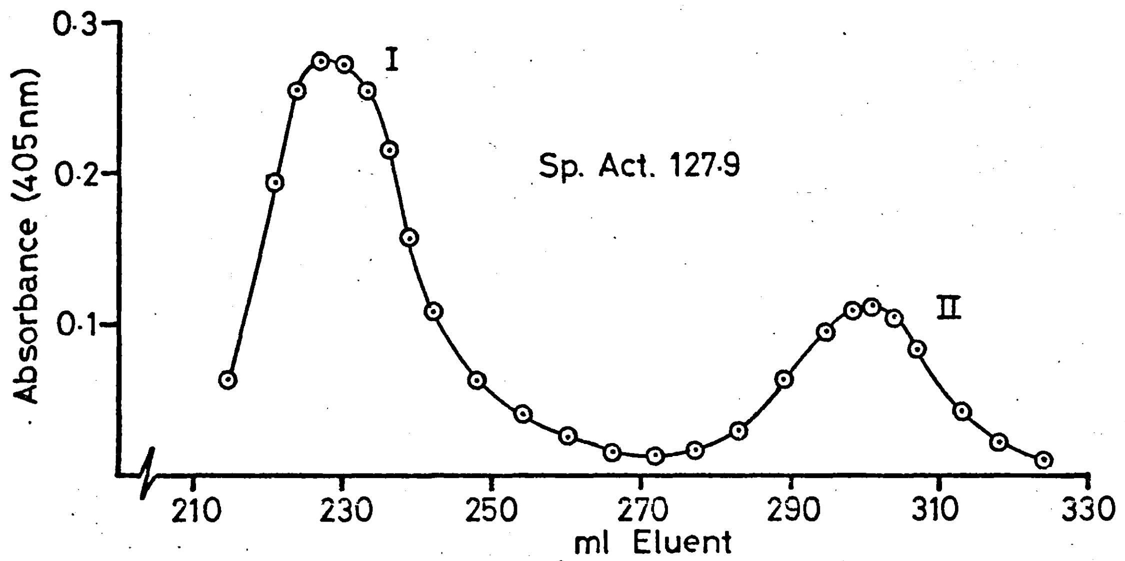
GRAPH 18.-Continued.

(iii) Ammonium Sulphate Fractionation



(iv) After Dialysis



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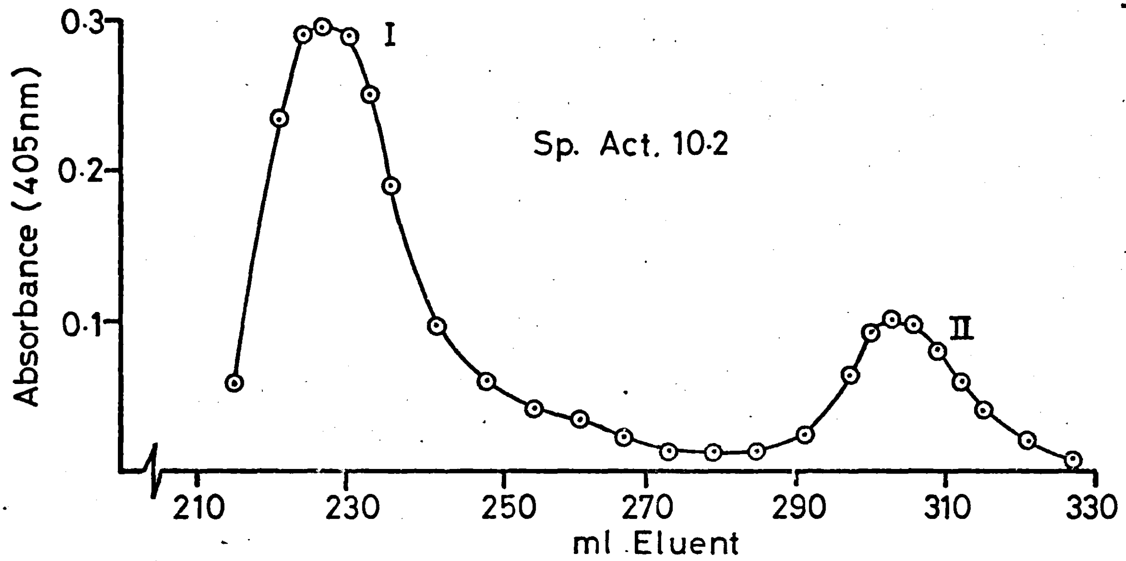
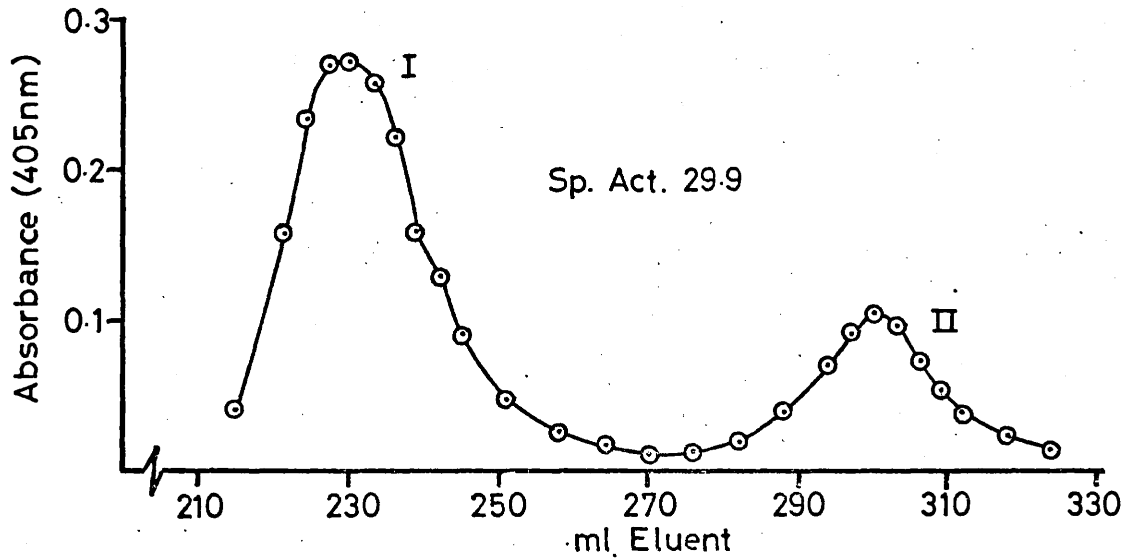
(b) Testas removed but replaced by a layer of cling film

(i) Crude Extract into Buffer



(ii) Citric acid Precipitation



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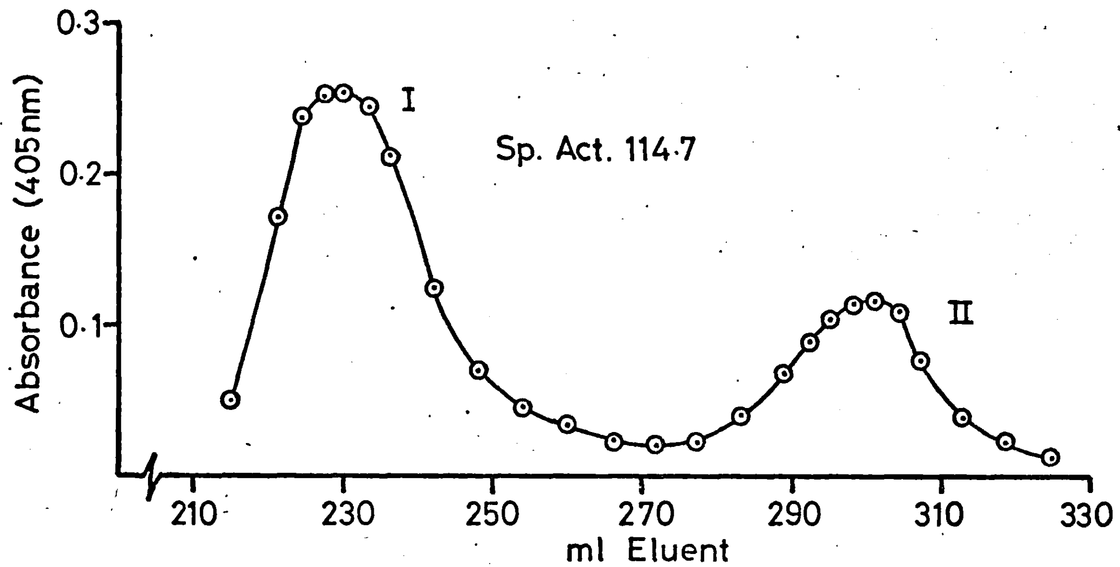
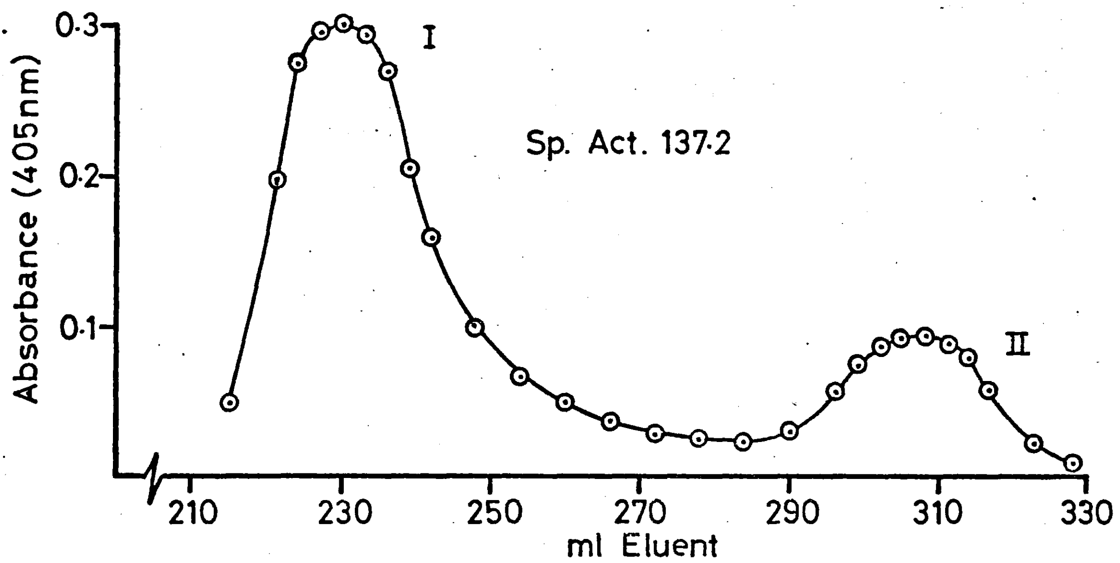
GRAPH 18.-Continued.

(iii) Ammonium Sulphate Fractionation



(iv) After Dialysis



in the conversion rate but the experiment where green seeds were covered with cling film (page 162.) does not support this idea. There remains, therefore, the postulate that some material which inhibits the $_{II}$ to $_{I}$ conversion is diffusing into the cotyledons or possibly something which activates the conversion is diffusing out. It might also be concluded that a conversion in the opposite direction i.e. $_{I}$ to $_{II}$, had been 'catalysed' by the presence of testa during the drying process. This is possible, although no other evidence, either *in vivo* or *in vitro*, for this type of conversion has been obtained.

A further possibility is that the low $_{I}$ / $_{II}$ activity ratios shown in Graph 17 a and b are caused by the testas contributing enzyme $_{II}$ to the extracts. However, this was not supported by an examination of testa material itself from beans at an equivalent stage of development. These tissues were extracted and the α-galactosidase purified as before and then examined by gel filtration. The results (Graph 19 a) showed that only low levels of enzyme $_{II}$ were present, and that the changes in specific activity on purification paralleled that of green beans (See Table 9); there was no formation of enzyme $_{I}$.

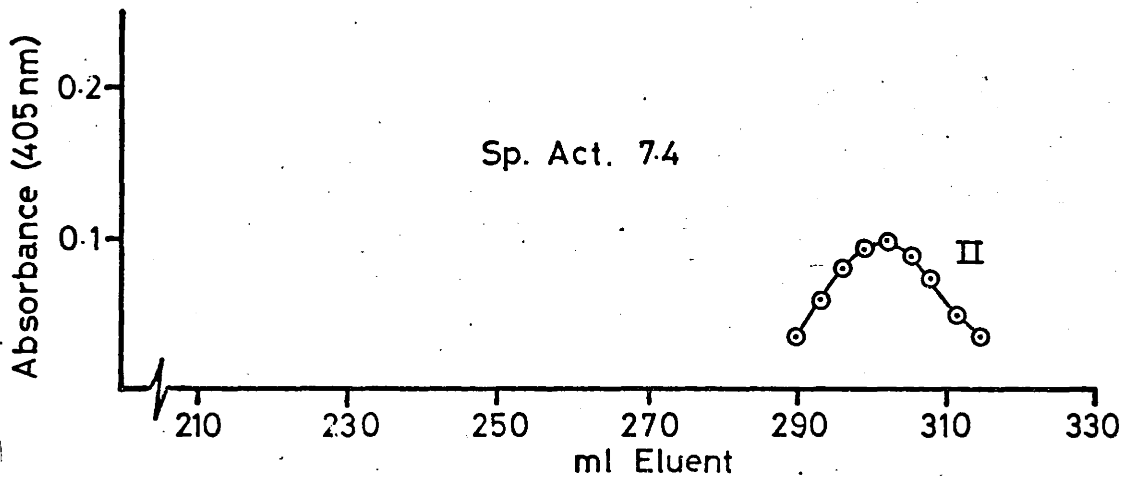
An examination of very young testa material from beans (5 - 15 mm long) with undifferentiated cotyledons was also

GRAPH 19.

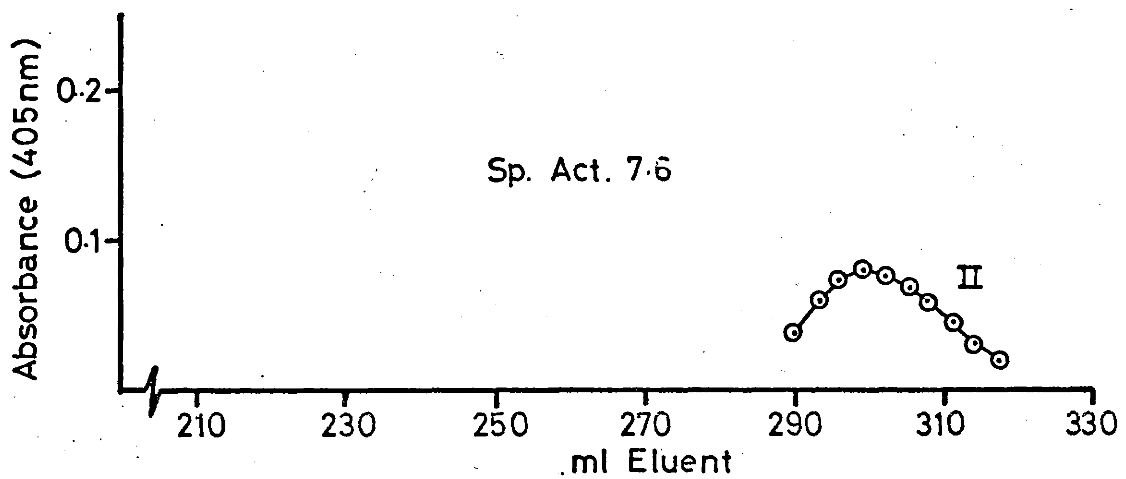
Sephadex G-100 Elution Patterns of α-Galactosidases from Extracts of Immature Beans after four purification Stages.

(a) Testa Material Alone

(i) Crude Extract into Buffer



(ii) Citric Acid Precipitation



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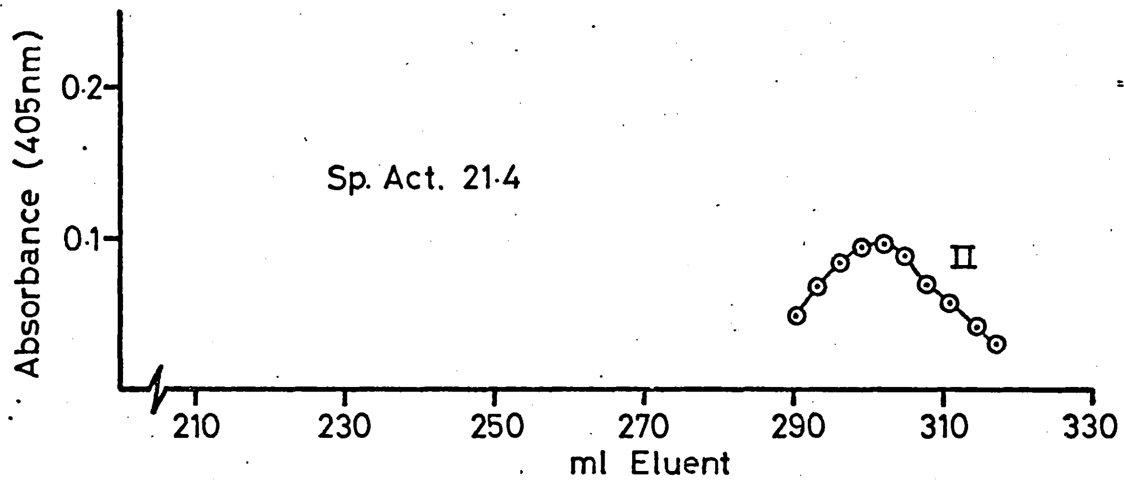
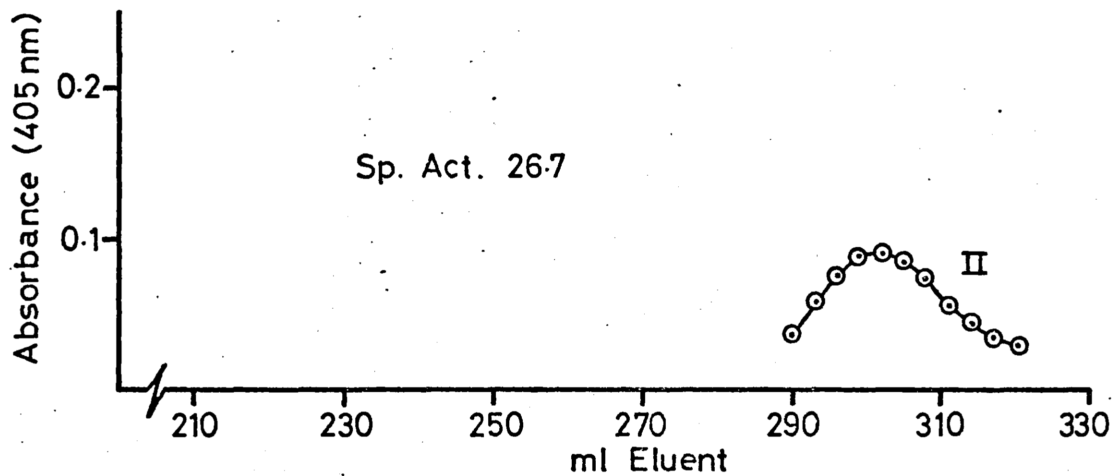
GRAPH 19.-Continued.

(iii) Ammonium Sulphate Fractionation



(iv) After Dialysis



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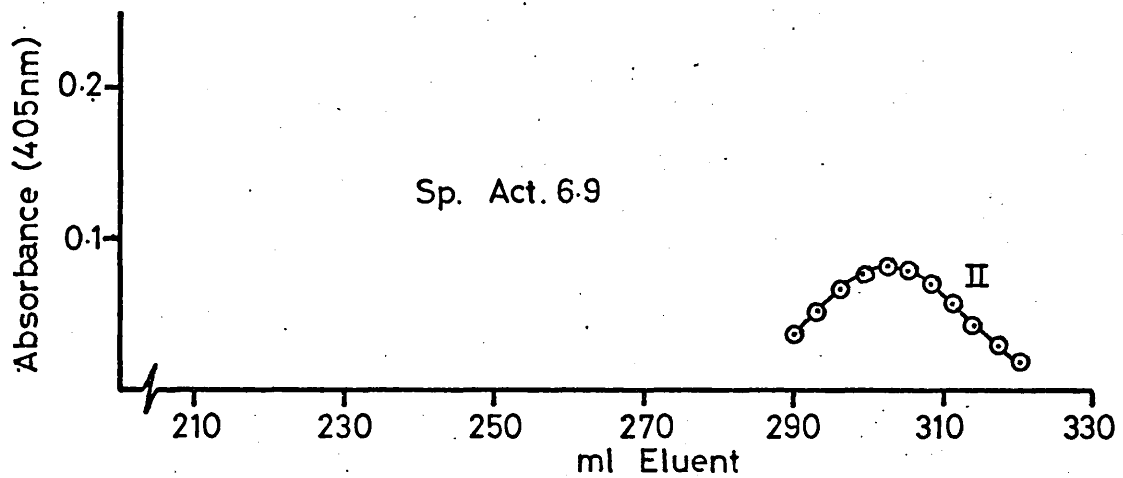
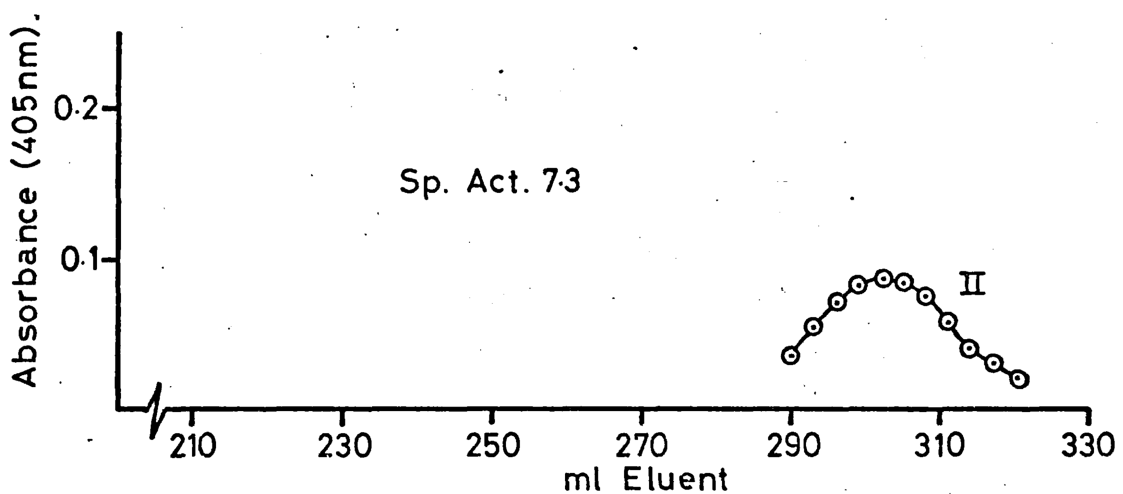
GRAPH 19.-Continued.

(b) Complete, very Young Beans where Cotyledons not yet formed.

(i) Crude Extract into Buffer



(ii) Citric acid Precipitation



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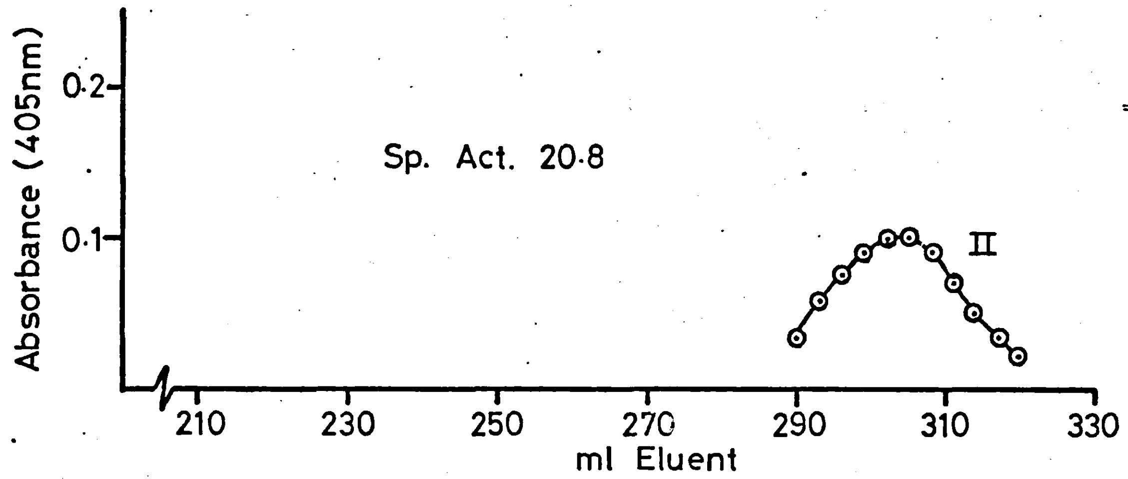
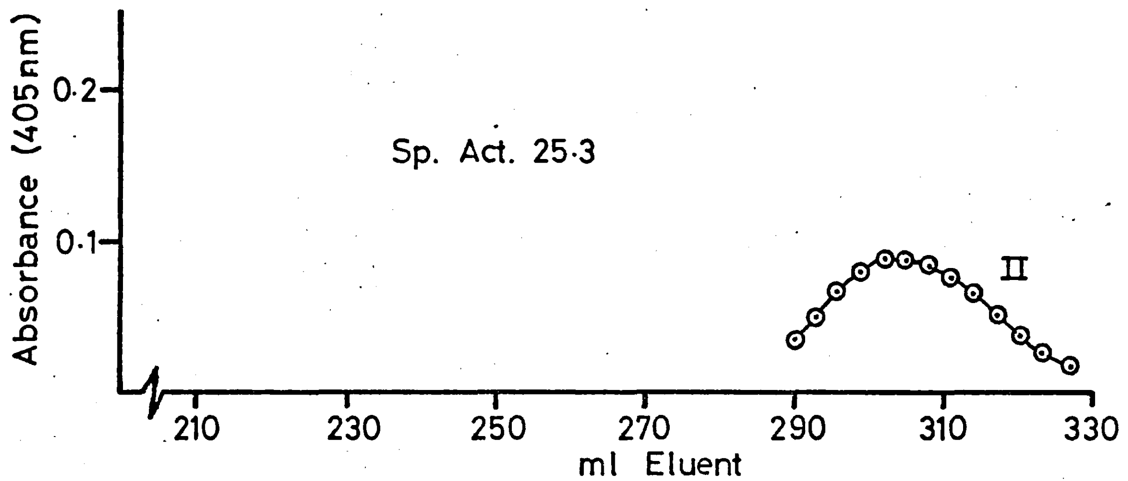
GRAPH 19.-Continued.

(iii) Ammonium Sulphate Fractionation



(iv) After Dialysis



carried out by the same procedure and, again, the results (Graph 19 b) were similar to those illustrated in Graph 19 a.

3. Observations on the Apparent Conversion of Enzyme II to Enzyme I.

The original hypothesis that, *in vivo*, high molecular weight α-galactosidase $_{I}$ is derived from enzyme $_{II}$ [10,316] was investigated further. Originally the model proposed to account for changing enzyme levels was based on the observation that whole green beans were rich in enzyme $_{II}$ (Graph 14(i)), and that drying the seed to the mature, brown stage, resulted in enzyme $_{I}$ production. This was supported by the fact that the molecular weight of enzyme $_{I}$ was a multiple of the value for enzyme $_{II}$ and that the amino acid composition for both forms were similar. In addition, the conversion of α-galactosidase $_{II}$ to $_{I}$ had been demonstrated *in vitro*. It is now apparent that the theory [316] that mature beans had a higher $_{I\ /\ II}$ activity ratio than green beans was incorrect and that the error was due to the fact that gel filtration patterns of the mature and immature seed extracts after different stages of purification had been compared (See page 154.). With reference to the results discussed

in the previous section, the situation *in vivo* is obviously very complex and relates, among other things, to the conditions of drying i.e. with or without the testa. Ignoring the changes occurring during the standard purification procedure, it would appear that, under physiological conditions, there is no major change in the levels of the isoenzymes during the drying out of the seed. Changes only appear to occur when 'maturation' proceeds in the absence of the seed coat or during extraction of green seeds minus testas. What, therefore, is the fuction of the testa in the drying out process?

In an attempt to answer this question a series of experiments on the effect green testa extracts on α-galactosidase $_{II}$ were carried out.

Crude, buffered (McIlvaine, pH 5.5) extracts of green tissues were incubated with the following α-galactosidase preparations, all from mature *Vicia faba* seeds:

(i) Crude Fraction (Stage (i));
(ii) 30 - 65% Ammonium Sulphate Fraction (Stage (iii));
(iii) Sephadex G-100 purified enzyme $_{II}$;
(iv) Enzyme $_{II}$ from a Sephadex G-100 column further purified on a δ-galactonolactonate affinity column. (For details of this purification step see Page 107.)

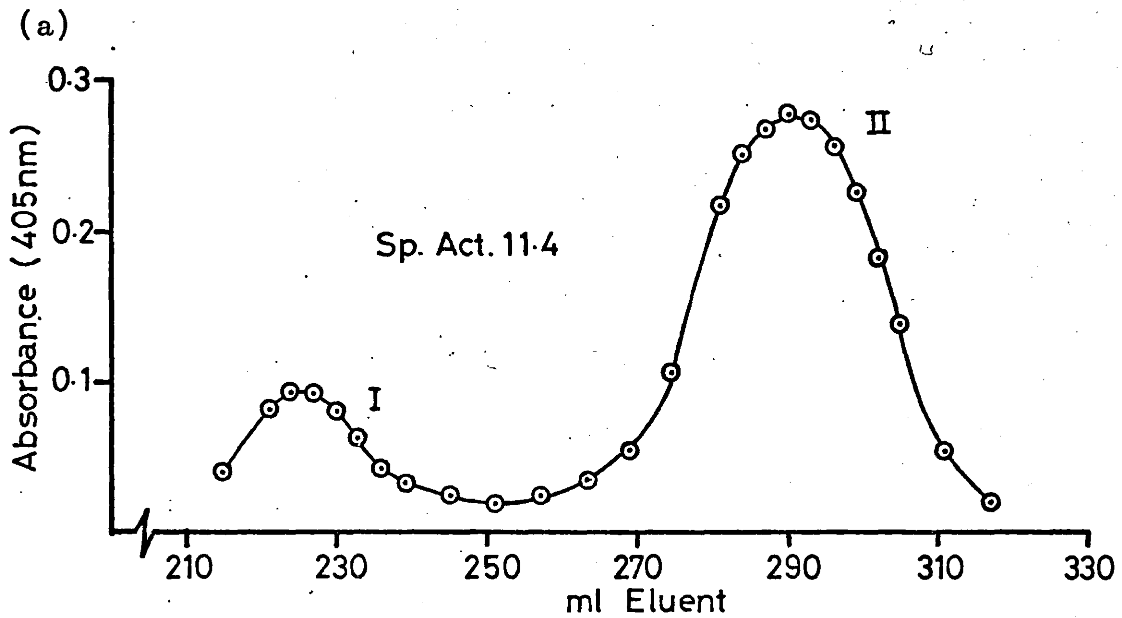
The effects of the testa extract on these fractions are shown in Graphs 20 - 23. In the graphs it can be seen that, in all cases, incubation of the mature seed enzyme preparations with testa extract either led to an increase in $_{I}$ relative to $_{II}$, or with the homogenous preparations of $_{II}$, to an appearance of $_{I}$. When the testa extract was boiled then it had no effect on enzyme $_{II}$. The testa extracts only appeared to be active when prepared from young, green beans, 10 - 25 mm in length. These results suggested that green testas contained a factor (X) which promoted the conversion of α-galactosidase $_{II}$ to $_{I}$ and, hence, further studies were initiated to try and determine the chemical nature of (X)

To see if the component was a heat labile factor testa extract was heated for various times at 50° before incubating with purified enzyme $_{II}$ (from the affinity column). The changes in specific activity were then measured (Graph 24). Graph 24 shows that the first 10 min. of heat treatment of the testa extract appears to 'activate' (X) resulting in an increase in the specific activity of the enzyme preparation. This was shown by gel filtration to be due to the formation of $_{I}$. Further heating at 50°, however, rapidly reduces the efficacy of testa factor and within 1hr. there is no activity. The active testa component is, therefore, heat labile.

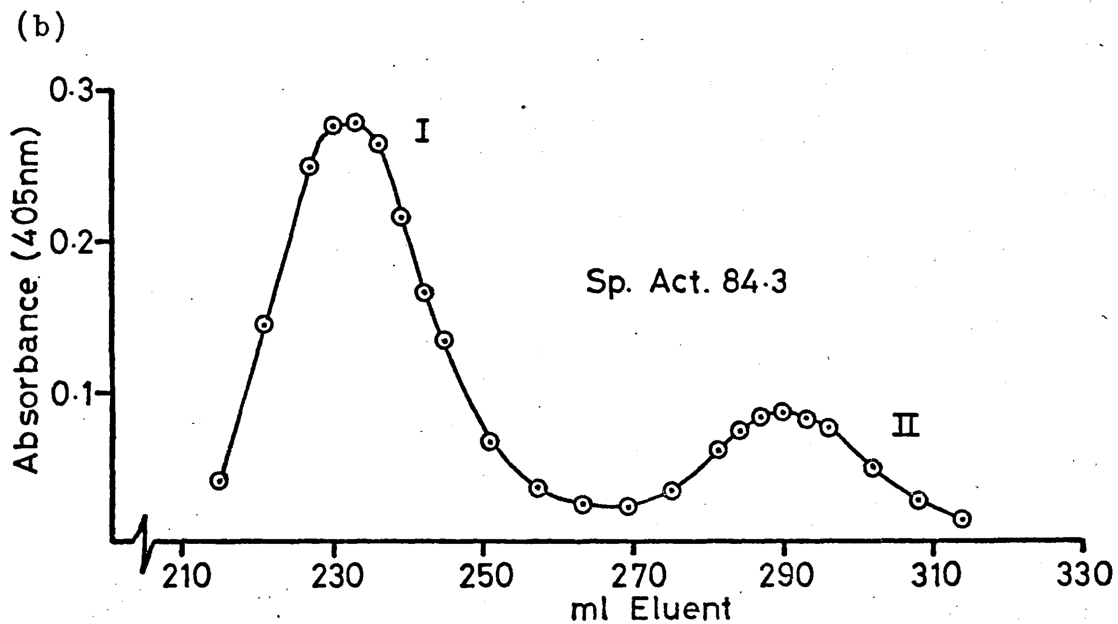
GRAPH 20.

Sephadex G-100 Elution Patterns of: (a), a crude (Stage(i)) α-Galactosidase preparation from mature seeds; (b), the crude preparation after treatment (4 hr. , 20°) with a McIlvaine buffer (pH 5.5) extract of green seed testas.

(a)



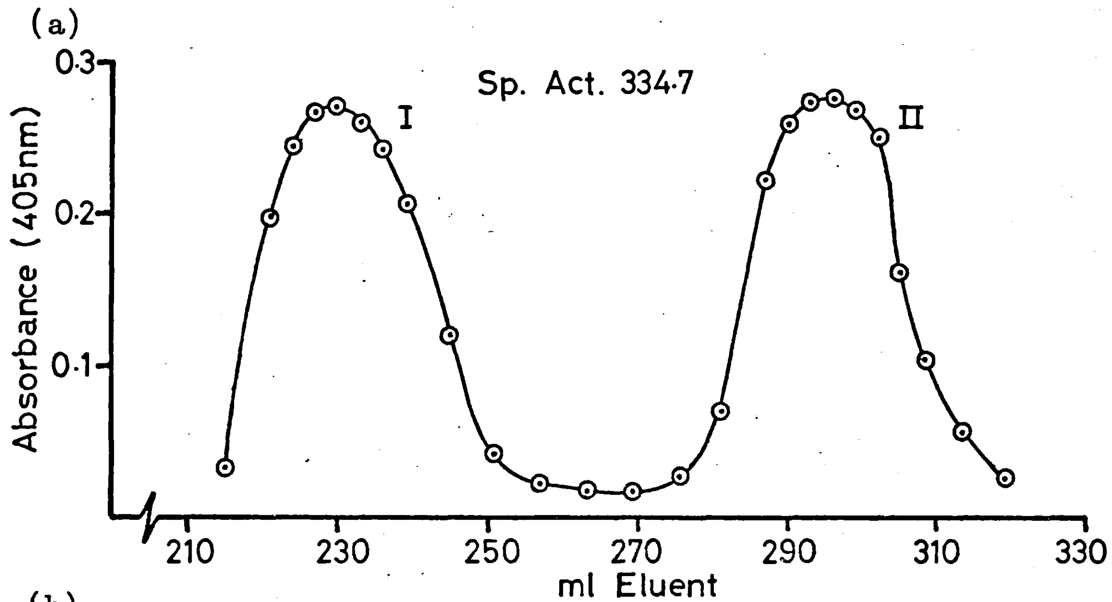
(b)



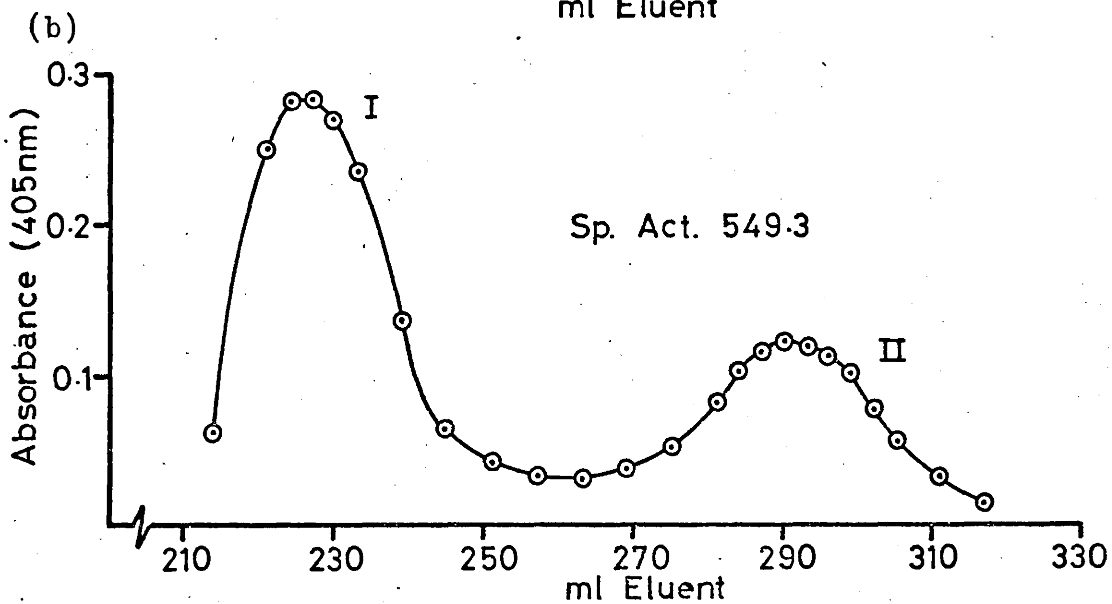
GRAPH 21.

Sephadex G-100 Elution Patterns of (a), a 30 - 65% ammonium sulphate fraction from mature seeds (Stage (iii)); (b), the 30 - 65% ammonium sulphate fraction after treatment (4 hr., 20°) with a McIlvaine buffer (pH 5.5) extract of green bean testas.

(a)

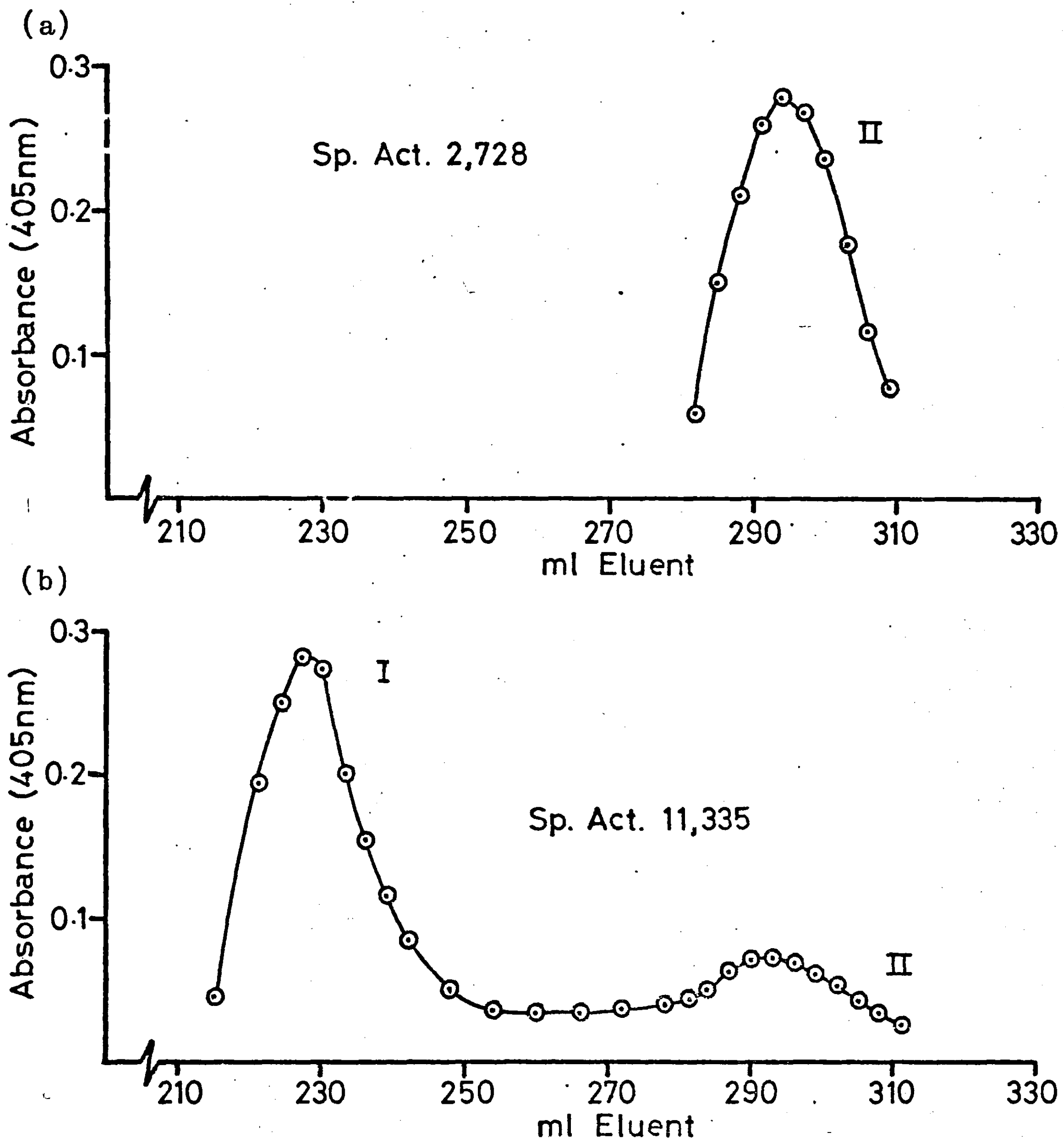


(b)



GRAPH 22.

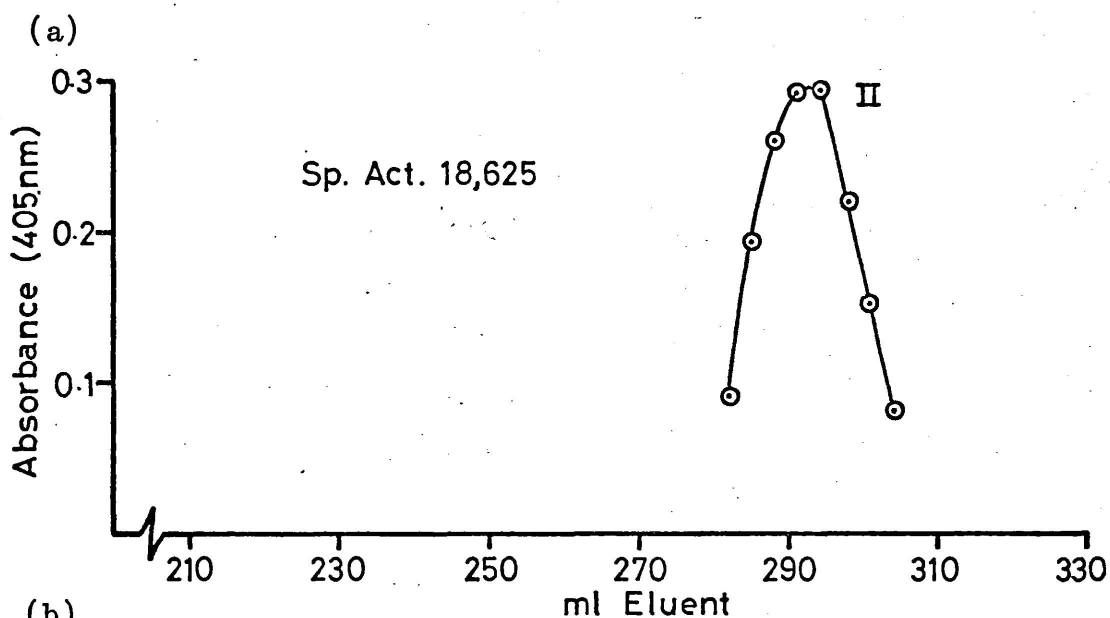
Sephadex G-100 Elution Patterns of: (a), a Sephadex G-100 purified enzyme $_{II}$ (Stage (v)) from mature seeds; (b), the same preparation after treatment (4 hr., 20°) with a McIlvaine buffer (pH 5.5) extract of green seed testas.



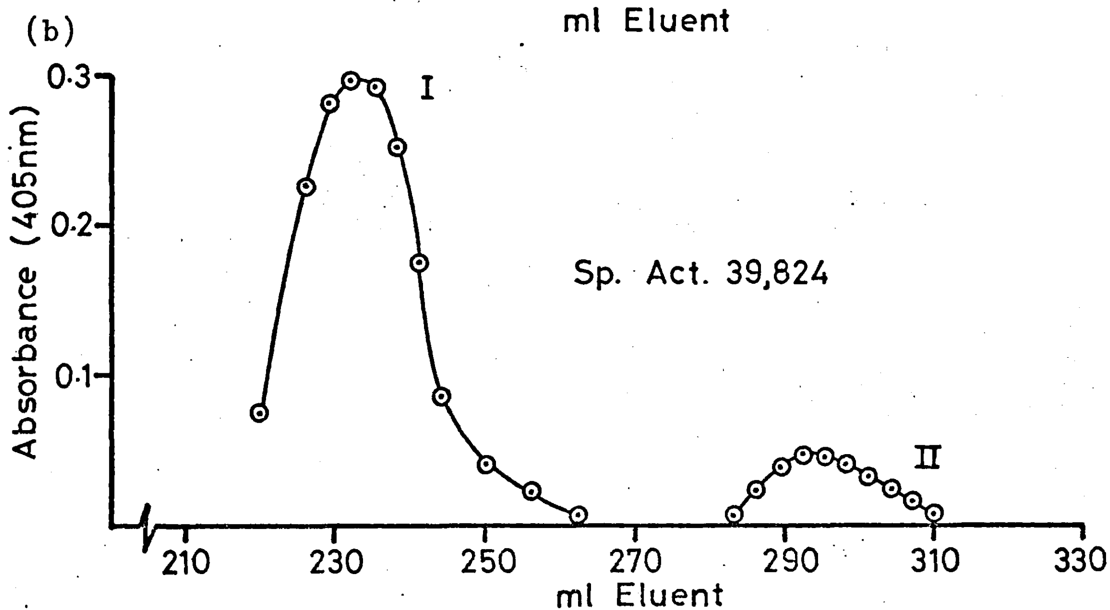
GRAPH 23.

Sephadex G-100 Elution Patterns of: (a), a α-galactosidase enzyme $_{II}$ further purified by affinity chromatography, from mature seeds; (b), the same preparation after treatment (4 hr., 20°) with a McIlvaine buffer (pH 5.5) extract of green seed testas.

(a)

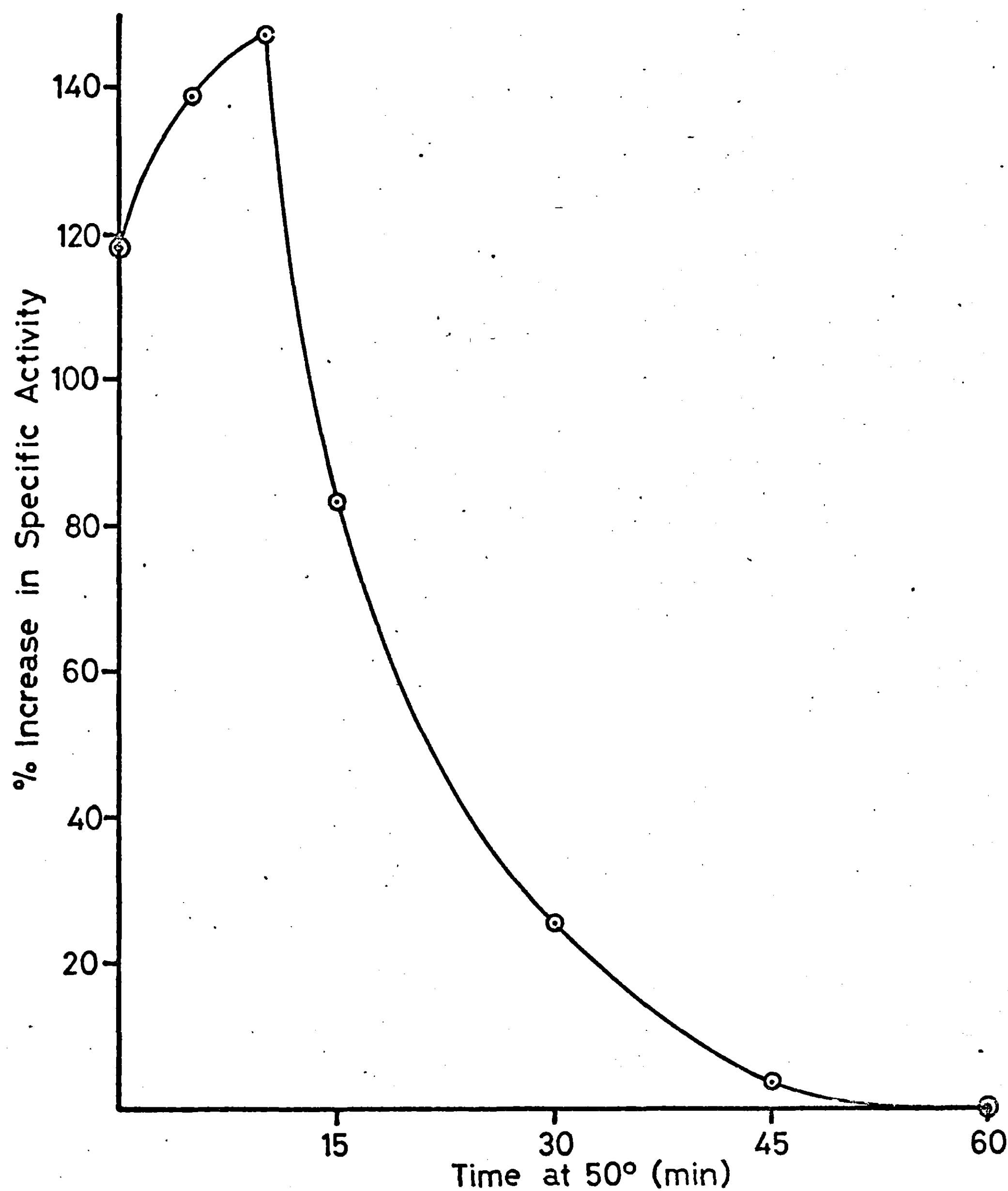


(b)



GRAPH 24.

Changes in Specific Activity of α-Galactosidase $_{II}$ on Incubation with heat-treated McIlvaine buffer (pH 5.5) extracts of green seed testas. Incubations of testa extracts with enzyme $_{II}$ were carried out for 4 hr. at 20°.

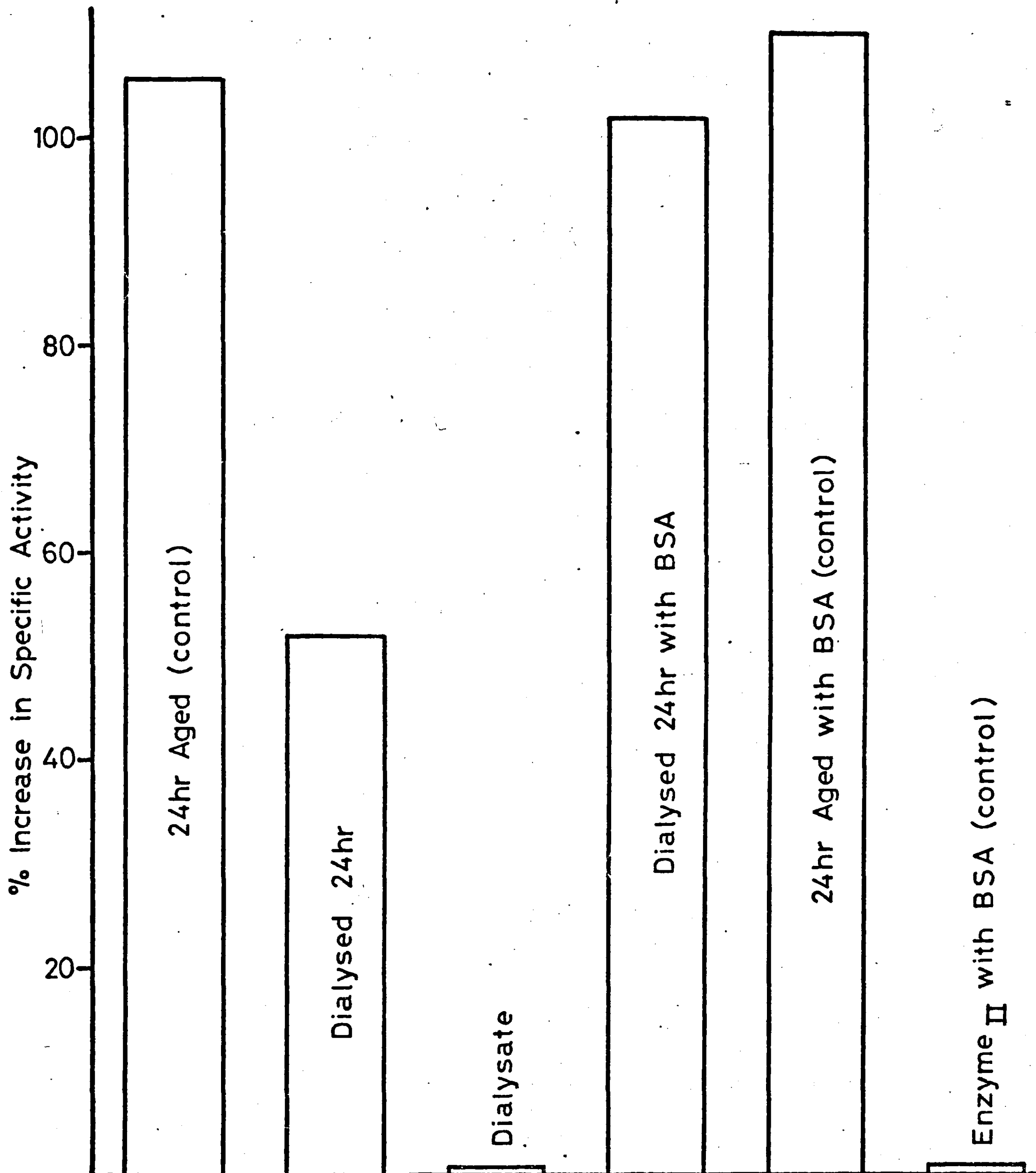


Dialysis of the testa material was next carried out at 4° against McIlvaine buffer (pH5.5) for 24 hr. At the end of this time an aliquot of the material remaining in the bag was added to pure enzyme $_{II}$ and the mixture incubated for 4 hr. at 20° before examining the specific activity. Undialysed testa material which had been allowed to stand at 4° for 24 hr. was used as a control. The dialysate was next freeze-dried, redissolved in buffer (McIlvaine, pH 5.5) and also tested to see if it would increase the specific activities of a preparation of enzyme $_{II}$ (Figure 9).

The dialysate had no effect on the specific activity of enzyme $_{II}$, whereas the dialysed material did increase the specific activity but the effect was about 50% less than in the case of the aged, undialysed control. Hence, it appeared that the factor (X) was unstable during dialysis and an attempt was, therefore, made to stabilise the system by adding bovine serum albumin to the testa material before dialysis. Aged controls and enzyme $_{II}$ both with added BSA were also examined. The results of these experiments are also shown in Figure 9, and it was clear that BSA had a stabilising effect and, therefore, it seemed probable that factor (X) was undialysable, and in view of its heat lability, probably proteinaceous.

FIGURE 9.

Effect of Testa Extract and Treated Extracts on the Activity of α-Galactosidase $_{II}$.

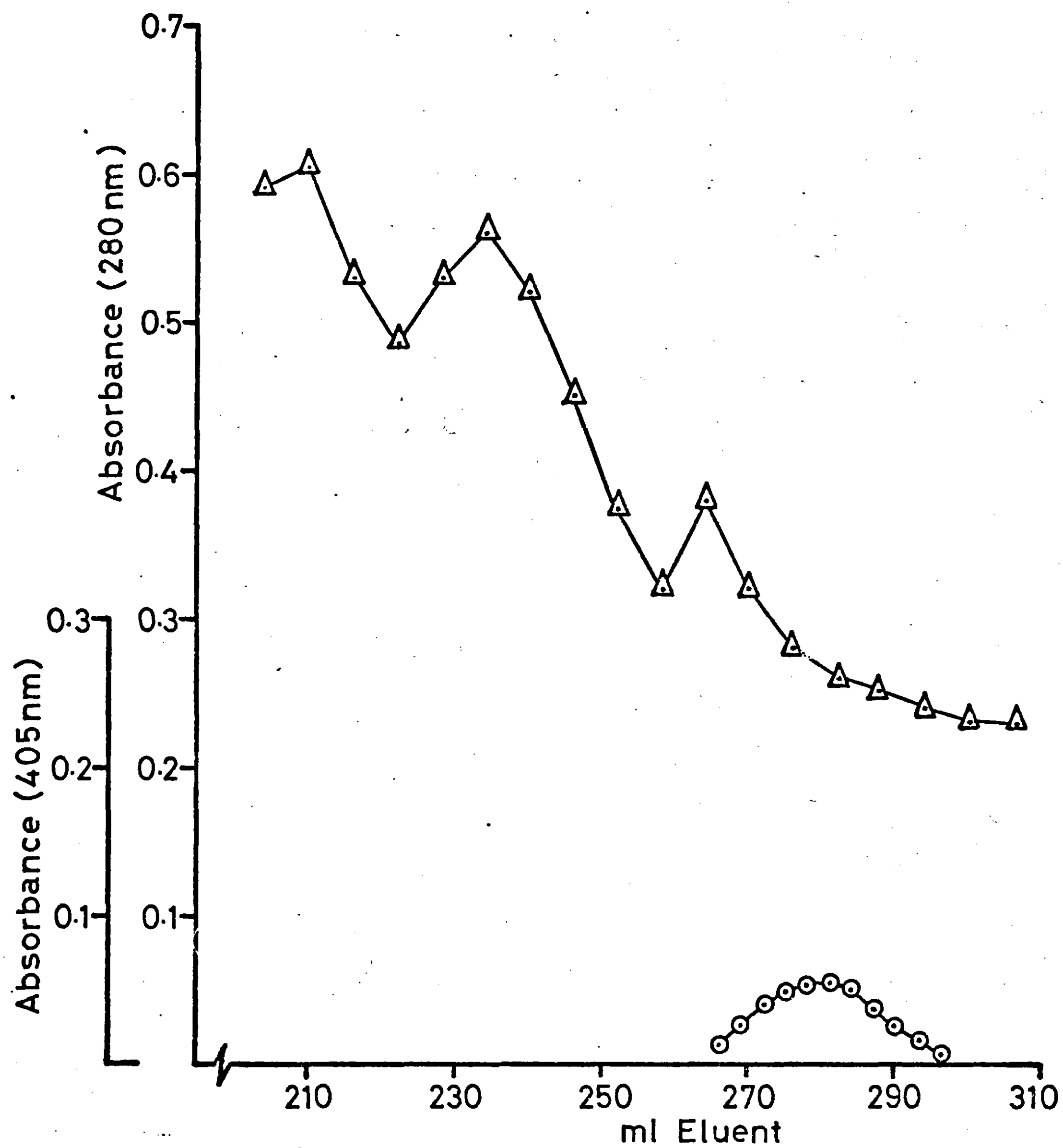


An attempt was next made to examine (X) by Sephadex gel chromatography. The gel filtration pattern produced by the testa extract on Sephadex G-100 is shown in Graph 25. No clear cut protein fractions, absorbing at 280 nm, were observed and a small amount of enzyme $_{II}$ activity was detected. Alternate 3 ml fractions from the Sephadex G-100 column were incubated with enzyme $_{II}$ and the specific activity of the digests examined. None of the column fractions were able to increase the α-galactosidase activity. The gel filtration chromatography was repeated using Sephadex G-50 and G-200, but again no fractions capable of increasing the specific activity of enzyme $_{II}$ could be located.

It was then decided to try and isolate the activity by taking a series of ammonium sulphate fractions. Ten fractions of testa material at 10% 'intervals' from 0 to 100% saturation were prepared and redissolved in buffer (McIlvaine, pH 5.5), dialysed and then added back to pure enzyme $_{II}$ and the specific activity checked after incubation for 4 hr. at 20°. The pellet obtained at 20 - 30% ammonium sulphate saturation gave a 70% enhancement of specific activity with α-galactosidase $_{II}$ (Unfractionated crude testa extract = 100%) and the 30 - 40% pellet gave a 5% enhancement. The other ammonium sulphate fractions were inactive. In the case of the

GRAPH 25.

Sephadex G-100 Elution Profile of Crude buffered Extract of Testa Material.



⊙—⊙ , α-Galactosidase activity

Δ—Δ , Protein at 280 nm

20 - 30% fraction the formation of enzyme $_{I}$ from $_{II}$ was also demonstrated by gel chromatography.

Aliquots of the 20 - 30% ammonium sulphate fraction of the testa were dialysed against McIlvaine buffers at pH values 4, 5, 6, and 7, as well as was the purified enzyme $_{II}$. The testa preparations at different pH values were then incubated with enzyme preparations at the same H^+ concentrations and specific activity changes measured after adjusting each pH to 5.5. The results obtained were as follows:

pH	% Increase in Specific Activity (Unfractionated crude extract = 100%)
4.0	28
5.0	90
6.0	65
7.0	25

This shows that the factor (X) present in the 20 - 30% ammonium sulphate fraction functions maximally at pH 5.0. The incubation mixture from the pH 5.0 trial was also applied to a Sephadex G-100 column which confirmed that enzyme $_{I}$ was present as the major form of α-galactosidase.

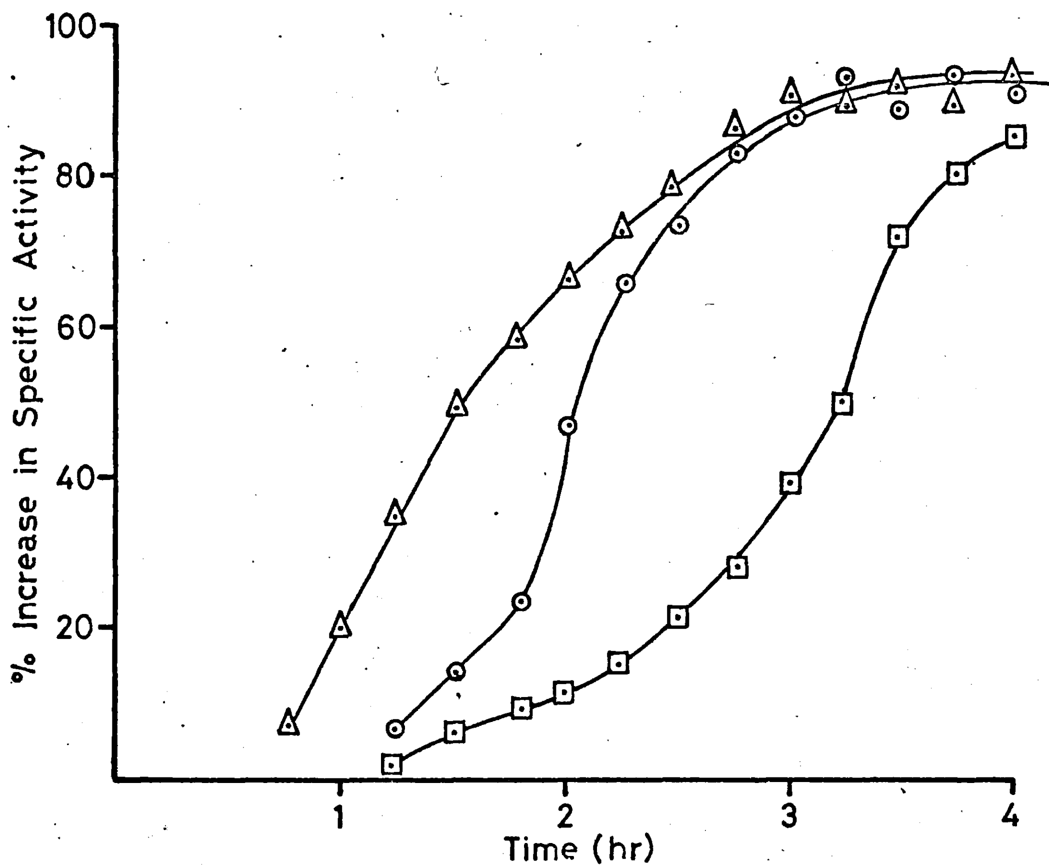
The effect of BSA and Iodoacetamide on the $_{II}$ to $_{I}$ conversion reaction catalysed by the 20 - 30% ammonium sulphate fraction of the testa (at pH 5.0) was next examined. It was considered that the rates of conversion might be modified by these agents with BSA possibly stabilising the system and iodoacetamide possibly modifying protein -SH groups which were involved. The results are shown in Graph 26 for different incubation times of the testa fraction with enzyme $_{II}$. Although the shapes of the curves vary for control, BSA and iodoacetamide-treated fractions (with lag phases in the case of the control and iodoacetamide) they all reached a very similar total percentage increase in specific activity after 4 hr. The G-100 elution patterns for each of these trials, after 4 hr. incubations, are shown in Graph 27. The activity ratio of enzyme $_{I}$ to enzyme $_{II}$ was 2 : 1 in each case.

In order to establish that the active factor in the 20 - 30% ammonium sulphate fraction was a protein, heat stability and dialysis trials were again carried out.

A time course to show the effect of heat (50°) on the ability of the 20 - 30% ammonium sulphate testa fraction to convert $_{II}$ to $_{I}$, i.e. increase the specific activity,

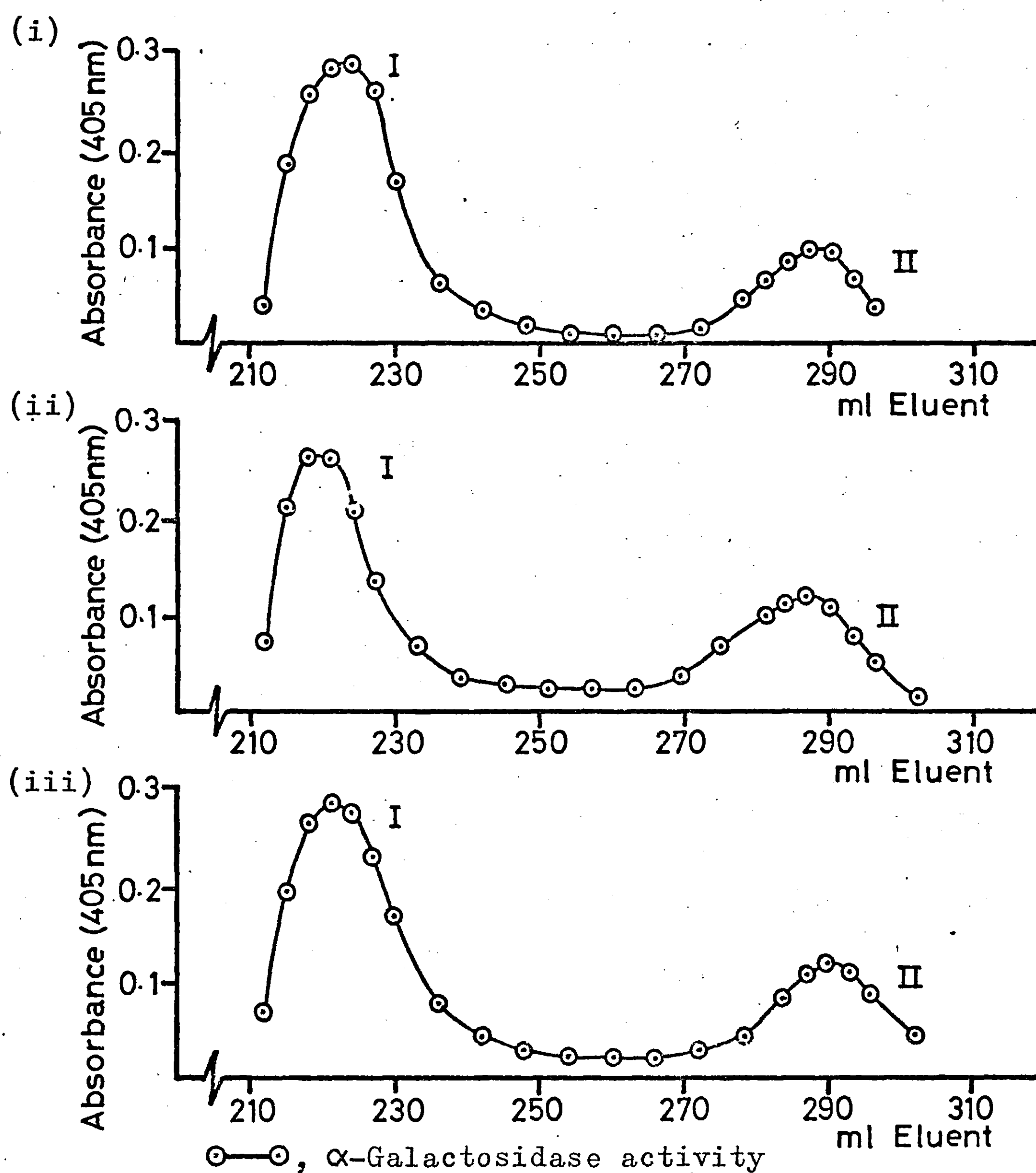
GRAPH 26.

Changes in Specific Activity of α-galactosidase $_{II}$ on incubation (4 hr., pH 5.0) with a 20 - 30% ammonium sulphate fraction from green seed testas. Incubations were carried out separately with: no additives, ⊙ ; in the presence of BSA (1%), △ ; in the presence of iodoacetamide (10^{-3}M), ⊡ .



GRAPH 27.

Sephadex G-100 Elution Patterns of α-galactosidase $_{II}$ after incubation with a 20 - 30% ammonium sulphate fraction of green seed testas (4 hr.,20°, pH 5.0). The incubation mixtures contained: (i), no additive; (ii), BSA (1%); (iii) iodoacetamide (10^{-3}M).



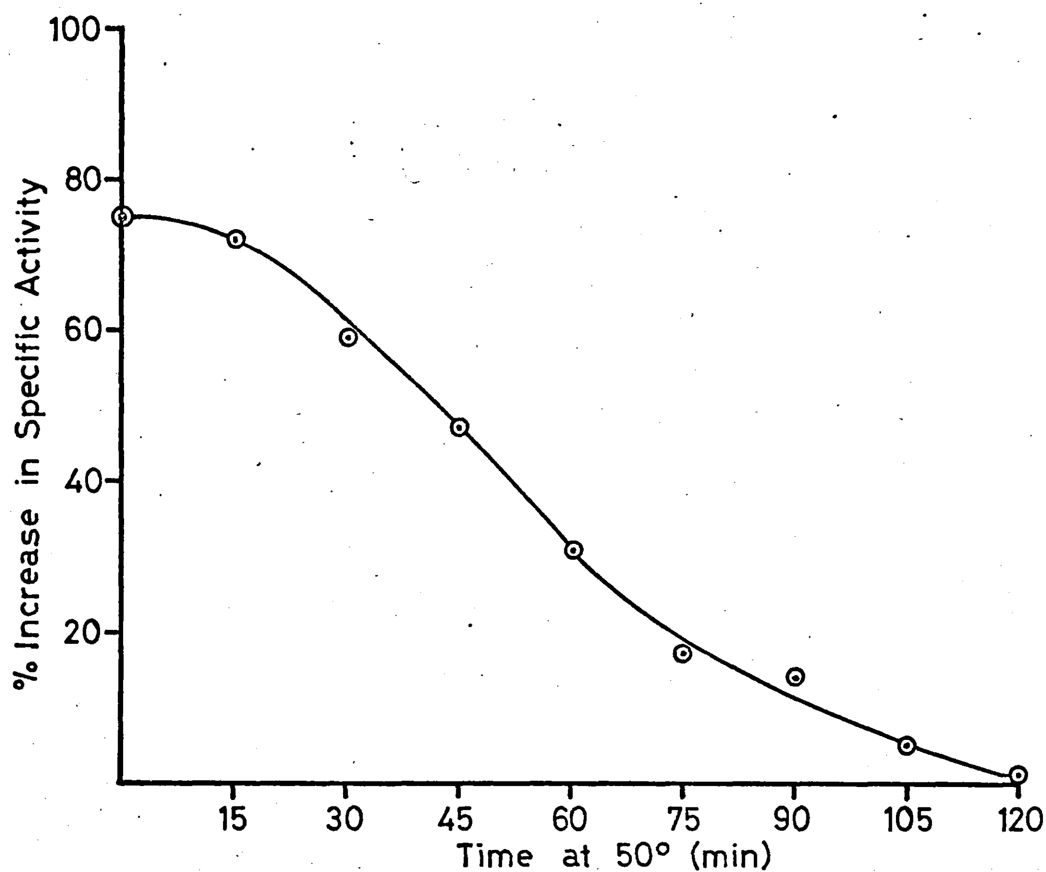
is shown in Graph 28. Comparing this with Graph 24, which shows a similar plot for the unfractionated testa material, the time taken to lose all activity is twice as long with the ammonium sulphate fraction as with the cruder preparation. Also, there is no initial enhancement of activity as was shown by the crude testa fraction.

As with the crude preparation, the effect of dialysis on the ammonium sulphate testa fraction over 24 hr. was examined. The result was very similar to that obtained with the crude preparation (See Figure 9). The dialysate had no effect on the specific activity of enzyme$_{II}$, while the dialysed material lost 50% of its ability to enhance $_{II}$ to $_{I}$ conversion when compared with the aged, undialysed control. Again, the loss of activity was almost totally prevented when BSA (1%) was added to the dialysis bag.

Finally, the enzyme$_{I}$ produced from $_{II}$ in the presence of the 20 - 30% ammonium sulphate fraction of the green testa was examined in more detail. For this purpose, incubation of enzyme$_{II}$ for 4 hr. with testa preparation was used to prepare a sample of enzyme$_{I}$ for comparison with purified enzyme$_{I}$ from resting seeds. The α-galactosidase $_{I}$ produced from $_{II}$ was purifed by Sephadex G-100 followed

GRAPH 28.

Changes in Specific Activity of α-Galactosidase $_{II}$ on incubation (4 hr., pH 5.0, 20°) with heat-treated 20 - 30% ammonium sulphate fraction of green seed testas (cf. Graph 24.).



by affinity chromatography. Then, the molecular weights and specific activities of both samples of enzyme $_{I}$ were compared (See Table 10). Both enzymes were eluted from Sephadex G-200 columns as single, symmetrical peaks of activity, and the activities of the preparations applied to the columns were completely recoverable. The protein peaks coincided exactly with the activity peaks in each case (Graph 29) and the specific activity of the enzyme $_{I}$ derived from enzyme $_{II}$ was of the same order as that of $_{I}$ from resting seeds.

Some attempt was made to follow the conversion of α-galactosidase $_{II}$ to $_{I}$ in the presence of testa factor by a less time consuming method than Sephadex gel column chromatography and a less equivocal procedure than following changes in specific activity. For this purpose a fluorescamine derivative of a Sephadex gel purified $_{II}$ of α-galactosidase was prepared in the hope that this would convert to a fluorescent form of $_{I}$ which could then be separated from fluorescamine $_{II}$ by thin layer Sephadex G-100 chromatography. By measuring the fluorescence of both derivatives it had been hoped to learn something of the stoichiometry of the process as well as develop a rapid assay technique. This study was abandoned, however, as the fluorescamine derivative of $_{II}$ could not be induced to convert to $_{I}$.

TABLE 10.

A Comparison of the Properties of Enzyme $_{I}$ from resting seeds and Enzyme $_{I}$ derived from Enzyme $_{II}$ in the Presence of a 20 - 30% ammonium sulphate testa fraction.

Property	Enzyme $_{I}$ from* resting seeds	Enzyme $_{I}$ derived from Enzyme $_{II}$**
Molecular weight (gel filtration)	209,000	209,000
Specific activity (milliunit/mg protein; substrate p-nitrophenyl α-D-galactoside	84,271	83,945
Carbohydrate Content (µg glucose/ mg protein)	49	42

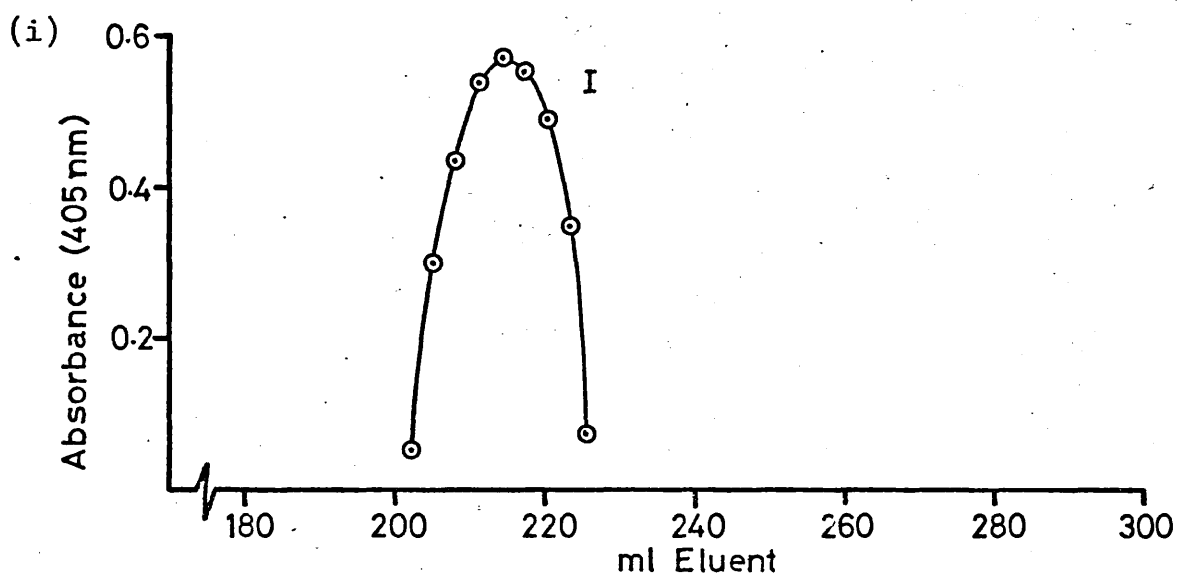
* Four stage purification ((i-iv), See page 124.)followed by Sephadex G-100 chromatography and passage of $_{I}$ through an affinity column (See Page 107.)

** Enzyme $_{I}$ isolated from the testa-enzyme $_{II}$ incubation mixture by Sephadex G-100 chromatography

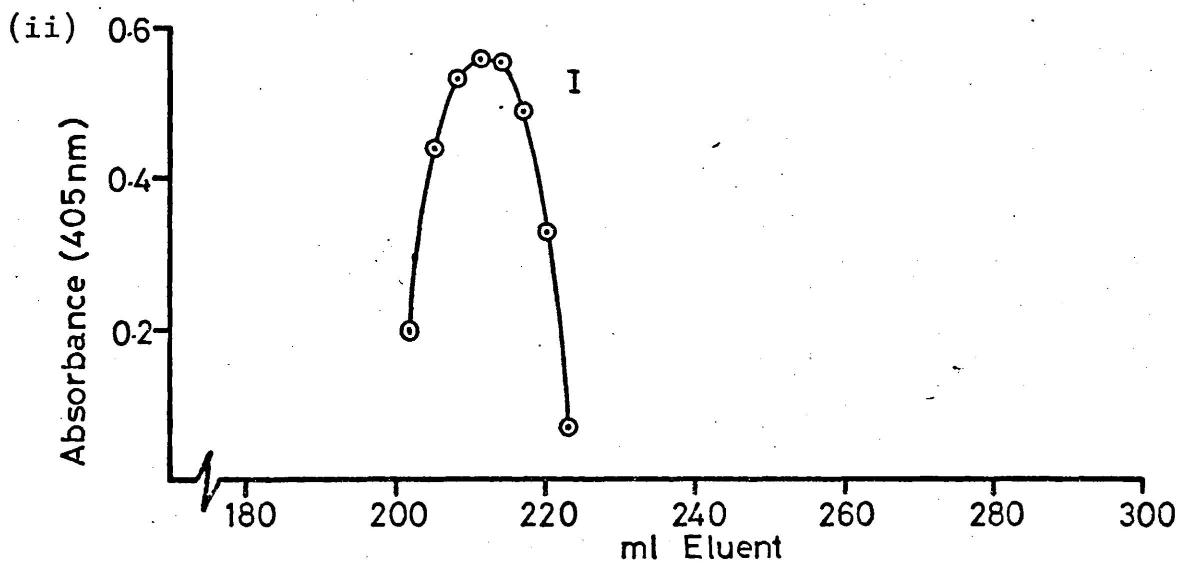
GRAPH 29.

Sephadex G-200 Elution Patterns of α-Galactosidase enzyme $_{I}$ from: (i) resting seeds; and (b) enzyme $_{I}$ derived from Enzyme $_{II}$ in the presence of a 20 - 30% ammonium sulphate testa fraction.

(i)



(ii)



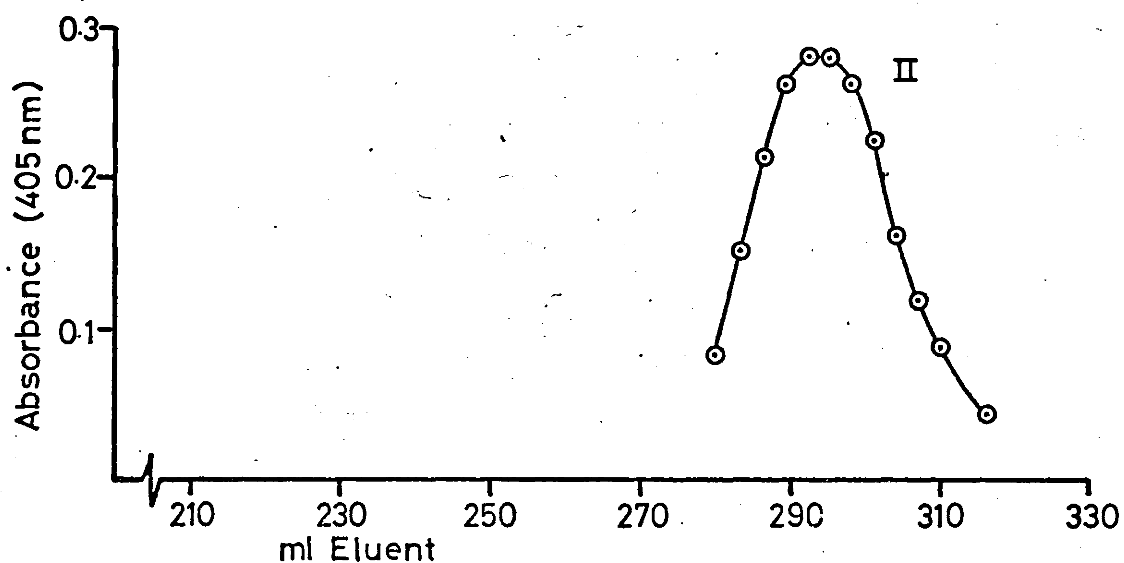
⊙—⊙, α-galactosidase activity

Having examined in detail seed maturation, attention was turned to the process of seed germination. Work[316] had shown that a change in the isoenzyme pattern took place when seeds were germinated, however, the patterns were examined after several stages of purification, and this suggested that the major isoenzyme present after germination was enzyme $_{II}$ and not enzyme $_{I}$. In view of the effect the purification had on the ratios of α-galactosidase forms which had already been observed with preparations from maturing and resting beans it was decided to re-examine the situation in germinating seeds i.e. following a 24 hr. soaking period and 72 hr germination. The results are shown in Graph 30. Stages (ii) - (iv) of the purification yielded patterns similar to those obtained by Khaleque[316] with enzyme $_{II}$ as the major form and a little of enzyme $_{I}$, but in stage (i), the crude buffered extract, only enzyme $_{II}$ could be detected. The protein from the enzyme $_{I}$ region of the crude α-galactosidase pattern was collected from three separate columns, pooled, concentrated and re-assayed for α-galactosidase, but there was no measurable activity. It appears, therefore, that some enzyme $_{I}$ is produced during the later stages of purification of germinated bean preparations, but in quantities much less significant than in the case of mature seed preparations (See Graph 6).

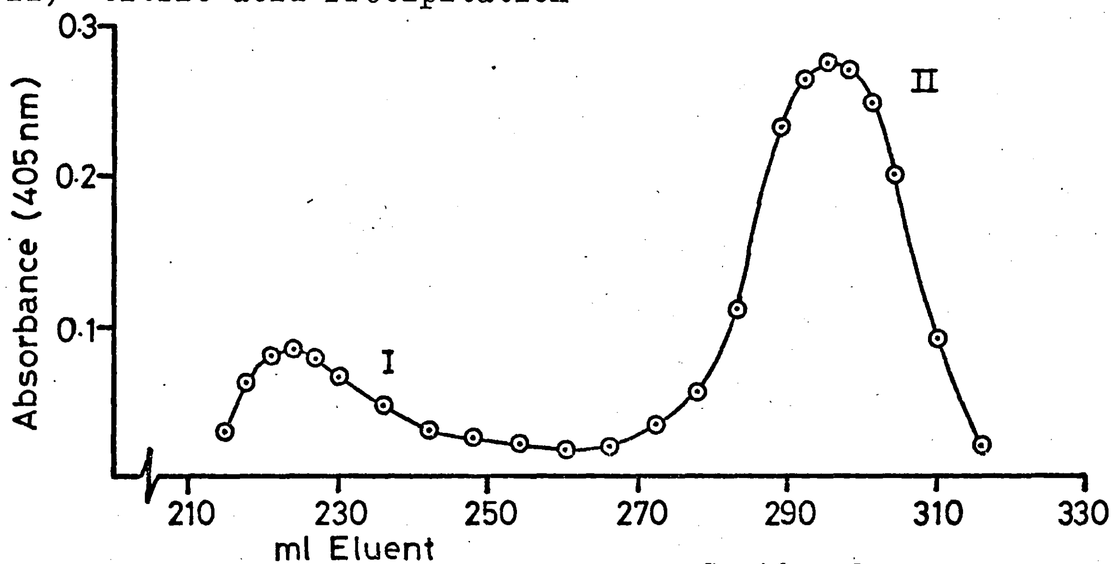
GRAPH 30.

Sephadex G-100 Elution Patterns of α-Galactosidases from Germinated beans (24 hr. soaking + 72 hr germination) after four stages of purification ((i-iv), See page 124.)

(i) Crude extract into Buffer



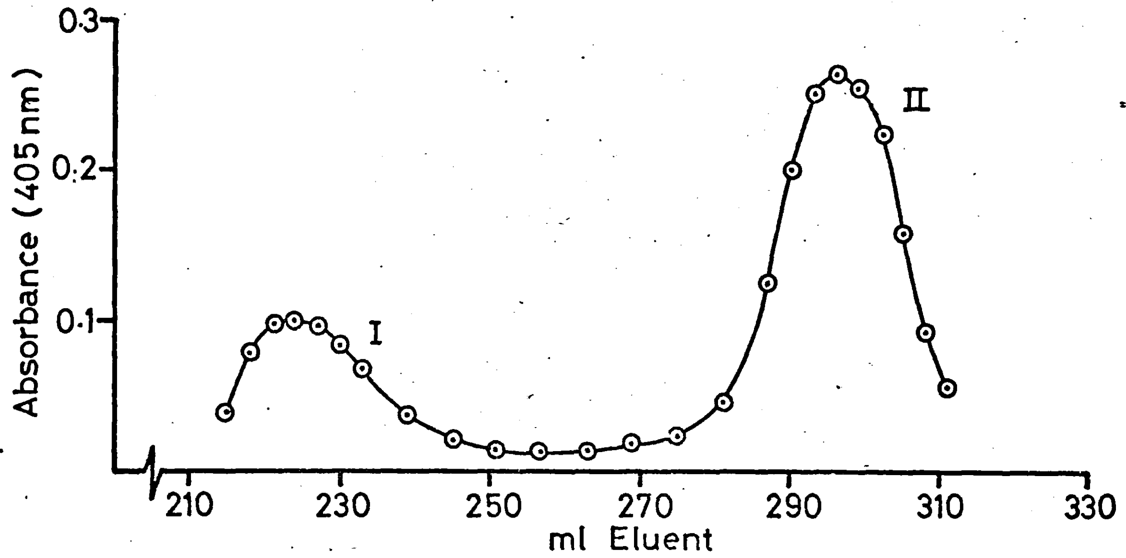
(ii) Citric acid Precipitation



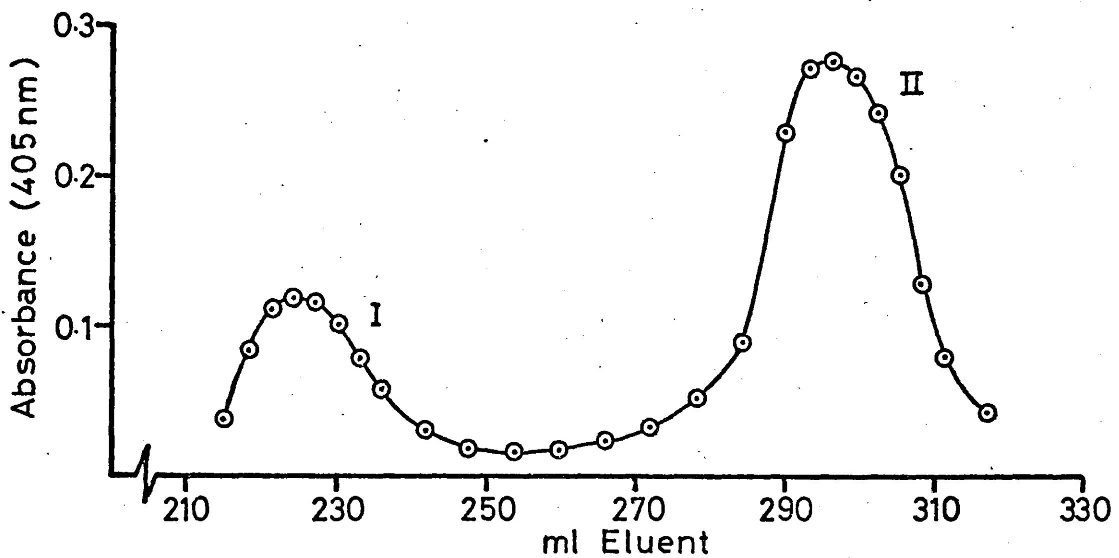
Continued over

GRAPH 30.-Continued.

(iii) Ammonium Sulphate Fractionation



(iv) After Dialysis



The changing enzyme patterns were examined over a shorter period i.e. after soaking the mature beans for 24 hr. Sephadex G-100 chromatography of a crude, buffered extract in this case again only showed the presence of enzyme $_{II}$. Further purification (Stages (ii-iv)) of this extract also resulted in patterns identical to those observed with preparations from fully germinated seeds (Graph 30 (ii,iii and iv)). Soaking seeds for 6 hr. followed by examination of a crude buffered extract, however, yielded an isoenzyme pattern similar to that obtained with dry, mature seeds i.e. enzyme $_{II}$ accompanied by a small amount of enzyme $_{I}$. Hence, it appears that *in vivo* enzyme $_{I}$ disappears sometime during the 6 - 24 hr. soaking period.

4. Interconversion of α-Galactosidases in Seeds and its Possible Physiological Importance.

The experimental results of this study confirm that there is probably a shifting pattern of α-galactosidase forms in seeds during changing physiological activity although the enzyme activity ratios of the forms are probably different from those reported in earlier studies[10, 316]. The differences almost certainly arise because of *in vitro* reactions which occur during the purification procedure.

Considering firstly the conversion of _Vicia faba_ α-galactosidase $_{II}$ to $_{I}$ _in vitro_ it is obvious that some crude preparations of enzyme containing both forms can convert with the formation of $_{I}$ at the expense of $_{II}$. The extent of conversion is proportional to time and it is facilitated by some purification procedures. For example, a crude, buffered extract of resting _Vicia faba_ seeds has a $_{I}$ / $_{II}$ activity ratio which is much less than unity (Graph 8(i)) and on standing for 60 hr. the ratio approximates to unity. This phenomenon can be achieved in about 2 hr., however, by lowering the pH of the preparation to 3.2 with citric acid. The stage of development of the seed tissue may complicate the situation as was seen with both germinating and immature seeds where further purification of crude extracts had only a small effect on the isoenzyme pattern.

Interconversion of isoenzymes _in vitro_ is not uncommon. _Streptococcus lactis_, for example, was reported to contain two forms of β-galactosidase[317,318], and when the partially purified enzyme preparation was incubated for 18 hr. at room temperature in ammonium sulphate solution (pH 7.0) and then analysed by Sephadex G-200 gel filtration only the higher molecular weight form was detected[319]. It has also been shown that β-galactosidase from _E. coli_ is made up of four sub-units[320,321] and

that temperature dependent association and dissociation can occur *in vivo* [322]. A similar phenomenon was reported with a proteinase from *Streptococcus lactis*[323] and α-chymotrypsin from pancreatic juice[324]. In the latter case, the conversion was dependent on protein concentration. Further, the interconversion of multiple forms of Tyrosine aminotransferase[325] has been reported. Here, incubation of the soluble enzyme with a microsomal fraction of liver produces a change in isoenzyme patterns. The effect is pH dependent.

Returning to the forms of α-galactosidase which exist *in vivo* in the cells of various stages of seed development, it is obviously impossible to be exactly sure what the pattern is as it may change immediately the tissues are disrupted. However, if this does not occur it would appear that the very immature seeds, where cotyledons are undifferentiated, contain only low levels of enzyme $_{II}$ (Graph 19 b) and that other unknown factors are absent which allow aggregation of $_{II}$ to form $_{I}$ *in vivo* or *in vitro*.

After the cotyledons have been formed the green seed appears to contain some α-galactosidase $_{I}$ but the activity relative to $_{II}$ varies according to whether the buffered extracts are made in the presence or absence of testa (Graphs 15 and 16). In the former case, $_{II}$ predominates

and in the latter, $_{I}$ is the major form. The α-galactosidase content of the testa itself cannot account for this difference, and again there is little tendency for $_{II}$ to convert to $_{I}$ in either case in vitro. The obvious inference is that the green testa contains a factor which during extraction prevents the conversion of $_{II}$ to $_{I}$. This hypothesis does, however, appear to be contradicted by the effect observed when green testa extract was added to crude mixtures of forms $_{I}$ and $_{II}$ or to purified $_{II}$ (See page 179.). In these cases the testa extract enhanced the conversion of $_{II}$ to $_{I}$ and the results suggested that this 'catalysis' was caused by a proteinaceous material which was designated (X). A further possibility is that (X) reacts with a factor which is normally present and which inhibits the conversion process. If the former theory is correct then it is conceivable that the green testa acts as a 'sink' during seed maturation hence drying the green seed with testa intact would allow (X) to pass into and accumulate in the testa and this in turn would tend to prevent conversion of $_{II}$ to $_{I}$ in the cotyledons as was observed (Page 163.). When seeds were allowed to mature after the testas had been removed, α-galactosidase $_{I}$ appeared as the major form in the crude extract and this might be explained by assuming that (X) remained in the cotyledons because the 'sink' had been removed.

The alternative theory derived from *in vitro* studies with testa extract and enzyme $_{II}$, i.e. that (X) removed an inhibitor of the conversion process, would be difficult to relate to the observations made with the maturing beans.

It is difficult to see any relationship between on the one hand the results obtained with the immature seeds, and on the other hand, the studies involving the effect of testa extract on enzymes from mature seeds and the maturation process itself.

As was originally claimed[10,316], the mature *Vicia faba* bean, in comparison with other developmental stages of this seed, possesses the greatest proportion of α-galactosidase $_{I}$ activity although the $_{I}$ / $_{II}$ activity ratio is probably lower than was previously believed. The presence of form $_{I}$ with a higher specific activity than $_{II}$ might be an obvious biological advantage if it allows a maximum rate of hydrolysis of reserves, such as raffinose and stachyose, to occur at the beginning of the germination period.

After about 24 hr., when, perhaps, utilization of seed starch has become significant, enzyme $_{II}$ appears to be removed from the tissues and, as in the case of the immature seeds this α-galactosidase shows little tendency

to convert to $_{I}$ *in vitro*. Here again it can be postulated that some factor which promotes aggregation of protein $_{II}$ units seems to have been lost.

PART II

ATTEMPTED ENZYMIC MODIFICATION OF GALACTOMANNANS

The occurrence and structure of the seed galactomannans has been discussed in the Introduction of this thesis together with their enzymic modifications both *in vivo* and *in vitro*.

It was originally thought that the ability to remove isolated galactose residues from the mannan chain would be advantageous commercially, for three reasons. Firstly, it might be possible (if economic) to upgrade poor batches of galactomannans (or cheap batches from poor sources) to give a uniform (and optimum) quality. Secondly, that the system could be employed to assess the quality of individual batches of galactomannans, and thirdly that it might lead to an aggregate understanding of the properties of the galactomannans and their relationship to block structure.

The present study arose mainly from the previous claim (10,316) that purified α-galactosidase $_{I}$ from *Vicia faba* (but not enzyme $_{II}$) released galactose from galactomannans (locust bean gum and tara gum) without degradation of the mannan backbone caused by traces of β-mannanase. It seemed appropriate to try and utilise the *Vicia faba*

α-galactosidase $_{I}$ to prepare a whole range of enzyme-modified galactomannans for gel formation studies. Work along these lines in the Unilever laboratories had previously shown that it was difficult to prepare α-galactosidase free of β-mannanase and that contamination of α-galactosidase with β-mannanase led to enzyme-treated products with 'antisynergistic' activities when used for mixed gel formation. e.g. with agarose or carrageenan. It had been deduced that this had arisen from splitting of the mannan backbone. (High molecular weight galactomannans have many 'junction zones' which create a more complex network of mixed polymers and hence a better gel).

In the present study α-galactosidase was extracted from mature <u>Vicia faba</u> seeds and purified through to stage (iv) of the purification scheme given on page 125. This preparation contained enzymes $_{I}$ and $_{II}$: it had previously been claimed that $_{II}$ had no β-mannanase activity as well as no action on the galactosyl residues of galactomannans.

Two galactomannans were then selected for attempted modification. These were locust bean gum (mannose/galactose ratio 3.75 : 1) and guar gum (mannose/galactose ratio 1.54 : 1) and they were dissolved separately in buffer (McIlvaine, pH 5.5) at concentrations of 0.5% , W/V. <u>Vicia faba</u> α-galactosidase was then added to both preparations

and incubations were carried out at 37° over 5 hr., during which changes in reducing power and viscosity were measured. At the end of all incubations low molecular weight sugars were dialysed away and the galactomannans remaining were freeze-dried, and the mannose/galactose ratios of each determined by gas chromatography using the alditol acetate procedure. (For details see Materials and Methods, page 116.) Table 11 gives the results from this set of incubations.

Initially, the results from this first set of incubations with *Vicia faba* α-galactosidase looked quite promising. The viscosity of the incubation mixtures seemed to be unaltered, indicating that little, if any, splitting of the mannan backbone had occurred. The percentage galactose released (based on the known content of the polysaccharide) was assayed by the Nelson method for reducing sugars. This is not a specific test for galactose, but it was assumed that the majority of the reducing sugar released would be galactose. Over the first hour of the incubation, both guar and locust bean gum incubation mixtures with *Vicia faba* enzyme showed a steady rise in reducing power. After 5 hr. incubation, however, this reducing power had disappeared in the case of the locust bean gum and had dropped to a low level with the guar digest. This could perhaps be explained by the fact that

TABLE 11.

Incubation (37°) of Galactomannans (Locust bean and Guar Gums) with Purified (Stage (iv)) α-Galactosidase from *Vicia faba*

Sample	Incubation Time (hr.)	Galactose %	Viscometry Times (min.sec., H_2O 11.9sec)	Mannose/ Galactose Ratio %
Guar	0	0	5.8	
"	0.25	11.4	5.7	
"	0.50	18.2	5.6	
"	1.0	22.7	5.14	68.8:31.2
"	5.0	5.3	4.47	
Locust Bean	0	0	4.2	
"	0.25	11.5	4.9	
"	0.50	15.4	4.17	
"	1.0	19.2	4.11	86.5:13.5
"	5.0	0	4.12	89.5:10.5
Controls:				
Guar	0	0	8.10	63.0:37.0
Locust bean	0	0	9.13	79.4:20.6
Guar	5.0	0	8.15	
Locust Bean	5.0	0	8.39	
Enzyme	5.0	0	0.12	

the enzyme preparation was a relatively crude fraction which contained another activity such as an oxidative enzyme. Such enzymes do occur in microorganisms, however, there has been no report in the literature of such a higher plant enzyme. A further possibility is that galactose was initially released and then, later, transferred to an acceptor molecule giving a non-reducing product. This is discussed on page 215.

The mannose/galactose ratio of the polysaccharide product after 1 hr. incubation of guar gum with <u>Vicia faba</u> α-galactosidase showed an increase from 63.0 : 37.0 to 68.8 : 31.2, while incubations of locust bean gum with the same enzyme appeared to increase the ratio from 79.4 : 20.6 to 86.5 : 13.5 and 89.5 : 10.5 after 1 hr. and 5 hr. , respectively.

In a second set of experiments some of the α-galactosidase from <u>Vicia faba</u> was purified further by Sephadex G-100 gel filtration which separated the isoenzymes $_{I}$ and $_{II}$. The following enzyme preparations were then used in an attempt to modify the galactomannans:

(i) Enzyme $_{I}$

(ii) Enzyme $_{II}$

(iii) A mixture of enzymes $_{I}$ and $_{II}$

(iv) A dialysed 30 - 65% ammonium sulphate fraction

(v) A commercial preparation of α-galactosidase from *Coffea sp.*

Each was incubated with both guar and locust bean gums for 5 days. This long period of time was chosen because viscosity measurements with earlier experiments had not indicated a substantial modification of the galactomannans. The monitoring of the progress of the reaction was modified by using two separate galactose assays. The Nelson method for free reducing sugars was carried out as before, and, in addition galactose was estimated enzymically. The latter involved the use of galactose dehydrogenase in the presence of NAD (See Materials and Methods, page 104.) The results for this set of incubations are given in Table 12

In these experiments with *Vicia faba* enzymes the results were quite different from those summarised in Table 11. The level of reducing sugar did not increase and the mannose/galactose ratios did not change significantly. The viscosities, however, did change markedly but this also occurred in the controls. It seems probable that in all cases no enzyme attack on the galactomannans occurred and that the drop in the viscosity was due to a 'shearing' effect which broke the mannan backbone. This has previously

TABLE 12.

Incubation (37°) of Galactomannans (Locust Bean and Guar Gums) with Various Purified α-Galactosidases.

Sample		Incub. Time	% Galactose Released (Nelson)	(Gal. Dehyd.)	Viscom. Time (min. sec.)	Man/Gal Ratio %
Vicia E_I	Guar	5 days	0	0	2.28	63:37
"	LBG	5 days	0	0	0.48	79:21
Vicia E_{II}	Guar	"	0	0	2.53	61:39
	LBG	"	0	0	0.49	81:19
Vicia E_I+_{II}	Guar	"	0	0	3.03	60:40
	LBG	"	0	0	0.53	80:20
Vicia Dial.	Guar	"	0	0	2.39	63:37
	LBG	"	0	0	0.44	79:21
Coffea	Guar	"	17	19	0.25	81:19
	LBG	"	14	13	0.20	91:9
Coffea	Guar	5 hr.	5	5	2.24	69:31
	LBG	"	5	5	1.40	87:13
Controls.						
Guar		5 days	0	0	3.32	63:37
LBG		"	0	0	0.54	80:20
Guar		0	0	0	7.05	63:37
LBG		0	0	0	7.45	80:20

been reported by Hui[300].

In an attempt to correlate the evidence from this second batch of incubations with the original trial (See Table 11), it seemed possible, as mentioned earlier, that galactose was released (increased reducing power) in the short term and then transferred to some acceptor, In this connection it is interesting to note that when α-galactosidase from Vicia faba was incubated with raffinose for about 10 hr. no free galactose could be detected in the incubation mixture but there did appear to be a transfer product present as judged by paper chromatography[326]. In the case of the guar and locust bean gums the galactose could have been derived from the polysaccharides themselves or from oligosaccharide impurities. The viscosity readings remained high over the 5 hr. incubations in the first trial (Table 11) which could be indicative of lack of enzyme activity towards the galactomannan substrates. The mannose/galactose ratios, however, did suggest that the enzyme had modified the polysaccharides but this was not so in the second set of experiments. Galactomannans are polydisperse with respect to molecular weight, and it is feasible that in the first experiments the polysaccharide was unintentionally fractionated during the work up procedure. In this case the enzyme incubations were stopped by boiling and the resulting precipitates centrifuged

down. This was not carried out in the later incubations where the solutions cooled, dialysed and were then freeze-dried. Hence, the apparent difference in the mannose/galactose ratios obtained in the first experiments could have been the result of examining different polysaccharide fractions which existed in the starting polysaccharide samples.

It was finally concluded that the Vicia faba enzyme was having little effect on the galactomannans and in particular producing no products of commercial interest.

Further attention was next directed to the commercial preparation of coffee α-galactosidase which appeared to have produced modified products from guar and locust bean gums (Table 12). Here, with both substrates, the incubation gave some insoluble product and up to 19% of the galactose appeared to have been removed. The viscosities also dropped from 7 - 8 min. to 20 - 30 sec. (Table 12). The formation of the insoluble product no doubt accounted for some decrease in viscosity. The mannose/galactose ratios in these experiments were raised from 63 : 37 to 81 : 19 in the case of guar (which is the average ratio for the untreated locust bean gum) and from 80 : 20 to 91 : 9 for locust bean gum. Paralleling the changes in the mannose/galactose ratios it was also observed that the

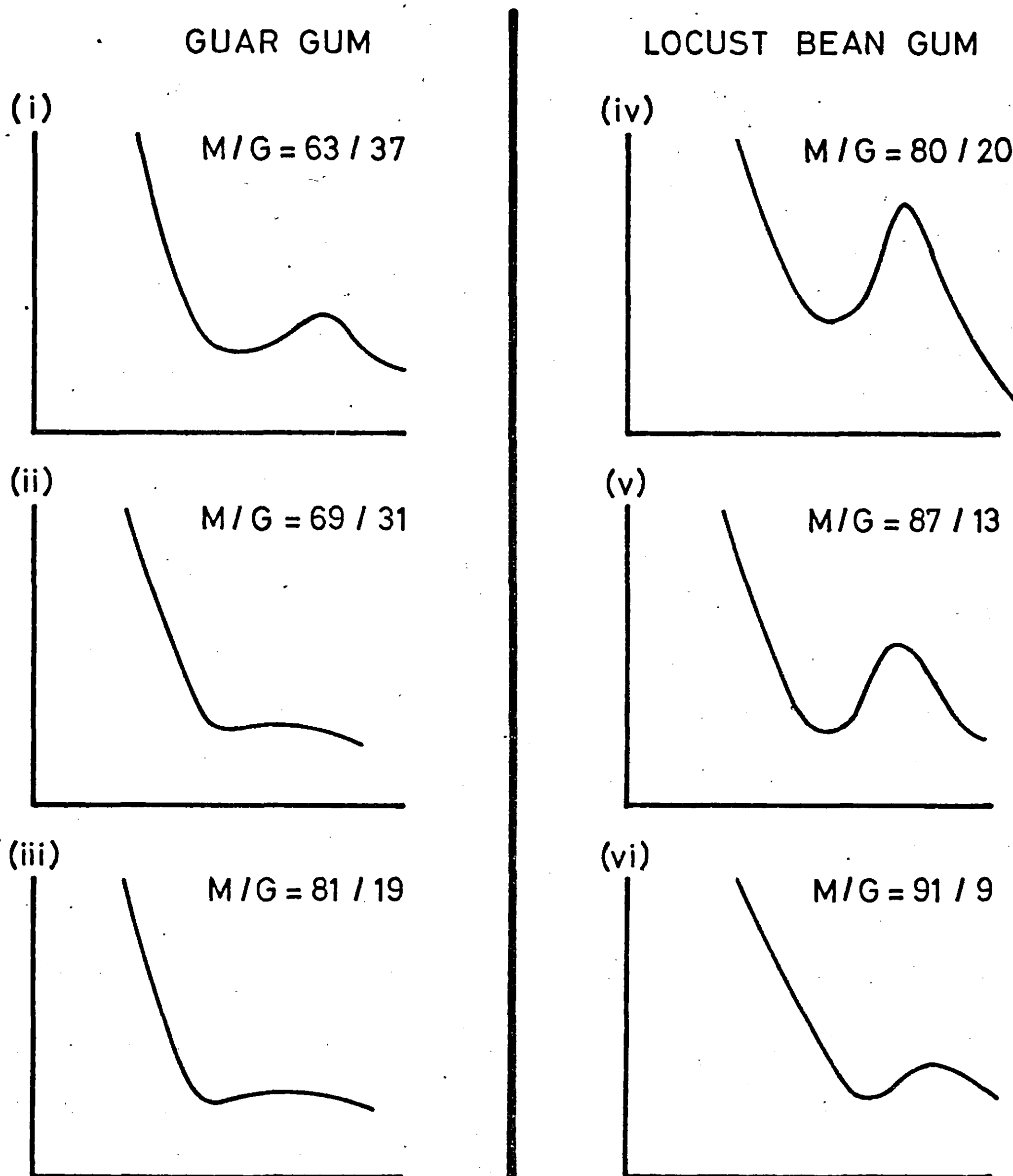
Coffea sp. enzyme released galactose (measured by two methods) from the polysaccharide. In a later study the molecular weights of these gums were determined. (See Appendix) In summary it appears that the normal rules of substrate specificity for α-galactosidases therefore apply to the enzymes from the two plant sources, i.e. Vicia faba and Coffea sp. That is, α-galactosidases which co-exist with galactomannans in vivo can hydrolyse these polysaccharides in vitro, but those from tissues not possessing these polysaccharides are unable to hydrolyse them. (See Introduction, page 24 .) Returning to the earlier work by Khaleque[10,316] who claimed that α-galactosidase I hydrolysed galactomannans; the evidence was based on increase in reducing power over 1 hr. and the analysis of the mannose/galactose ratio by measurement of optical rotation[149] which presumably gave incorrect results. The apparent galactose release over a short period reported by Khaleque parallels data obtained in the present study and an attempt to account for this has already been made.

The effect of the Coffea sp. α-galactosidase-modified galactomannans on the strength of κ-carrageenan gels was next investigated using an Instrom tensile strength tester. In these experiments the effects of native guar and locust bean gums were compared with polysaccharide samples which had been treated with Coffea sp. α-galactosidase

for 5 hr. or for 5 days. Figure 10 shows the Instrom curves obtained (See Materials and Methods, page 113.) Figures 10(i) and 10(iv) show the curves for the untreated galactomannan/carrageenan gels. Locust bean gum (10(iv)) having fewer galactose side chains displayed higher synergistic characteristics than guar with many side chains which impair interaction with the double helices of the κ-carrageenan. This difference is shown in the traces by the height of the peaks. The higher the peak, the firmer the gel and, under these test conditions, the greater the synergism conferred by the galactomannan. Untreated guar (10(i)) slightly increased the strength of the carrageenan gel, and the two modified gums produced even weaker gels (10(ii),(iii)) despite the fact that the mannose/galactose ratios were decreased which in theory should have increased the heights of the peaks. The modified gums were, therefore, antisynergistic in these systems. The three locust bean gum samples (10(iv),(v),(vi)) showed a parallel trend with all the gels exhibiting a somewhat greater strength than the corresponding samples of guar gum. The most likely explanation for the antisynergistic effects of the modified galactomannans is that the enzyme preparation also contained β-mannanase which was capable of cleaving the β-(1→4) links in the mannan chain. One can see that a single break in the mannan chain would reduce the molecular weight of the galactomannan

FIGURE 10.

Instrom Curves of α-Galactosidase (*Coffea* *sp.*)-Treated Galactomannans. (i) and (iv) untreated gums; (ii) and (v) treated (37°) for 5 hr. with enzyme; (iii) and (vi) treated for 5 days with enzyme.



M/G = mannose/galactose ratio

by half, and according to Rees *et al*[149], high molecular weight polysaccharides are necessary to allow sufficient hydrophobic interactions leading to the formation of a mixed gel network in the system. A very low level of β-mannanase activity could, therefore, completely destroy any improvements brought about by α-galactosidase removing isolated galactose stubs and, hence, extending the smooth regions of the gum.

A literature search for α-galactosidases free of β-mannanases was made without success. Attention was next directed towards the further purification of an α-galactosidase free of β-mannanase. McCleary and Matheson[172] had reported that by a combination of chromatographic techniques using DEAE and CM Cellulose it was possible to purify α-galactosidase of guar "substantially" free of β-mannanase but this was considered insufficient for the modified galactomannan/κ-carrageenan gel studies. It seemed then that several lines of approach were open for future work.

Firstly, it might have been possible to have removed the β-mannanase by affinity chromatography but this was abandoned in view of the likely difficulty of preparing an affinity gel with the enzyme substrate, β-mannan, which is very insoluble. Another possible approach was

to selectively inactivate the mannanase by carefully controlling the extraction conditions. In this connection Deuel _et al_[327] had reported that β-mannanase was less stable at alkaline pH values than α-galactosidase.

In the present study an α-galactosidase preparation from clover (_Trifolium repens_) containing a β-mannanase contaminant was examined. This source was chosen as it was readily available and it was known to yield α-galactosidases which readily hydrolysed galactomannans[14].

The preparation was purified by the method described on page 125 as far as the first Sephadex G-100 gel filtration stage. After Amicon concentration the pH of the fraction was raised to 8.0 (_cf._ Deuel _et al_ [327]) and the solution, after adjustment to pH 5.5, passed down a column of ivory nut mannan in an attempt to remove β-mannanase. All fractions possessing α-galactosidase activity were pooled. Incubation trials using locust bean gum and guar gum as substrates were set up, using this enzyme. Galactose was released from both substrates and the polysaccharide products had higher mannose/galactose ratios than the native gums. (See Table 13). However, the incubations substantially lowered the viscosities of the gums and on making carrageenan water gels with the freeze-dried hydrolysis products, anti-synergistic behaviours were

TABLE 13.

Incubation (37°, 5 days) of Galactomannans (Locust bean and Guar Gums) with α-Galactosidase from Clover.

Sample	Viscosities (min.sec.)	% Galactose Released (Gal. dehyd.)	Mannose/ galactose ratio %
Guar	0.16	26	83 : 17
Locust bean Gum	0.18	17	93 : 7
Controls			
-Time 0			
Guar	7.38	0	63 : 37
Locust bean Gum	8.08	0	80 : 20

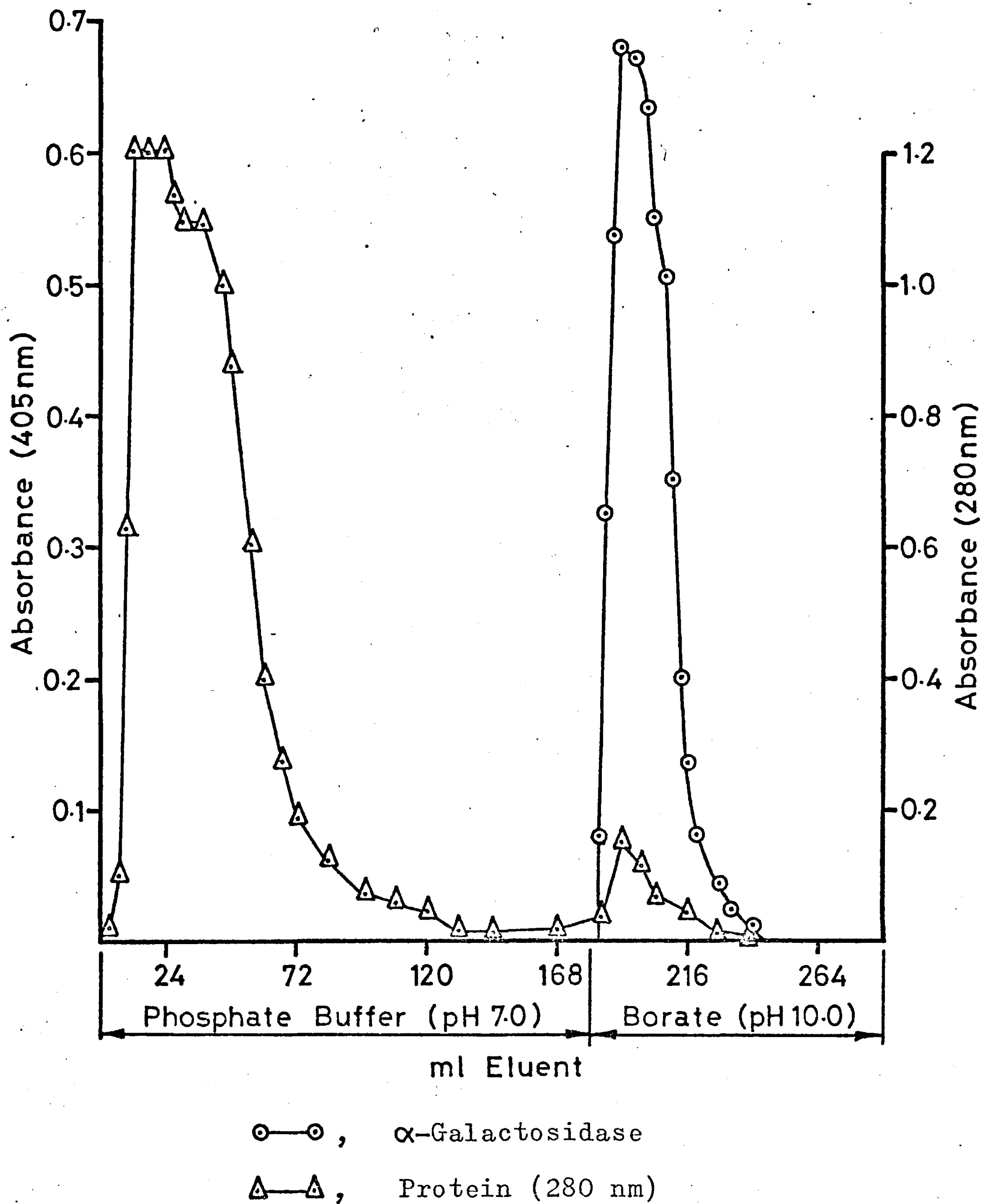
again observed. Subsequently, it was shown that the clover enzyme increased the reducing power of a Salep (*Orchis intacta*) glucomannan solution, i.e. the preparation presumably still possessed β-mannanase activity (N.B. β-mannosidase which would also hydrolyse glucomannan should have been removed by Sephadex G-100 chromatography.)

A second approach was the preparation of an affinity column for α-galactosidase. Two materials capable of binding the enzyme had been previously synthesised[21,27]. Eventually a galactonolactone-benzidine-Sepharose affinity column, as described by Kanfer *et al* [21] was chosen because of the relative ease of preparation.

A dialysed ammonium sulphate fraction (See page 125.) of α-galactosidase from clover which had been further fractionated on Sephadex G-100 was applied to the affinity column. The column was then thoroughly washed with buffer to remove non-enzymic protein; the α-galactosidase remaining on the column was subsequently eluted with a borate buffer (pH 10) (Graph 31) This procedure increased the specific activity of the preparation 150-fold. The presence of β-mannanase in the fraction could not be detected by incubation with glucomannan, followed by reducing power measurements but incubation of the fraction with galactomannans again produced sharp decreases in the

GRAPH 31.

Purification of Clover α-Galactosidase by Affinity Chromatography on 'Galactonate' Sepharose[21]



⊙—⊙, α-Galactosidase

△—△, Protein (280 nm)

viscosities of these gums which were typical of β-mannanase activity. The enzyme-treated products exhibited increased mannose/galactose ratios, and effects when mixed with κ-carrageenan were antisynergistic.

In a final attempt to purify an α-galactosidase free of β-mannanase, advantage was taken of the fact that β-mannanase, present in clover α-galactosidase (purified as far as the Sephadex G-100 stage), disaggregates to a lower molecular weight form when the preparation is allowed to stand at 4° for about 7 days. This allows a better separation of β-mannanase and α-galactosidase on gel columns. The clover was therefore left at 4° then passed through a column of Sephadex G-100 and the α-galactosidase isolated. This procedure was then repeated twice; the specific activity of the final α-galactosidase preparation was only 10% of that of the original preparation.
The α-galactosidase was then concentrated by ultrafiltration, subjected to alkaline treatment (5 hr. at pH 8) and the preparation applied to the affinity column. The α-galactosidase absorbed on to the column, and was eluted off with alkaline buffer. No β-mannanase could be detected in this fraction, but the α-galactosidase was totally inactive against guar and locust bean gum, and, in addition, exhibited only weak activity with PNPG.

In conclusion, it appears to be very difficult to obtain an α-galactosidase preparation which hydrolyses galactomannans and which is completely free of contaminating β-mannanase activity. McCleary and Matheson[172], however, claim to have prepared α-galactosidases substantially free of β-mannanase from guar seeds and completely free from carob and soybean seeds.

In the present study the inability to obtain a suitable α-galactosidase preparation resulted in mannan backbone cleavage in all cases when galactomannans were incubated with the enzyme preparations. Hence, the relatively low molecular weight hydrolysis products were not found to be suitable 'gel-strengthening agents'.

APPENDIX.

Molecular Weight of Gums and Enzyme-Modified Gums.

Samples of guar and locust bean gum which had been modified by α-galactosidase from *Coffea sp*. (See page 217.) were subjected to molecular weight determinations using two different methods, i.e. intrinsic viscosity and ultra-centrifugation. The results of these determinations are given in the Table overleaf.

Allowing for normal discrepencies between values obtained by the two different methods, it is clear that the molecular weights of the enzyme-treated guar and locust bean gums are very much smaller than those of the corresponding native polysaccharides. It must be concluded that this difference is a function of a β-mannanase contaminant in the *Coffea sp* α-galactosidase.

The Molecular Weights of Native and Enzyme*-Modified Guar and Locust bean Gums.

Sample	Molecular Weight	
	Intrinsic Viscosity Method (Mz)	Sedimentation Equilibrium Method (Mz)
Native Guar Gum	196,600	
Native Locust Bean Gum	141,600	
Modified Guar Gum	23,000	23,000 (0.2)+ 33,000 (0.6)+
Modified Locust Bean Gum	22,700	48,000 (0.2)+ 40,000 (0.6)+

* Coffea sp. α-galactosidase

+ Numbers in parenthesis refer to polysaccharide concentration (mg/ml)

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