

GLYCOSIDASES IN SEEDS

A Thesis submitted by

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a candidate for the Degree of

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ABSTRACT

Two forms of α -galactosidases (I and II) have been shown to exist in Vicia faba seeds and these have been purified 3660- and 337- fold, respectively. They behaved as homogeneous preparations when examined by ultracentrifugation, disc electrophoresis and gel filtration. The apparent molecular weights of enzyme I and II, as determined by gel filtration, were 209,000 and 38,000, respectively. The carbohydrate contents of enzyme I and II were 25.0% and 2.8%, respectively, and the enzymes differed in their aromatic amino acid compositions. Enzyme I was split into six inactive sub-units in the presence of 6M-urea. α -Galactosidases I and II showed different pH optima and K_m and V_{max} values with various natural and synthetic substrates and also differed in their thermal stabilities. The effect of temperature on various kinetic parameters has also been examined. Both enzymes are inhibited by excess substrate (p-nitrophenyl α -D-galactoside): with enzyme I this is competitive and is caused by the galactosyl moiety. Enzyme I is inhibited by various metal ions and by oligosaccharides possessing two terminal non-reducing galactose residues and to a lesser extent by L-arabinose and D-fucose. The effect of pH on K_m and V_{max} values suggests that carboxyl and imidazole (histidine) groups are involved in the catalytic activity of enzyme I. Photo-oxidation experiments with I also suggest that an imidazole group is present at the active site.

Histochemical and tissue fractionation studies show that the enzyme is widely distributed in seedling tissues and is non-particulate. Aerobic conditions increase the level of α -galactosidase during germination and both gibberellic acid and kinetin enhance synthesis of the enzyme in vivo. As germination proceeds, the concentration of α -galactosyl sucrose derivatives decreases with a simultaneous decrease in the level of enzyme I and an increase in that of II.

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ABBREVIATIONS

tris, Tris (hydroxymethyl) aminomethane.

PNPG, p-Nitrophenyl α -D-galactoside.

DMF, N,N¹-Dimethyl formamide.

Residual activity, $A/A^{\circ} \times 100$ where A is the enzyme activity after inactivation and A^o is the activity before the inactivation.

K_m, Michaelis constant.

K_s, Dissociation constant of enzyme-substrate complex.

K_i, Inhibitor constant.

V_{max}, Maximum velocity.

PCMB, p-Chloromercuribenzoic acid.

IAA, Indoleacetic acid.

GA, Gibberellic acid.

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

Glycosidases (also termed carbohydrases) catalyse the hydrolysis of glycosidic bonds. The specificity of these enzymes may be defined in terms of the structural factors that determine whether a given glycosidase will act on a particular linkage. Such factors are mainly :

- a) The hydroxyl configuration of the glycosyl residue, b) The configuration (alpha or beta) of the anomeric carbon atom of the glycosyl residue,
- c) The size of the heterocyclic ring.

The glycosidases may be divided into two broad groups; those which catalyze the hydrolysis of glycosidic bonds in simple glycosides or in oligosaccharides, and those which catalyze the hydrolysis of glycosidic bonds in polysaccharides. (A few glycosidases can utilize both simple glycosides and polysaccharides as substrates.) The term 'glycosidase' is frequently assigned to the first group only whereas the other group of enzymes are often termed polysaccharidases. Amongst polysaccharide hydrolyzing enzymes, there are endo- and exo- enzymes. For example, α -amylase, an endo-enzyme cleaves internal glycosidic links in some glucans, whereas β -amylase, an exo-enzyme, removes maltose residues starting from the non-reducing ends of α -1 \rightarrow 4 linked chains of glucose residues.

Glycosidases are widely distributed in animals (1-5), microorganisms (6-9) and plants (10-13). Most of the research with these enzymes has centered around the amylases, glucosidases and invertases. β -Galactosidases have also been studied extensively with respect to the mechanism of action (14) and induced enzyme-formation. On the other hand, α -galactosidases have so far received little detailed attention.

Oligosaccharides containing one or more β -galactopyranosyl groups in their structure are widely distributed in nature, especially in the plant kingdom. (The structural aspects of these compounds have been reviewed by French (15) and Courtois *et al.* (16). It is, therefore, not unexpected that α -galactosidases which hydrolyse these oligosaccharides are also ubiquitous in nature. α -Galactosidase is a well known microbial enzyme. It was first discovered in bottom yeast in 1895 simultaneously by Bau (17) and by Fischer *et al.* (18). The enzyme was originally named

melibiase and studied further by Weidenhagen et al (19) and Adams et al (20). Later α -galactosidase activity was detected in various fungi and bacteria (Table 1), higher plants (Table 2) and in the animal kingdom (Table 3)

Table 1. Occurrence of α -galactosidase in Fungi and Bacteria

Source of α -galactosidase	Reference
<u>Aspergillus niger</u>	21
<u>Aspergillus oryzae</u>	19
<u>Aspergillus fumigatus</u>	22
<u>Aspergillus luchuensis</u>	22
Several species of Aspergillaceae including some Penicillia	23,24
<u>Trichomonas foetus</u>	25
<u>Saccharomyces carlsbergensis</u>	26,27
<u>Lactobacillus delbruckii</u>	28
Sulphur ^r bacteria	28
Kefir bacteria	29
<u>Escherichia coli</u>	30,31
<u>Aerobacter aerogenes</u>	32
<u>Colstridium maebashi</u>	33
A bacterial strain from guinea-pig intestine	34
<u>Streptococcus bovis</u>	35
<u>Diplococcus pneumoniae</u>	36
Several species of Streptomy ^{ce} ces	37

Table 2. Occurrence of α -galactosidase in the plant kingdom

Source of α -galactosidase	Reference
<u>Amygdalus</u> sp. (Almond)	38
<u>Medicago sativa</u>	39

<u>Coffea</u> sp.	40
Several conifer seeds	41, 42
<u>Gossypium</u> sp.	43
<u>Triticum</u> sp.	44
<u>Citrullus vulgaris</u>	45
<u>Vicia faba</u>	46
<u>Vicia sativa</u>	47
<u>Vicia dumitorum</u>	48
<u>Pisum sativum</u>	48
<u>Acer pseudoplatanus</u>	48
<u>Zea</u> sp. seeds	19
Guar seeds	49
<u>Phaseolus vulgaris</u>	50
<u>Trigonellum foenum</u>	51
Cabbage	52
Raddish	52
Castor oil beans	52
Aspen soft phloem	53
Papain commercial preparation	54
<u>Poppyra umbilicalis</u>	55

Table 3. Occurrence of α -galactosidase in the animal kingdom

Source of α -galactosidase	Reference
Rabbit semen	56
Horse semen	56
Ram semen	57
Various species kidney tissues	58
Various human and rat tissues	59, 60
<u>Helix pomatia</u> digestive juice	61
<u>Helix laeda</u> liver	62
<u>Patella vulgata</u>	63

Oyster	64
<u>Arion ater</u> (slug) digestive tract	65
Insects, various species	66-78
<u>Epidinium caudatum</u> Rumen ciliate	79

To date only a limited number of carbohydrases have been obtained in a very pure form. The known examples of crystalline carbohydrases are β -galactosidase (80), α -amylase (81-87), β -amylase (88,89), cellulase (90), xylanase (91) and ~~oligo-1,6-glucosidase~~ ^{limit dextranase} (84).

Although α -galactosidase has been obtained from a variety of sources and recognized for many years, it has not, until recently, been highly purified; it has normally been studied in the presence of other related glycosidases. Helferich et al (92) partially (10-fold) purified the enzyme by treatment of an almond emulsion solution with silver hydroxide. Separation of α -galactosidase from β -glucosidase and chitinase in this emulsion was achieved on a bauxite column by Zechmeister et al (93). Shibata and Nisizawa (94) have shown that it is possible to separate α -galactosidase from β -glucosidase in tannin precipitated apricot emulsion by paper electrophoresis. The α -galactosidase of Diplococcus pneumoniae was purified (about 100-fold) by Li et al (36,95); one of the purification steps was absorption of the enzyme on red blood cells. α -Galactosidase from water melon seeds has also been partially (10-fold) purified (45). Much later, extensive purification of α -galactosidases from sweet almonds (96,97), Medicago sativa (98) and Vicia sativa (47) was reported and the homogeneity of the preparations demonstrated by various physical methods.

Helferich and Vorsatz (40) made an α -galactosidase preparation from coffee beans and Petek and To Dong (99) reported the isolation of two α -galactosidase forms from the same source by chromatography on an alumina column. The two active forms showed similar physical properties (100). The French workers also demonstrated a similar isolation of two α -galactosidases from Plantago ovata seeds (101).

The pH optima of α -galactosidases vary somewhat with the source of the enzyme and with the substrate used (14). The optima for most of the α -galactosidases lie within the range 3.5 - 6.7 (14,50,98,102,103). It is interesting to note that whereas a crude enzyme preparation from coffee seeds showed two pH optima (3.0 and 6.0) using phenyl α -D-galactoside as substrate (104), the two forms of α -galactosidase obtained by alumina column fractionation had optima of 5.3 and 6.0, respectively (99). It is not yet clear whether the two optima exhibited by the crude extract are due to the presence of two separate enzymes or to other factors. Delvin and Gianetto (105) showed a similar phenomenon of two pH optima (4.7 and 5.35) with a partially purified β -glucuronidase from rat liver lysosomes. The pH-activity curve in this case, however, showed a single optimum when the ionic strength of the buffer was raised. Crystalline β -galactosidase also produced a double peak when pH was plotted against V_{\max} using p-nitrophenyl β -D-galactoside as substrate (14).

Some α -galactosidases have been shown to require thiol groups for activity (32,36,106). A few other glycosidases have also been shown to have this requirement (14,107,108). On the other hand the enzyme obtained from sweet almonds required histidine and carboxyl groups for catalytic activity (109).

The heat stabilities of α -galactosidases varied with the enzyme source. The lucerne (Medicago sativa) enzyme (39) lost 80% of its activity in 3 hr. at 45°, snail enzyme (110) was completely inactivated in 30 min. at 70° and Pneumococcal enzyme (36) lost 30% activity in 1 hr. at 50°. The lucerne enzyme was also inactivated with D-galactose (competitive K_i , $2.4 \times 10^{-3} M$) (104) but not inhibited by D-galactone - 1 \rightarrow 4-lactone (39) in contrast to the powerful inhibition of other glycosidases by aldonolactones of corresponding structure and configuration (111). The specificity and high affinity of such lactones (also 1 \rightarrow 5 lactones) for glycosidases probably arise from ease of inhibitor-binding due to the flexibility of the lactone ring and/or the stereochemical similarities between the lactone and the substrate in the transition state which is probably in the half chair configuration (112).

A great deal of work has been done on the specificity of a number of glycosidases (EC.3.2.1.20-26) and details of this can be found in reviews by Helferich (113), Pigman (114), Gottschalk (115) and Wallenfels (14). In general, change of configuration of hydrogen and hydroxyl on any single carbon atom of a glycoside substrate is sufficient to prevent the action of the corresponding enzyme. In the case of the epimers involving carbon atoms, 1, 2 and 4 of an aldohexose ring, specific enzymes exist for the hydrolysis of the corresponding glycosides. With β -glucosidase any substitution of H or OH on carbon atoms 2, 3 or 4 completely prevents hydrolysis. Substitution on C-6, however, normally only lowers the rate of hydrolysis thus replacement of the primary alcohol group in a glucopyranosyl ring by -H (i.e. conversion to a β -xyloside) produces a 200-fold reduction in rate. A similar state of affairs exists with β -galactosidase (14). Phenyl-, methyl- and ethyl β -L-arabinosides, in comparison with the corresponding α -D-galactosides, are hydrolysed at a reduced rate by both almond (92, 116, 117) and yeast (20) α -galactosidases. The α -galactosidases from Streptococcus bovis (35) Epidinium ecandatum (118) and Diplococcus pneumoniae (6) however had no effect on arabinosides. Phenyl D-glycero- α -D-galacto-heptopyranoside is not hydrolysed by almond (119) or yeast (20) enzyme.

The aglycon group of a substrate may or may not have a marked effect on hydrolysis by glycosidases. Normally the group will not completely prevent the hydrolysis but after the rate, most of the available data are expressed in terms of 'enzyme efficiencies' which are essentially rates of hydrolyses (cf. 120). Thus the variations might be due to the effect of aglycon structure on either enzyme-substrate affinity or the catalytic step. Here it appears that the electron-attracting power of the group, rather than its size, is the operative factor (121). In general with α -galactosidase, aryl α -D-galactosides are better substrates than alkyl derivatives or disaccharides (14, 117). In homologous series of α -D-galactosides (35, 118, 122) the rate of hydrolysis seems to be

reduced by increase in chain length. Reduction of reducing groups, eg. conversion of melibiose to melibitol and manninotriose to manninotriitol, also reduces the rate of hydrolysis (35,118). Oxidation of the reducing group, for example conversion of melibiose to melibionic acid does not appear to affect the rate (123). In oligosaccharides α -galactosyl residues linked (1 \rightarrow 6), (1 \rightarrow 4) and (1 \rightarrow 2) are all hydrolysed. O- α -D-galactose-(1 \rightarrow 4)-D-galactose is cleaved at a similar rate to melibiose (118,124).

In addition to hydrolysing ^{terminal} galactosyl residues, almond β -galactosidase is able to attack the internal galactosidic linkage in stachyose, forming galactobiose and sucrose (125,126). Courtois *et al* (127) observed that D-galactose residues from lucerne galactomannan were partially removed by the action of coffee α -galactosidase. This is a property of several other plant α -galactosidases (51,128). Rees and Shibata (22) observed that β -mannanase preparations from several microbial sources possessed α -galactosidase activity and that the preparations removed galactose from guar and carob gums. It is, however, not known whether highly purified α -galactosidase preparations hydrolyse galactomannans.

Glycosidases catalyse glycosyl transfer reactions as well as hydrolysis. (Hydrolysis can of course be regarded as a special glycosyl transfer to H₂O). The first observation of transferase action by α -galactosidases was made by Blanchard and Albon (129) who observed the formation of two unknown compounds of lower chromatographic mobility than melibiose in a digest of melibiose and yeast- α -galactosidase. One of the new compounds was later identified as manninotriose (15). Thereafter, α -transgalactosylation was studied extensively especially by Courtois and his co-workers with respect to acceptor specificity, effect of acceptor concentration, pH, source of enzyme and effect of temperature (14,51,100,130). The results obtained so far show that not only the hydrolysis and transfer reactions are catalysed by the same enzyme but also that they take place on the same active site of the enzyme molecule.

With β -galactosidases, the synthesis of glycosides from free sugars and alcohols has also been shown to occur in addition to transfer reactions from glycosides and hydrolysis (14). Here again one and the same enzyme is responsible for all three activities (131).

The acceptor specificity of α -galactosidases from coffee, lucerne and fenugreek has been studied (14,51,100,130) with phenyl α -D-galactoside as the galactosyl donor and it has been shown that the following compounds serve as acceptors: D-galactose, D-glucose, D-mannose, lactose, maltose, cellobiose, gentiobiose, sucrose, trehalose, gentianose, glycerol, D-mannitol, myo-inositol, methyl α -D-glucoside, methanol, ethanol, 1-propanol, 2-propanol, 1-butanol, 2-pentanol. No transfer to the following compounds could be detected: D-ribose, L-arabinose, D-xylose, L-xylose, L-rhamnose, D-fructose, L-sorbose, D-glucosamine, amygdalin, methyl β -D-glucoside and methyl α -D-mannoside. No correlation was observed between acceptor efficiency and the number of primary alcoholic groups or the molecular size. However, it was suggested that transfer occurred preferentially to the primary alcoholic group of the acceptor molecule. The complete identification of transfer products was accomplished only in a few cases (6,25,95,101,132,133,134,135).

A galactobiose and a galactotriose were isolated from incubation mixtures of D-galactose and yeast α -galactosidase and α -1,6-linkages were assigned to these compounds. Other oligosaccharides were produced in much smaller amounts (15). Clancy and Whelan (26,27) using the same enzyme and substrate also obtained high yields of a α 1 \rightarrow 6 linked galactobiose together with small amounts of α -1,5-, α -1,4- and α -1,3-linked disaccharides and higher oligosaccharides. It should be noted that β -galactosidase also shows similar properties and Wallenfels *et al.* (14) demonstrated the formation of β -1,6-, β -1,4- and β -1,3-linked galactosylglucoses by β -galactosidase transfer reactions.

The physiological role of α -galactosidase in plants is presumably the hydrolysis of galactose containing carbohydrates, which occur primarily in seeds, roots and underground stems as reserve carbohydrates. It has been shown that a number of α -galactosyl derivatives, such as the

raffinose family of oligosaccharides, and polysaccharides, such as galactomannans and galactans, occur in many plant species (eg. 15, 16). Such compounds are degraded during germination of seeds (43, 136-141) to provide energy and intermediates for early embryonic growth. Pridham *et al.* (142) suggested that the concerted action of α -galactosidase and sucrose : UDP glucosyltransferase may form an important mechanism for the rapid utilization of raffinose and other galactosyl-sucrose reserves in germinating seeds. Pazur *et al.* (138) suggest that the liberation of galactose by the action of α -galactosidase is followed by phosphorylation by galactokinase. The latter reaction must be assumed to be rapid as free galactose does not normally accumulate in germinating seeds (43).

α -Transgalactosylation by α -galactosidases has often been said to be involved in the biosynthesis of galactosyl sugars in plants (46, 134, 143, 144). This type of reaction is, however, of doubtful biological importance and it is perhaps more reasonable to assume that the galactosyl residue in raffinose and related sugars *in vivo* is derived from a nucleoside diphosphate D-galactose derivative (145, 146). Recent publications (141, 147-150), however, indicate that galactinol (O- α -D-galactosyl-(1 \rightarrow 1) myo-inositol) might be the galactosyl donor for the biosynthesis of stachyose and verbascose. Sastry and Kates (151) believe that α -galactosidase is responsible for the hydrolysis of digalactosylglycerol to the monogalactosyl derivative in runner-bean leaves.

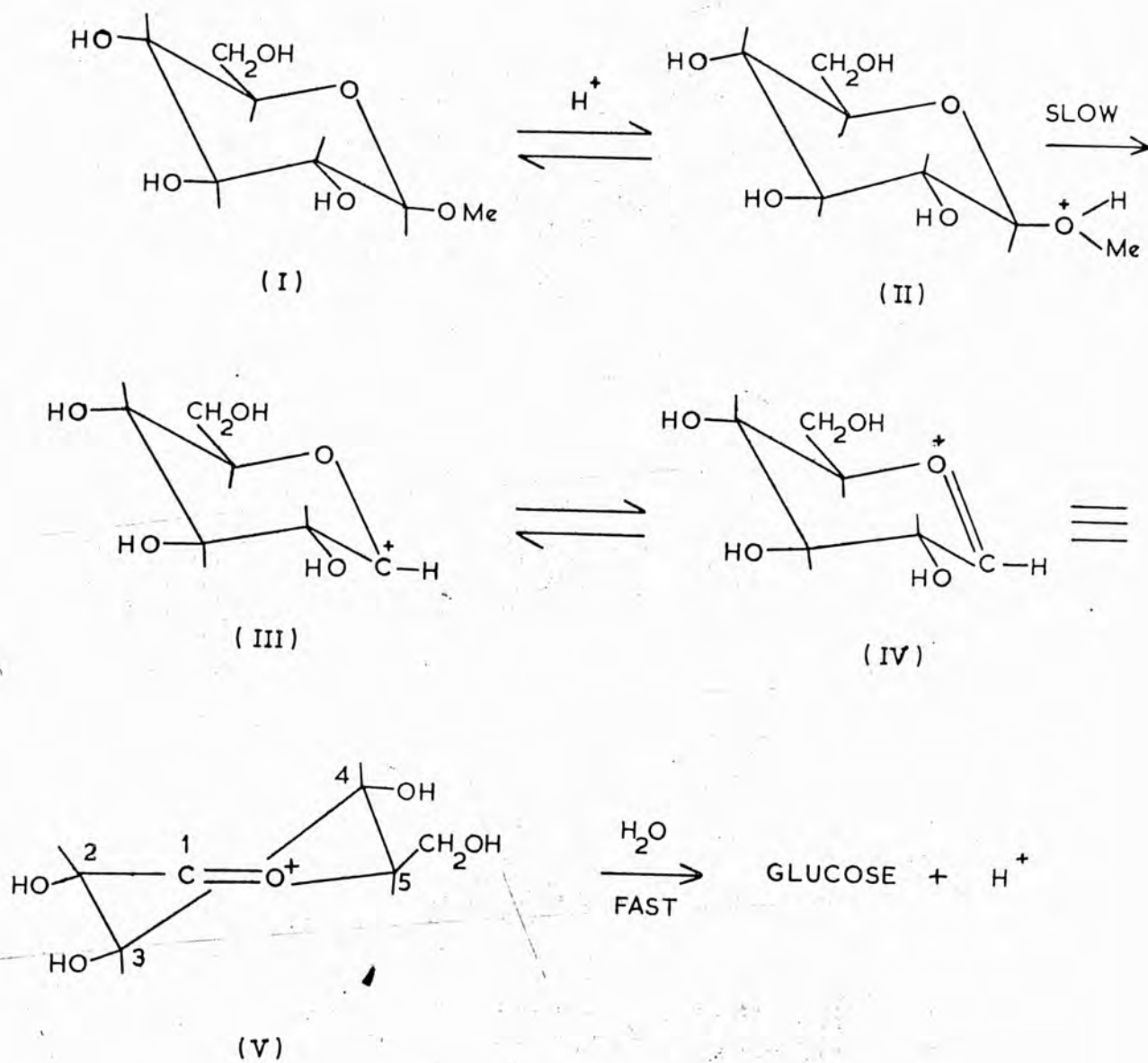
Both the acid-catalysed and enzyme-catalysed hydrolysis of glycosidic linkages₁₈ ^{have been} studied in detail, but whereas the former is now well understood, the mechanisms involved in the latter are still largely unknown.

The acid-catalysed hydrolysis of a number of D-glucopyranosides has been examined using H_2O (152) and in each case there is hexose-oxygen bond fission. This course of reaction is, no doubt, the general case, the only exception being the acid hydrolysis of t-Butyl β -D-glucopyranoside which, because of the facile formation of the t-butyl carbonium ion,

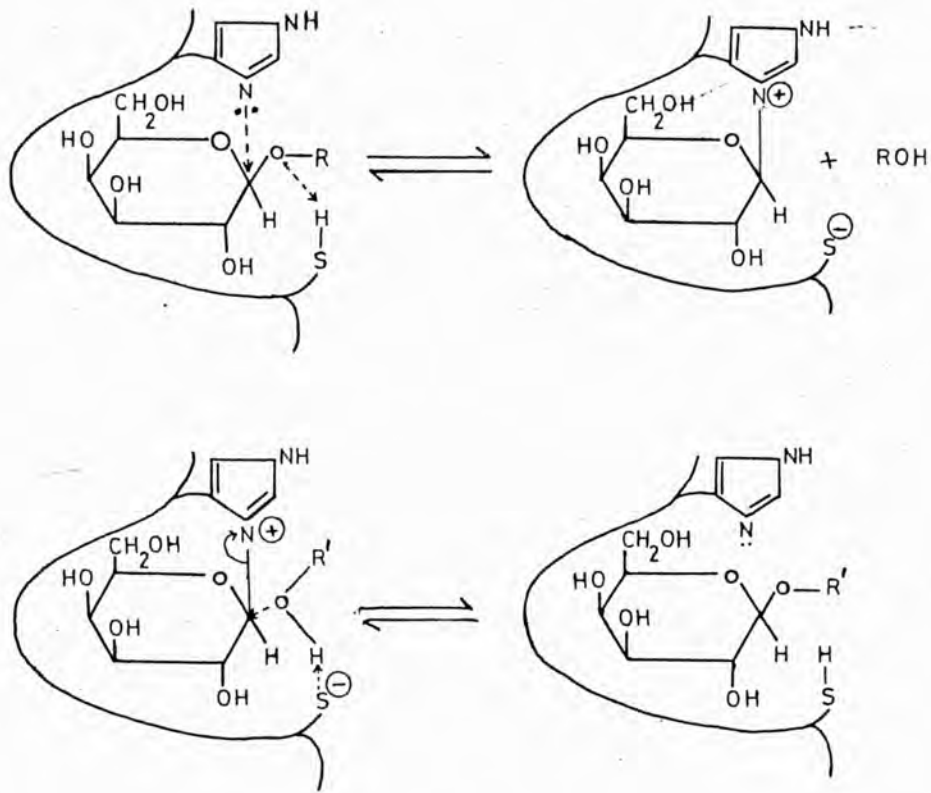
proceeds by alkyl-oxygen bond fission (153). Hydrolysis of α -glucosides by yeast α -glucosidase, β -glucosides by almond β -glucosidase (154) and β -galactosides by E. Coli β -galactosidase (14) have also been shown to occur by hexose-oxygen fission and with little specificity towards the aglycon.

The mechanism of acid-hydrolysis of glycosidic linkages was first suggested by Edwards (156) and has been verified by several groups (157, 158). As illustrated in Scheme I with methyl β -D-glucopyranoside (I) it involves a rapid protonation of the glycosidic oxygen to give the corresponding conjugate acid (II). This is followed by a slow rate-determining step in which the glycosidic bond is broken with the production of methanol and carbonium (III) and oxonium (IV) forms of the glucosyl which probably exist as the resonance hybrid (V) in the half-chair conformation. This is followed by the rapid nucleophilic attack of water to give glucose and a proton. Vernon (158) mentions that the carbonium ions probably react with solvent before achieving the half-chair conformation and, stereochemically, inversion at C-1 predominates. Hydrolysis of glycosides by glycosidases might, as Koshland (155) has suggested, proceed by such a mechanism. This would involve two bimolecular nucleophilic substitutions at the C-1 atom with first displacement of the aglycone by the nucleophile in the enzyme leading to a glycosyl-enzyme intermediate, and, second displacement of the enzyme by a water molecule (Scheme II). The scheme has the advantage of correctly predicting the stereochemical course of the reaction (overall retention of configuration). Such a type of covalent catalysis was suggested for β -galactosidase action (14). However, Wallenfels et al. (14) pointed out that an alternative one-step mechanism namely a "switch-over" mechanism (Scheme II) may also be operative resulting in retention of configuration of the product. In this case the authors postulated the existence of two similar sites (for glycone and acceptor, respectively) near to each other.

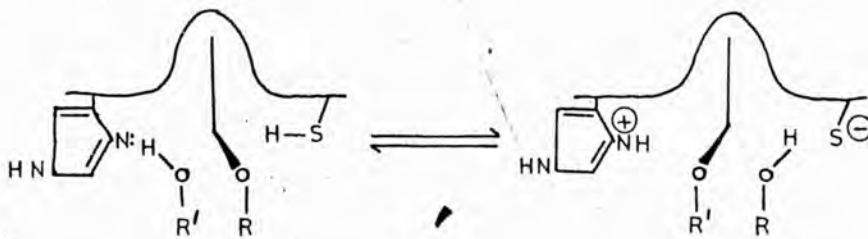
In the case of lysozyme-catalysed hydrolysis of glycosidic linkage Vernon (158)¹⁵⁹ points out that covalent bond formation between the substrate



Scheme I Acid hydrolysis of methyl β -D-glucopyranoside



Two-step mechanism of β -galactosidase action



One-step mechanism of β -galactosidase action

Scheme II

and the enzyme is not very likely. The most satisfying hypothesis is that heterolysis of the C-O⁻ bond occurs under the influence of the negative charge of the carboxyl group Asp52. In other words the reaction proceeds, as it does in solution, by the formation of a carbonium ion. The difference is that, whereas in free solution the carbonium ion is stabilized by interaction with the dipoles of the solvent molecule, in the enzyme it is stabilized by a negative charge held at a distance of about 3Å. The ion pair so formed cannot collapse to give a normal covalent bond, because the substrate fragment is held more or less rigidly in position by the geometry of the protein.

EXPERIMENTAL

(1) Preparation of substrates: Alkyl α -D-galactosides were prepared by the method of Dale and Hudson (160) and the aryl α -D-galactosides, by a reported method (117,161). *p*-Nitrophenyl β -L-arabinoside (162) and *p*-aminophenyl α -D-galactoside (163) were prepared as reported in the literature and *p*-nitrophenyl α -D- and β -D-glucosides, by the method of Montgomery *et al.* (164). *p*-Nitrophenyl α -D-fucoside was a gift from Dr. G.A. Levvy, Rowett Research Institute, Bucksburn, Aberdeen. The rest of the substrates were pure products obtained from commercial suppliers.

The physical constants of 6-bromo-2-naphthyl α -D-galactoside were found to be different from those reported by Morris *et al.* (165). The results are compared as follows; 6-bromo-2-naphthyl tetra-*o*-acetyl α -D-galactoside, m.p. 155-156°, $[\alpha]_D^{20} + 175^\circ$ in CHCl_3 (C, 1.0). (Found: C, 52.02; H, 4.63; Br, 14.46. Calc. for $\text{C}_{24}\text{H}_{25}\text{O}_{10}\text{Br}$: C, 52.08; H, 4.52; Br, 14.46%). The values reported by Morris *et al.* (165) were, m.p. 75-85°, $[\alpha]_D^{24} + 94^\circ$ in dioxan (C, 1.3). 6-Bromo-2-naphthyl α -D-galactoside, m.p. 179-180°, $[\alpha]_D^{20} + 200^\circ$ in methanol (C, 1.0). (Found: C, 50.2; H, 4.46; Br, 21.42. Calc. for $\text{C}_{16}\text{H}_{17}\text{O}_6\text{Br}$: C, 49.88; H, 4.46; Br, 20.74%). The literature values were, m.p. 225°, $[\alpha]_D^{28} + 60^\circ$ in pyridine (C, 1.2). The high optical rotation of the compounds obtained by us is characteristic of the α -anomer and is comparable with those of other aromatic α -D-galactosides (161).

(2) Enzyme assay: In all the cases only initial rates of substrate hydrolyses were measured. α -Galactosidase was assayed by addition of appropriately diluted enzyme solution (0.1 ml) to a mixture of McIlvaine buffer, pH 5.5 (0.25 ml) and 12mM PNPG (0.05 ml) preheated to 30°. This temperature was maintained for 15 min. and the reaction was then stopped by the addition of 0.1M - Na_2CO_3 solution (5 ml). The release of *p*-nitrophenol was measured at 405 nm. α -glucosidase, β -glucosidase and β -galactosidase activities were determined using the corresponding *p*-nitrophenyl glycosides under conditions identical with those used for α -galactosidase. Similarly, 'alkaline' invertase activity was determined at pH 7.8 with sucrose as substrate (46).

A unit of enzyme activity is defined as the amount that hydrolyses one micromole of substrate/min. under the conditions specified above.

Specific activities are expressed as enzyme units/mg. of protein. The protein determinations were made by the method of Lowry et al. (166), with crystalline bovine albumin as a standard.

For the substrate specificity experiments initial rates of hydrolyses were measured in McIlvaine buffer, pH 4.0 under the assay conditions described earlier.

Hydrolyses of all nitrophenyl glycosides were followed by estimating the liberated nitrophenol at 405 nm.; the extinction coefficients of various nitrophenols under the assay conditions were, o-nitrophenol, 4.05×10^3 ; m-nitrophenol, 1.56×10^4 ; p-nitrophenol, 1.53×10^4 cm²/m.mole.

The release of p-aminophenol from the corresponding galactoside was measured by the method of Furth et al. (167).

To follow the enzymic hydrolysis of 6-bromo-2-naphthyl α -D-galactoside, a 10 mM substrate solution was prepared in methanol. This was diluted 10-fold in McIlvaine buffer, pH 4.0 prior to assay. This solution (0.05 - 0.3 ml) and the properly diluted enzyme (total vol. of digest, 0.4 ml) were incubated for 30 min. at 30°. The reaction was stopped by adding 0.1N NaOH (4ml) and the liberated bromonaphthol determined at 296 nm. (Fig.1). This method of estimation was 10 times more sensitive than that described by Tsou et al. (105).

The hydrolysis of melibiose was followed by estimating the liberated glucose with glucose oxidase as described by Huggett et al. (168).

Hydrolysis of α -D-galactose-1-phosphate was detected qualitatively by paper chromatography using butan-1-ol-ethanol-water (40:11:19, by vol.) solvent and the liberated galactose was located using silver nitrate reagent (169).

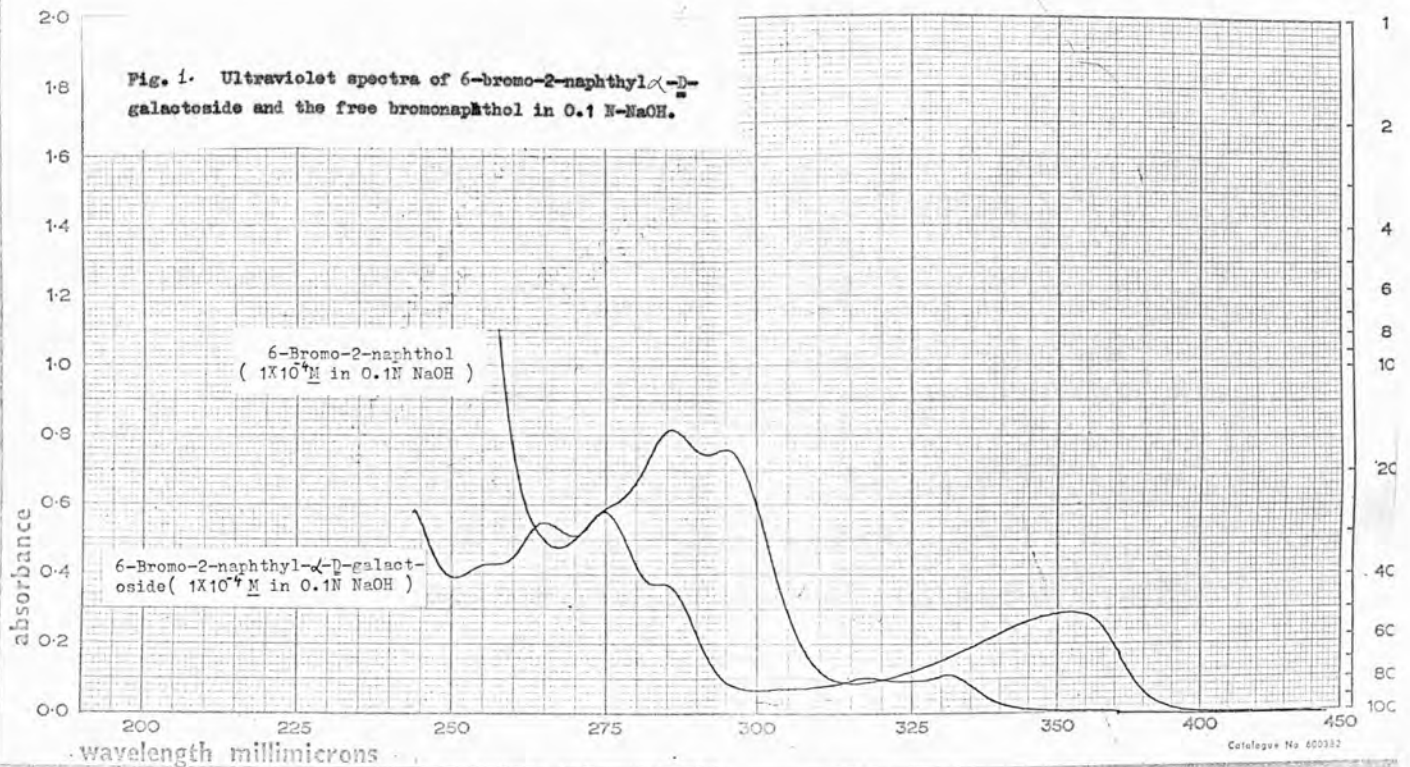
The release of galactose from other galactosides was measured by the micro-cuprimetric method of Nelson (170) using arseno-molybdate reagent (171). The procedure is described below;

Cu-reagent A : Na_2SO_4 , 20g. + NaHCO_3 , 2g. + Rochelle salt, 2g. + 100 ml water.

Cu-reagent B : 15% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$

25 parts of A and 1 part of B were mixed together. One ml. of this solution

Fig. 1. Ultraviolet spectra of 6-bromo-2-naphthyl- α -D-galactoside and the free bromonaphthol in 0.1 N-NaOH.



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was added to stop the reaction of the enzyme digest. The test tubes were heated in boiling water bath for 20 min., cooled, 1 ml. dilute arsenp-molybdate reagent (1 vol. diluted with 2 vol. of 1.5 N H_2SO_4) added, finally 3 ml. water was added and the blue colour measured at 560 nm. A linear calibration curve was obtained for 0.1 to 0.6 μ mole of galactose.

The Lineweaver-Burk plots (172) were constructed to calculate the K_m and V_{max} values of each substrate. The range of substrate concentration used for this purpose is shown in Table 6.

(3) Sephadex-gel filtration: Sephadex G-100 and G-200 columns (2.4 cm. X 50 cm.) were prepared as described by Andrews (173). The columns were eluted with McIlvaine buffer, pH 5.5, containing 0.1M-KCl, at a flow rate of 30 ml./hr., and fractions (4ml.) were collected. The columns were calibrated by determination of the elution volumes of a number of reference proteins of known molecular weight. Sephadex G-100 columns were calibrated at 20° and Sephadex G-200 columns at 4°. The results were expressed in terms of R_{SA} values (i.e. elution volume of bovine serum albumin/elution volume of the protein). The recovery of enzyme activity on gel-filtration was always 80-90%. The fractions were concentrated in dialysis sacs using Aquacide (Calbiochem, California) as the absorbent.

(4) Disc electrophoresis: Electrophoresis on polyacrylamide columns was carried out by the method of Steward *et al.* (174) with a Shandon apparatus. The Reservoir buffer (tris-glycine, pH 8.3) was prepared as described by Davis (175). The enzyme preparations (25 μ g. of protein) in 4% (w/v) sucrose solution were applied to the polyacrylamide columns and electrophoresis was carried out for 90 min. at 100v and 3 mA/tube.

(5) Ultracentrifugation study: Sedimentation-velocity experiments were carried out by Dr. S.P. Spagg of the University of Birmingham with a Spinco model E ultracentrifuge. The enzymes were examined in McIlvaine buffer, pH 5.5, containing 0.1M-KCl, and the concentrations of enzymes I and II were 0.27% and 0.31% respectively.

(6) Inhibition experiments: The inhibition studies were carried out with α -galactosidase I in McIlvaine buffer, pH 4.0 using PNPG (0.6 mM final

concentration) as substrate. The inhibition of enzyme I by sugars, metal ions and group specific reagents was instantaneous and thus no pre-incubation with inhibitor was required. In the case of *p*-hydroxymercuri-benzene sulphonic acid, the enzyme-inhibitor digest was pre-incubated until "equilibrium" was attained (20 min.) i.e. there was no further change in activity.

(7) Photo-oxidation: In this experiment, a test tube (1cm. X 5 cm.) containing 2 ml. of a solution which consisted of 1 μ g. α -galactosidase I, 0.02% methylene blue and McIlvaine buffer (pH 6.0) was placed at a distance of 10 cm. from two Shandon "photopol" fluorescent lamps. The solution was kept cool with ice-water during illumination. Aliquots (0.2 ml.) were withdrawn at various times and assayed for enzyme activity using PNPG as substrate. Parallel experiments were performed while bubbling air through the reaction mixture. In all cases, control experiments were carried out in the absence of light.

(8) Estimation of sugars during germination: Dormant broad beans (500g.) were treated with a bleaching powder suspension and washed thoroughly with water. A beaker containing the seed was left for 12 hr. under running water, which was continuously aerated, and then transferred to an incubator at 28° with continued aeration. The water in the beaker was changed once every 24 hr. to inhibit microbial contamination. Samples of seeds (100g.) were taken out at various time intervals, the testas removed and cotyledons and embryos then crushed into fine slurries. These were washed three times with 200 ml. volumes of ether. The defatted powders were refluxed for 30 min. in boiling ethanol (500 ml. 70%) and the extracts filtered off and concentrated to 25 ml. The residues on the filter paper were dried under vacuum and weighed. Aliquots (100 μ l.) of each concentrated extract were streaked on Whatman No. 3 papers (washed) and the papers developed for 48 hr. with ethylacetate : propane-1-ol : water (1:7:2, by vol.) solvent. Standard sugars, *ie.* verbascose, stachyose, raffinose, melibiose, sucrose, glucose and galactose, were also applied to each chromatogram. The separated sugars were eluted

off the papers with water and then estimated by the phenol-sulphuric acid method (176).

(9) Levels of α -galactosidase during germination: The seeds were germinated as described above and samples of 15-20 g. seeds were taken at various times for enzyme extraction. The testas were removed and the remaining tissues macerated with McIlvaine buffer, pH 4.8 (5 ml.) and the suspensions then allowed to stand at 5° overnight. After centrifugation the enzyme activity in the supernatant solutions was estimated (at pH 4.8) using PNPG as substrate (see p. 21). Activities were determined in the embryos and the cotyledons separately. Similar estimations were made when the seeds were 'germinated' under nitrogen. The effects of gibberellic acid, indole-acetic acid and kinetin on the levels of α -galactosidase during ^{normal} named germination were also examined. In these experiments the seeds were soaked in solutions of the growth promoters.

For determination of the levels of α -galactosidase I and II during normal germination, 6 seeds (without testas) of medium size were crushed in McIlvaine buffer, pH 5.5, with a pestle and mortar. The extract, after adjustment of the pH to 5.5, was centrifuged and 2.0 ml. of the supernatant solution applied to a Sephadex G-100 column. Gel-filtration was effected as described on p. 24.

(10) Histochemical localization of α -galactosidase: A stock solution of the chromogenic substrate, 6-bromo-2-naphthyl α -D-galactoside, was made in ^{N,N}dimethylformamide (10 mg. in 0.3 ml.). The standard incubation medium, prepared in a small beaker, consisted of McIlvaine buffer, pH 5.5, (2.5 ml.) and distilled water (7.5 ml.). Into this mixture, at 70°, was pipetted 0.1 ml. of the stock substrate solution. The mixture was cooled to about 37° and sections of Vicia faba seed tissues * were floated on to the surface and incubated for 30 min. keeping the temperature at ca 37°.

*Before cutting the sections, the beans were fixed at 0-4° in 3% neutral formalin overnight.

The sections were rinsed with distilled water and placed in a beaker containing Fast blue B salt (tetrazotized diorthoanisidine (1mg.), in McIlvaine buffer, pH 7.5 (1ml.)). The stained sections were washed with water and mounted in glycerine for microscopic examination.

(11) Purification of α -galactosidases: The following steps were carried out at 0-4°, unless stated otherwise.

(a) Extraction: In a typical experiment, 500g. of testa-free dormant broad beans were powdered in a coffee grinder, soaked overnight in McIlvaine buffer, pH 4.8 (300 ml.) and then centrifuged (5000-8000 r.p.m. for 30 min.) to obtain a clear extract. The residue was extracted twice in the similar way.

(b) Variation of pH: The pH of the enzyme solution was lowered to approximately 3.0 with 1M-citric acid. Efficient stirring was essential at this step. The mixture was kept overnight at 4° and this produced a heavy precipitate of inactive proteins, which were removed by centrifugation at 1000g. for 20 min. The pH of the supernatant solution was adjusted to 4.8 with 1M-disodium hydrogen phosphate solution.

(c) Protamine sulphate treatment: Protamine sulphate solution was added to the enzyme preparation (final conc. 10 mg./ml.) and any nucleic acid precipitated over 24 hr. was removed by centrifugation.

(d) Acetone precipitation: Throughout this step, the temperature was held between -5° and -10°. Acetone at -20° was gradually added to the enzyme solution (at -5°) with stirring, up to a final concentration of 30% (V/V). After 45 min., the precipitated inactive protein was removed by centrifugation at 1000g. for 5 min. The acetone concentration of the supernatant was increased to 60% (V/V) and the resulting active precipitate was centrifuged down and dissolved in McIlvaine buffer, pH 4.8.

(e) Dialysis: The enzyme solution as obtained above was dialysed against water (1L.) and then against McIlvaine buffer, pH 3.5 (diluted 1:5 with water), for 4 days. During this period the buffer (1L.) was changed daily. An active precipitate was formed and this was isolated and dissolved in McIlvaine buffer, pH 4.8. The recovery of activity at this

step was variable with different preparations. The highest recovery was 60%.

(f) First Sephadex G-100 gel filtration: The enzyme solution from step (e) was dialysed against McIlvaine buffer, pH 5.5, containing 0.1M-potassium chloride, and then passed through the sephadex column.

(g) Second Sephadex G-100 gel filtration: The enzyme preparations were then recycled through the sephadex column.

A summary of the whole purification procedure is given in Table 4.

Table 4. Purification of *Vicia faba* α -galactosidases ^c

Recovery was calculated on the basis of the total enzyme activity from the preceding step. The overall recoveries of enzymes I and II were 8.75% and 7.25% and the overall purifications were 3660- and 337-fold respectively.

Step No.	Purification step	Volume (ml)	Enzyme activity (milliunits/ml.)	Protein (mg./ml.)	Sp. activity (milliunits/mg. of protein)	Recovery (%)		
1	Extraction	750	450	37.50	12	-		
2	Variation of pH	800	274	4.00	68.5	65		
3	Protamine sulphate treatment	810	270	3.55	76	100		
4	Acetone fractionation	20	6900	42.00	168	63		
5	Dialysis	5	16560	19.48	850	60		
	Enzyme form	I	II	I	II	I	II	
6	First Sephadex G-100 gel filtration and concentration	2	18837	0.43	43800	2700	45	37
7	Second Sephadex G-100 gel filtration and concentration	2	14768	0.335	44000	4050	78	80

M A I N S E C T I O N

(R E S U L T A N D D I S C U S S I O N)

Purification of α -galactosidases: Several glycosidases other than α -galactosidase have been shown to occur in Vicia faba seeds. The attempts at purification were, therefore, directed to remove the contaminating activities and ultimately to obtain a homogeneous protein preparation. A summary of the purification results is presented in Table 4 and the procedure is described in the experimental section.

The solution obtained at each step of purification was tested for α -glucosidase, β -galactosidase, 'alkaline' invertase, β -glucosidase and α -galactosidase. The ratio of these enzyme activities in the crude extract was found to be 1.0 : 2.0 : 2.1 : 3.0 : 5.0. The acidification stage (step 2) resulted in the complete removal of all glycosidase activities other than α -galactosidase from the crude extract. At this stage it was essential to remove nucleic acids present in the enzyme preparation by protamine sulphate treatment. Some extraneous proteins seemed to be co-precipitated which resulted in a slight increase in the specific activity (Table 4). This treatment proved helpful in achieving efficient purification in later steps.

Step 4, acetone precipitation, increased the specific activity of α -galactosidase two-fold. It also helped in removing fats, phenolic materials and oligosaccharides which were also present in the enzyme preparation. Traces of low molecular weight substances which were still present after acetone precipitation were finally removed in the dialysis step (step 5). As the enzyme was preferentially precipitated by dialysis against a buffer of pH 3.5, it is probable that isoelectric point of the protein is approximately 3.5.

The first Sephadex G-100 gel filtration (step 6) produced two protein fractions with α -galactosidase activity (peaks I and II; Fig. 2). Peak I came off the column close to the void volume whereas II lay within the fractionation range of sephadex G-100. It was therefore possible that peak I might be fractionated further. However, later studies with sephadex G-200 suggested that this peak was, in fact, a single active enzyme (Fig. 3). The fractions of peaks I and II with high specific

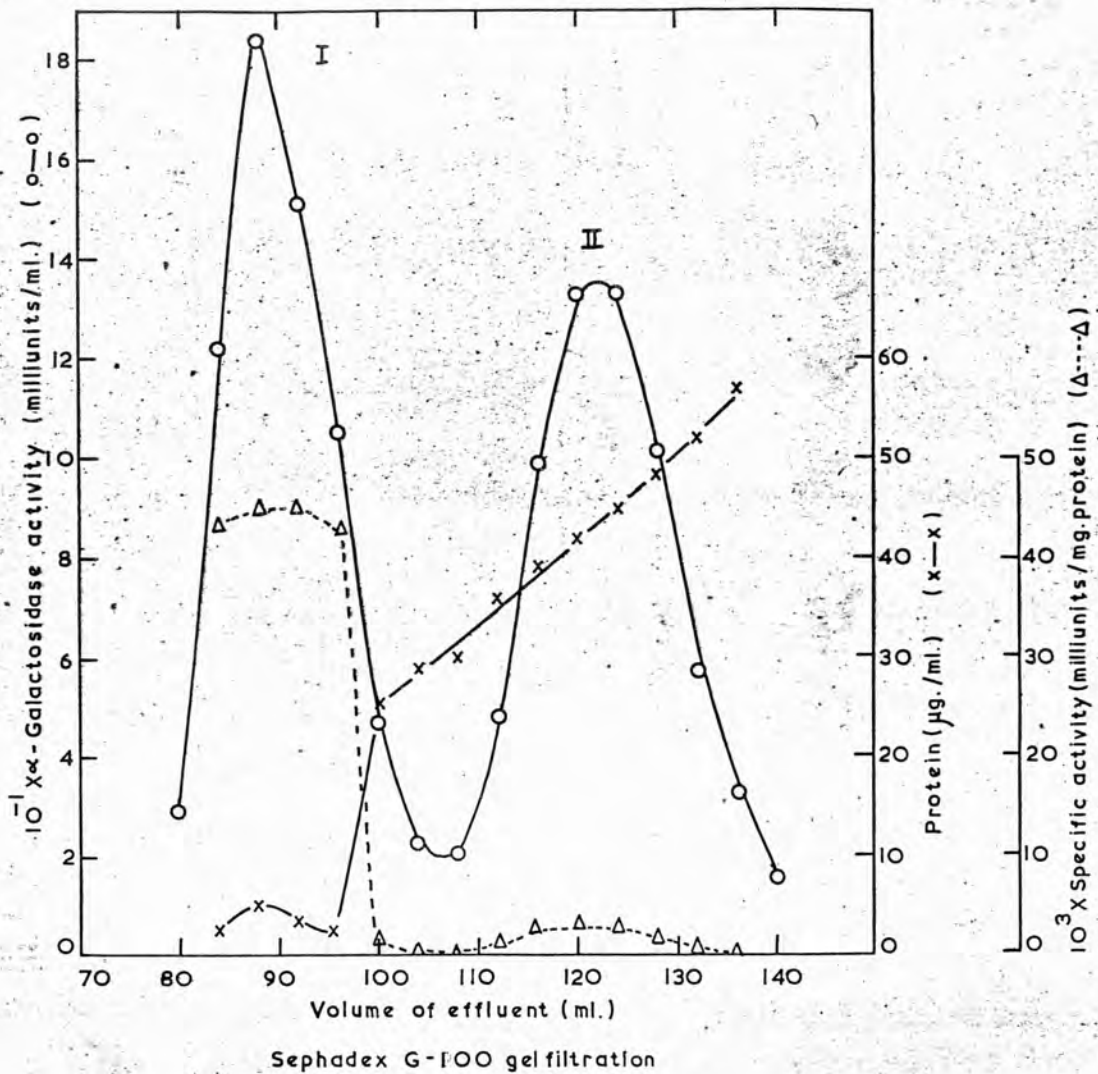
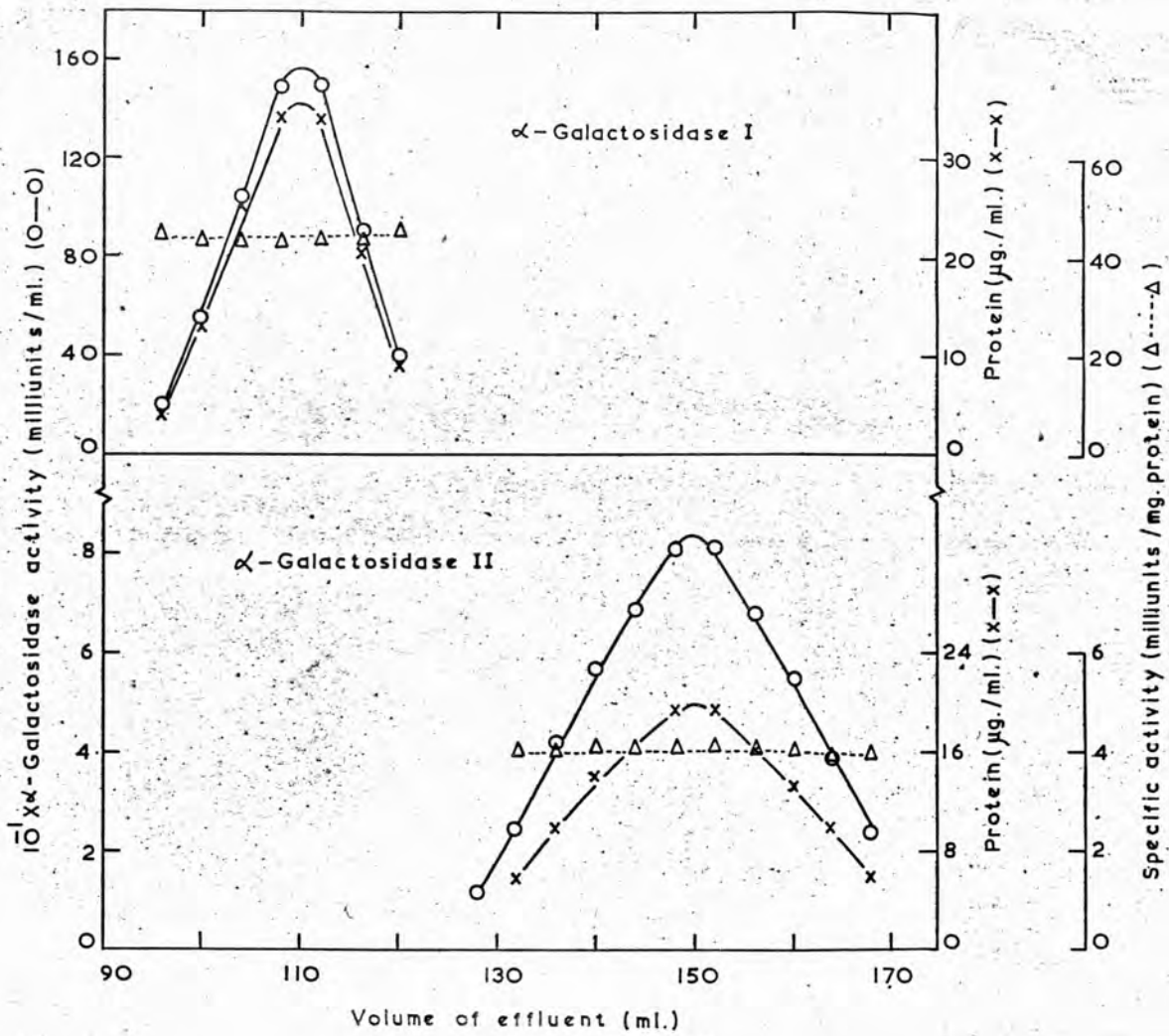


Fig. 2. Separation of two α -galactosidases (I and II) by Sephadex G-100 gel filtration. Enzyme (5000 milliunits) from purification step 5 (see Table 4) was applied to the column. Experimental conditions are given in the text. O, α -Galactosidase activity; X, protein; Δ , α -galactosidase specific activity.



Sephadex G-200 gel filtration

Fig. 3. Sephadex G-200 gel filtration of purified α -galactosidases I (a) and II(b). The procedure and the conditions were the same as those described for Sephadex G-100 filtration. The recoveries of enzymes I and II were 97% and 95% respectively. O, α -Galactosidase activity; X, protein; Δ , α -galactosidase specific activity.

activities were pooled separately and concentrated using Aquacide. Enzymes I and II thus obtained were recycled separately through a well washed Sephadex G-100 column and the fractions with high specific activities were again pooled and concentrated. There was a very little increase in the specific activity of I in this final step (Fig.4). The specific activity of II was one tenth of that of I and further experiments showed that both of these enzymes were homogeneous. The high efficiency of enzyme I could be due to the presence of more than one active centre or a different catalytic mechanism from that of II.

This work has produced the first reported highly purified and homogeneous preparation of α -galactosidases. The two forms of the enzyme do not appear to be artifacts of the purification procedure, as both fractions are found when gel filtration of a crude extract of beans is carried out. In addition purified I and II do not appear to be interconvertible when re-chromatographed on Sephadex G-100 (Fig.4).

Homogeneity of the enzyme preparations: When the final fractions of α -galactosidases I and II were re-chromatographed on Sephadex G-200, protein and activity peaks coincided exactly and were symmetrical (Fig. 3) and the specific activities of the preparations applied to the columns were completely recoverable. Thus it would appear that any extraneous proteins if present in the enzyme preparations, must be of the same molecular weights as those of the enzymes.

In the ultracentrifuge, α -galactosidases I and II both sedimented as single components and gave symmetrical Schlieren peaks (Fig. 5). The sedimentation coefficients were 5.56S and 3.49S, respectively. The two enzyme preparations also gave single bands when examined by polyacrylamide-gel electrophoresis (Fig. 6). Enzyme I had a lower electrophoretic mobility than II.

All the above results support the homogeneity of the enzymes and that I has a higher molecular weight than II.

Molecular-weight determination by Sephadex-gel filtration: The method of Andrews (173), which is based on the elution volumes of proteins of known molecular weights, was followed with both Sephadex G-100 and G-200

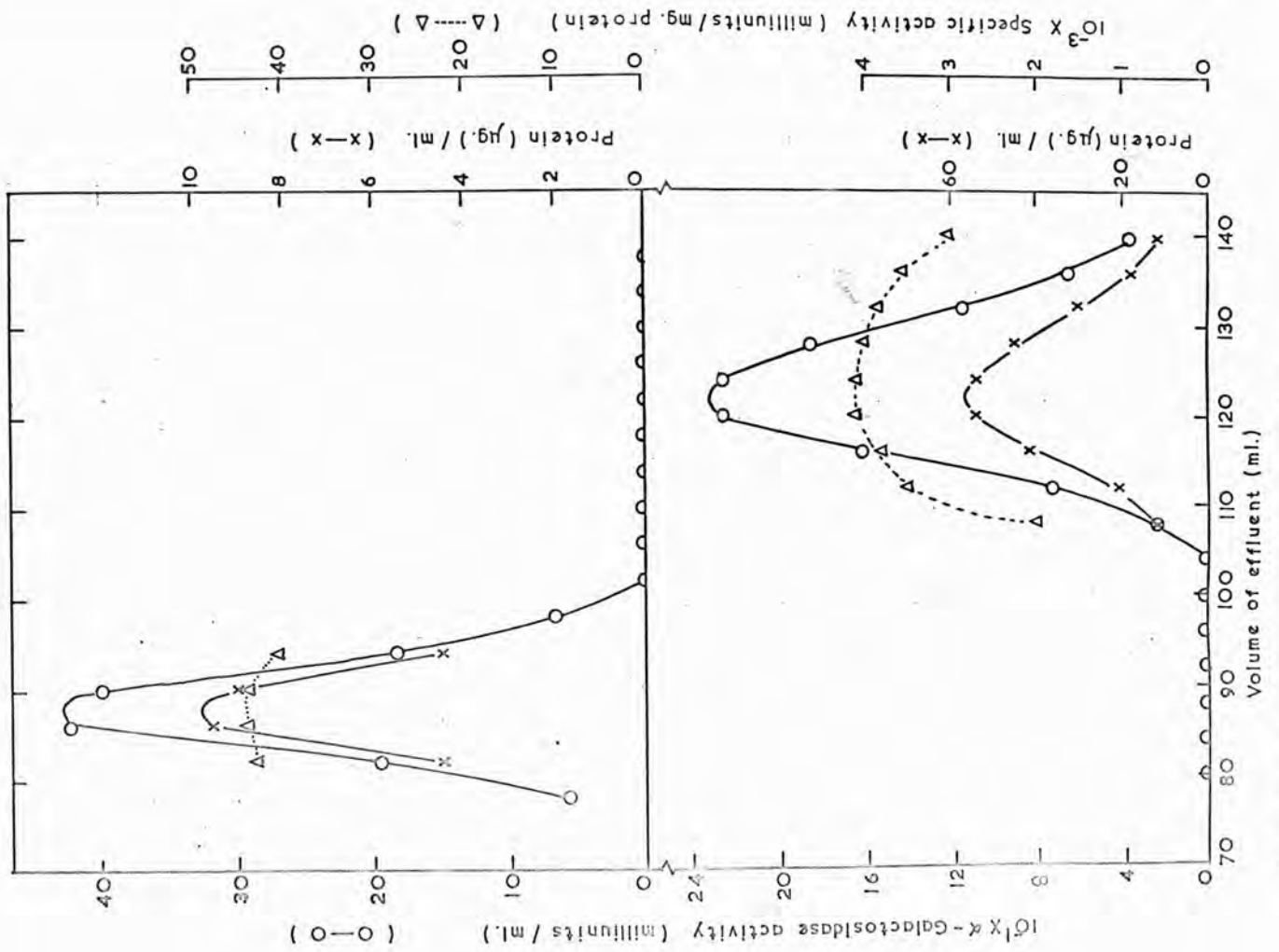


Fig. 4. Second Sephadex G-100 gel filtration



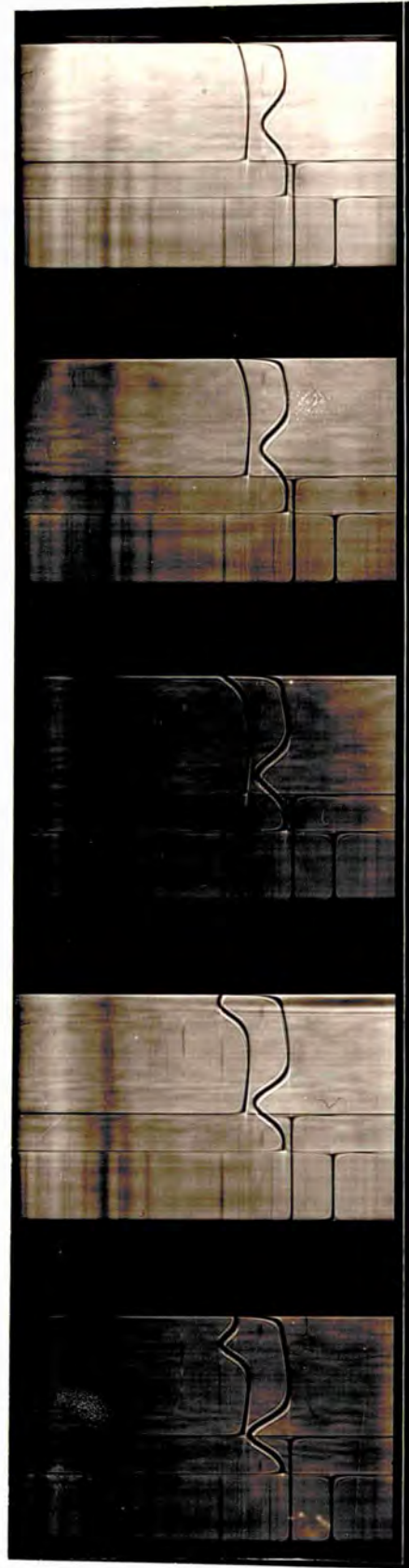
16 min.

24 min.

32 min.

40 min.

48 min.



80 min.

96 min.

112 min.

128 min.

144 min.

Fig. 5 Schlieren pattern of purified α -galactosidase I (top) and α -galactosidase II (bottom) sedimenting from left to right at 20°. The photographs were taken at various time intervals after the rotor had attained maximum speed (50740 rev./min.). Enzymes were examined in McIlvaine buffer, pH 5.5, containing 0.1 M-KCl, and the concentrations of enzymes I and II were 0.27% and 0.31% respectively.

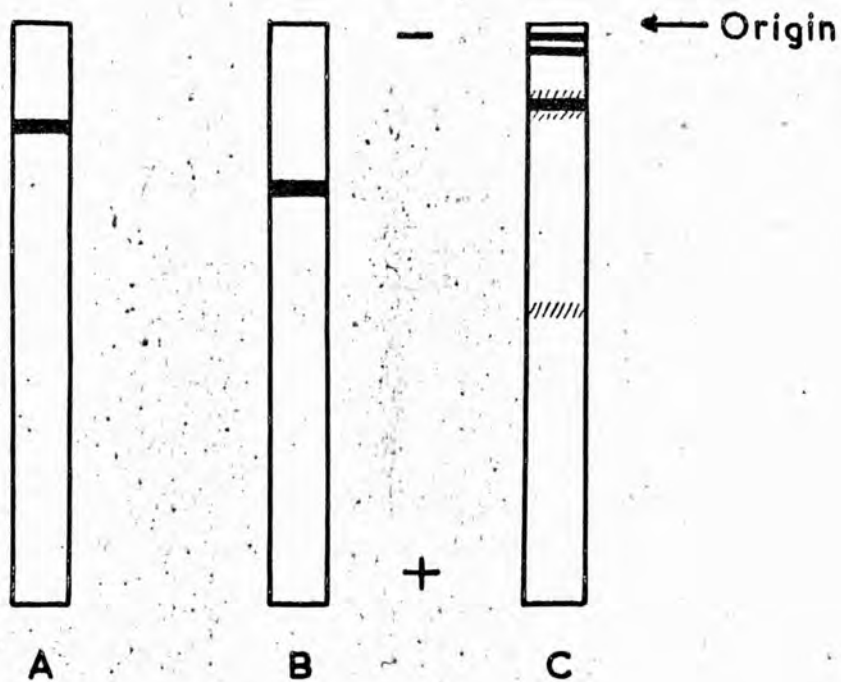


Fig. 6. Polyacrylamide-gel electrophoresis of the purified enzyme preparations. α -Galactosidase I (A), α -galactosidase II (B), α -galactosidase I in presence of 6M-Urea (C). Experimental conditions are described in the text.

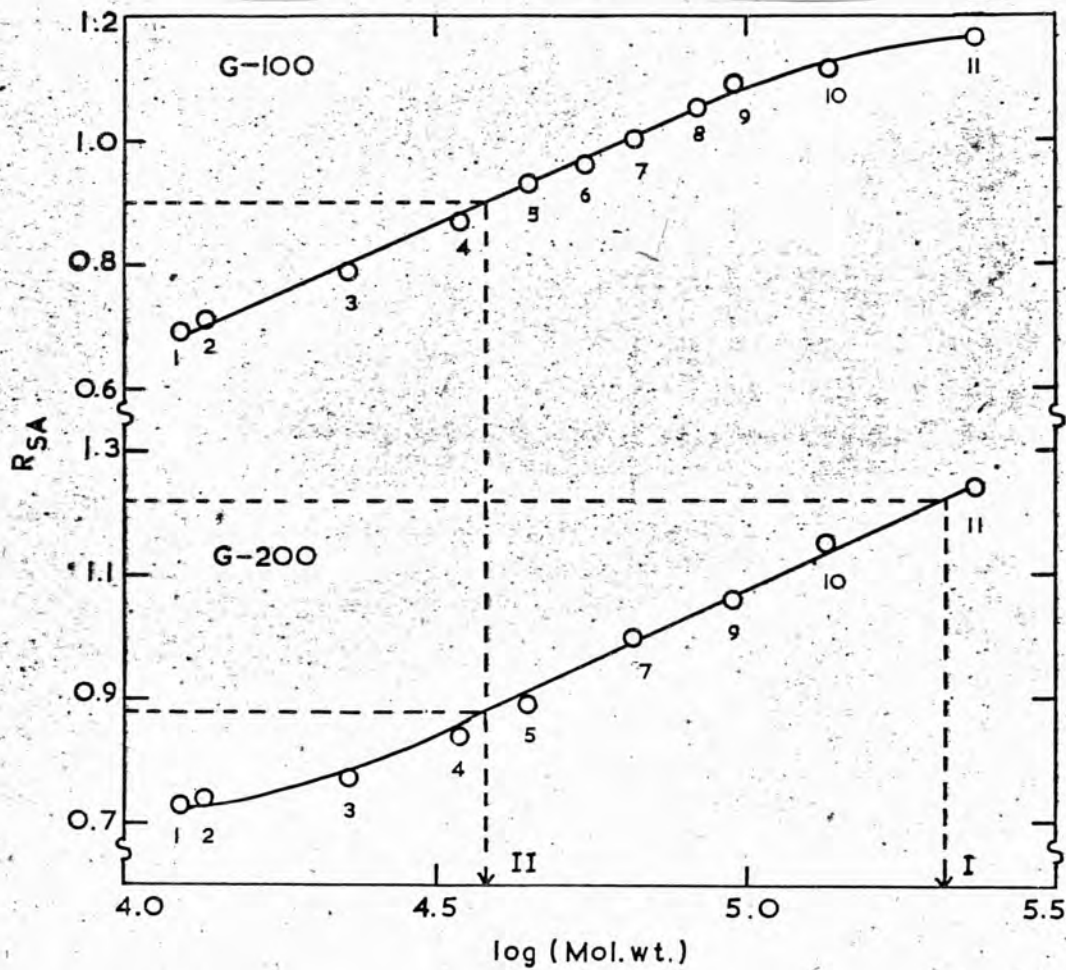
columns. The R_{SA} values were plotted against \log_{10} (molecular weight) of the corresponding proteins as shown in the calibration curves (Fig.7). R_{SA} values were plotted rather than the elution volumes because the latter are influenced by the conditions of experiment and column characteristics which may not be always identical. Difference in these characteristics are more or less eliminated when the R_{SA} values are compared. Moreover R_{SA} shows a direct relationship to \log_{10} (molecular weight) rather than the inverse relationship which occurs with elution volumes.

Assuming that the molecular shape of the α -galactosidases is similar to those of the marker proteins, the apparent molecular weight of enzymes I and II as determined from the calibration curves are 209,000 and 38,000, respectively. The molecular weight of enzyme I was beyond the separation limit of Sephadex G-100 and therefore only G-200 column was used for this purpose.

The carbohydrate contents of enzyme I and II were measured by the phenol-sulphuric acid method (176) and expressed as glucose equivalents; they were 25.0% and 2.8%, respectively. The presence of a high proportion of carbohydrate in enzyme I, therefore, could conceivably interfere with the molecular-weight determination by gel filtration.

α -Galactosidase I, when subjected to Sephadex G-100 gel filtration in the presence of 6M-urea, showed six protein peaks of which two were major (B and C in Fig. 8). Peak A had an elution volume corresponding to that of the native enzyme. The apparent molecular weights of components B and C (cf. 177) were 18,200 and 14,400, respectively. Peaks D, E and F seemed to be low-molecular-weight components and were not within the range of the calibration curve for the particular column used. These estimates, based on gel-filtration data, should, however, be regarded as tentative, as the chromatographic behaviour of proteins on Sephadex gel can be appreciably altered in the presence of urea (178). None of the above protein peaks showed enzymic activity, before or after dialysis against water. The enzyme activity was also not restored by

Fig. 7. Determination of the molecular weights of α -galactosidases I and II by Sephadex G-100 and Sephadex G-200 gel filtration. The procedure is described, and $R_{S.A.}$ is defined, in the text. Proteins used for calibration were: 1, cytochrome c; 2, ribonuclease; 3, α -chymotrypsin; 4, pepsin; 5, ovalbumin; 6, haemoglobin; 7, bovine serum albumin; 8, alcohol dehydrogenase; 9, hexokinase; 10, lactate dehydrogenase; 11, pyruvate kinase.



Calibration of sephadex columns

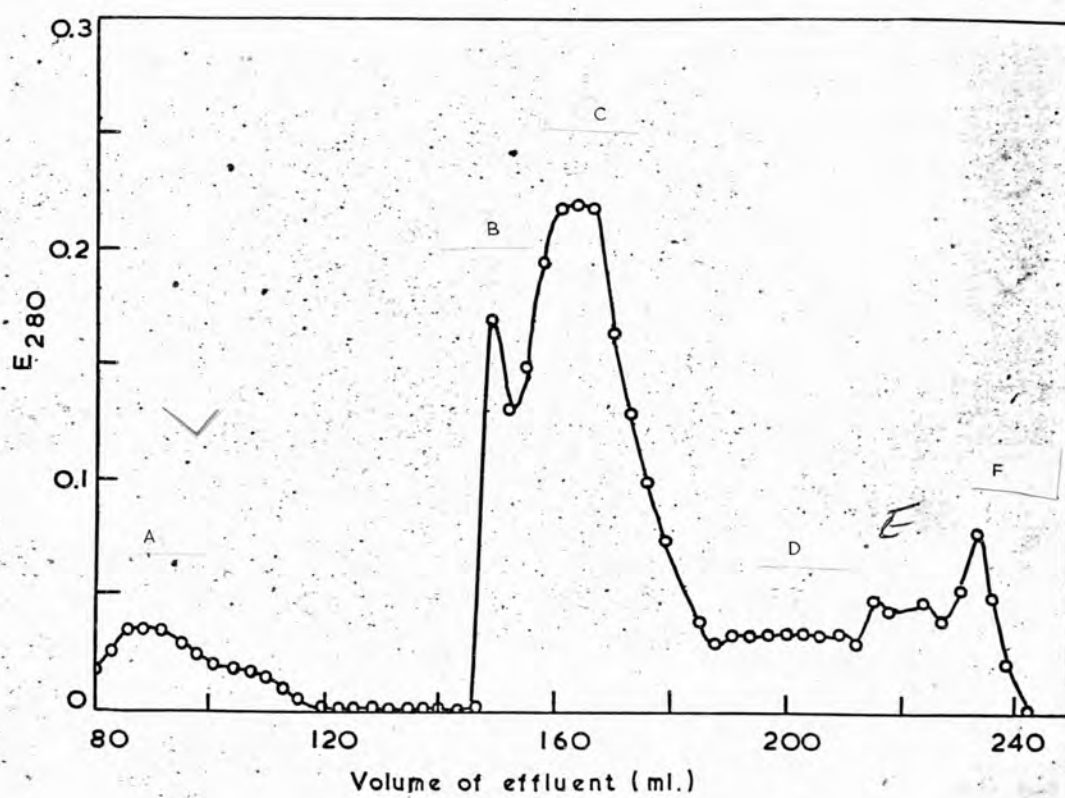


Fig. 8. Gel filtration of α -galactosidase I on a Sephadex G-100 column in the presence of 6 M-urea. The procedure is described in the text.

pooling the fractions followed by dialysis and concentration. This was expected as it was observed that inactivation of this enzyme with 6M-urea was irreversible even on removal of urea by dialysis.

When the enzyme was examined by disc electrophoresis at pH 8.3 in the presence of 6M-urea, three distinct and three diffused protein zones were observed (Fig.6). All these results taken together leads to the conclusion that enzyme I is composed of subunits. The molecular relationship between enzyme I and II, if any, is at present not apparent: the presence of carbohydrate in enzyme I complicates the issue.

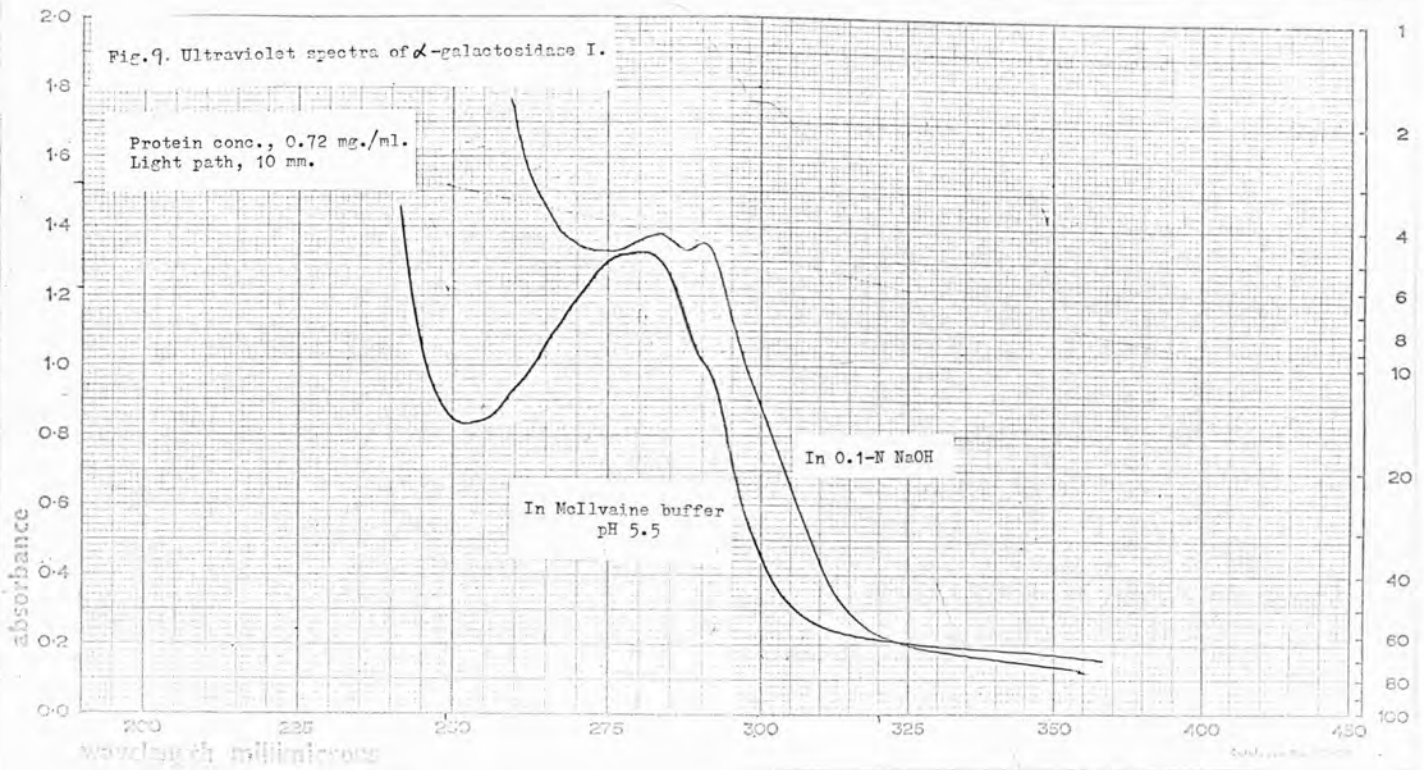
Ultraviolet spectrum: α -Galactosidase I showed an absorption maximum at 280 nm. and an inflexion at 291 nm. (Fig.9). The $E_{280}^{1\%}$ was 18 and the E_{280}/E_{260} ratio was 1.4. α -Galactosidase II had an absorption maximum at 278 nm. with an inflexion at 284 nm. (Fig.10). The $E_{278}^{1\%}$ and $E_{280}^{1\%}$ were 20 and 19, respectively, and the E_{280}/E_{260} ratio was 1.3.

Tyrosine and tryptophan residues were determined spectrophotometrically by the method of Beneze and Schmid (179), and the respective values were 51 and 47 moles/mole of α -galactosidase II,* If proper correction for the carbohydrate content in enzyme I is made, the respective values of the two amino acids will be then, 13 and 12 moles/mole of enzyme.

Thermal stability: The results of thermal inactivation of α -galactosidase I and II are presented in Table 5 and the patterns for a 15 min. incubation at the given temperature are shown in Fig.11. Enzyme I is heat-resistant over a wider range of temperature than enzyme II, but with the former there is an initial 15% loss of activity, whereas enzyme II appears to be very stable within the range 35-45°. The glycoprotein nature of enzyme I could account for its thermal stability. Speculations on the function of the polysaccharide moiety include the concept that it stabilizes an enzymatically active conformation in the polypeptide chain (180). Arnold (181), working on yeast invertase showed that there was a correlation between the heat stability and the polysaccharide content of the enzyme preparation.

* and 13 and 9 moles/mole of α -galactosidase II.

Fig. 9. Ultraviolet spectra of α -galactosidase I.



CONCENTRATION	RECORDED BY	RECORDED DATE	RECORDED TIME
WAVELENGTH			

10

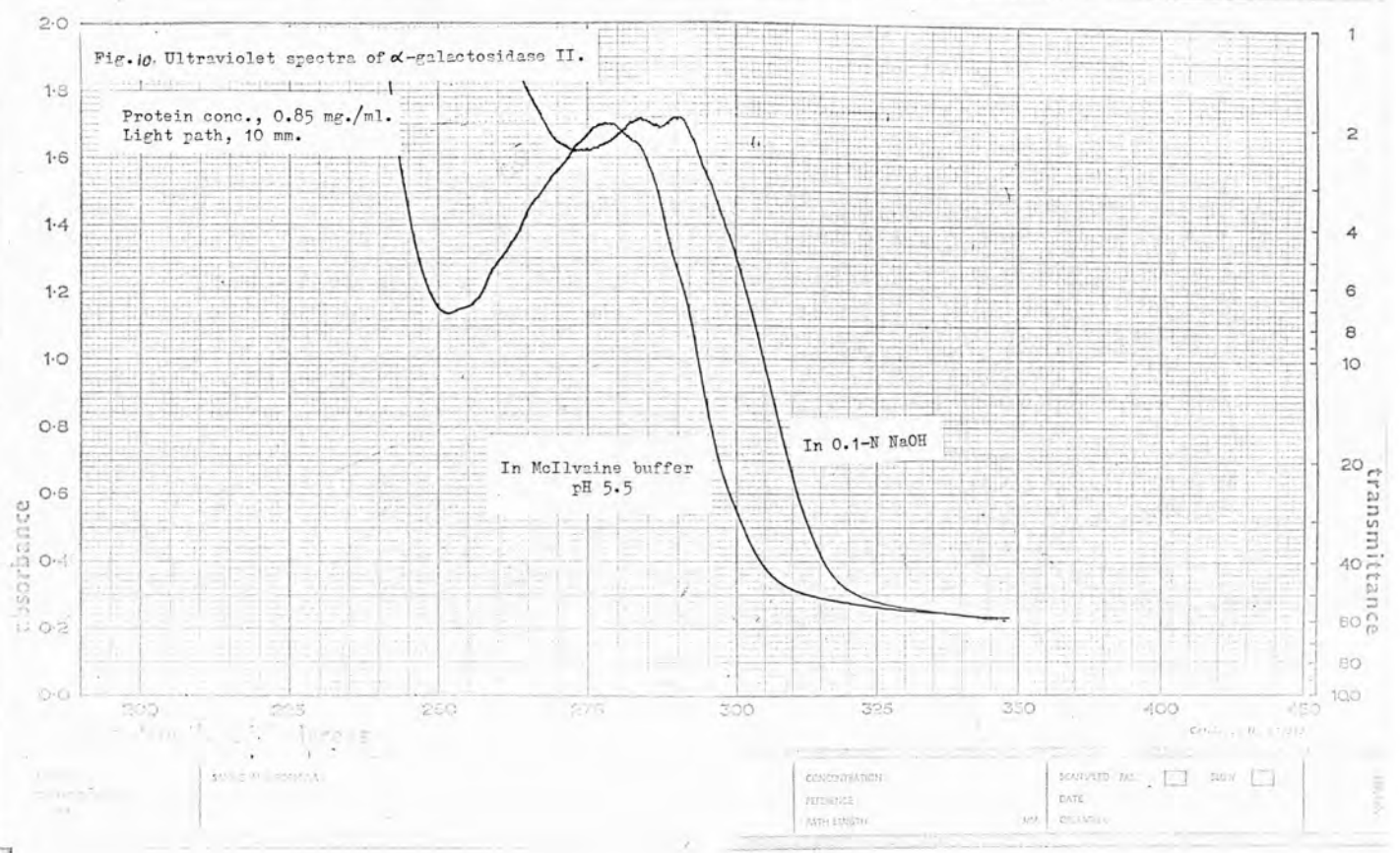


Table 5. Residual activity ($\frac{A}{A} \times 100$) of α -galactosidases I and II at various temperatures. Experimental conditions are described in Fig. 11.

Time (min.)	35°		40°		45°		50°		55°		60°		65°		70°		75°	
	I	II	I	II	I	II	I	II	I	II	I	II	I	II	I	II	I	II
5	91.8	97.4	89.7	100	87.7	100	85.7	97.2	83.6	94.4	82.6	65.0	65.3	34.8	49.0	2.1	10	0
10	86.7	97.4	85.7	100	85.7	100	77.5	94.4	81.6	79.0	77.5	39.0	59.1	14.1	33.6	0	0	0
15	81.6	97.4	79.5	100	79.5	100	73.4	90.2	77.5	75.0	66.3	33.0	53.0	7.6	20.0	0	0	0
20	77.5	95.0	75.5	98.6	75.5	97.2	72.5	86.0	73.4	69.0	62.2	27.7	53.0	0	14.0	0	0	0
30	74.5	97.4	73.4	96.0	69.3	96.0	70.4	84.7	67.3	64.0	58.1	19.4	51.0	0	7.1	0	0	0
40	74.5	97.4	73.4	94.4	67.3	94.4	65.3	83.3	63.2	61.0	54.0	14.0	45.0	0	0	0	0	0
60	73.4	97.4	71.4	94.4	66.3	93.0	63.2	82.0	61.2	58.0	53.0	9.7	38.7	0	0	0	0	0

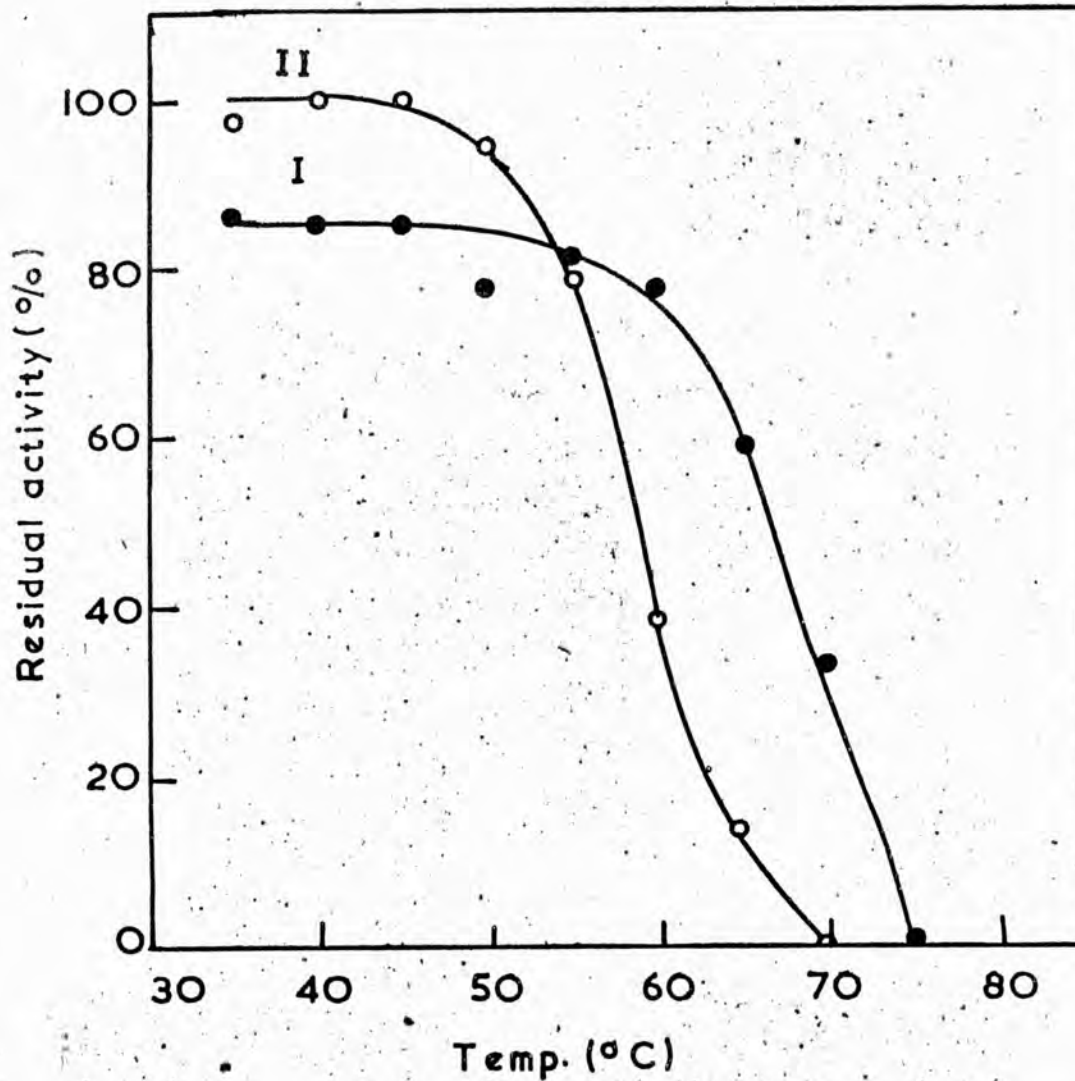


Fig. 11. Thermal inactivation of α -galactosidases I and II. Enzyme I (50 $\mu\text{g./ml.}$) and enzyme II (600 $\mu\text{g./ml.}$) both in McIlvaine buffer, pH4.0, were maintained for 15 min. at the given temperature and suitable samples were withdrawn and assayed spectrophotometrically with p-nitrophenyl α -D-galactoside as substrate.

Substrate specificity: The V_{\max} and K_m values for the hydrolysis of various substrates by α -galactosidases I and II were determined as described in the Experimental section and the results are presented in Table 6. Both the enzymes were unable to hydrolyse *p*-nitrophenyl β -D-galactoside and *p*-nitrophenyl α - and β -D-glucosides. This shows that the configuration at C-1 and C-4 of the substrate molecule is important. On the other hand, the structure at C-6 of the galactose moiety of an α -D-galactoside seems to be relatively less important in the case of enzyme I as change from $-\text{CH}_2\text{OH}$ to $-\text{CH}_3$ (ie. α -D-galactoside to α -D-fucoside) does not cause any appreciable decrease in the V_{\max} value. Enzyme II hydrolyses *p*-nitrophenyl α -D-fucoside almost three times as fast as the corresponding galactoside. However, complete removal of C-6 (as in β -L-arabinoside) reduces the rate of hydrolysis by enzyme I but has little effect in the case of enzyme II. The affinity ($1/K_m$) of substrate for both the enzymes is largely dependent on the structural changes in the glycone moiety and follows the order, α -D-galactoside $>$ α -D-fucoside $>$ β -L-arabinoside. This suggests that one of the specific points of binding of the substrates with the enzymes may be through the primary alcohol group of the galactose moiety.

The variation in the structure of the aglycone residues of the α -D-galactosides affected the K_m and the V_{\max} values for both of the enzymes. The hydrolyses of aromatic galactosides proceeded more readily than that of alkyl derivatives. Relationships between K_m and V_{\max} values were highly irregular with changing aglycon^es. For example, in Table 6 comparisons of the values for raffinose, melibiose and *n*-propyl α -D-galactoside and of phenyl and *o*-nitrophenyl α -D-galactosides indicate that a high affinity of the enzyme for the substrate is not an essential factor for rapid hydrolysis and vice versa.

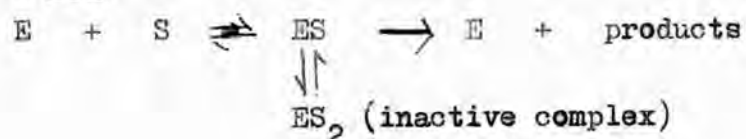
Among the aromatic α -D-galactosides, a bulky bromonaphthyl aglycon^e residue does not reduce the affinity or the rate of hydrolysis to a great extent with either enzyme. With the exception of *m*- and *o*-nitrophenyl galactosides, the nature of the substituent in the phenyl ring does not

Table 6. Specificity of α -galactosidases from *Vicia faba*.

Substrate	α -galactosidase I		α -galactosidase II		V_{max} ratio (I/II)	K_m ratio (I/II)	[S] in assay (mM)
	V_{max} (μ moles min. ⁻¹ mg. ⁻¹)	$10^3 \times K_m$ (M)	V_{max} (μ moles min. ⁻¹ mg. ⁻¹)	$10^3 \times K_m$ (M)			
<u>Glycosyl and stereochemical specificity:</u>							
p-Nitrophenyl α -D-galactoside	25.53	0.38	2.39	0.45	10.68	0.84	0.25 - 2.5
p-Nitrophenyl α -D-fucoside	24.10	4.76	6.96	5.88	3.46	0.81	1.25 - 7.5
p-Nitrophenyl β -L-arabinoside	16.40	14.30	2.39	12.50	6.86	1.14	1.4 - 13.475
p-Nitrophenyl α -D-glucoside	Not hydrolysed		Not hydrolysed		-	-	1.5
p-Nitrophenyl β -D-glucoside	Not hydrolysed		Not hydrolysed		-	-	1.5
p-Nitrophenyl β -D-galactoside	Not hydrolysed		Not hydrolysed		-	-	1.5
Sucrose	Not hydrolysed		Not hydrolysed		-	-	15
<u>Aglycosyl specificity:</u>							
Methyl α -D-galactoside	1.66	7.13	0.29	14.30	5.72	0.49	6.25 - 25.0
Ethyl α -D-galactoside	1.66	8.93	0.28	8.00	5.93	1.11	6.25 - 25.0
n-Propyl α -D-galactoside	2.20	6.13	0.27	5.88	8.14	1.04	6.25 - 25.0
Phenyl α -D-galactoside	20.30	1.11	4.36	1.25	4.65	0.88	0.25 - 2.5
p-Cresyl α -D-galactoside	26.00	1.33	2.90	0.78	8.96	1.70	0.25 - 2.5
m-Cresyl α -D-galactoside	24.30	1.38	2.80	2.00	8.68	0.69	0.25 - 2.5
p-Cresyl α -D-galactoside	23.00	1.54	2.43	1.00	9.40	1.54	0.25 - 2.5
p-Aminophenyl α -D-galactoside	26.60	0.95	2.72	0.87	9.77	1.09	0.25 - 2.5
p-Chlorophenyl α -D-galactoside	20.60	0.83	3.30	1.17	6.24	0.79	0.25 - 2.5
o-Nitrophenyl α -D-galactoside	42.10	1.14	2.80	0.69	15.03	1.65	0.25 - 2.5
m-Nitrophenyl α -D-galactoside	5.86	10.00	0.31	2.50	18.90	4.00	0.25 - 2.5
p-Nitrophenyl α -D-galactoside	25.53	0.38	2.39	0.45	10.68	0.84	0.25 - 2.5
6-Bromo-2-naphthyl α -D-galactoside	22.70	1.80	1.66	0.62	13.67	2.90	0.25 - 0.75
α -D-Galactose-1-phosphate	Hydrolysed		Hydrolysed		-	-	10
Galactinol	2.15	0.13	0.72	0.69	2.98	0.18	0.50 - 5.0
Melibiose	2.54	0.96	0.41	0.77	9.16	1.24	0.50 - 50.0
Raffinose	28.40	4.00	4.18	5.00	6.79	0.80	0.25 - 25.0
Stachyose	9.00	7.50	1.36	5.26	6.61	1.42	2.5 - 12.5

seem to have a large effect on the V_{\max} . However, the affinity ($1/K_m$) for both enzymes shows some tendency to increase when electron-attracting substituents are present in the phenyl ring of the substrate. A plot of pK_m against the Hammett substituent constant (σ') (182,183) is shown in Fig. 12. In a study on α -glucosidase, Hall *et al.* (182) obtained similar results and explained that the attachment to the enzyme side chains -X through the hydrogen atoms of the sugar hydroxyl groups of the substrate would be facilitated by electron-attracting substituents whereas the bond between glucosidic oxygen and -YH would be weakened; this does not appear to be a very satisfactory explanation however, it is depicted for α -galactosidase in Fig.13. Electronic effects at the hydroxyl on C-4 would be expected to be very weak, for example, and in addition the decrease in the negative charge on the glycosidic oxygen would not favour binding. It would however assist attack by a nucleophile at the active site and hence facilitate the catalytic step. The high degree of scatter of the points in Fig.12 suggests that factors other than electronic effects play some role in enzyme-substrate binding, for example, orientation, molecular size and the degree of hydration of the substituents in the phenyl ring of the substrate molecule.

Effect of substrate concentration: p-Nitrophenyl α -D-galactoside was inhibitory at higher concentrations with both of the α -galactosidases (Fig.14). No substrate inhibition occurred with raffinose and classical Michaelis-Menten kinetics were followed (Fig.15). α -Galactosidase I - catalysed hydrolysis of PNPG has been further examined in more detail in an attempt to explain the abnormal behaviour. One possible explanation of substrate inhibition (*cf.* p.71 in *ref.* 184) may be that for effective enzyme-substrate complex formation, a single substrate molecule must associate with a binding site on the enzyme. At higher substrate concentrations, a second molecule may associate with the enzyme to form an inactive complex *i.e.*



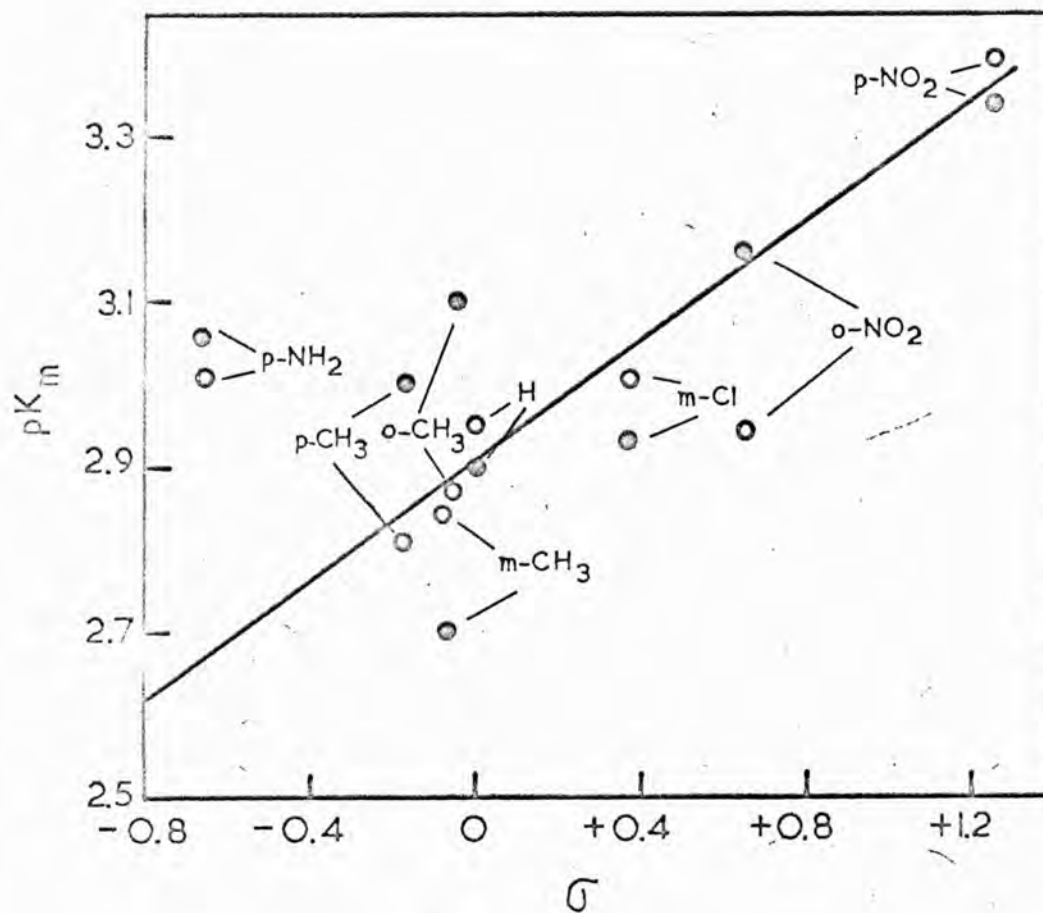


Fig. 12. Effect of substituents on the Michaelis constant of aryl α -D-galactosides. The Hammett's substituent constants (σ) were taken from ref. 122. α -Galactosidase I (○), α -galactosidase II (●).

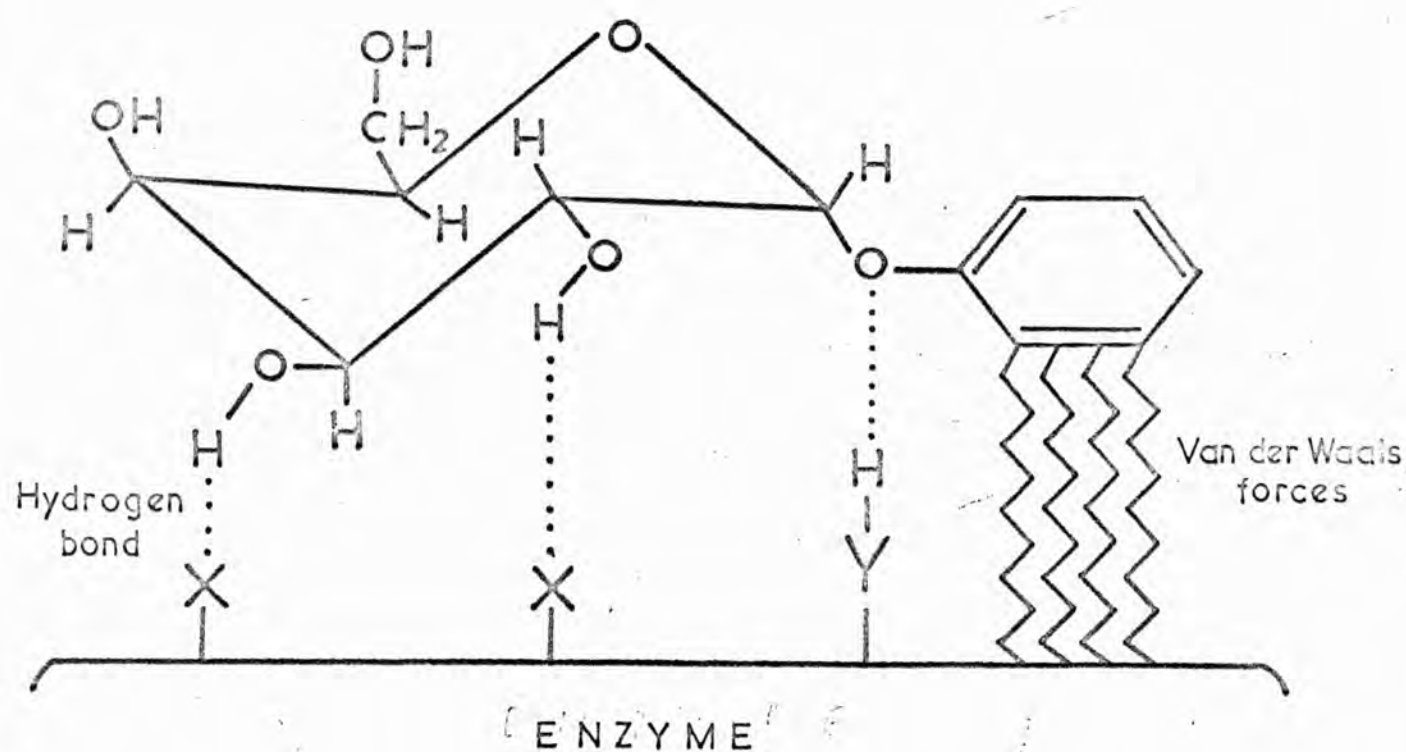


Fig. 13. A possible mode of attachment of phenyl α -D-galactoside to the enzyme (cf. ref. 121). X and Y represent the groups on the enzyme.

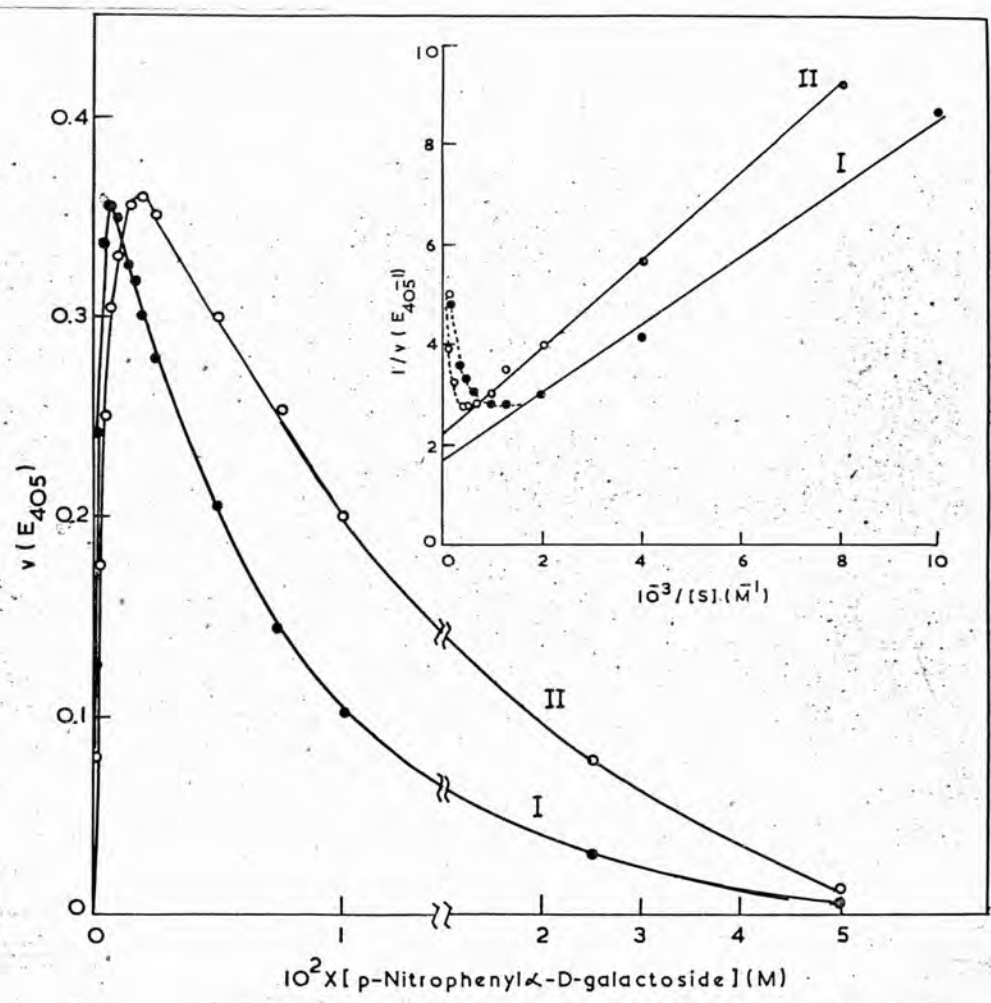


Fig. 14. Effect of substrate concentration on the initial rate of hydrolysis of p-nitrophenyl α -D-galactoside in McIlvaine buffer, pH4.0. α -Galactosidase I (0.5 μ g.) (O) and α -galactosidase II (5.0 μ g.) (O) were incubated under standard conditions with various amounts of substrate and the extent of hydrolysis was measured as described in the Materials and Methods section. A Lineweaver-Burk double-reciprocal plot is given as an inset.

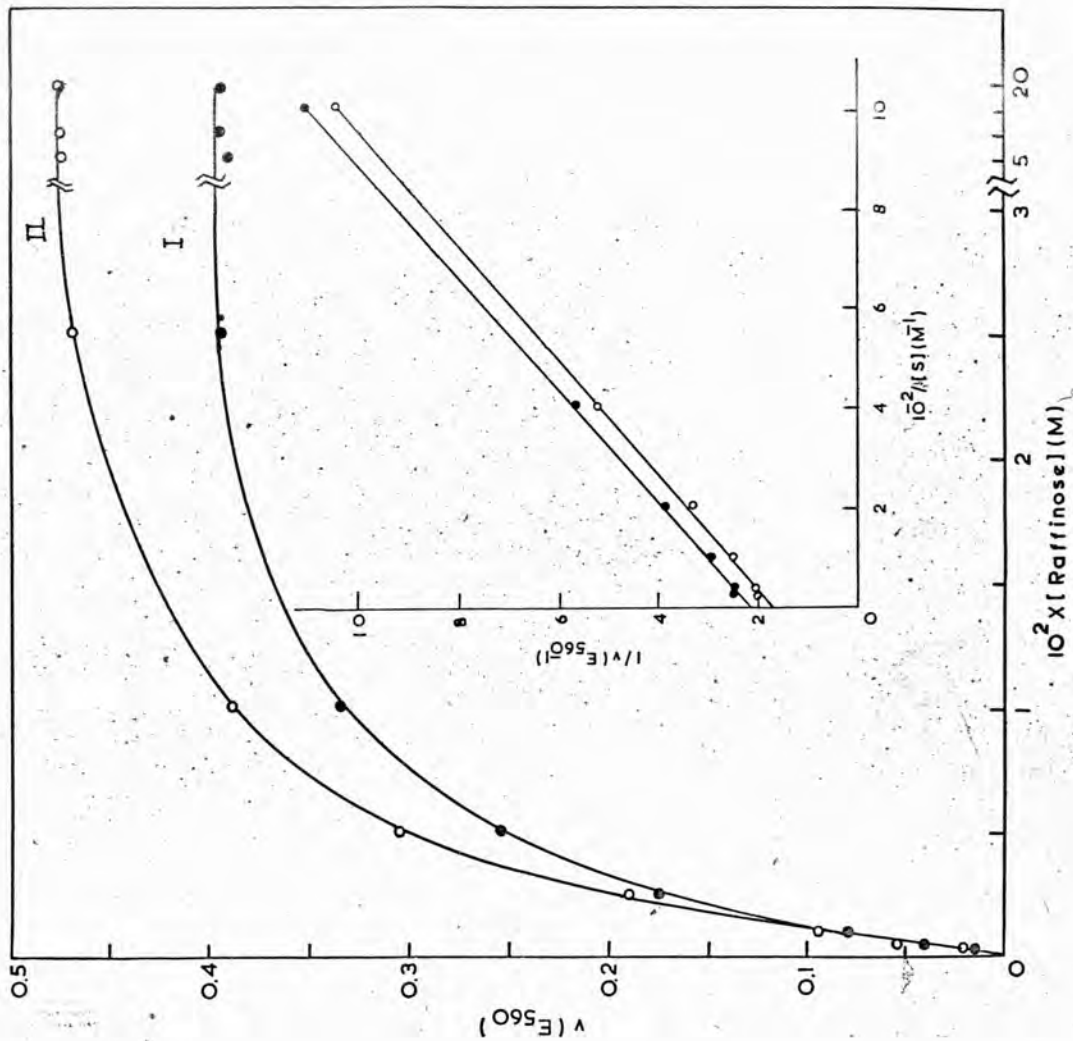


Fig. 15. Effect of substrate concentration on the initial rate of hydrolysis of raffinose. α -Galactosidase I (0.62 μ g.) (●) and α -galactosidase II (5.5 μ g.) (○) were incubated with various amounts of the substrate; conditions were as that in the case of PFG. Liberated galactose was determined by a micro-cuprimetric method (Nelson, 1944) with arsenomolybdate reagents (Nestrin, Feingold & Schramm, 1955). A Lineweaver-Burk double-reciprocal plot is given as an inset.

Hence at very high $[S]$ the rate of product formation would approach zero. Haldane (185) has treated this possibility theoretically and stated that two substrate molecules may be bound by different parts of a single site that should normally bind different parts of the same substrate molecule. Neither molecule is therefore correctly bound so the complex is inactive. In the present study, a plot of the reaction rate against $\log_{10}[PNPG]$ (Fig.16) results in a symmetrical "bell-shaped" curve (open circles) (cf. ref.184, p.72) with little inhibition occurring until $[S]$ reached $7.5 \times 10^{-4}M$. Whilst holding the $[S]$ at this level, galactose was added in increasing concentrations and this resulted in an inhibition curve (closed circles) paralleling inhibition by PNPG. In a separate set of experiments it was found that galactose inhibited the enzyme competitively without requiring a pre-incubation (Fig. 17; $K_i^{-1} = 1.41 \pm 0.05 \text{ mM}$). Glucose did not act as an inhibitor under the same conditions (Fig.16). This discounts the possibility as stated by Dixon and Webb (186)p.78) that the reaction rate of an enzyme acting in an aqueous medium may be reduced at high $[S]$ due to the reduction in the concentration of water. Laidler and Hoare (187) have also excluded such a possibility in their studies with urease. It is, therefore, probable that PNPG-inhibition is a competitive substrate-inhibition. Liberated galactose is unlikely to have a marked effect as it would only be present in low concentrations during the measurements of initial rates.

Methyl α -D-galactoside which exhibits a low V_{\max} with enzyme I, also competitively inhibits the hydrolysis of PNPG (Fig.18). There was no detectable hydrolysis of the inhibitor itself under the experimental conditions used. The K_i for methyl α -D-galactoside ($7.6 \pm 0.2 \text{ mM}$) is very similar to the K_m (7.13 mM) which suggests that both the galactosides (and possibly the whole range of substrates for α -galactosidase reported in Table 6) are bound to the same active site on the enzyme molecule and that the K_m is almost a true representation of dissociation constant (ie. K_s) of the ES complex. Asp and Dahlqvist (188), working with rat

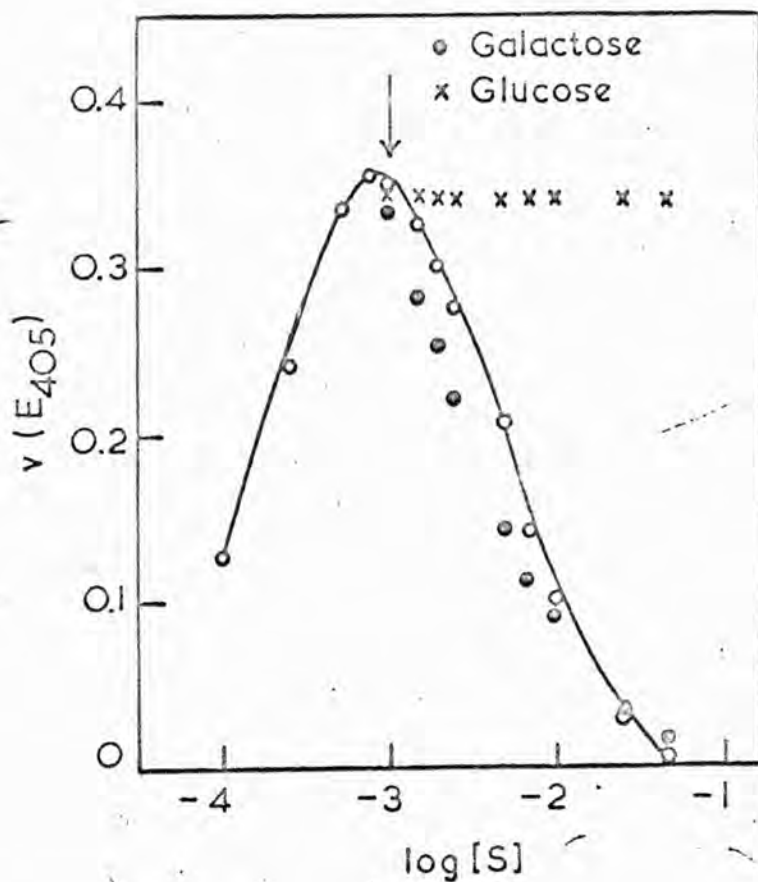


Fig. 16. Inhibition of α -galactosidase I at high concentrations of p-nitrophenyl α -D-galactoside and the effect of galactose and glucose. o, p-Nitrophenyl α -D-galactoside (data from Fig. 14); e, galactose; x, glucose. Details are described in the text.

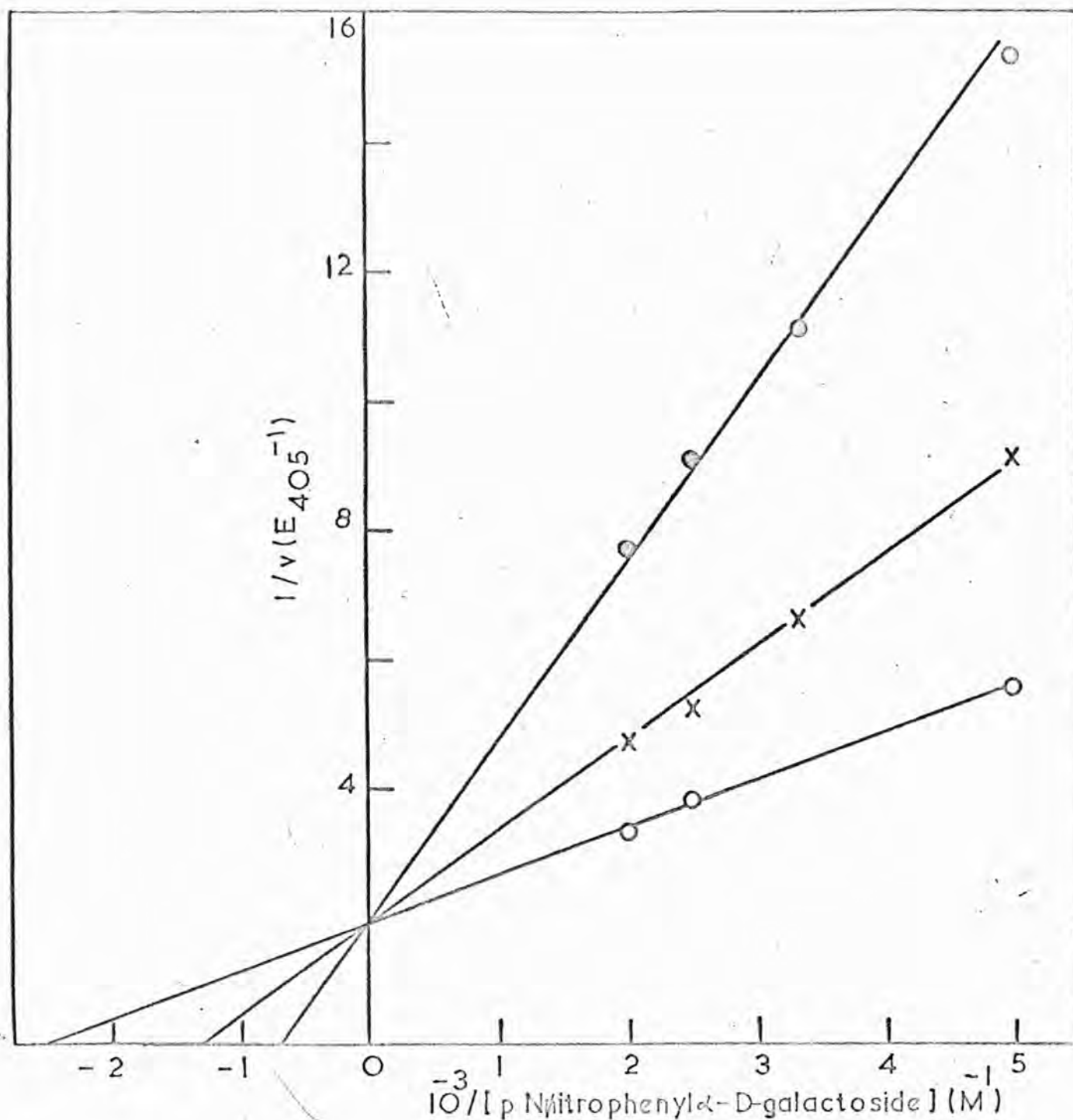


Fig. 17. Inhibition of α -galactosidase I-catalysed hydrolysis of p-nitrophenyl α -D-galactoside by D-galactose. Conditions of assay and procedure are described in the Experimental part. Enzyme activities were determined at different galactose concentrations (o, nil ; X, 1.25 mM ; •, 3.75 mM).

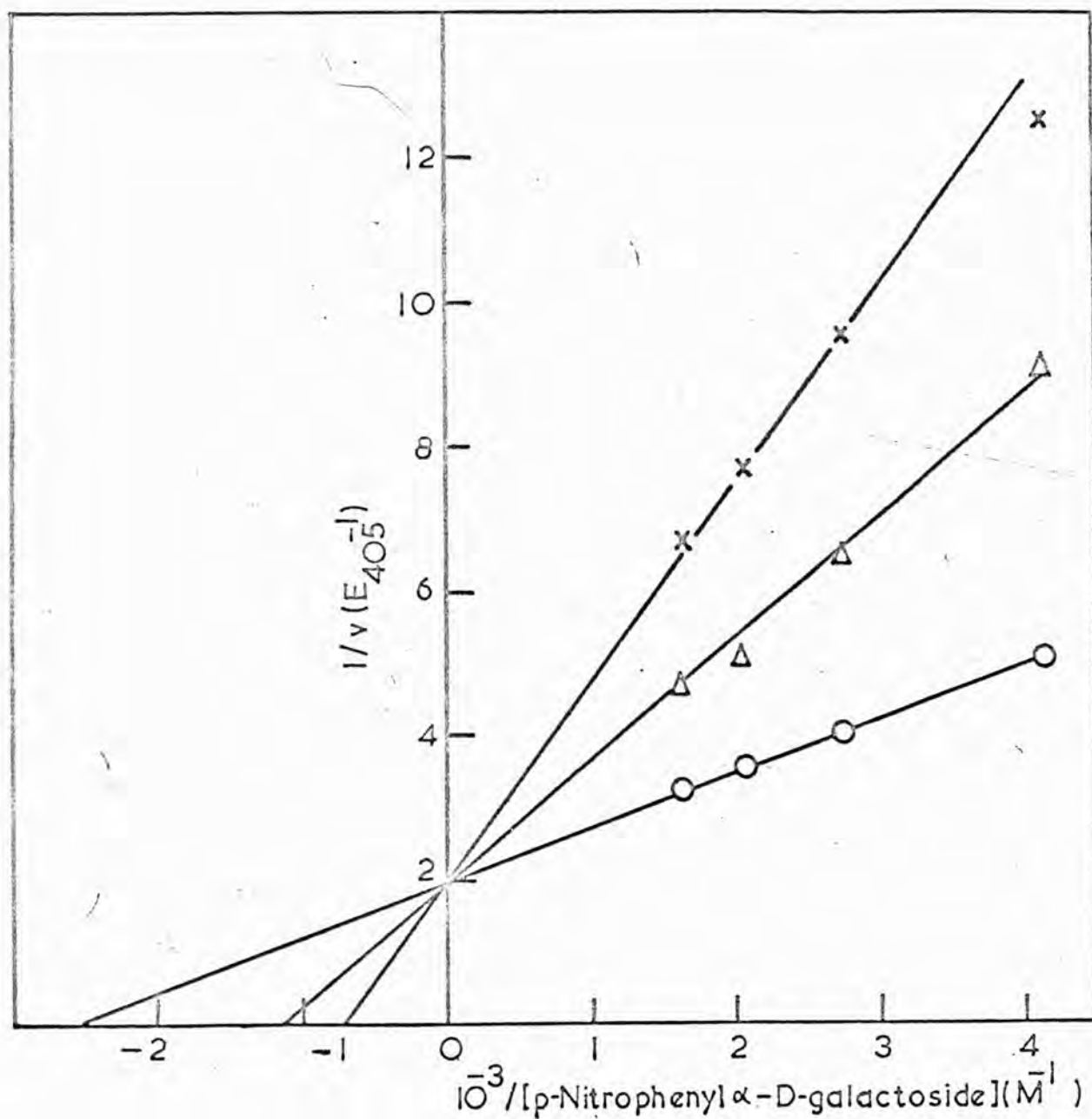


Fig. 18. Inhibition of α -galactosidase I-catalysed hydrolysis of p-nitrophenyl α -D-galactoside by varying concentrations of methyl α -D-galactoside (o, nil ; Δ , 10 mM, X, 20 mM). Conditions of assay and procedure are described in the Experimental part.

intestinal β -galactosidases, have shown that lactose acts as a competitive inhibitor when phenyl β -D-galactoside is used as a substrate and vice versa. The K_i values are approximately equal to the K_m values in the case of both compounds. It was suggested that the β -galactosidases hydrolysed lactose and phenyl β -D-galactoside at the same active site.

Effect of temperature on enzymic action: A rise of 10° caused about 2-fold increase in both V_{\max} and K_m for α -galactosidase I, and about a 4-fold and 3-fold increase in these values, respectively, for α -galactosidase II. The rise in the K_m (i.e. lower affinity) with the rise of V_{\max} in an independent way supports the results obtained from the substrate specificity studies i.e. the nature of the substrate affects K_m and V_{\max} differently. It is, therefore, quite likely that K_m represents the dissociation constant of the enzyme-substrate complex.

The values of $\log V_{\max}$ and $\text{p}K_m$ (i.e. $-\log K_m$) at different temperatures were plotted against $1/T$ and straight lines were obtained for both the enzymes (Fig.19). The linear relationship between the kinetic constants and $1/T$ suggests that K_m and V_{\max} are simple constants rather than complex functions of several velocity constants and that in the case of the α -galactosidases the breakdown of a single intermediate complex is rate determining. The energies of activation calculated from Fig.19a are 15.4 and 27.1 kcal./mole for enzymes I and II, respectively, and the corresponding ΔH values (from Fig.19b) are -13.28 and -16.5 kcal./mole. The ΔS values for I at 30° is, therefore, -32.4 and for II, -43.6 cal./deg./mole. If the K_m is regarded as the dissociation constant of the ES complex (as is indicated by specificity and substrate inhibition studies) the values of ΔH and ΔS would represent the changes occurring during the formation of the Michaelis complex. The negative entropy changes suggest that profound changes in both the enzymes may occur during combination with the substrate and the large change cannot be explained only in terms of electrostatic interactions. The results, therefore, suggest that considerable conformational changes probably occur in the enzymes during the course of reaction.

Effect of pH: Fig.20A shows the pH-activity profile of the α -galactosidases

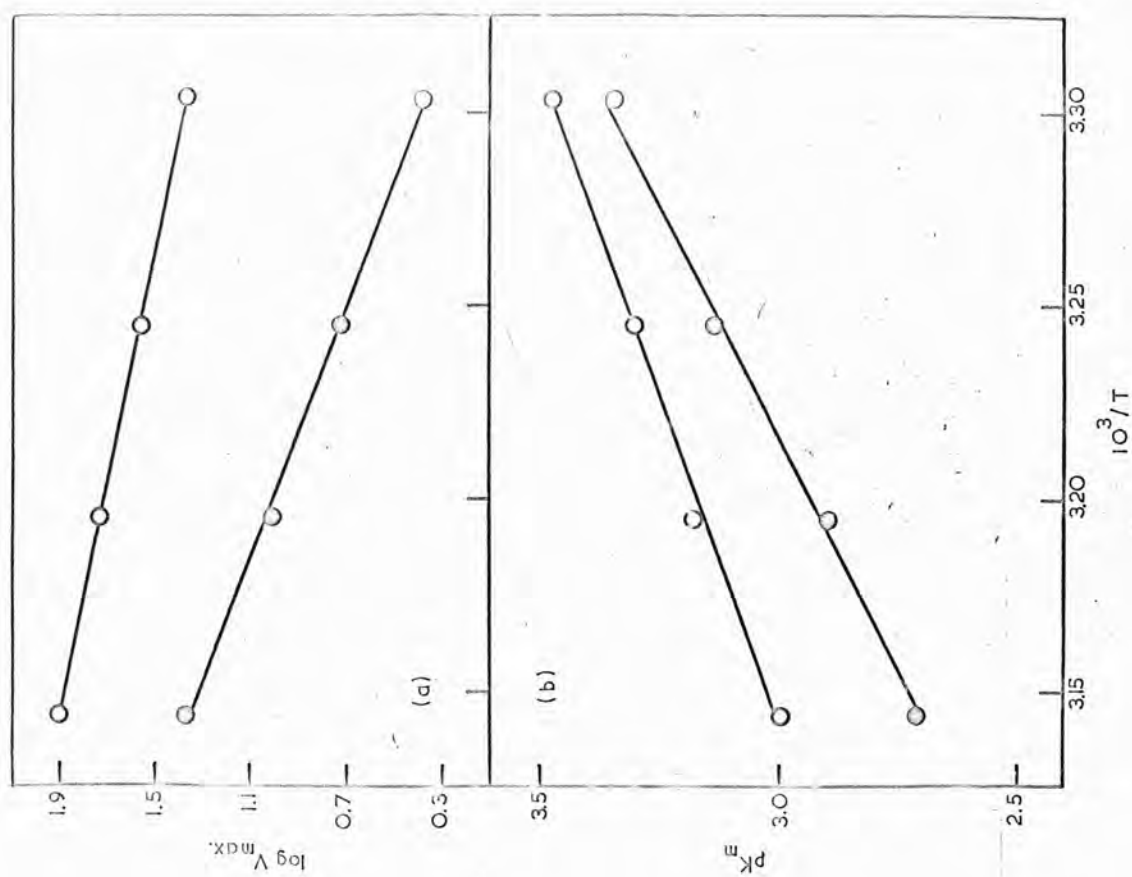


Fig. 19. Effect of temperature on V_{max} and K_m for the hydrolysis of *p*-nitrophenyl α -D-galactoside by α -galactosidase I (a) and II (b) in McIlvaine buffer, pH 4.0.

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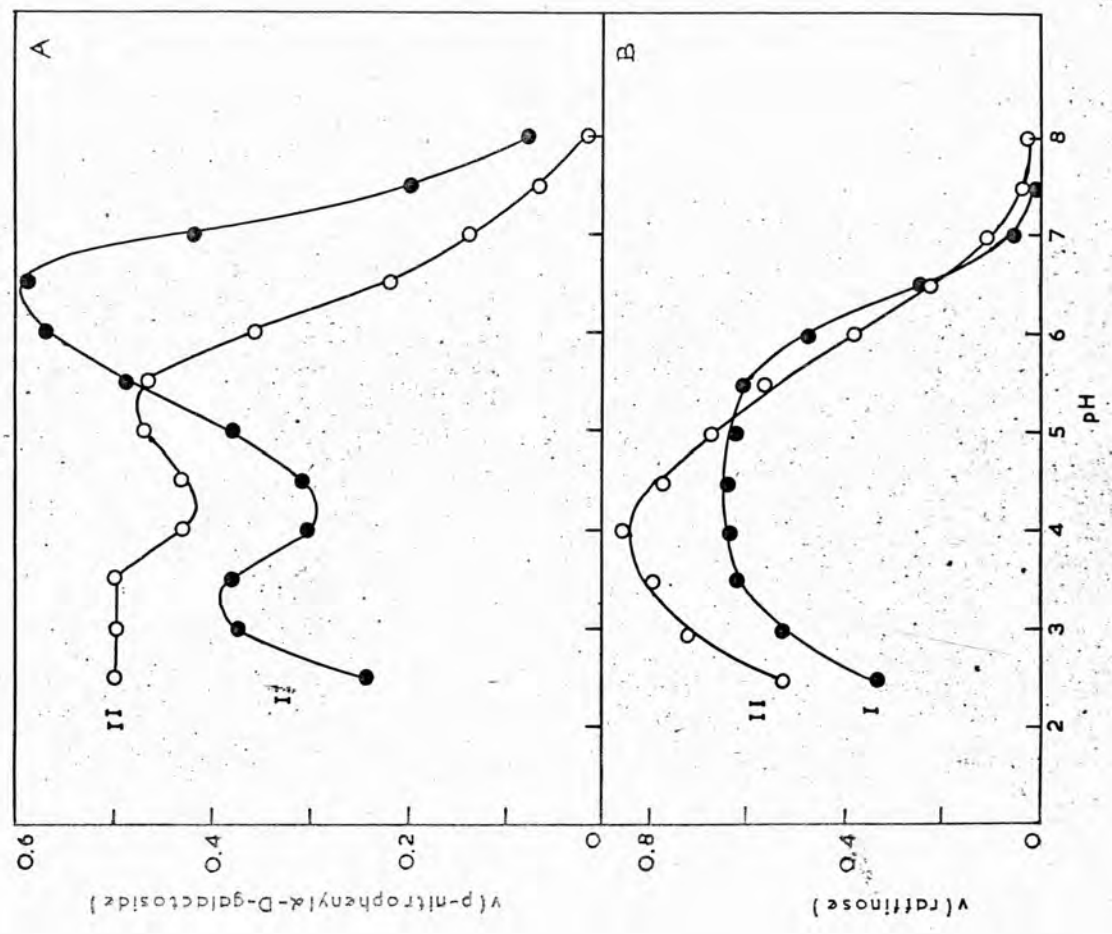


Fig. 20. Effect of pH on the activity of α -galactosidases I and II. (a) Hydrolysis of p-nitrophenyl- α -D-galactoside (1.5mM) measured spectrophotometrically at 405 nm.; (b) hydrolysis of raffinose (5mM) followed by determination of the liberated galactose. McIlvaine buffer with a pH range of pH2.5-8 was used.

with PNPG as substrate. The activity patterns of the enzymes were different when raffinose was used as the substrate (Fig. 20B). α -Galactosidase I has been investigated in more detail. The V_{\max} and the K_m values for both of the substrates were determined over the pH range 2.5-7.5 and the results are presented in Fig. 21 and 22 in the form recommended by Dixon (189). In the pK_m -pH plots the straight line portions (with slope +1, 0, -1) of the curves intersect when they are extended. The application of Dixon's rules (186, p. 134; 189) show that with both substrates the pK values of the ionizing groups existing in the free enzyme (downward bend of the curves) are 2.9-3.4 and 4.7-4.8 and that in the enzyme-substrate complex (upward bend of the curves) there is a group with pK 5.5-5.25. Groups present in the free enzyme with pK values 2.9-3.4 and 4.7-4.8 also appear in the $\log(V_{\max}/K_m)$ -pH plots. The group with pK 4.7-4.8 does not seem to take part in the substrate binding but its pK is shifted (to pK 4.15-5.25) presumably due to the proximity of the substrate in the ES complex. This latter group corresponds quite closely with the pK value (5.1-5.2) obtained from $\log V_{\max}$ -pH curves. The second pK (6.3-6.6) shown in this latter curve would appear also to represent a group present in the ES complex but it does not appear in the pK_m plots and is present in the $\log(V_{\max}/K_m)$ -pH plots (pK , 6.35-6.7). This group, therefore, which exists in the free enzyme, can still ionize when present in the ES complex without any change in pK value. It is therefore not involved in substrate binding, although concerned inactivity. A comparison of the above pK values with those listed by Dixon and Webb (ref. 186, p. 144) indicates the possible participation of an α -carboxyl group (pK , 3.0-3.2), an aspartyl carboxyl group (pK , 3.0-4.7) and a histidine imidazolium group (pK , 5.6-7.0) in the enzyme-catalysed hydrolysis of both PNPG and raffinose.

Photo-oxidation: The pH studies suggest the presence of histidyl group in the active site of the enzyme. This possibility was further examined* in the presence of methylene blue (cf. 190-193). As shown in Fig. 23 irradiation of the enzyme in the presence of this dye results in some loss of activity and this is markedly enhanced when air is bubbled through

* by subjecting the enzyme to photo-oxidation

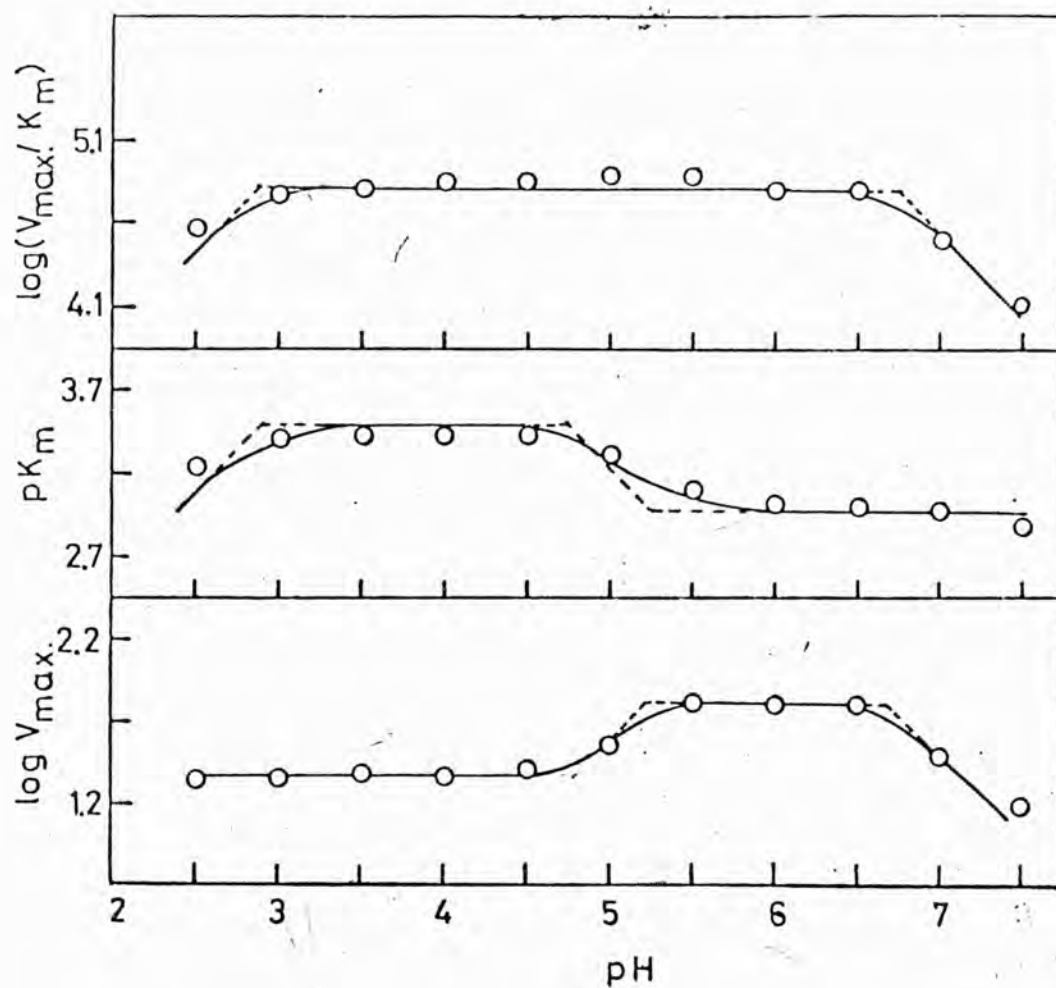


Fig. 21 K_m and V_{\max} as a function of pH for the hydrolysis of p-nitrophenyl α -D-galactoside by α -galactosidase I.

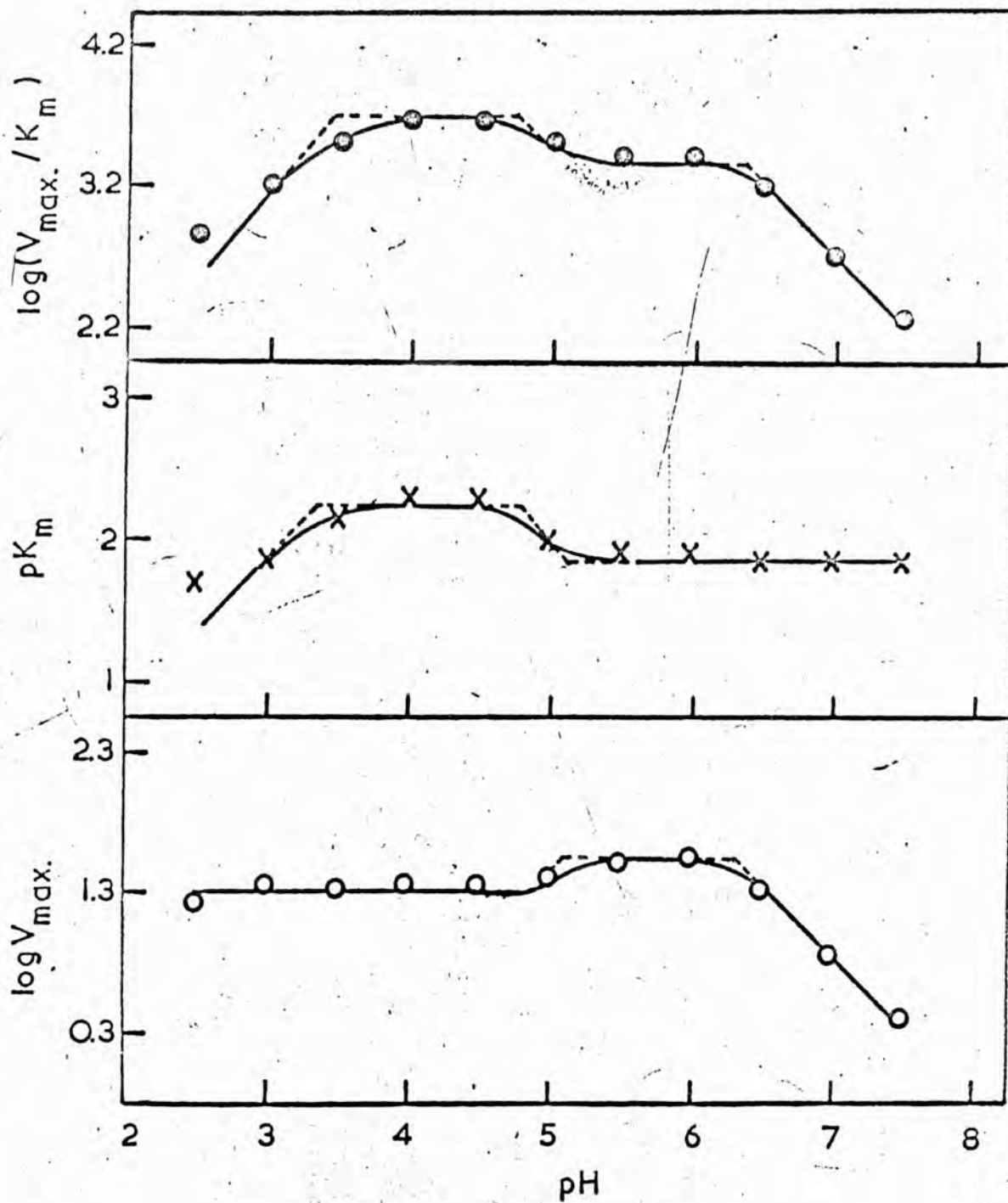


Fig. 22. K_m and V_{\max} as a function of pH for the hydrolysis of raffinose by α -galactosidase I.

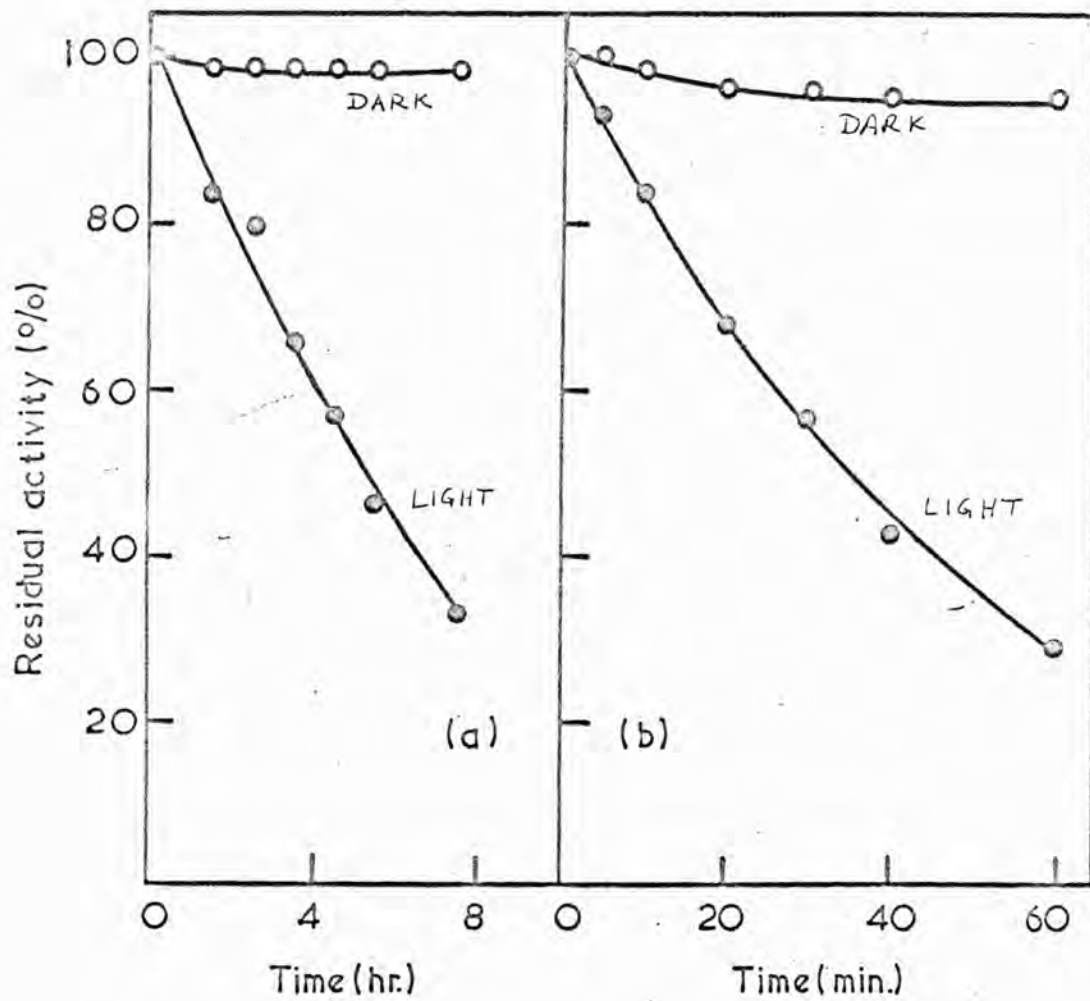


Fig. 23. Effect of photo-oxidation on α -galactosidase I in the presence of methylene blue (a) un-aerated, (b) aerated. Conditions are described in the Experimental section.

the mixture. However, the possible destruction of other residues such as tryptophan and tyrosine by photo-oxidation cannot be ruled out (194-196). In conclusion, it seems highly probable that histidine and carboxyl groups are involved in α -galactosidase I catalysed hydrolyses. Inhibition by -SH specific reagents and heavy metal ions: The results presented in Table 7 show that α -galactosidase I is highly sensitive, without pre-incubation, to heavy metal ions such as Ag^+ and Hg^{2+} but much less sensitive to Cu^{2+} . Although the high sensitivity to these metal ions suggest the participation of a thiol group in the enzyme catalysis, typical thiol reagents (Table 7) failed to inhibit the activity even after a 20 min. pre-incubation. In addition, when the enzyme was titrated with 5,5¹-dithiobis (2-nitrobenzoic acid) (197) no thiol groups could be detected. If thiol groups are present in the protein they must, therefore, be highly inaccessible.

In the absence of -SH at the active site the inhibition by Ag^+ may possibly be attributed to its combination with carboxyl and/or histidine residues. This possibility has been suggested by Dixon and Webb (ref. 186, p.346) and Myrbäck (198). The Ag^+ inhibition was competitive at both pH 4.0 (K_i 4.0 μM) and pH 6.0 (K_i 0.59 μM) (Fig.24). The large decrease in K_i with the rise of pH suggested that the ions were binding with a group that lost protons over this range, i.e. one that was not largely unprotonated at pH 4.0. The fact, however, that the decrease was much less than 100-fold suggested that the group did not have a pK above 6.0. Ag^+ -inactivated enzyme at pH 6.0 regained 83% of its activity when dialysed against McIlvaine buffer, pH 6.0. Dialysis against a buffer containing 0.1M-cysteine resulted in 98% restoration of activity. A similar result was obtained when inactivation followed by dialysis was carried out at pH 4.0.

As shown in Table 8, the inhibitory effect of Ag^+ was reduced when low concentrations of galactose were present. The protection was observed only when galactose was added before the addition of Ag^+ . This confirms that Ag^+ reacts with the active site.

Inhibition by Hg^{2+} usually suggests reaction with thiol groups.

Table 7. Inhibition of α -galactosidase I by heavy metallic ions and thiol specific reagents in McIlvaine buffer at 30°. The experimental conditions are described in the "Experimental" section.

Inhibitor	10^4 x Inhibitor concentration (M)	Inhibition (%)
Ag^+	0.250	76.0
	(pH4.0) 0.125	32.0
	(pH6.0) 0.025	87.9
	0.010	22.8
Hg^{2+}	(pH4.0) 10.00	55.0
	0.60	39.4
	(pH6.0) 0.003	79.3
	0.001	42.7
Cu^{2+}	(pH6.0) 250	45.7
	500	32.7
p-Hydroxymercuribenzene sulphonic acid	(pH4.0) 0.050	57.0
	0.010	29.8
	(pH6.0) 0.200	67.9
	0.100	39.1
<u>N</u> -Ethyl maleimide	(pH4.0) 700	0
	(pH6.0) 700	0
Iodoacetamide	(pH4.0) 250	5.5
	(pH6.0) 250	1.7
Iodoacetic acid	(pH4.0) 250	2.0
	(pH6.0) 250	1.7
5,5'-Dithiobis (2-nitrobenzoic acid)	(pH6.0) 250	6.0

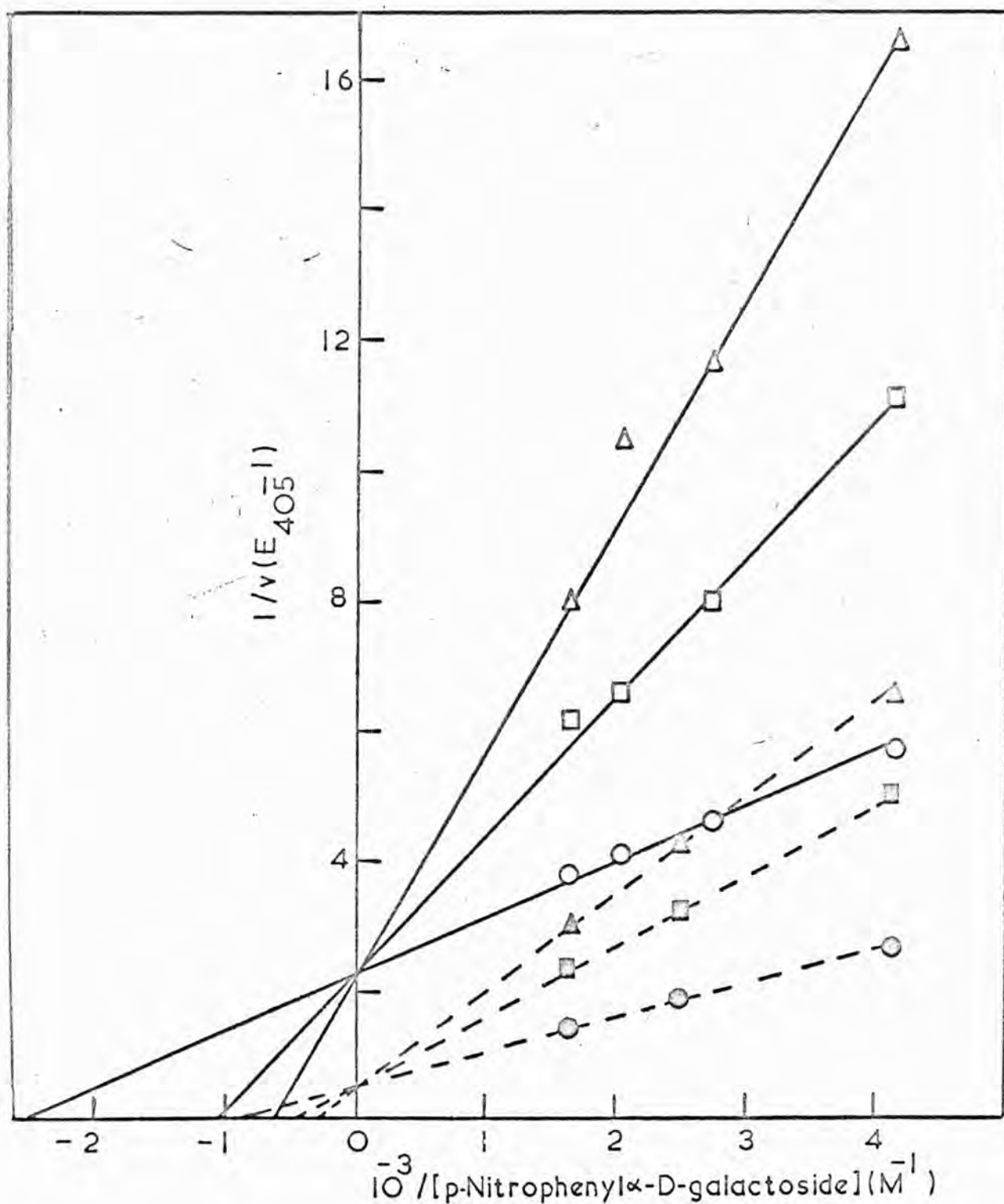


Fig. 24. Inhibition of α -galactosidase I-catalysed hydrolysis of p-nitrophenyl α -D-galactoside by varying concentrations of silver nitrate (\circ, \circ , nil; \blacksquare , 0.75 μM ; \blacktriangle , 1 μM ; \square , 6.25 μM ; \triangle , 12.5 μM). Continuous lines represent hydrolysis at pH 4.0 and broken lines at pH 6.0. Conditions of assay and the procedure are described in the Experimental section.

Table 8. Protective effect of galactose on the inhibition of α -galactosidase I by Ag^+ . Purified enzyme (1 μg protein) was incubated for 5 min. with D-galactose in McIlvaine buffer, pH 6.0 at 30^o and then silver nitrate was added (final concentration 1 μM) to the solution (0.4 ml). Enzyme activity was determined with p-nitrophenyl α -D-galactoside as substrate. Control, experiments contained galactose but no silver nitrate.

Conc. of Galactose (<u>mM</u>)	Inhibition (%)
0.5	45
1.0	45
2.0	40
4.0	23
5.0	18

However, Webb (199, p.737) has pointed out the possibility of co-ordination of Hg^{2+} between carboxyl and amino groups i.e.,



Hg^{2+} is also known to react with amino and imidazolium groups of histidine (200) and with peptide linkages (201). In the present study the degree of inhibition by Hg^{2+} was less than that of Ag^+ at pH 4.0 (Table 7) and it was non-competitive in nature (K_i 75 μM) (Fig.25). This discounts the possibility of Hg^{2+} complexing with carboxyl or histidine residues at the active site of the enzyme at pH 4.0. Therefore it is possible that Hg^{2+} reacts with these groups at sites other than the active site of the enzyme. Inhibition by Hg^{2+} at pH 6.0, on the other hand was much greater and competitive (Fig 25; K_i 0.035 μM). Presumably reaction is thus occurring with the histidine group at the active site in this instance. The activity of the Hg^{2+} -inhibited enzyme at pH 6.0 was restored to 42% on dialysis against McIlvaine buffer, pH 6.0 containing 0.1M-cysteine.

The inhibition by p-hydroxymercuribenzenesulphonic acid was not instantaneous and a preincubation (20 min.) with the enzyme was required for completion of inhibition (Fig. 26). The inhibition was non-competitive both at pH 4.0 (K_i 6 μM) and pH 6.0 (K_i 10 μM).

Inhibition by sugars and related compounds: The inhibitory effects are summarized in Table 9. The inhibition by galactosides follows the order, galactinol > raffinose > melibiose > stachyose. D-galactose is a very powerful inhibitor.

The structural analogues of D-galactose, i.e. L-arabinose and D-fucose, also inhibit the hydrolysis of p-nitrophenyl α -D-galactoside by enzyme I whereas their enantiomers are ineffective. This is in accordance with the results of the substrate specificity experiments (Table 6), i.e. that p-nitrophenyl β -L-arabinoside and α -D-fucoside served as substrates.

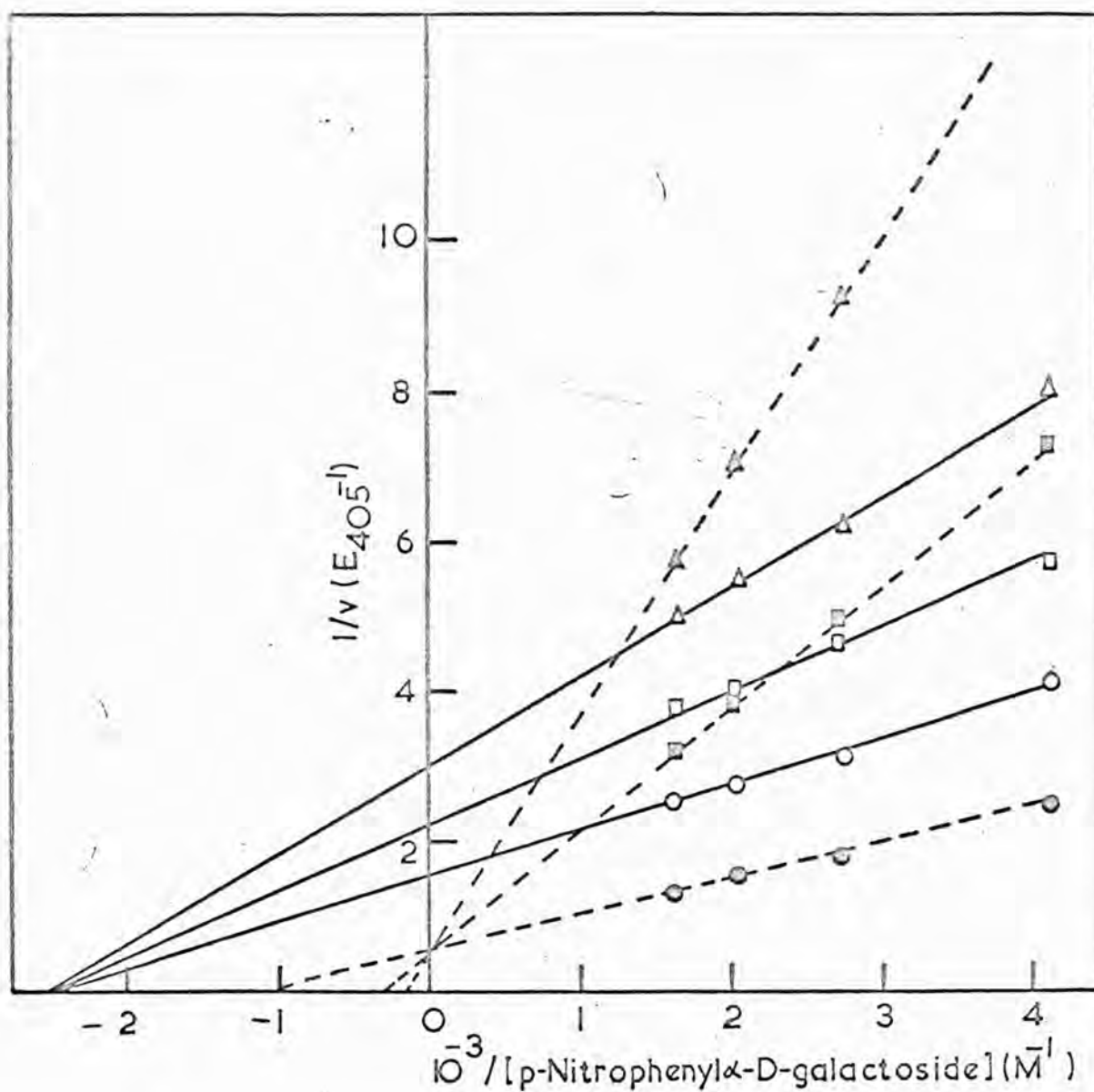


Fig. 25. Inhibition of hydrolysis of p-nitrophenyl α -D-galactoside by α -galactosidase I using varying concentrations of mercuric chloride (○, ◌, nil ; ◻, 0.1 μ M ; ▲, 0.15 μ M ; ◻, 0.1 mM ; △, 1mM) Continuous lines represent results at pH4.0 and broken lines at pH6.0. Conditions of assay and procedure are described in the Experimental section.

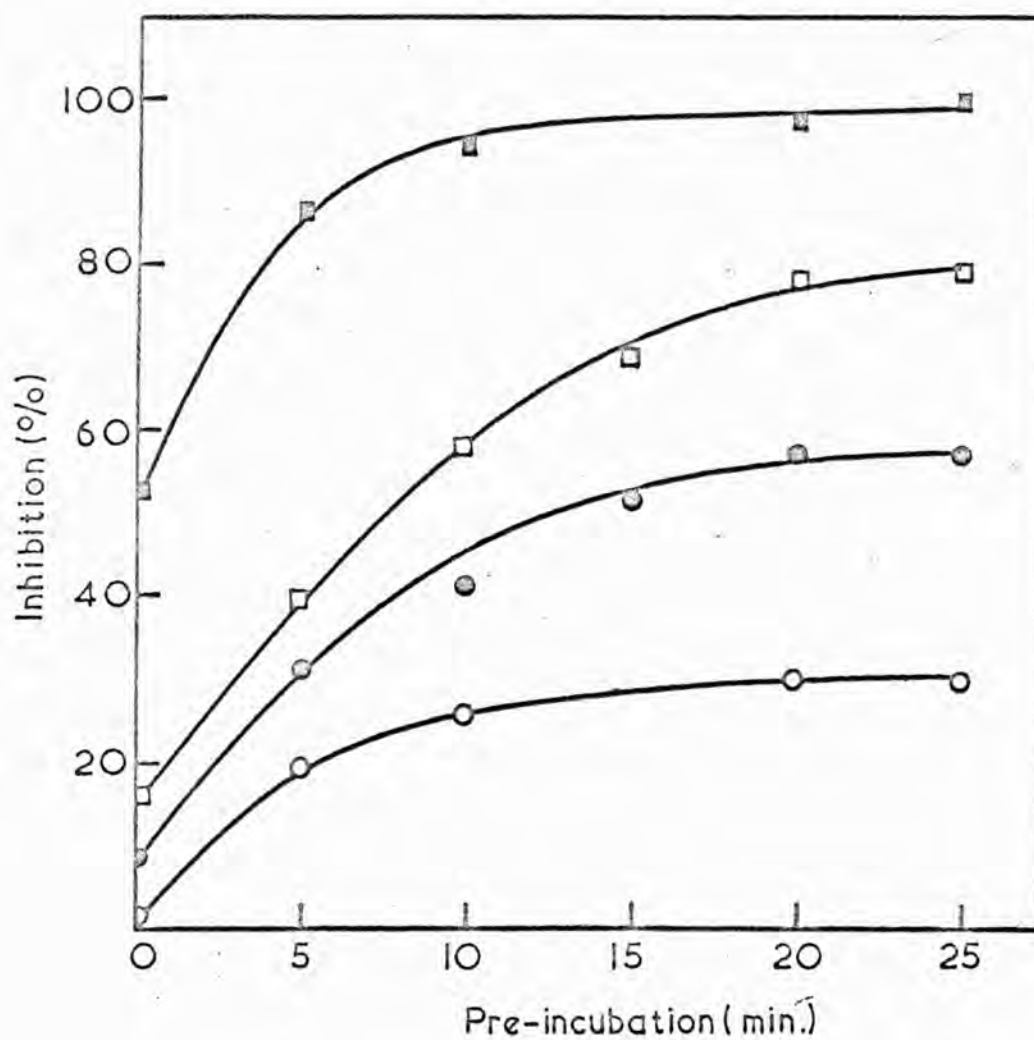


Fig. 26. Dependence of inhibition on pre-incubation time of α -galactosidase I with different concentrations of p-hydroxy-mercuriphenyl sulphonic acid (0, 1 μ M ; ●, 5 μ M ; ■, 10 μ M ; □, 50 μ M) in McIlvaine buffer, pH4.0.

Table 9. Inhibition of α -galactosidase I by sugars and related compounds at 30° in McIlvaine buffer, pH6.0. Experimental conditions are described in the "Experimental" section.

Inhibitor	Inhibitor concentration (mM)	Inhibition (%)
L-Arabinose	25.00	16
	6.25	0
D-Arabinose	25.00	0
D-Fructose	25.00	0
D-Fucose	25.00	14
	6.25	4
L-Fucose	25.00	0
Galactinol	25.00	74
	6.25	44
D-Galactose	25.00	90
	6.25	70
α -D-Galactose-1-phosphate	25.00	8
	6.25	0
D-Galactose-6-phosphate	25.00	4
	6.25	0
D-Glucose	25.00	8
	6.25	0
α -D-Glucose-1-phosphate	25.00	8
	6.25	0
Melibiose	3.75	39
	1.50	21
Myo-inositol	25.00	44
	6.25	16
Raffinose	8.00	57
	2.00	34
Stachyose	50.00	34
	25.00	14
Sucrose	25.00	8

Detection of α -galactosidase activity *in vivo*: A histochemical method (cf.165) for the location of α -galactosidase in various parts of germinating seeds was used for this study. The histological reagent was 6-bromo-2-naphthyl- α -D-galactoside and this on hydrolysis liberated ^{an} insoluble aglycon which can be converted into a blue pigment by treating with tetrazotized ^{an} o-~~an~~anisidine. With every preparation untreated control sections were carefully prepared for comparison. The enzyme was found to be readily removed from the cells by aqueous system and significant prevention of solubilization was noted only after formalin-fixation of the samples for 24 hr. at 4°. The use of 10% neutral formalin (in buffer, pH 4.8) brought about distortion of the cell contents. The use of 3% formalin solution was best suited. α -Galactosidase activity appeared to be intracellular and located all over the cytoplasmic parts of cells of young stems, cotyledons or embryos of germinating ^{seeds} seeds.

A tissue fractionation study of soaked beans by differential centrifugation was also carried out in 0.4M sucrose solution according to the method of De Duve (202). The results shown in Table 10 indicate that the enzyme activity is not located in any particulate fraction. Levels of total α -galactosidase activity during germination: Fig. 27 shows that the enzyme levels in the whole seeds and in cotyledons are approximately the same and remained constant up to the sixth day of anaerobic "germination". A similar pattern occurred in the embryo but the total activity was higher than that in the cotyledons on a dry weight basis. The enzyme activity in the embryo constituted only 10% of the total activity present in the whole seed. On the other hand, the enzyme level during the germination (aerobic conditions) rose until the third day of germination and then dropped slowly over the next three days finally reacting its initial level (Fig.28). The rate of depletion of activity was greater in the embryo than in the cotyledon or whole seed. This decrease could be due to protease activity which might destroy α -galactosidase.

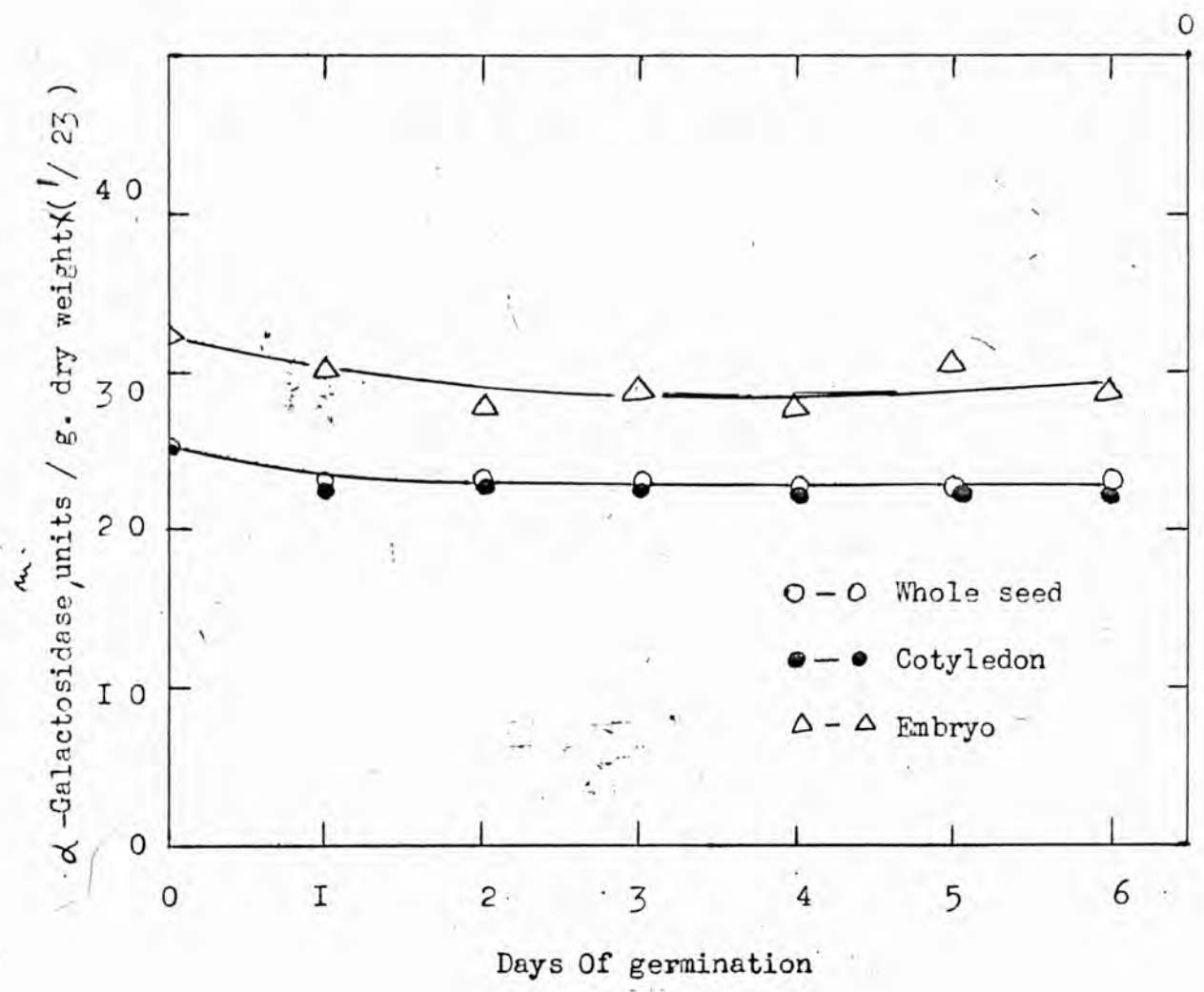


Fig. 27. α -Galactosidase level during ^{an}aerobic germination* of V.faba

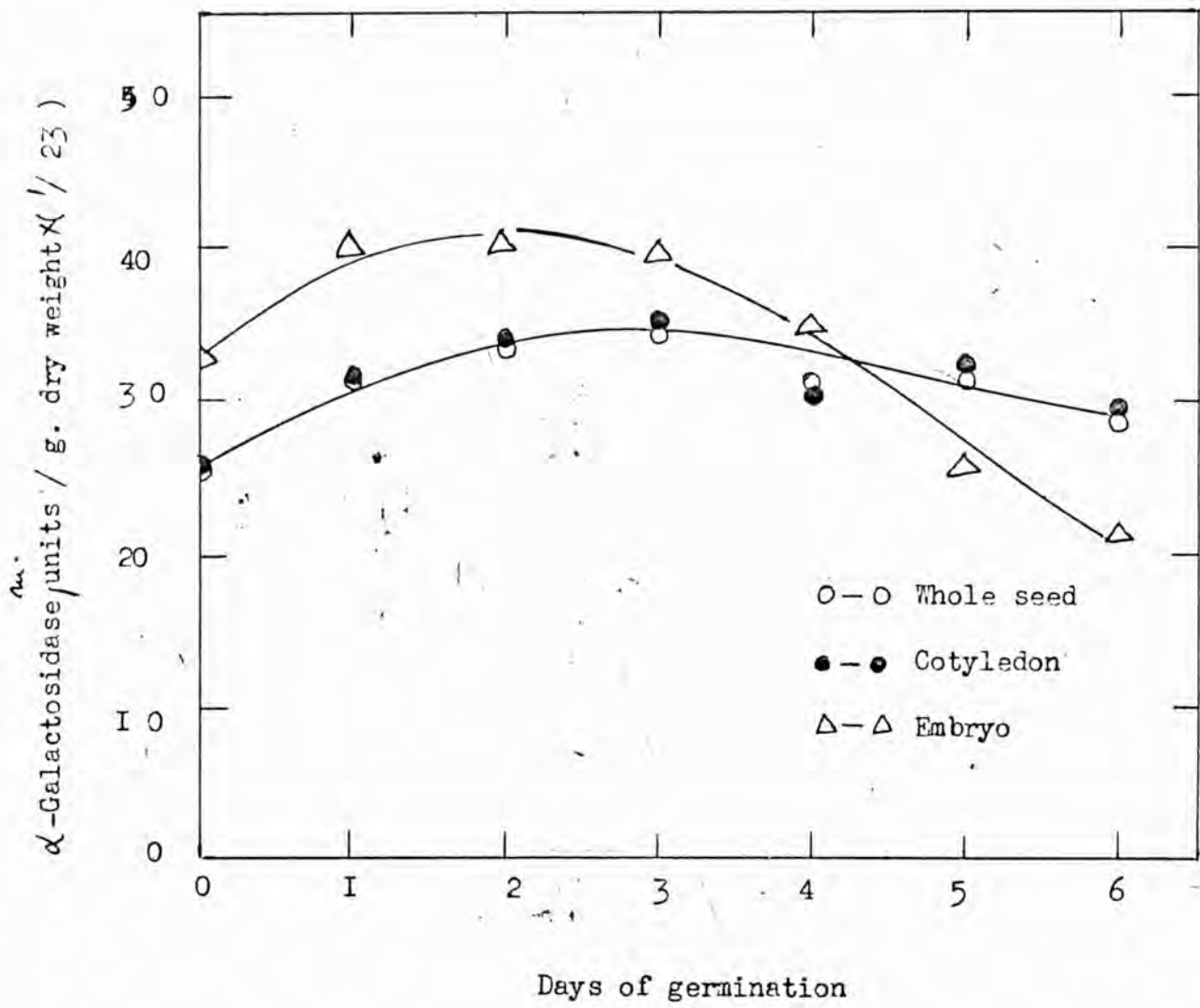


Fig. 28 α -Galactosidase level during ~~anaerobic~~ germination of V.faba

The effect of three growth promoters *i.e.* indoleacetic acid (IAA), kinetin and gibberellic acid (GA), on the level of α -galactosidase was examined during germination of seeds. No apparent effect was shown by IAA at two concentrations (*ie.* 1 $\mu\text{g/L}$ and 10 $\mu\text{g/L}$) (Fig.29). The effect of kinetin (Fig.30) was more pronounced on the embryo enzyme than on the enzyme in the cotyledons; in the former case the activity, however, decreased very sharply after the third day of germination. In addition, it was observed that 10 $\mu\text{g/L}$ of kinetin was more effective a promoter than a concentration of 1 $\mu\text{g/L}$. Enhancement of α -galactosidase activity was observed also with GA, but in this case a higher concentration decreased the activity (Fig. 31). As is generally implicated (203-205) the increase in the enzyme level produced by these hormones may be due to increased protein synthesis. The individual levels of α -galactosidases I and II were also determined during germination and the results are presented in Fig.32. It is evident that the level of enzyme I decreased with a probable simultaneous increase in the level of II. The changes in the levels of the two enzymes during germination could be accounted for by a), interconversion of I and II; b), proteolysis of I; c), irreversible inactivation of I or d), a combination of these effects. Fig.33 reveals that the raffinose family of sugars rapidly decrease during the initial stages of germination. It is quite likely that enzyme I plays an important role in this regard; of the two activities it is the one with the greatest hydrolytic activity and is present at a higher level than II in the dormant seed. At early germination stages it is therefore available for rapid utilization of sugars. The formation of galactose by α -galactosidase-catalyzed hydrolysis of galactosyl sugars during germination would be expected eventually to inhibit the further utilization of the oligosaccharides. The seed can, however, convert galactose to galactose-1-phosphate (142) and this is a very weak inhibitor of α -galactosidase.

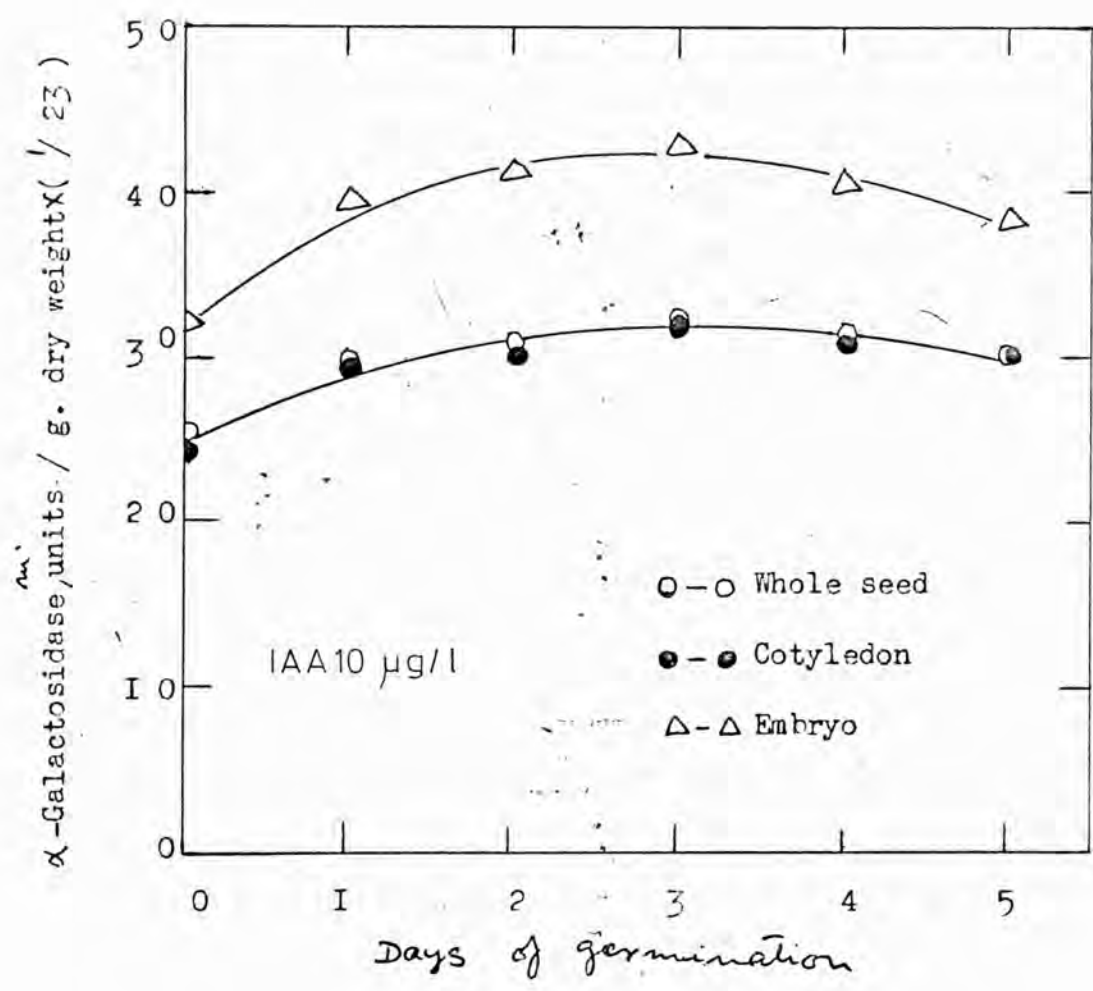


Fig. 29. Effect of IAA on α -galactosidase level during ~~early~~ germination of V.faba. (cf. Fig. 28)

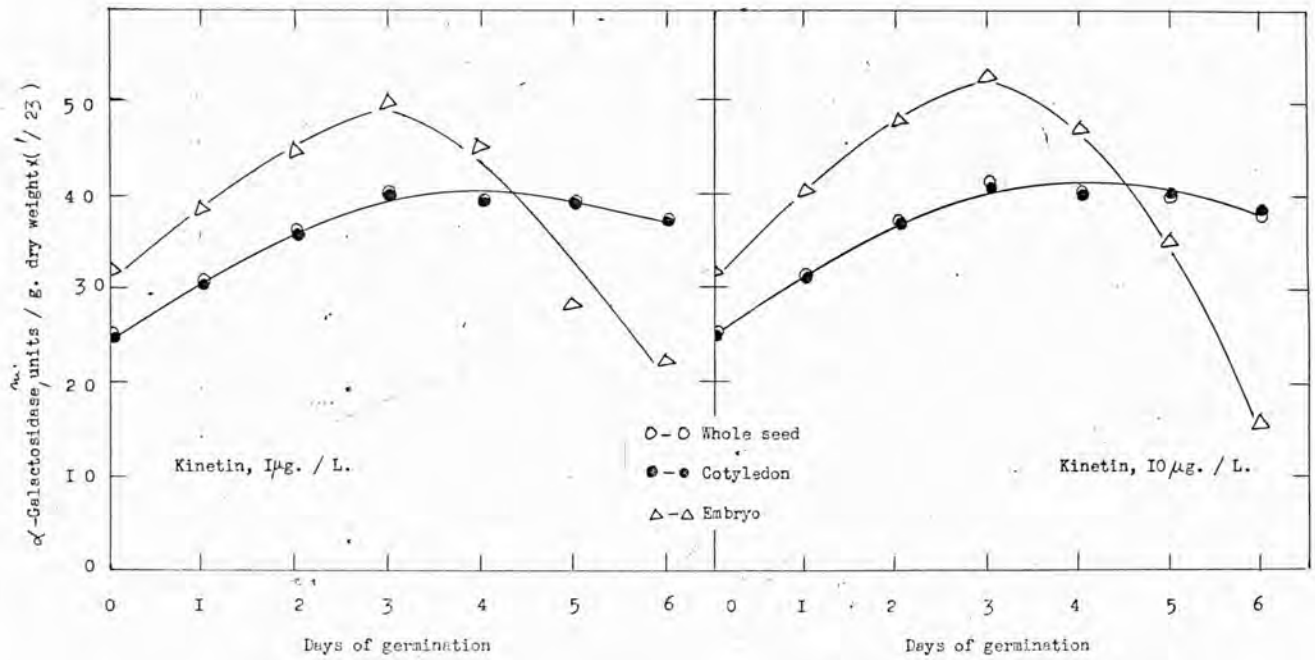


Fig. 30. Effect of kinetin on α -Galactosidase level during ~~the~~ germination of *V. faba*

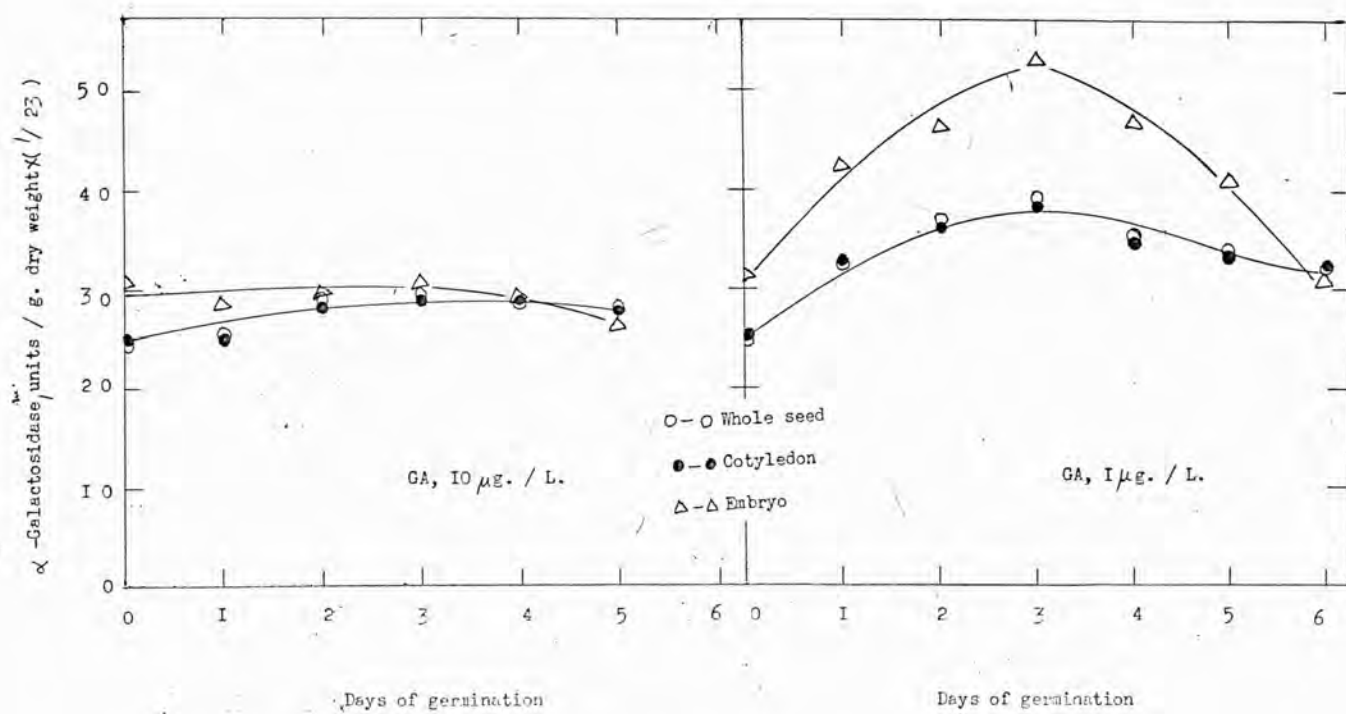
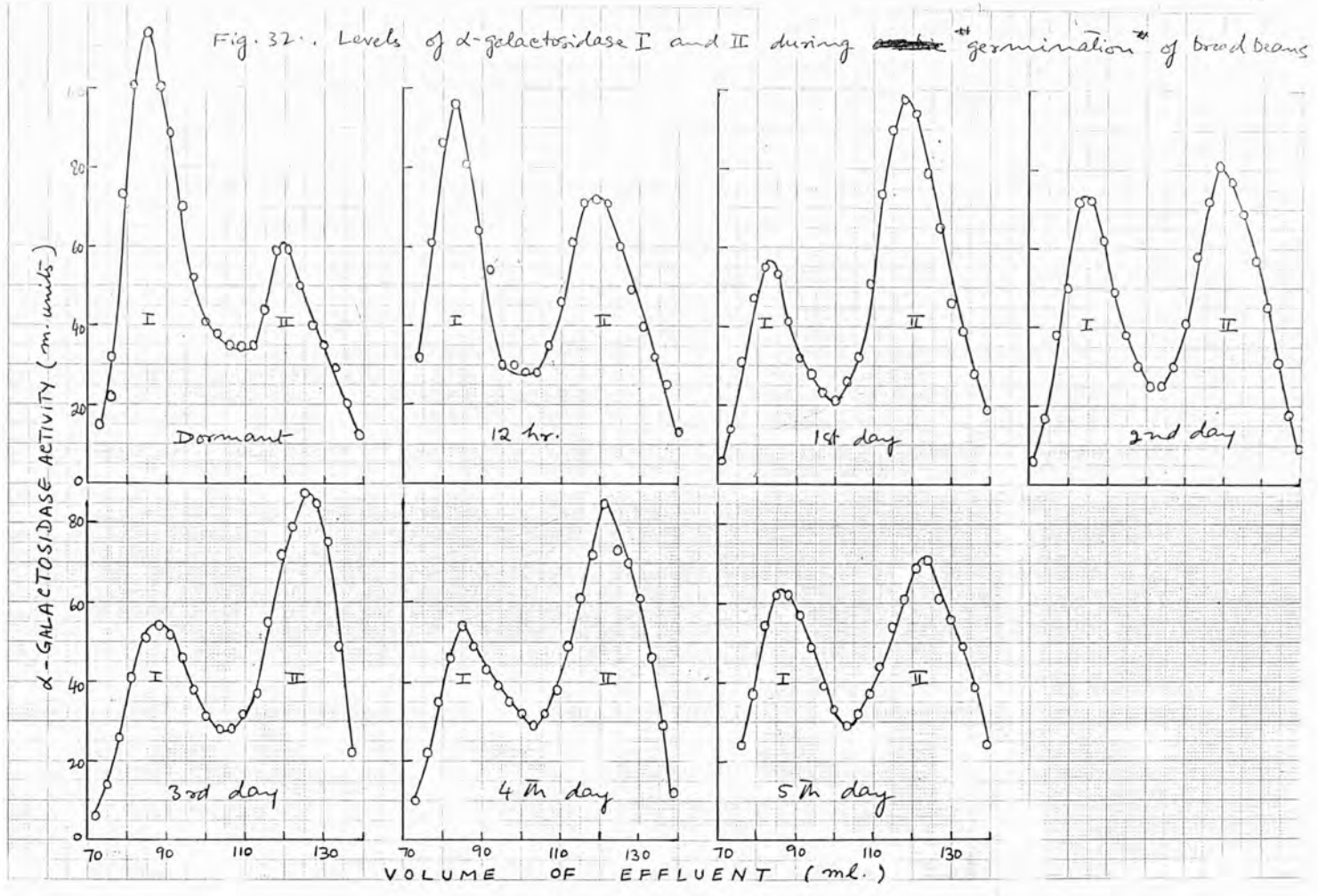
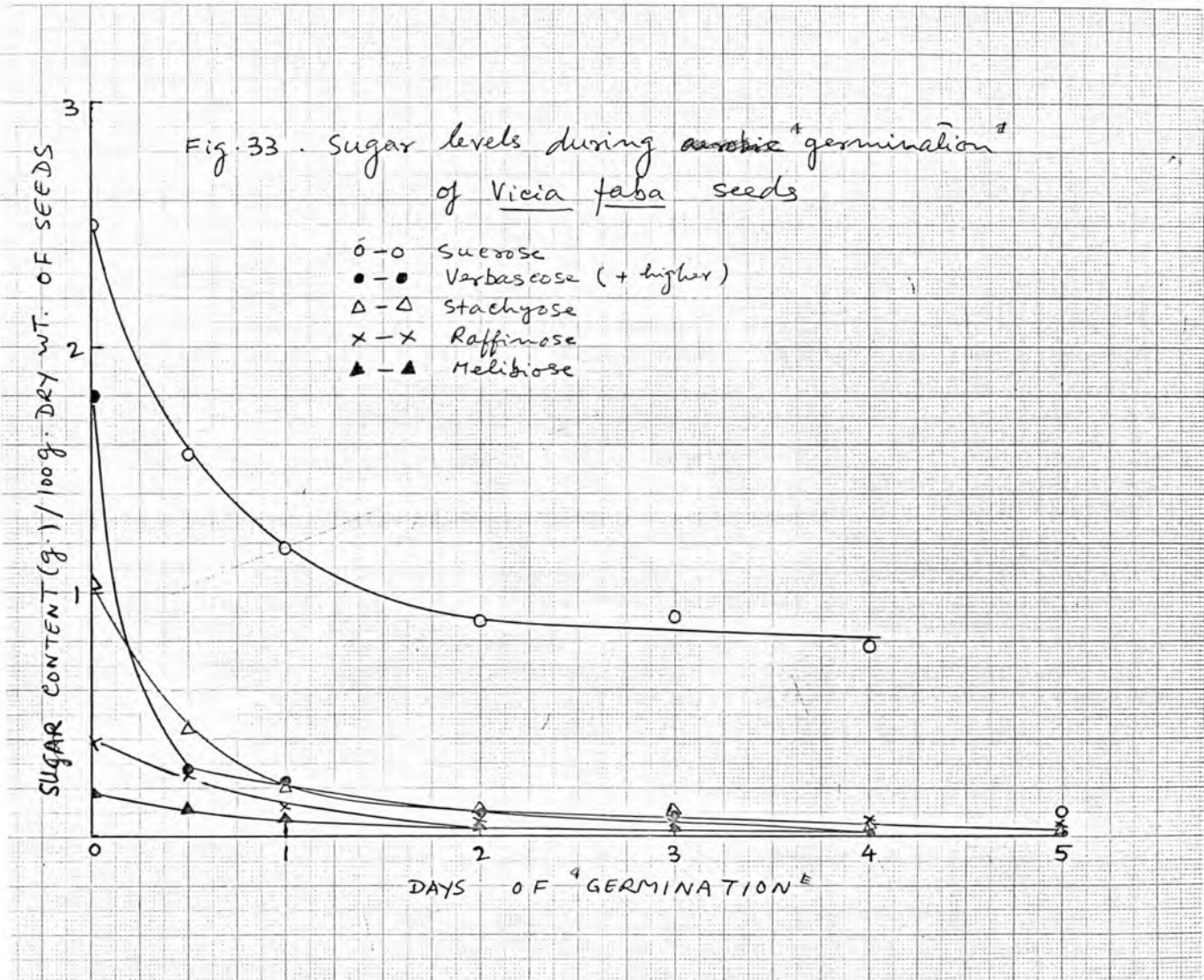


Fig. 31. Effect of GA on α -Galactosidase level during ~~the~~ germination of *V. faba*.

Fig. 32. Levels of α -galactosidase I and II during ~~and~~ germination of broad beans





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