STUDIES ON

MULTIPLE FORMS OF α-GALACTOSIDASE

FROM *VICIA FABA*

A thesis submitted by

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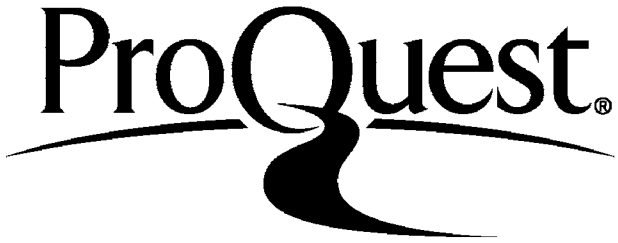

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ABSTRACT

The various molecular forms of α-galactosidase from dormant and germinated Vicia faba seeds have been studied. The enzymes have been purified by multistage procedure and have been examined by ultracentrifugation and gel-filtration. The molecular weights of the enzymes, as determined by ultracentrifugation, were 160,000 ± 2850 for enzyme I from dormant seeds, 54,340 ± 5225 for enzyme II from dormant seeds, and 47,060 ± 2941 for enzyme II from germinated seeds. There was some discrepancy between these values and those obtained by gel-filtration and this is discussed. Enzyme II from dormant seeds can be resolved on CM-cellulose into two active fractions (II^1 and II^2) together with four other inactive protein fractions. Enzyme II^1 and II^2 have molecular weights of 45,730 ± 3073 and 43,390 ± 1409, respectively, as shown by ultracentrifuge measurements. Gel filtration measurements give similar values. Enzyme II from germinated seed can also be resolved into similar fractions by ion-exchange chromatography.

Amino acid analysis of all the molecular forms of the enzyme together with the molecular weight data, suggest that enzyme I is composed of subunits of II; the latter enzyme may be composed of a mixture of II^1 and II^2 together with other inactive proteins. This theory is to some extent supported by the fact that a preparation of enzyme II was observed to convert in vitro to an enzyme with very similar and physical properties to enzyme I from dormant seeds. Some factors affecting this conversion have been investigated.

The kinetic properties of the various forms of α-galactosidase have been examined including substrate specificity, effect of pH, Vmax and Km determinations with p-Nitrophenyl

α-D-galactopyranoside as substrate and activation by K^+ and other alkali metal ions.

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

ABBREVIATIONS

IUPAC	International Union of Pure and Applied Chemistry
IUB	International Union of Biochemistry
DEAE	Diethyl aminoethyl
CM	Carboxymethyl
Km	Michaelis constant
Vmax	Maximal velocity
UDP	Uridine diphosphate
UTP	Uridine triphosphate
NAD	Nicotinamide adenine dinucleotide
PNPG	p-Nitrophenyl α-D-galactoside

CONTENTS

I. INTRODUCTION

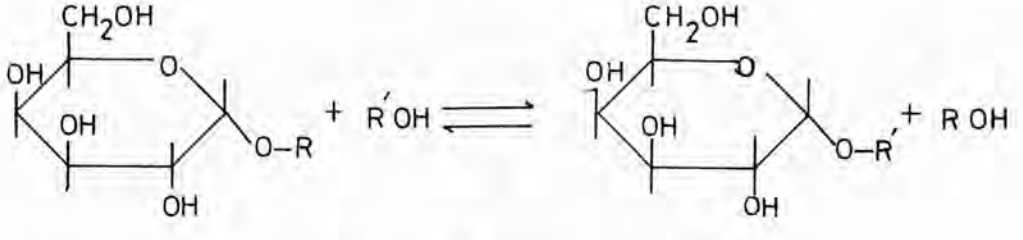
A. GENERAL CONSIDERATIONS

Carbohydrates with terminal α-D-galactosyl residues are widely distributed in nature. In particular, oligo- and polysaccharides containing α-D-galactopyranosyl units are common reserve metabolites in plants. The occurrence and structure of these compounds have been reviewed by French (1), Courtois and Percheron (2) and Whistler and Smart (3). Enzymes, α-galactosidases, which hydrolyse the α-D-galactosidic linkages of these carbohydrates are also widespread in nature (4).

The main purpose of the research described in this thesis was to examine the multiple forms of α-galactosidase occurring in the leguminous seed, Vicia faba, at various physiological stages. The physical and catalytic properties and interconversion of these forms was investigated and an attempt made to relate these observations to the state of development of the tissue.

B. PROPERTIES OF α-GALACTOSIDASE

α-D-Galactosidase (α-D-galactosyl galactohydrolase; E.C. 3.2.1.22) was first discovered in bottom yeast in 1895, simultaneously by Bau (5) and Fischer and Lindner (6). This enzyme was originally given the name melibiase, because of its ability to hydrolyse the disaccharide melibiose.



R = Glucose ; R'OH = Hydroxylic acceptor.

The name melibiase was later changed to α-galactosidase by Weidenhagen (7,8) who studied the substrate specificity of the enzyme and found that it could hydrolyse a number of α-D-galactosides possessing various aglycon residues.

α-Galactosidases are ubiquitous in all the major groups of organisms. The occurrence and cellular location of these enzymes have been discussed in a recent review (4). Although the enzymes have been isolated from a large variety of sources, particularly seeds, highly purified and apparently homogeneous preparations have only been obtained in a few cases. These include α-galactosidases from Diplococcus pneumoniae (9,10), Prunus amygdalus (11,12), Medicago sativa (13) and Vicia sativa (14). The first crystalline preparation of α-galactosidase was obtained by Suzuki et al. (15) from the fungus Mortiarella vinacea.

To add to the complexity of the α-galactosidase group of enzymes recent studies have shown that in some organisms two or more forms of the enzyme may exist. The existence of different molecular forms of an enzyme derived from the same organism which show essentially similar catalytic activities is common. These forms or isozymes may be located in the different tissues of the organism or in different parts of the cell (16, 17, 18). The existence of multiple forms of an enzyme may be caused by a variety of factors and these have recently been reviewed by the IUPAC - IUB commission (see Table 1) (19).

Petek and To Dong (20) were the first to demonstrate the existence of multimolecular forms of α-galactosidase. They showed that the enzyme from coffee bean could be separated into two active forms by chromatography on an alumina column (20). Courtois and Petek (21) later demonstrated that these enzymes (20) had similar substrate specificities. A similar isolation of two isozymes of α-galactosidase from Plantago ovata was also reported (22).

Table 1.

Multiple forms of enzymes

Group	Reason for Multiplicity	Examples
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 chymotrypsinogen.
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.

Suzuki et al. (15) detected three isozymes of α-galactosidase in the fungus Mortierella vinacea. The separation in this case was accomplished by DEAE-Sephadex column chromatography; the molecular weights were not examined but one of the forms was crystallized. The presence of α-galactosidase in Vicia faba was first demonstrated by Pridham (2,3). It was later shown that dormant Vicia faba seed possessed two molecular forms of α-galactosidase (24, 25, 26) (I and II) which could be separated by Sephadex G-100 gel filtration. Using the same technique Dey and co-workers (27) have shown the presence of two molecular forms of α-galactosidase in seeds of several other plant species. The two forms of α-galactosidase from coffee bean seeds which were isolated on alumina by Petek and To Dong (20) were not apparent when extracts of this seed were examined by Sephadex G-100 gel filtration (27). α-Galactosidase obtained from A. niger (28, 29) gave only one enzymically active peak when examined by chromatography on Sephadex G-200, Bio-gel P-200, DEAE-Sephadex and DEAE-cellulose. However, when this enzyme preparation was passed down a column of CM-cellulose, three active forms were resolved (29). The existence of these three ionic forms of α-galactosidase (29) was also confirmed by iso-electric focusing. Dey and co-workers (27) have determined the molecular weights of the multiple forms of α-galactosidase from various dormant seeds by gel-filtration (30) and observed that thelevel of the higher molecular weight form of the enzyme was 2-6 times greater than that of the lower molecular weight form in most of the species. The authors have also stated that seeds which are not mature and/or have too high a water content may not exhibit this difference in enzyme levels. It was also shown that the higher molecular weight form of α-galactosidase from V. faba possessed a sub-unit structure (25) as on treatment with

6 M-urea six protein peaks were produced none of which possessed enzymic activity.

It is known that 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. In particular, all glycosidases show absolute specificity for the configuration at the anomeric carbon atom of the glycoside. 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 (31), Pigman (32), Gottschalk (33) and Wallenfels (34). In case of β-glucosidase any substitution of hydrogens or hydroxyl groups on carbon atoms 2, 3 or 4 of the β-D-glucopyranoside substrate, completely prevents enzymic hydrolysis. However, substitution at C-6, only decreases the rate of hydrolysis by β-glucosidase. Similarly, replacement of the primary alcohol group of the pyranoid ring by -H (i.e. conversion to β-D-xylopyranoside) produces a 2oo-fold reduction in the rate of hydrolysis. In case of α-galactosidase, in addition to the configuration of the anomeric carbon, two main factors govern the rate of hydrolysis of the substrate. Firstly, the ring structure must be pyranoid and secondly, for the maximum hydrolysis rate the configuration of -H and -OH groups on carbon atoms 2, 3 and 4 must be the same as that of D-galactose. Like other glycosidases, such as β-galactosidase (35, 36, 37), β-glucosidase (35, 37) and α-mannosidase (35), changes at C-6 of the glycosyl moiety of the substrate are normally tolerated by α-galactosidase. Hence, β-L-arabinosides are normally hydrolysed by the enzyme (15, 25, 35, 37, 38, 39). However, α-galactosidase from Streptococcus bovis (40),

Epidinium ecaudatum (10), Diplococcus pneumonae (9) and Calvatia cyanthiformis (41) cannot use β-L-arabinosides as substrates. Pigman (42) stated that D-glycero-α-D-galactoheptosides should serve as substrates for α-galactosidase. However, this could not be demonstrated with either sweet almond or yeast (43) α-galactosidase. Pigman (43) was able to show that β-galactosidase from the former source could hydrolyse D-glycero-β-D-galactoheptoside. Dey and Pridham (44) studied the specificity of the two molecular forms of α-galactosidase from V. faba and showed that neither enzyme (I or II) was able to hydrolyse p-nitrophenyl β-D-galactoside or p-nitrophenyl α- and β-D-glucosides. This confirmed the expected absolute specificity towards the α-configuration at C-1 and in addition, indicated the importance of the configuration at C-4. Substitution at C-6 of the galactose moiety of an α-D-galactosides seemed to be relatively less important in the case of the higher molecular weight form of the enzyme (I); changing $-CH_2OH$ to $-CH_3$ (i.e. α-D-galactoside to α-D-fucoside) did not cause any appreciable decrease in the rate of hydrolysis. On the other hand, the lower molecular weight form of α-galactosidase (II) from V. faba hydrolysed p-nitrophenyl α-D-fucoside almost three times as fast as the corresponding galactoside. Complete removal of the primary alcohol group from a galactoside (i.e. conversion of α-D-galactoside to β-L-arabinoside) decreased the rate of hydrolysis by enzyme I but had little effect with enzyme II. The authors have also shown that the affinity of the enzyme for substrate is largely dependent on the structure of the glycon moiety and for both forms of enzyme (I and II), follows the order: α-D-galactoside > α-D-fucoside > β-L-arabinoside. This may suggest that one of the specific points of binding of the substrate to the enzyme may be through primary alcohol group of the galactose moiety.

The replacement of the anomeric oxygen atom of an α-galactoside by a sulphur atom on the effect of hydrolyzability by α-galactosidase is still in question. In the case of E. coli β-galactosidase, O and p-nitrophenyl 1-thio-β-D-galactosidase have affinities for the enzyme that are similar to those of the corresponding O-galactoside, although O-nitrophenyl β-D-galactoside is hydrolysed 7 x 10^5 times faster than the corresponding thio-galactoside (34, 45-48).

The variation in the structure of the aglycon residue of the substrate may or may not have a marked influence on hydrolysis by glycosidases. Wallenfels and Malhotra (49) have shown that the aglycon residue of a β-D-galactoside exerts a strong influence on the enzyme - substrate affinity ($\frac{1}{Km}$) in the case of E. coli β-galactosidase. Variation in the structure of aglycon residues of α-D-galactosides and their effects on the affinity and the rate of hydrolysis by the two forms of α-galactosidase from V. faba was examined by Dey and Pridham (44). These workers have shown that the hydrolysis of aromatic glycoside derivatives proceeds more rapidly than those of the corresponding alkyl derivatives. The relationships between Km and Vmax values exhibited a highly irregular pattern with changing aglycon structure. It was suggested that a greater affinity of the enzyme for the substrate may not be the essential factor for rapid hydrolysis and vice versa. Amongst the aromatic α-D-galactosides studied, it was shown that a bulky bromonaphthyl aglycon residue did not change the affinity or the rate of hydrolysis to a great extent with either form of α-galactosidase from V. faba. However, the affinities for both forms of enzyme have been shown to have some tendency to to increase when electron-attracting substituents are present in the phenyl ring of the substrate. The factors effecting the

affinity are probably complex and include the position and the size of the aromatic substituents, their electronic effects and the degree of hydration (44). On the other hand, Vmax values with α-galactosidase from sweet almond have been shown to increase with both increasing electron-releasing and electron-withdrawing properties of the phenyl ring substituents of the substrate (12).

In case of galactose-containing oligo-saccharides such as melibiose and manninotriose, Bailey and co-workers (40, 50, 51) have shown that reduction of the terminal reducing group (producing melibiitol and manninotriitol, respectively) decreases the rate of enzymic hydrolysis by Streptococcus bovis (40) α-galactosidase. On the other hand, oxidation of the terminal reducing group, as in the case of conversion of melibiose to melibionic acid, does not appear to effect the rate (52). The rate of hydrolysis of a homologous series of α-linked oligogalactosides was reported to decrease with increasing chain length (4, ref therein). However, Courtois et al.(53) and Bailey (40) have shown in the case of α-galactosidases obtained from penicillium sp. and Streptococcus bovis, that the rate of hydrolysis increases with the increasing chain length of the oligosaccharides. In addition to the hydrolysis of the terminal galactosyl residue, almond α-galactosidase has been reported to split the internal galactosidic linkage in stachyose forming galactobiose and sucrose (48, 54). α-Galactosidase from coffee bean on the other hand, can only hydrolyse stachyose (55, 56) and verbascotetraose (57) in a stepwise fashion starting from the nonreducing end. α-Galactosidase from Streptococcus bovis (40, 51) and Epidinium ecaudatum (50) acting on verbascose and ajugose behaves in a similar fashion.

In addition to hydrolytic activity glycosidases are also known to catalyse transgalactosylation reactions (58, 59).

Hydrolysis and transglycosylation are essentially similar reactions; in the former case glycosyl residues are transferred to water and in the latter to other hydroxyl groups such as those attached to simple alcohols or carbohydrates. The first observation of transferase activity by α-galactosidase was made by Blanchard and Albon (60); the enzyme was prepared from yeast. The authors showed that galactose could be transferred from melibiose to a second melibiose acceptor molecule with the formation of mannino-triose (5, 61). Following this report, α-transgalactosylation was studied extensively, especially by Courtois and his co-workers, with respect to acceptor specificity and the effect of acceptor concentration, temperature, pH and source of enzyme (21, 34, 62, 63). The results obtained so far, show that the hydrolysis and the transfer reactions are catalysed by the same enzyme, and also that they take place on the same active site of the enzyme molecule. With β-galactosidase, the synthesis of glycosides from free sugars (acting as donors) and simple alcohols has also been reported to occur in addition to transfer and hydrolytic reactions (64, 65). Wohnlich (66) reported that α-galactosidase from wheat bran had a high transferase/hydrolase ratio. Shiroya (67), on the other hand, found that the reverse was true with α-galactosidase from cotton seed using raffinose as both donor and acceptor. Tanner and Kandler (68), however, have isolated an enzyme from Phaseolus vulgaris seeds that transfers galactose from galactinol to raffinose with the formation of stachyose; this enzyme possessed very little hydrolytic activity. The specificity of transgalactosylation catalysed by α-galactosidases from various sources has recently been reviewed by Dey and Pridham (4). Li and Shetler (10) have shown that phosphorylation, oxidation or reduction of either C-1 or C-6 of D-glucose or D-galactose destroys the acceptor properties of these hexoses when pneumococcal α-galactosidase is used as catalyst.

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 destroys the acceptor properties of this hexose.

Galactosidases are also known to synthesize oligosaccharides when incubated with high concentrations of galactose or other monosaccharides (de novo synthesis) (69, 70). This procedure has been used for the synthesis of several glucose and galactose derivatives (71, 72, 73). When yeast α-galactosidase was incubated with D-galactose, a galactobiose and a galactotriose were isolated from the reaction mixture, and α-(1→6)-linkages were assigned to these compounds. Other products, also formed in small amounts, were α-(1→3), α-(1→4) and α-(1→5)-galactobioses (1, 74). Clancy and Whelan (74, 75) using the same enzyme and substrate obtained a high yield of α-(1→6)-linked galactobiose together with smaller amounts of α-(1→5), α-(1→4)- and α-(1→3)-linked disaccharides and higher oligosaccharides. β-Galactosidase has similar properties and Wallenfels and Malhotra (34) have demonstrated the formation of β-(1→6), β-(1→4) and β-(1→3)-linked galactosyl glucose by allowing glucose and galactose to react together.

C. SEED DEVELOPMENT AND GERMINATION

The metabolism of carbohydrates in seeds is very much dependent on the physiological state of the tissues. Seeds in higher plants develop from ovules present in the ovary of young flowers, as a result of changes following the reception of pollen by the stigma. The mature seed contains materials which are

utilized during the process of germination. Gould and Greenshields (76) have examined the metabolism of glucose, fructose, sucrose and galactosylsucrose derivatives during the process of maturation of Phaseolus vulgaris seeds. It was observed that the hexoses tended to disappear from the cotyledons and embryo but the sucrose content remained unaffected. On the other hand the galactose-containing oligosaccharides, such as raffinose, stachyose and verbascose, began to appear in the seed during the final stages of maturation. Reid and Meier (77) made a similar observation in the case of Trigonella foenum Graecum.

Many seeds do not germinate when placed under conditions which are normally favourable for germination. Such seeds can be shown to be viable, as they can be induced to germinate by special treatments. Some seeds will only germinate under natural conditions if they are kept for a certain period of time. These seeds are said to be in a state of dormancy. Dormancy may be defined as the period during which changes occur in the seed which allow it to become viable and hence germination becomes possible. Dormancy can be due to various causes, such as immaturity of the embryo, impermeability of the seed coat to water and gases, special temperature or light requirements or the presence of chemicals which inhibit germination. The possible causes of dormancy have been reviewed by Mayer and Poljakoff-Mayber (78). Dry seeds are characterized by a markably low rate of metabolism. This is probably a direct result of their low water content (5-10%). The dry seed is usually well equipped with functional units, which can carry out a large number of biochemical reactions provided the initial hydration of the enzymic protein can take place.

Germination of seeds may be regarded as those consecutive steps which cause a quiescent seed, with a low water content, to

increase its metabolic rate which in turn initiates the formation of the seedling from the embryo. The chemical changes which occur in the seed at the onset of germination are complex in nature. They consist of three main types: (a), the breakdown of reserve materials; (b), the transport of metabolites from one tissue to another especially from cotyledons to developing points and (c), the biosynthesis of new materials from the breakdown products from the reserves. The changes in the storage reserve during germination from several leguminous species has been extensively studied by many workers (79-86). Mayer and Poljakoff-Mayber (78) recently reviewed the whole phenomenon and conclude that some of the storage products undergo very marked metabolic changes but those substances which are present in the greatest amounts are not necessarily broken down first. The metabolic changes occuring in the early stages of germination are the results of the activities of various enzymes. The enzymeswhich have hydrolytic or transferase properties are either present in the dry seed or very rapidly appear as the seed imbibes water.

D. FUNCTION OF α-GALACTOSIDASE

1. In Plants

In considering the biochemical role of α-galactosidase in plants one may begin by examining the metabolism of commonly occuring sugars which contain α-galactosyl units. Raffinose is one such sugar and this consists of a D-galactopyranosyl residue joined by an α-(1→6)-linkage to the glucose moiety of a sucrose molecule. The raffinose family of oligosaccharides is in fact, the most widely occuring group of galactosyl sucrose derivatives in plants (87 - 90). The lower members of the series, raffinose

and stachyose, have been reported to occur in many plant organs, such as roots, seeds, shoots, underground tubers, etc (89, 91-94), in amounts usually equal to or greater than that of sucrose. The higher members of the series, however, have been found only in seeds and roots. In leguminous seeds it has been reported that the raffinose family of oligosaccharides constitute the soluble reserve carbohydrates during dormancy (76, 95, 96). In Vicia faba (97) raffinose and stachyose were found to be accumulated and increase in concentration during seed maturation.

The possible biosynthetic pathways for the formation of raffinose in several plant species has been examined by a number of workers and appears to consist of the transfer of a galactose moiety from UDP-galactose to sucrose (97, 98, 99).

UDP-galactose + sucrose $\longrightarrow$ raffinose + UDP.

UDP-galactose has been detected in many plant tissues, including Vicia faba seeds (97), and may rise from UDP-glucose by the action of UDP-glucose-4'-epimerase (100), or from galactose via galactose-1-phosphate which involves galactokinase and UDP-galactose pyrophosphorylase. Duperon (101) has shown that ^{14}C-labelled glucose rapidly gives rise to labelled sucrose as well as raffinose in Phaseolus vulgaris.

In vitro, raffinose has been synthesized by allowing sucrose to react with p-nitrophenyl α-D-galactoside or melibiose in the presence of α-galactosidase (102 - 106). This enzyme is predominantly a hydrolase and in order to obtain significant amounts of transfer product high concentrations of sucrose and melibiose have to be used. The physiological importance of such reaction is not clear. The thermodynamics of the process are unfavourable although it is conceivable that high concentration of sucrose could be present in the cell to overcome the problem.

There are no known endogenous galactose donors for such reaction, however. Tanner and Kandler (107, 108) have discovered a specific transferase with poor hydrolytic properties in ripening seeds of Phaseolus vulgaris. In vitro this enzyme catalyse the transfer of a galactosyl moiety from galactinol to raffinose with the production of stachyose. The author suggests that this reaction can occur in vivo and that galactinol is regenerated from free inositol by the transfer of a galactosyl residue from UDP-galactose.

UDP-gal + inositol → galactinol + UDP

Galactinol + raffinose → stachyose + inositol

UDP-gal + raffinose → stachyose + UDP

In subsequent work Sensor and Kandler (109) showed that galactinol functions as a galactosyl donor in all plants containing raffinose and stachyose. 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(Fig. 1).

Metabolism of Galactose by Higher Plants

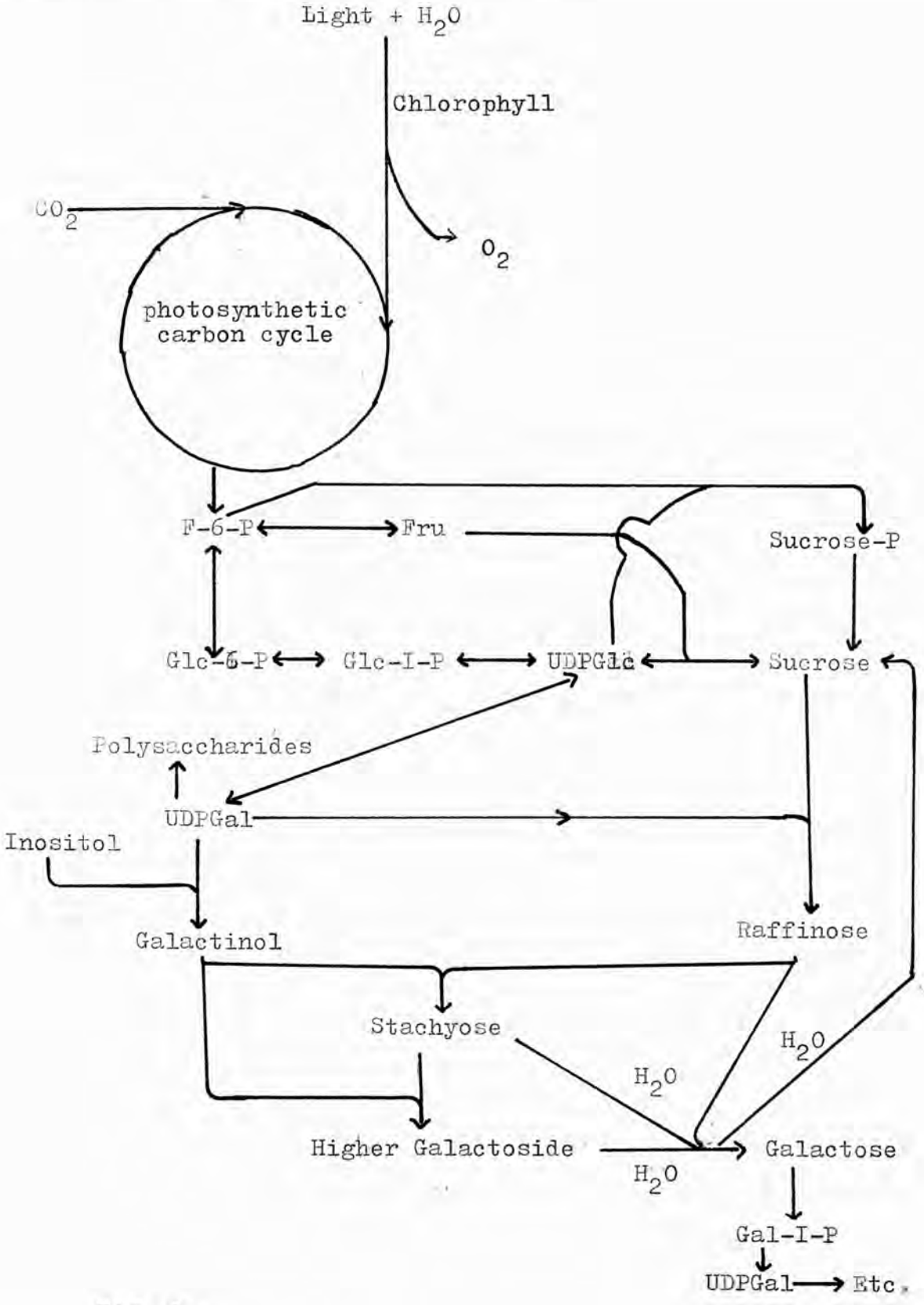


Fig. 1.

A similar observation was reported by Shiroya (67) who showed that when $^{14}CO_2$ was fed to the needle of Pinus strobus, sucrose and raffinose in the phloem became labelled after 8 hours. Study of the kinetics of the labelling in the leaves of Lamium macalatum after feeding $^{14}CO_2$ (109) reveals that the galactinol pool is saturated with ^{14}C more rapidly than the oligomers of the raffinose series and that under conditions of continued photosynthesis, label accumulates in raffinose and stachyose and decreases in galactinol. During the process, label accumulates initially in the galactosyl portion of the galactinol and then in the galactosyl residues of raffinose and stachyose. This shows that in this case raffinose and stachyose is produced by the specific transfer of a galactose moiety from galactinol to raffinose. It is likely that by a repetition of this process the whole series of raffinose oligosaccharides is produced (110). In case of, stachyose, the highest specific activity during a short period of exposure to $^{14}CO_2$, was found in the terminal galactosyl residue (111). A study of this biosynthetic process in the ripening seeds of Phaseolus vulgaris confirms the work with Lamium leaves, and reveals that the synthesis of raffinose and stachyose takes place in the seeds not in the pod (112). During ripening of Phaseolus vulgaris seeds, the pool of inositol and sucrose which accumulates during the early stage of seed development disappears as galactinol is formed. Later the level of galactinol decreases as galactinol - raffinose - 6 - galactosyl transferase (68, 113) activity rises and stachyose is formed (112). The formation of stachyose *in vitro* was demonstrated by Shiroya (76) who incubated raffinose with a cotton seed extract which contained α-galactosidase. In V. faba seeds the biosynthesis of verbascose, a member of raffinose series of

oligosaccharides, was shown to occur from stachyose and galactinol (110).

Gould and Greenshields (76) studied the change in the levels of galactose-containing oligosaccharides in ripening and germinating seeds (Phaseolus vulgaris) and demonstrated that on germination these oligosaccharides disappear leaving mainly sucrose in the cotyledons and sucrose, glucose and fructose (76) in the embryo. No free galactose was detected in any part of the germinating seeds. Shiroya (67) reported similarly that raffinose and stachyose, which are contained in resting cotton seeds, disappear rapidly and completely in the early stage of germination. Neither galactose nor melibiose were detected at any stage of germination. This author also observed that the activity of α-galactosidase in resting, soaked cotton seeds is much higher than that of invertase, and hence raffinose and stachyose are hydrolysed by the former enzyme and give rise to sucrose and galactose. The failure to detect free galactose in tissues where active hydrolysis of galactosylsucrose derivatives is occurring suggests that galactose utilization is very efficient and that the rate of utilization of the monosaccharide is at least as high as the rate of removal of galactose from the oligosaccharides by α-galactosidase. The galactose utilization system was examined in the cotyledons of germinated cotton seeds by Shiroya (67) who showed that seeds infiltrated with raffinose metabolized the trisaccharide but no galactose was detected in the tissues. The actual pathway of the metabolism was not explained by the author. Pridham et al. (114) have examined the fate of raffinose in germinating V. faba seeds. They suggest that the concerted action of α-galactosidase and sucrose-UDP-glucose glucosyl transferase may form an important

mechanism for the rapid utilization of raffinose and other galactosylsucrose derivatives. The V. faba seeds have also been shown to possess galactokinase activity which is capable of converting the free galactose, liberated from galactosylsucrose by the action of α-galactosidase, to galactose-1-phosphate (115). This latter reaction presumably is at least partly responsible for the failure of the seeds to accumulate free galactose during germination. Galactose-1-phosphate are, no doubt, be converted to UDP-galactose by UTP-galactose-1-phosphate uridylyl transferase, a ubiquitous enzyme which has been detected in germinating V. faba seeds (J.B. Pridham and M.W. Walter, unpublished work).

Pridham and co-workers conclude that the metabolism of the raffinose family of oligosaccharides and sucrose provide a ready source of sugar nucleotides for cell wall synthesis and other important biosynthetic processes. Pazur et al. (116) examined the metabolism of galactose-containing oligosaccharides in soya-bean (Glycin max) and have come to a similar conclusion regarding the conversion of galactosylsucrose derivatives to UDP-glucose and UDP-galactose. Further, along with other workers, they point out that these two nucleotides are interconvertible via UDP-glucose-4'-epimerase.

The utilization of galactose in connection with cell wall formation has been demonstrated with several plant species (117, 118). Altermatt and Neish (119) examined the utilization of D-galactose for cell wall formation in wheat plants and the fate of galactose in Avena coleoptiles was examined by Ordin and Bonner (120) and by Thimann and co-workers (121). In both cases, by feeding D-[^{14}C] galactose, it was observed that little $^{14}CO_2$ was produced and that most of the label was incorporated into insoluble products,

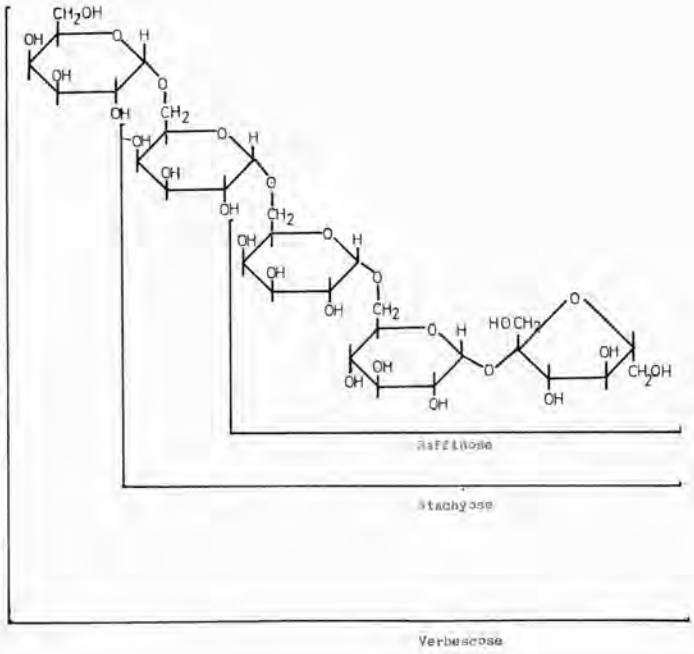
many of them being associated with the cell wall. *In vitro* experiments by McNal *et al.* (122) and more recently Panayotos and Villemez (123) have shown that the galactose from UDP-galactose can be incorporated into *Phaseolus aureus* cell walls and that β-(1$\rightarrow$4)-linkages are produced. Of particular interest is the evidence that suggests that growth of the galactan chain, in this case is on the reducing end.

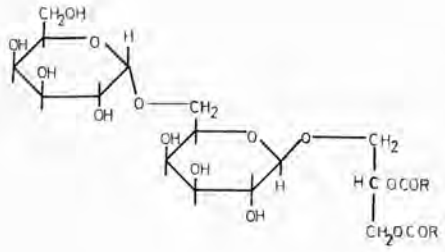
Endogenous sucrose, whether free or derived from the breakdown of galactosylsucrose, may also be metabolized in higher plants by coupled hydrolysis and phosphorylation reactions. Hydrolysis of sucrose in higher plants may be effected by invertase (usually β-fructofuranosidase) resulting in the production of glucose and fructose. The importance of such reaction *in vivo* is in some doubt: invertase activity is widespread in plant tissue. The hydrolysis of sucrose in germinating *Phaseolus vulgaris* seeds has been examined by Gould and Greenshields (76) and Cooper and Greenshields (124) who have shown that the situation is complex. The authors suggest that the cotyledons of steeped and germinated beans possess sucrose activity (alkaline sucrase; optimum pH 7.7). The embryos of steeped seed also possess a similar enzyme activity but after three days of germination another sucrose hydrolysing enzyme (optimum pH 4.9) appears in these tissues in addition to the alkaline sucrase. This 'acid' enzyme appears to be a β-fructofuranosidase, whilst the alkaline sucrase appears to be a true sucrase which only hydrolyses sucrose. Regarding the functions of these two hydrolytic enzymes *in vivo*, the authors (76, 124) observed that before the appearance of the β-fructofuranosidase there was a lack of free reducing sugars in the tissues although sucrose was present. The appearance of this enzyme in the embryo, on germination, coincides with the

appearance of glucose and fructose.

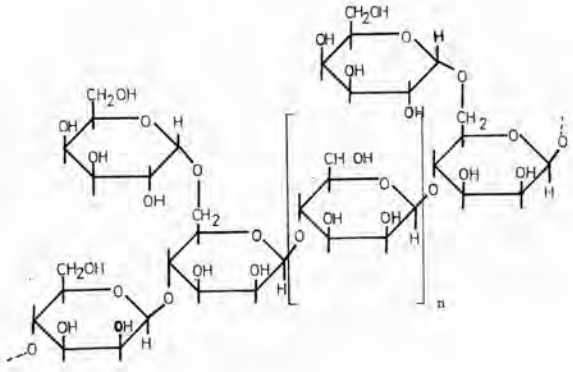
Glucose and fructose released by hydrolysis can undergo further metabolism involving the enzymes of the glycolytic pathway and can be reconverted to sucrose and other oligo- and poly-saccharides or used as a course of energy.

In addition to galactose-containing oligosaccharides, photosynthetic tissues of plants also contain galactosylglycerides (cf. Fig.2) (125-132). The total galactolipid content of the chloroplast is related to the chlorophyll content of the leaf tissue (129). Rosenberg and his co-workers (133, 134, 135) demonstrated that in the cells of Euglena gracilis, the appearance and disappearance of chlorophyll is accompanied by the simultaneous appearance or disappearance of galactosyl diglycerides in 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. The biosynthetic pathway to galactolipids in plants is not yet clear. Ferrary and Benson (136) observed a rapid incorporation of ^{14}C into monogalactosyl diglyceride and a slower entry into digalactosyl diglyceride during the growth of Chlorella pyrenoidosa in $^{14}CO_2$: they conclude that the digalactosyl diglyceride is synthesized by galactosylation of monogalactosyl diglyceride and that both galactose transfers involve UDP-galactose although in the first the linkage formed is β and in the second case α. Neufeld and Hall (137) have demonstrated that spinach chloroplasts catalyse the transfer of galactose from UDP-galactose to an uncharacterized endogenous acceptor with the appearance of mono-, di-, tri- and possibly tetragalactosyl diglycerides. Chang and Lundin (138) found a stimulatory effect of galactolipids on the rate of cytochrome C photoreduction by intact spinach chloroplast. Moreover, both Ferrary and Benson (136) and Kates (131) found that galactolipids have a high rate of turnover. Helmsing (139) believes that galactolipids play an important role in the photosynthetic apparatus of plants. Sastry





Digalactosyl Diglyceride
(R = Fatty acid)



Galactomannan

Fig. 2 . Structure of some galactose-containing compounds in higher plants.

n = 2 - Guar gum
n = 3-4 - Locust bean and Tara gum

and Kates (140) and Helmsing (141) 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 cleaved to glycerides and galactose by the action of β-galactosidase. Several plant species have been tested for the presence of galactolipase, specific enzymes for the hydrolysis of galactolipids (139, 140, 141, 142). These have been found in spinach leaves (141) and runner-bean leaves (139) and they catalyse the following reactions.

Digalactosyl diglyceride $\rightarrow$ digalactosyl monoglyceride + fatty acid.

Digalactosyl monoglyceride $\rightarrow$ digalactosyl glyceride + fatty acid. Further hydrolysis of the digalactosyl glycerides has been reported to catalyse by α- and β-galactosidase.

One of the major galactose-containing reserves in leguminous seeds is galactomannans (143-145). Galactomannans are composed of chains of β-(1$\rightarrow$4)-linked D-mannopyranosyl residues carrying a varying proportion of single D-galactopyranosyl residues joined by α-(1$\rightarrow$6)-linkages to the mannan chain (see fig.2). The galactose/mannose ratio is also reported to vary depending on the source of the galactomannans (147, 148, 149, 150). Formation of reserve galactomannans during the development of *Trigonella foenum graecum* seeds has been examined recently by Reid and Meier (77). Their results demonstrate that the galactomannans begin to be laid down in the seeds at the early stage of development and continue to be formed until the seeds reach their full size and yellow. After seed maturation the galactomannan content appears to decrease. Courtois and his co-workers (151, 152, 153) have shown with seeds of *G. ferox* and *G. tricanthos* that no changes in the proportion of galactose to mannose residues occur during the formation of the

polysaccharide. The authors suggested that the galactose and mannose residues in galactomannans are probably laid down at the same time by a specific mechanism (77) rather than by random attachment of D-galactosyl residues to a preformed mannose chain (154). When the galactomannans begin to form in vivo (77), the low molecular weight carbohydrate in the seed are mainly glucose, fructose, sucrose, galactinol and a trace of stachyose. However, after the deposition of galactomannans is complete, the most abundant sugar then present is stachyose which is accompanied by small amounts of raffinose, sucrose, galactinol and hexoses. Reid and Meier (77) suggested (in the case of Trigonella F-G) that as stachyose and galactomannans are synthesized in the seeds at the same time, that galactinol may be involved in the formation of both. Here it may be recalled that the biosynthesis of stachyose in the seeds of Phaseolus vulgaris a legume, which does not contain reserve galactomannans, has been shown to proceed by the transfer of an α-galactopyranosyl residue from galactinol to raffinose (108). The authors (77) suggested that the apparent decrease in polysaccharide may be due to a decrease in the solubility of the polysaccharide as the seeds dry out. Germination of seeds usually results in a rapid degradation of the galactomannans. For example Percheron and his co-worker (118) have shown that the galactomannan in Trigonella foenum graecum seeds is utilized first by removal of galactose residues which is reflected in the changes in galactose/mannose ratio of the polysaccharide isolated from the seeds at various stages of germination. At later stages the mannose backbone is cleaved. In vitro α-galactosidases from A. niger (28), P. vulgaris (155) and coffee species (151, 152) have all been reported to liberate galactose from galactomannans of leguminous

seeds. Courtois et al. (151, 152) showed that when the galactose/mannose ratio of the polysaccharide is less than 1, then coffee bean α-galactosidase can remove all galactose residues from the mannose chains. An insoluble mannan precipitates out when the galactomannan from Gleditschia ferox which has a galactose/mannose ratio of ca 0.25 is treated with α-galactosidase.

2. In microorganisms

A limited amount of literature is available on α-galactosidases in microorganisms (cf. Dey and Pridham (4)). Wild strains of many bacteria (64, 155) contain no α-galactosidase, but the enzyme can often be induced in these organisms by feeding various α-D-galactopyranosides (157, 158, 159) and in some cases β-D-galactopyranosides (156, 160). Melibiose can induce both α- and β-galactosidase in E. coli and A. aerogenes (45, 157, 160-166). In Streptococcus sp. (167) an α-galactosidase, which can hydrolyse blood group substance B, can be induced by galactose, lactose, raffinose and melibiose. Hogness and Battley (160) and Sheimen and Crocker (159) observed that phenyl and o-nitrophenyl α-D-galactosides induced the formation of α-galactosidase in A. aerogenes and E. coli.

3. In animals

The α-galactosidases in animal tissues have received relatively little attention compared with those of plants. The main potential substrates for α-galactosidase in animal tissues are galactose-containing lipids and oligosaccharides.

Ceramide trihexoside, a glycolipid, possesses an α-linked terminal galactosyl residue (168, 169). The accumulation of this compound (α-D-Gal-(1 → 4)-β-D-Gal-(1 → 4)-β-D-

Glc-(1 → 1)-ceramide) in Fabry's disease, in various organs of the human body has been examined by Wise et al. (170). The characteristic symptoms of the disease are skin lesion, angiokeratoma, cerebral artery haemorrhage and renal failure. The disease was initially reported to be due to a lack of a ceramide hexosidase, an α-galactosidase which removed the terminal α-galactosyl residues from ceramide trihexoside (171, 172, 173). It was later shown that normal human tissue contained two α-galactosidase isozymes. The predominant form, α-galactosidase A, is thermolabile, has an acidic isoelectric point, and is able to hydrolyse the terminal α-galactosyl residue of ceramide trihexoside (171, 172). This isozyme appears to be absent in patients with Fabry's disease. The other isozyme B, which is thermostable and has a neutral isoelectric point, cannot remove the terminal sugar residue: this isozyme is present in cases of Fabry's disease (and in normal individuals) (174, 175). Kint (176) observed that the α-galactosidase A contains nuraminic acid and that removal of this acid converts it to isozyme B. Beutler and Kuhl (177) suggested that the basic defect in patients with Fabry's disease might be the failure to convert the precursor (α-galactosidase B) into the active enzyme (α-galactosidase A) i.e. that nuraminic acid cannot be transferred to enzyme B (178, 179). This phenomenon may be analogous to the possible defect in Tay-Sachs disease (180-183) in which it has been suggested that the lack of the hexosaminidase A is due to the inability of the tissue to convert hexosaminidase B to the A form (184, 185).

Human erythrocyte plasma membrane contains α-galactosidase (186) along with other glycosidases. The glycosidases can remove carbohydrate from intact red blood cells and in vivo this could be an important mechanism for modifying the erythrocyte plasma membrane.

Blood group substances isolated from human secretion are well-known glycoproteins. The major sugars present are L-fucose, D-galactose, N-acetyl D-glucosamine, N-acetyl D-galactosamine and N-acetyl nuraminic acid (187, 188). The serological specificity of blood is associated with the pattern of sugar residues at the terminal non-reducing ends of the carbohydrate chains of the blood group substances (187, 188, 189). Blood group substance B is known to contain L-fucosidic and D-galactosidic residues as terminal end groups. α-Galactosidase from coffee beans has been shown to hydrolyse the terminal galactose residue from the B substance and reduce serological activity (190). This has also been demonstrated with α-galactosidases extracted from Clostridium maebashi (191), Trichomonas foetus (192) and Streptococcus sp. (67). It should be noted that not all α-galactosidase can act on B substance in this way: such as the case with α-galactosidases from brewer's yeast, almond emulsin, Patella vulgata (193) and Mortierella vinacea (15).

II. MATERIALS AND METHODS

General: Analar grades of reagents and only glass distilled or deionized water was used. All operations involving enzymes, unless specified, were carried out at 4°.

Vicia faba seeds, variety Bunyard exhibition (long pod) were used as a source of enzyme preparations.

Enzyme Assays: Enzyme activities were determined by measuring only the initial rates of substrate hydrolysis. α-Galactosidase was assayed by addition of appropriately diluted enzyme solution (0.1 ml) to a mixture of McIlvaine buffer (194), pH 5.5 (0.6 ml) and 2.0 m M - PNPG (0.3 ml) preheated to 30°. This temperature was maintained for 15 min and the reaction was then stopped with 0.1 M - sodium carbonate solution (5.0 ml) and the P-nitrophenol released measured using a Unicam SP. 500 spectrophotometer at 405 nm. The extinction coefficient of P-nitrophenol, under the assay conditions used is $\longrightarrow$ 1.53×10^{4} cm /m mol (44).

A unit of activity is defined as the amount of enzyme that hydrolyses one μ mol substrate per min. under the condition specified.

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

The galactose released from galactomannans was measured by the micro-cuprimetric method of Nelson (195) using an arsenomolybdate reagent (196). The reagent was prepared as follows:-

Copper reagent A: Na_2So_4 (20.0g) $NaHCo_3$ (2.0g) and Rochelle salt (2.0g) in water (100 ml).

Copper reagent B: 15% aqueous $CuSo_4.5H_2o$ solution.

35 volumes of A and 1 volume of B were mixed together. One ml of this solution was added to each digest at the completion of the enzyme reaction. The mixture was then heated in a boiling water

bath for 20 minutes, cooled and arsenomolybdate reagent (1.0 ml; diluted 1:2, v/v, with 1.5 N H_2SO_4) and water (3.0 ml) then added. The resulting colour was measured at 560 nm (Unicam SP.500). The amount of galactose released was determined from a calibration curve prepared using D-galactose (Fig. 3).

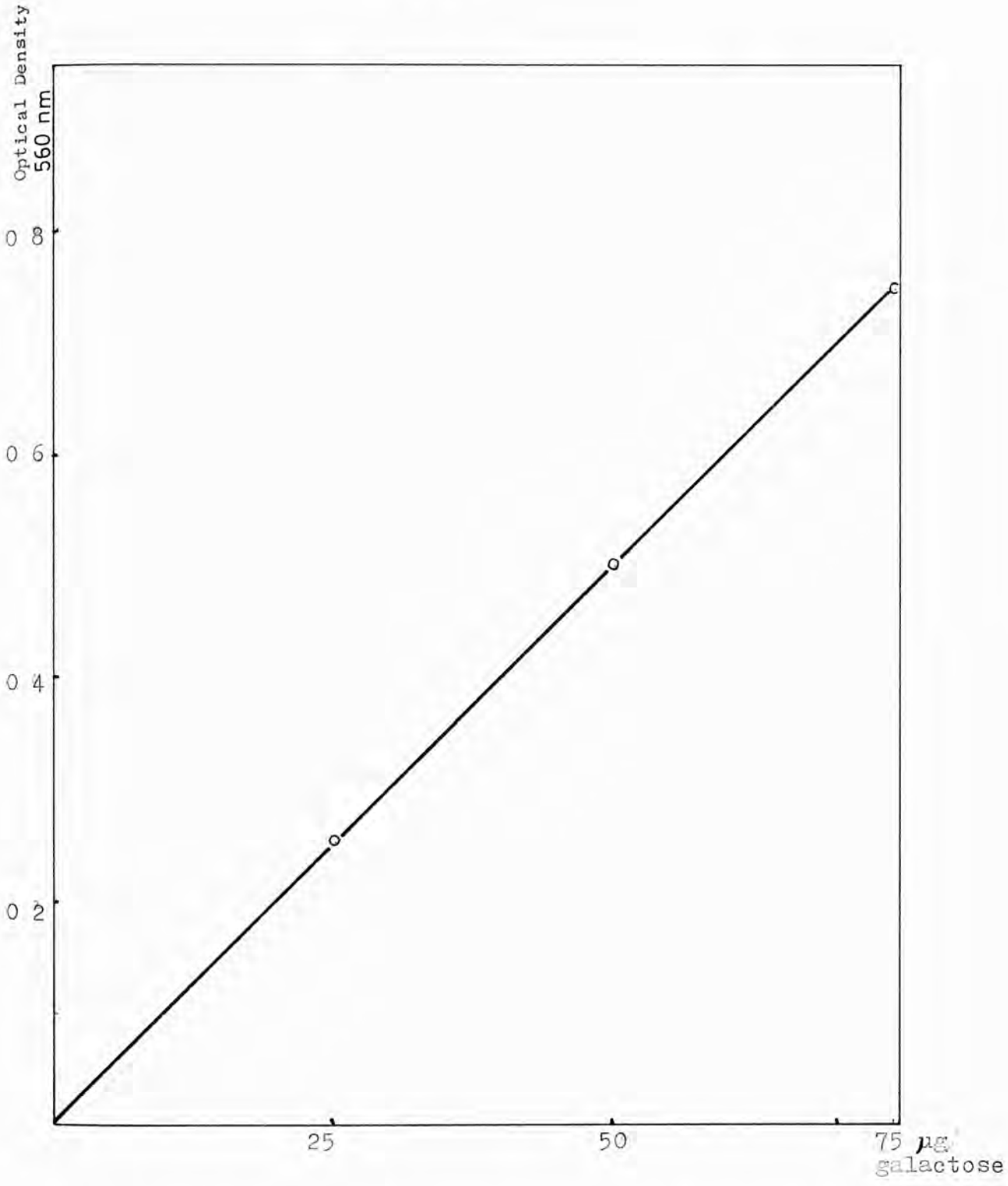


Fig. **3.** Standard curve for determination of galactose by the Nelson(I95) method.

Determination of Protein: This was carried out by the method of Folin and Ciocalteu (197) as modified by Lowry et al (198). The procedure was as follows:

Reagent A: 2.0% Na_2CO_3 in 0.1 N-NaOH.

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

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

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

3M - Trichloroacetic acid (0.4 ml) was added to the appropriately diluted enzyme solution (1.0 ml). The precipitated protein was the collected by centrifugation at 5000 r.p.m. using a MSE bench centrifuge and the supernatant solution was discarded. The precipitate was then suspended in water (0.9 ml) using a Rotamixer, 1N-NaOH (0.1 ml) was then added. After solubilization of the protein, solution C (5 ml) was added followed 10 minutes later by the diluted Folin-Ciocalteu reagent (0.5 ml). After 30 minutes the absorbance of the solution was measured at 750 nm (Unicam SP. 500). Crystalline bovine serum albumin was used to prepare a calibration curve (Fig. 4).

Estimation of Carbohydrate: The total carbohydrate content in enzyme preparations was measured by phenol/sulphuric acid method as described by Dubois et al. (199). Glucose was used as standard (Fig. 5) and the results expressed as glucose equivalents.

Ammonium sulphate fractionation: Powdered ammonium sulphate was added in ca. 0.5g quantities with gentle and constant stirring at 4^{o} and pH 5.5. Percent saturation was calculated from the nomogram described in Methods in Enzymology (200, 201).

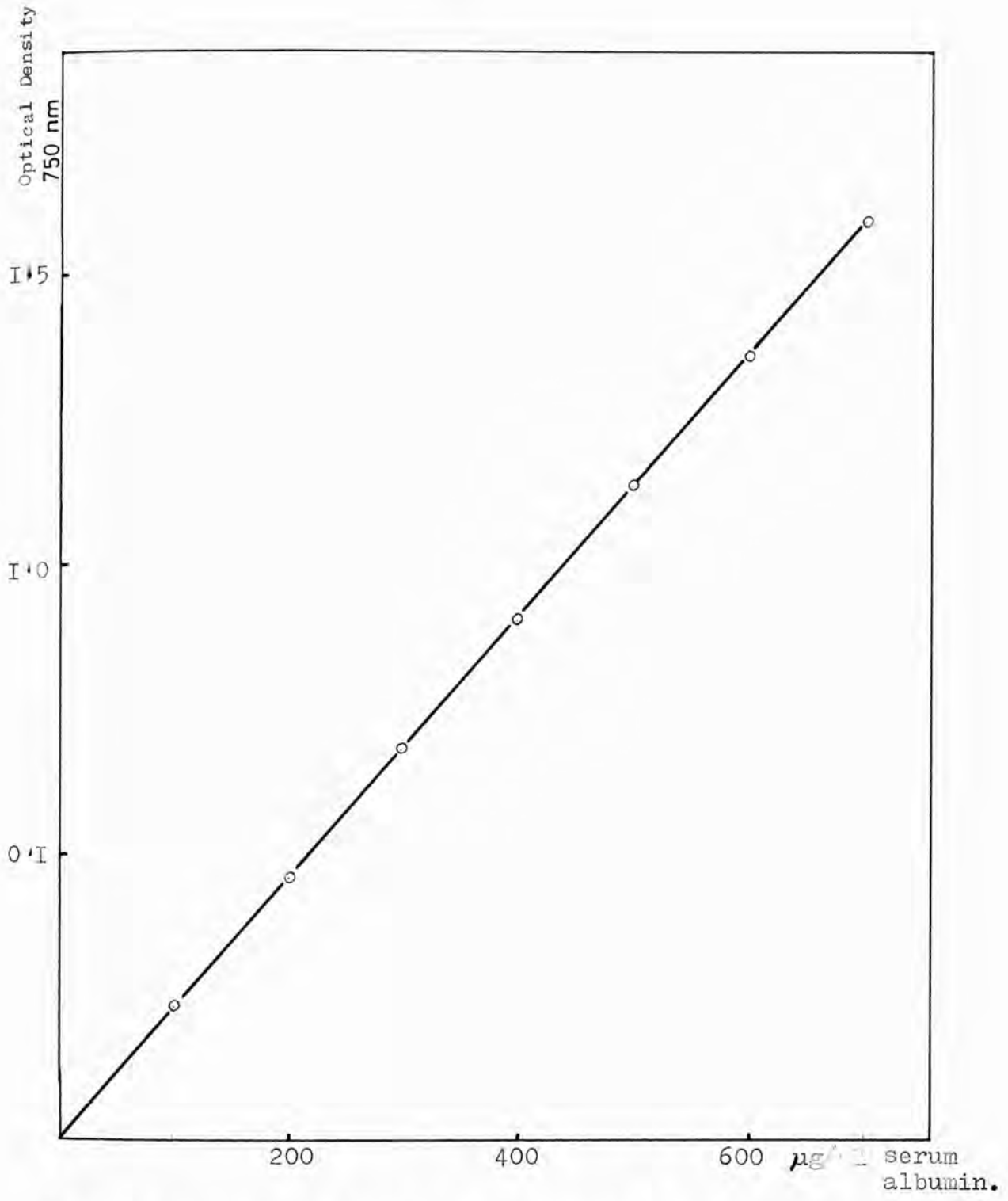


Fig. 4. Standard curve for determination of protein by the Lowry et al. (197) method.

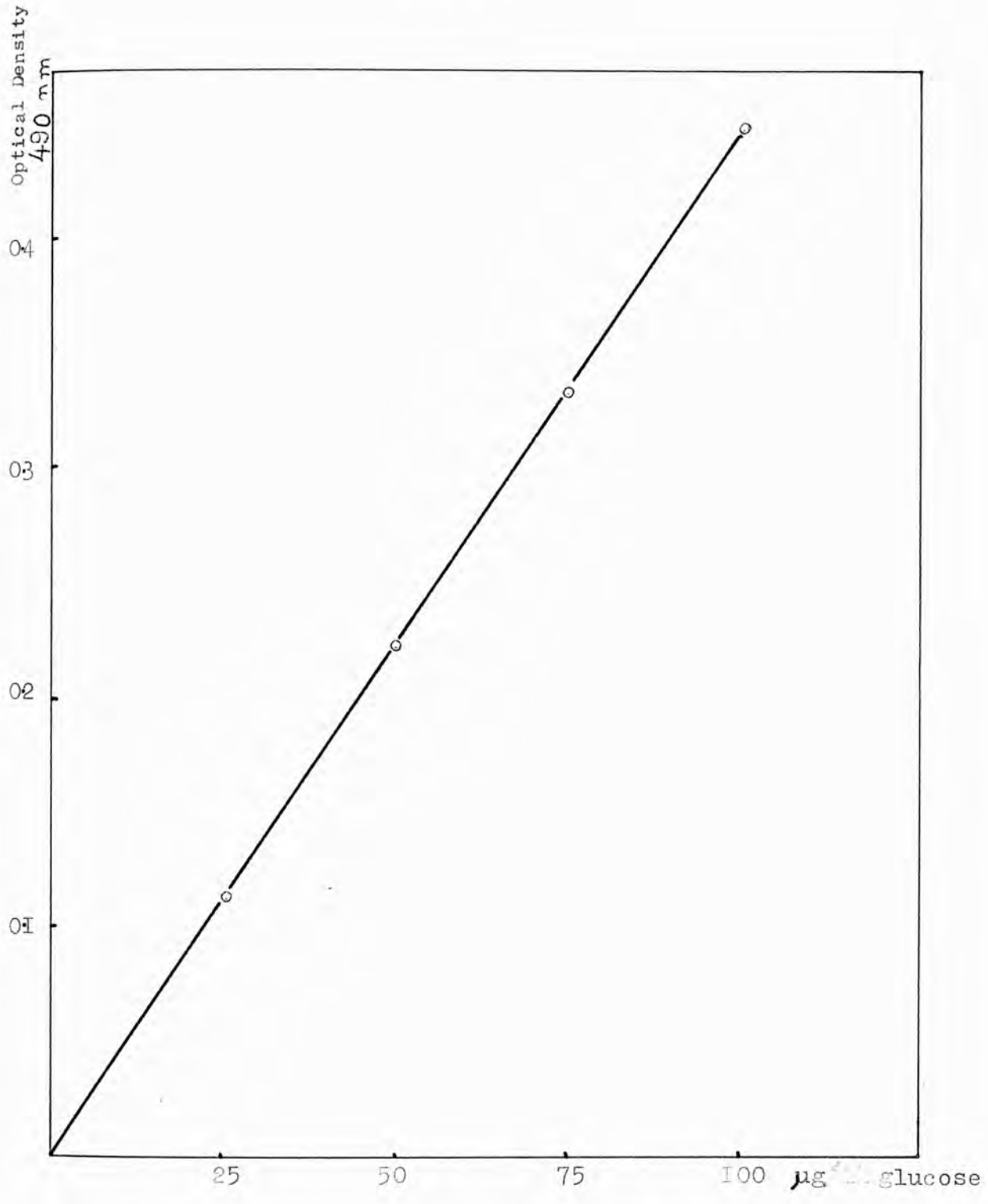


Fig.5. Standard curve for determination of glucose by the method of Dubois et al. (I99)

Sephadex gel filtration: Sephadex G-100 and G-200 columns (86 cm x 2.5 cm internal diameter) was prepared by the method described by Andrews (30). The columns were eluted with McIlvaine buffer, pH 5.5, at a flow rate of 30 ml per hour and 3 ml fractions were collected. The packing of the column was checked and the void volume (Vo) determined by using 0.2% blue dextran 2000 (Pharmacia chemicals, Uppsala, Sweden). Columns were calibrated at 4°. 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 XM-100 A and PM-30 membranes (Amicon Corporation, Lexington, Massachusetts, U.S.A.) for enzyme I and II respectively.

Ion exchange chromatography: (a) Anion-exchange chromatography - Whatman chromedia DE 11 anion-exchange cellulose was pre-cycled according to the method described by Mikes(202). The precycled resin was then equilibriated with McIlvaine buffer, pH 8.0 (194) and then deaerated and a column (20 cm x 1.4 cm internal diameter) prepared. The enzyme preparation (dialysed against McIlvaine buffer, pH 8.0) was then applied to the column and a linear salt gradient (0.05 M-Nacl in McIlvaine buffer, pH 8.0, 300 ml - 0.5 M-NaCl in McIlvaine buffer, pH 8.0, 300 ml) used for elution. Fractions (3 ml) were collected at a flow rate of 15 ml/hr.

(b) Cation-exchange chromatography - Whatman ion-exchange cellulose, CM 32, was pre-treated according to Mikes(202) and equilibriated with McIlvaine buffer (pH 3.5), deaerated and the column was then packed (1.5 cm x 20 cm internal diameter). The enzyme preparation was dialysed against the same buffer and was applied to the column. A linear NaCl gradient in McIlvaine buffer, pH 3.5, as described for DEAE-cellulose chromatography, was used for elution. In some cases a stepwise, pH

gradient was also used for elution. This was carried out with successive 60 ml volumes of McIlvaine buffers pH 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0. Three ml fractions were collected at a flow rate of 15 ml/hr. both with linear salt and stepwise pH gradients.

Ultra centrifugation study: Sedimentation equilibrium experiments were carried out by Dr. S.P. Spragg, of the Department of Chemistry, University of Birmingham, using Kelf standard Spinco Model E Ultra-centrifuge with a RITC rotor unit. Sedimentation was measured by using a UV absorption optical system at 284 nm. Optical density was checked 19 times in each experiment and the data were edited and programmed by Computer. The sedimentation experiments were carried out at 20°-21°. The enzymes were examined in McIlvaine buffer pH 5.5. The concentration of enzymes were 1.20, 1.72, 3.80 and 4.10 mg/ml for enzyme I, enzyme II, enzyme II^{1} and enzyme II^{2} respectively.

Amino acid analysis: Amino acid compositions of enzymes were examined at the Centre for Material Services, University of Birmingham, with a GEOL 5AH automatic amino acid analyser using the standard two column technique (Table 2). Tyrosine was not estimated in any case because of the method used for hydrolysis the proteins (6 N-HCl for 24 hours at 110°). Same enzyme solutions used for the ultra centrifugation study were used for amino acid analysis.

Circular dichroism: Circular dichroism measurements were carried out by the University of London Intercollegiate Research Services at Westfield College, using a Roussel Jouan Dichrograph 180. The enzyme (EI from dormant seeds; 1.2 mg/ml) was examined in McIlvaine buffer, pH 5.5 using a path length of 0.5 cm for 250 - 330 nm and 0.05 cm for 200 - 260 nm.

Ultraviolet spectrum: Ultraviolet spectra of purified enzyme preparations were examined with a Unicam SP. 800 spectrophotometer,

Table 2

Amino Acid Compositions of various forms of α-galactosidase from Vicia faba seed

Amino Acid	Enzyme I (Dormant Seed) Recovery 71.5%	Enzyme II (Dormant Seed) Recovery 90.1%	Enzyme II[1] (Dormant Seed) Recovery 81.7%	Enzyme II[2] (Dormant Seed) Recovery - 90.3%	Enzyme I (Converted from Enzyme II in vitro) Recovery - 90.3%	Enzyme II (Germinated Seed) Recovery - 92.1%
	micro mol per 100 mg of protein					
Lysine	14.5	15.5	14.7	15.1	15.6	15.2
Histidine	3.9	6.9	3.8	5.6	4.6	5.9
Arginine	10.8	12.0	10.3	10.3	7.7	10.0
Aspartic Acid	34.8	32.4	30.0	32.6	32.4	33.0
Threonine	15.7	18.0	13.0	16.0	18.3	17.0
Serine	26.1	26.7	23.0	25.1	26.9	26.4
Glutamic Acid	22.9	23.8	21.8	24.2	24.2	24.6
Proline	12.7	16.6	19	20.6	20.5	20.8
Glycine	25.5	26.0	24.1	25.1	30.3	25.4
Alanine	16.8	16.8	14.7	18.4	22.0	18.3
Cysteine	8.4	10.4	15.1	9.9	10.2	13.3
Valine	14.5	16.8	14.0	15.0	16.4	15.8
Methionine	1.1	1.7	1.7	2.7	1.0	3.0
Isoleucine	9.6	11.2	10.4	11.4	10.7	13.0
Leucine	19.9	19.6	16.8	19.6	24.1	21.8
Tyrosine	7.4	8.0	8.7	11.4	9.5	10.7
Phenyl Alanine	9.6	11.0	11.0	12.3	10.6	13.5

using 1 cm path length cells. McIlvaine buffer, pH 5.5, was used as blank.

Purification of α-galactosidases: The following steps were carried out in succession:-

Stage 1. Extraction: Mature and dry (referred to as dormant) testa-free Vicia faba seeds (Long pod, Bunyard Exhibition type) are finely powdered in a grinder and extracted for 30 minutes, three times with McIlvaine buffer, pH 5.5 (1 kg/1000 ml buffer). The extract was then centrifuged at 10,000xg for 30 minutes and the clear supernatant solution used as a source of crude enzyme.

Stage 2. Variation of pH: The pH of the solution was lowered to 2.5-3.0 with 1.0 M - citric acid solution. Slow addition of citric acid and efficient stirring was maintained and the mixture was then kept overnight at 4° with constant, gentle stirring. The resulting inactive proteins were removed by centrifugation at 22,000xg for 20 minutes and the pH of the supernatant solution was readjusted to 5.5 with 1.0 M - disodium hydrogen orthophosphate solution.

Stage 3. Protamine sulphate treatment: Protamine sulphate solution was added to the enzyme preparation to give a final concentration of 10 mg/ml and any nucleic acid precipitated over 24 hours was removed by centrifugation.

Stage 4. Acetone precipitation: Analar acetone (precooled to -20°) was gradually added to the enzyme solution, which was also kept between -4° and -10°, to a final concentration of 30% (v/v). Constant and gentle stirring was maintained all through the procedure. After 20 minutes, the inactive protein precipitate was removed by dentrifugation at 27,000xg for 3 minutes. The acetone concentration in the supernatant solution was then increased to 60% (v/v) and the resulting active precipitate centrifuged down, collected and dissolved

in McIlvaine buffer, pH 5.5. This solution was dialysed first against 3-4 changes of water for 6 hours (each change) at 4°, to remove traces of acetone, then against McIlvaine buffer, (pH 5.5; diluted 1:4, v/v with water), for 24 hours. The turbidity was removed by centrifugation.

Stage 5. Ammonium sulphate fractionation: The active enzyme solution was fractionated with ammonium sulphate using the procedure already described. Precipitates obtained by 0-30% saturation with ammonium sulphate were spun down and discarded and the concentration of ammonium sulphate in the supernatant solution then increased to 70%; the active precipitate was centrifuged off, collected and dissolved in McIlvaine buffer pH 5.5. The solution was then dialysed against 3-4 changes of buffer, pH 5.5, for 6-8 hours (each change) to remove ammonium sulphate.

Stage 6. Sephadex gel filtration: The dialysed enzyme solution was then passed through a Sephadex column (see page 42). Eluted enzyme fractions were pooled separately then concentrated and dialysed against McIlvaine buffer, pH 5.5.

Stage 7. Recycling through Sephadex column: The isolated dialysed enzyme was recycled twice through a Sephadex column and the various fractions obtained treated as in Stage 6.

Stage 8. Cation-exchange chromatography: The enzyme solution from stage 7 were dialysed against McIlvaine buffer, pH 3.5 and then passed through a CM-cellulose column, which had been pre-cycled and equilibriated at pH 3.5, as described in the earlier section. The resulting 'peaks' of activity were pooled separately and concentrated.

In one instance enzymes I and II from dormant seeds were examined in DE 11 anion-exchange columns (see page 42) after stage 7. The enzymes were not absorbed by the exchanger and no further examination was undertaken.

A summary of the complete purification procedure is given in Table 3

Table 3

Purification of dormant Vicia faba seed α-galactosidases

Purification Stage	volume (ml)	Enzyme Activity (milliunits/ml)	Protein (mg/ml)	Sp. Activity (milliunits/mg of protein)	Recovery* (%)
1. Extraction	3,700	183	12.0	15.2	-
2. Acidification (pH 2.5)	4,200	143	3.2	44.7	87
3. Protamine Sulphate Treatment	4,300	140	2.1	66.4	83
4. Acetone Fractionation	120	3,392	20.5	165.4	81
5. Ammonium Sulphate Fractionation	45	9,223	26.5	342	101
	I II	I II	I II	I II	
6. Sephadex G-100 Gel Filtration and Concentration	2.5 5.0	103,225 30,510	2.5 2.3	41,290 13,260	99
7. Recycling through Sephadex G-100 and G-200 Gel Column and Concentration	2.5 5.0	99,000 27,880	2.1 1.9	47,143 14,300	93
	II^1 II^2	II^1 II^2	II^1 II^2	II^1 II^2	
8. CM-cellulose Chromatography and Concentration	1.0 1.0	92,540 31,200 28,500	1.4 2.8 4.2	65,631 11,119 6,753	75

* Recovery was calculated on the basis of the total enzyme activity from the proceeding stage

The overall purifications were 4316, 441, 731 and 444 fold for enzyme I, II, II^1 and II^2 respectively.

III. RESULTS AND DISCUSSION

A. α-GALACTOSIDASE FROM DORMANT VICIA FABA SEEDS

The occurrence of multiple forms of α-galactosidase in Vicia faba seeds has been discussed in the introduction of this thesis (see page 11). In the present study, further work on the purification and properties of these forms is described.

1. Purification

Initially attempts were made to improve the purification methods of Dey and Pridham (25) and to obtain α-galactosidases free from other contaminating glycosidases and in higher yields.

The extraction of α-galactosidases from dormant seeds was accomplished by soaking the powdered seed in McIlvaine buffer, pH 5.5, for approximately 1.5 hours then centrifuging the suspension immediately. This procedure was found to be better than extending the soaking for longer periods (24 hours; Dey and Pridham, ref. 25) and resulted in higher total and specific activities.

The second stage of purification which consisted of lowering the pH of the enzyme extract, resulted in complete removal of all the contaminating glycosidase activities (25).

In the third stage the nucleic acid was removed by protamine sulphate. Some protein seemed to be co-precipitated along with the nucleic acid and this resulted in a slight increase in the specific activity. The protamine sulphate treatment was found to be helpful in achieving effective purification in later stages.

Fractionation of the proteins with acetone in stage 4 removed fats, phenolic compounds and other acetone soluble

materials from the preparation together with some soluble carbohydrates. A two-fold increase in the specific activity was obtained at this stage.

Following stage 4 preliminary further fractionation with ammonium sulphate, ethanol and acetone (second acetone fractionation) were attempted and the results are shown in Table 4. On the basis of those investigations it was decided to proceed with the main fractionation using ammonium sulphate in view of the relatively high activity yields obtained and the simplicity of the technique.

The ammonium sulphate fractionation, stage 5, of the enzyme preparation from stage 4 resulted in a further two-fold increase in the specific activity with a recovery of 85%.

The first Sephadex G-100 gel filtration, stage 6, produced two protein fractions (enzyme I and II; Fig. 6) with α-galactosidase activity (cf Dey and Pridham, ref. 24). These were pooled separately and concentrated by using an Amicon ultra-filtration cell. The elution volume of enzyme I was very close to the void volume of the column but enzyme II was well within the fractionation range of Sephadex G-100. It was thought that enzyme I might consist of more than one enzymically active protein and, therefore, fraction was next passed through a Sephadex G-200 column (Fig. 7). The elution pattern showed a single active peak.

Enzyme I and II thus obtained were recycled, stage 7, separately, through Sephadex G-200 and G-100 columns respectively and the fractions with high specific activity pooled and concentrated as before.

Ion-exchange chromatography was used, stage 8, to achieve further purification of enzyme I and II. When α-galactosidase I from stage 7 was applied to a cation exchange (CM-cellulose)

Table 4

Fractionation of α-galactosidase from dormant Vicia faba seed. Enzyme (16,000 milliunits) in 50 ml was fractionated by using Ammonium Sulphate, Ethanol and Acetone. The resulting precipitates were then dissolved in buffer (5.0 ml) and assayed.

Precipitant	Percent Concentration	Enzyme (milli-α-units/ml)	Protein (mg/ml)	Sp. Activity (milliunits/mg of protein)
Ammonium Sulphate	0 - 30	179	8.3	21.5
	30 - 50	1196	22.0	54.4
	50 - 70	1450	20.5	72.5
Ethanol	0 - 30	1490	22.0	68.0
	30 - 50	426	8.0	53.3
	50 - 70	136	7.5	18.1
Acetone	0 - 30	1407	22.3	63.1
	30 - 50	1023	25.0	41.0
	50 - 70	136	7.5	18.2

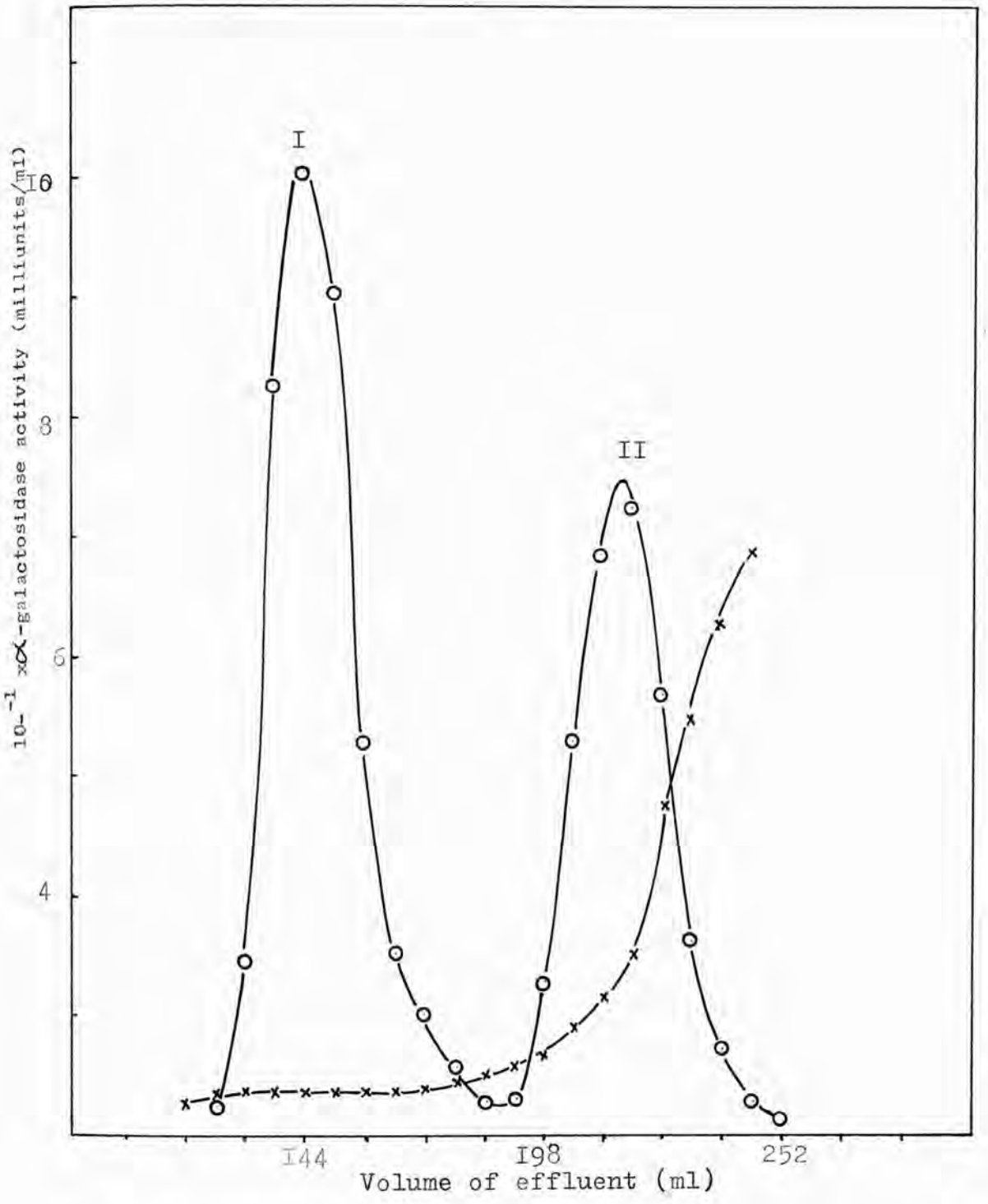


Fig. 6. Separation of two forms of ∝-galactosidase (I and II) from mature Vicia faba seed, by Sephadex G-100 gel filtration. Enzyme from purification stage 5 was applied to the column (2.5cm X 86cm). Experimental conditions are given in the text, p.42. o—o , ∝-galactosidase activity; x—x , protein.

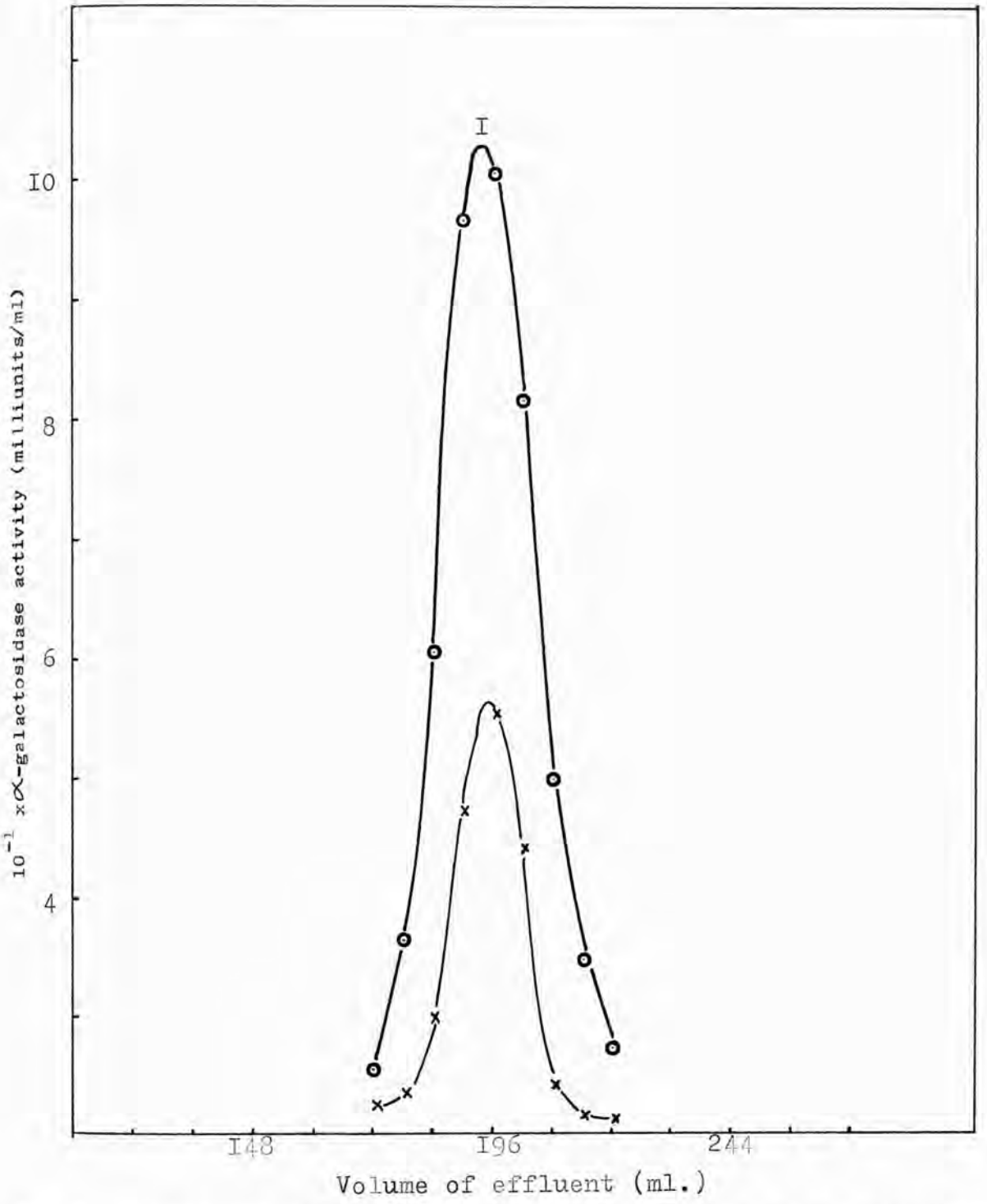


Fig.7. Sephadex G-200 gel filtration of purified ∝-galactosidase I from mature Vicia faba seed. The procedure and the conditions were the same as those described in Fig. 6 .o—o, ∝-galactosidase activity; x——x , protein.

column which was then eluted stepwise with McIlvaine buffers of different pH, two protein peaks were obtained but only one of these which was eluted at pH 4.5, was enzymically active (Fig. 8). Enzyme II when passed through the CM-cellulose column gave rise to six protein peaks but only two, which came off the column at pH 6.0 and 6.5, possessed α-galactosidase activity (enzyme II^1 and II^2 respectively; Fig. 9). The total protein associated with the two active peaks was approximately equal but the activity of enzyme II^1 was three times that of enzyme II^2. When the two molecular forms (enzyme II^1 and enzyme II^2) from CM-cellulose column were combined in two different ratios and passed through a Sephadex C-100 column, in both cases a single peak resulted with an elution volume the same as that of the unresolved enzyme II (Fig.10). Elution of both enzymes I and II from CM-cellulose columns was also carried out using a linear salt (NaCl) gradient (0.04M - 0.5M; pH 3.5). Enzyme I came off the column as a single peak at a salt concentration of 0.35M-NaCl (Fig.11). Enzyme II was resolved, as before, into two enzymically active proteins (Fig.12) with enzyme II^1 eluting at a NaCl concentration of 0.12M and enzyme II^2 at 0.17M. The identification of the two molecular forms of enzyme II for comparison with enzyme II^1 and enzyme II^2 obtained from CM-cellulose using a pH gradient was achieved by kinetic studies (see page 77) and observation of the relative peak areas.

Enzymes I and II derived from the Sephadex columns were also subjected to DEAE-cellulose column and elution was effected with McIlvaine buffer (pH 8.0;) with a linear salt gradient. Enzyme I was eluted off as a single peak and was unretarded by the column: no noticeable increase in the specific activity of this enzyme resulted from this procedure (Fig.13). Enzyme II was not further resulved by DEAE-cellulose.

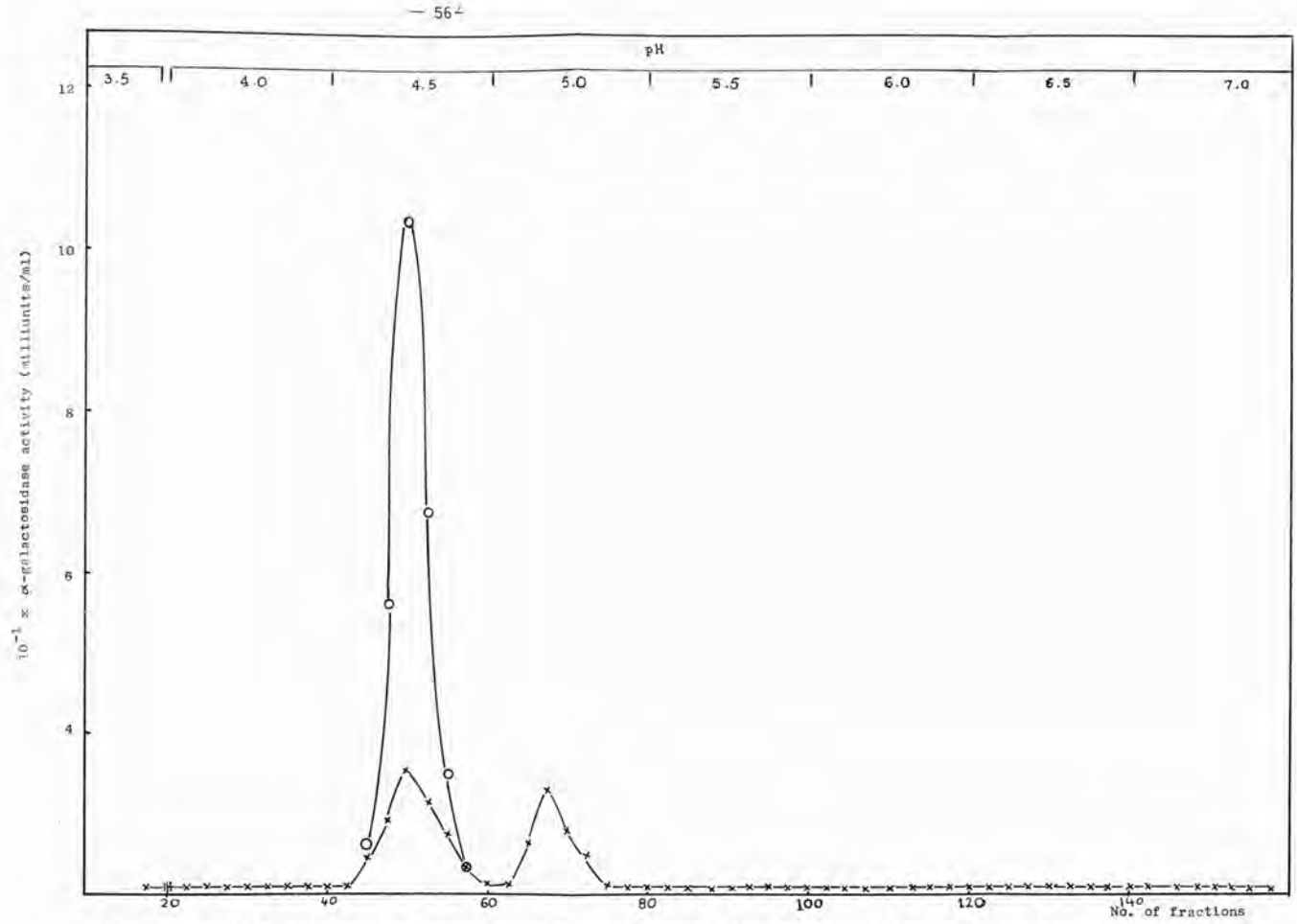


Fig. 8. CM-cellulose cation exchange, chromatography of purified enzyme I from dormant Vicia faba seeds. The elution was effected with a stepwise pH gradient ranging from pH 3.5 - 7.0. The procedure and conditions are described in the text, p. 42. o—o, enzyme activity; x——x, protein.

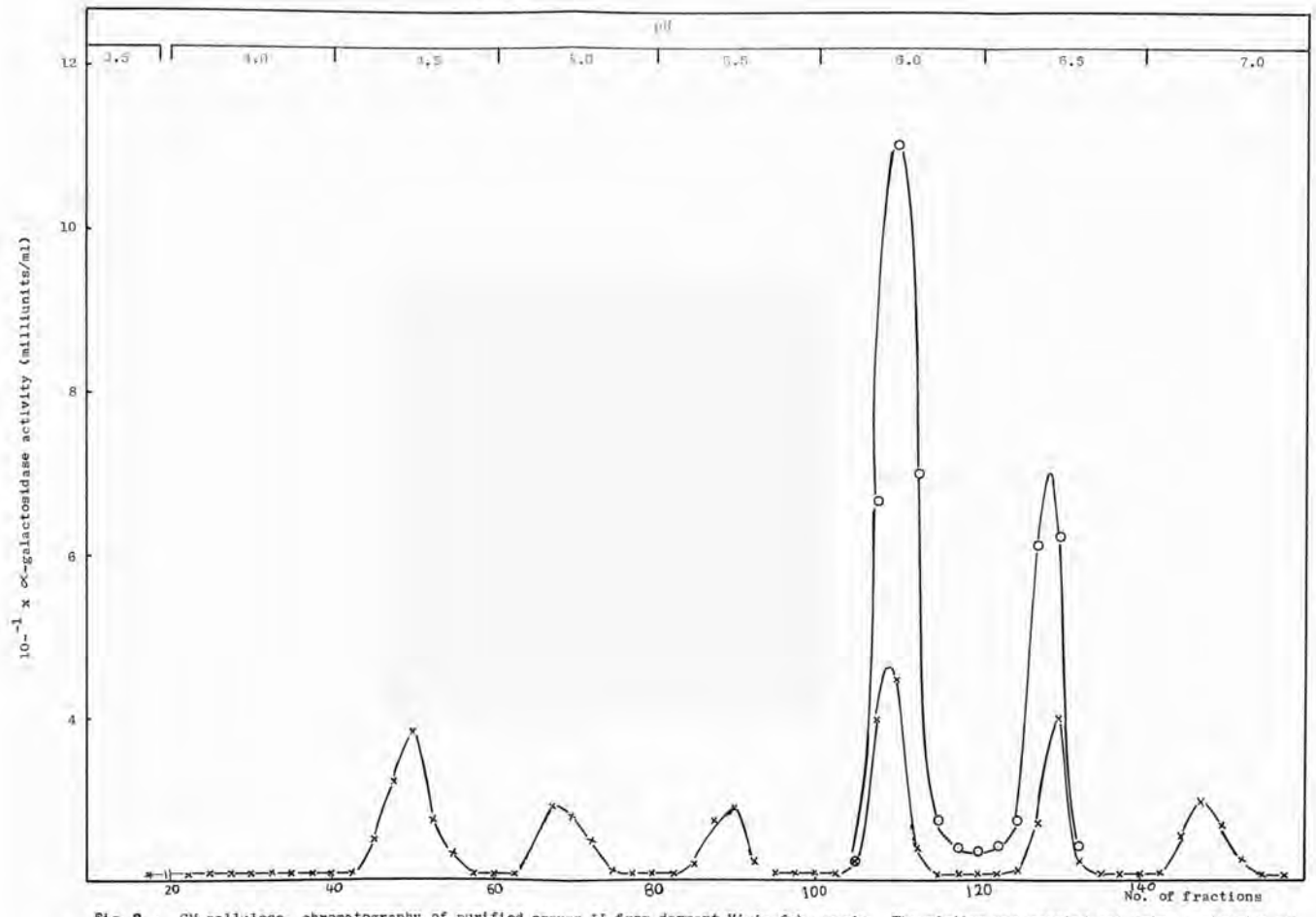


Fig. 9 . CM-cellulose, chromatography of purified enzyme II from dormant Vicia faba seeds. The elution was carried out using a stepwise pH gradient ranging from pH 3.5 - 7.0. The procedure and conditions are described in the text, p.42 •-•,α-galactosidase activity; x—x,protein.

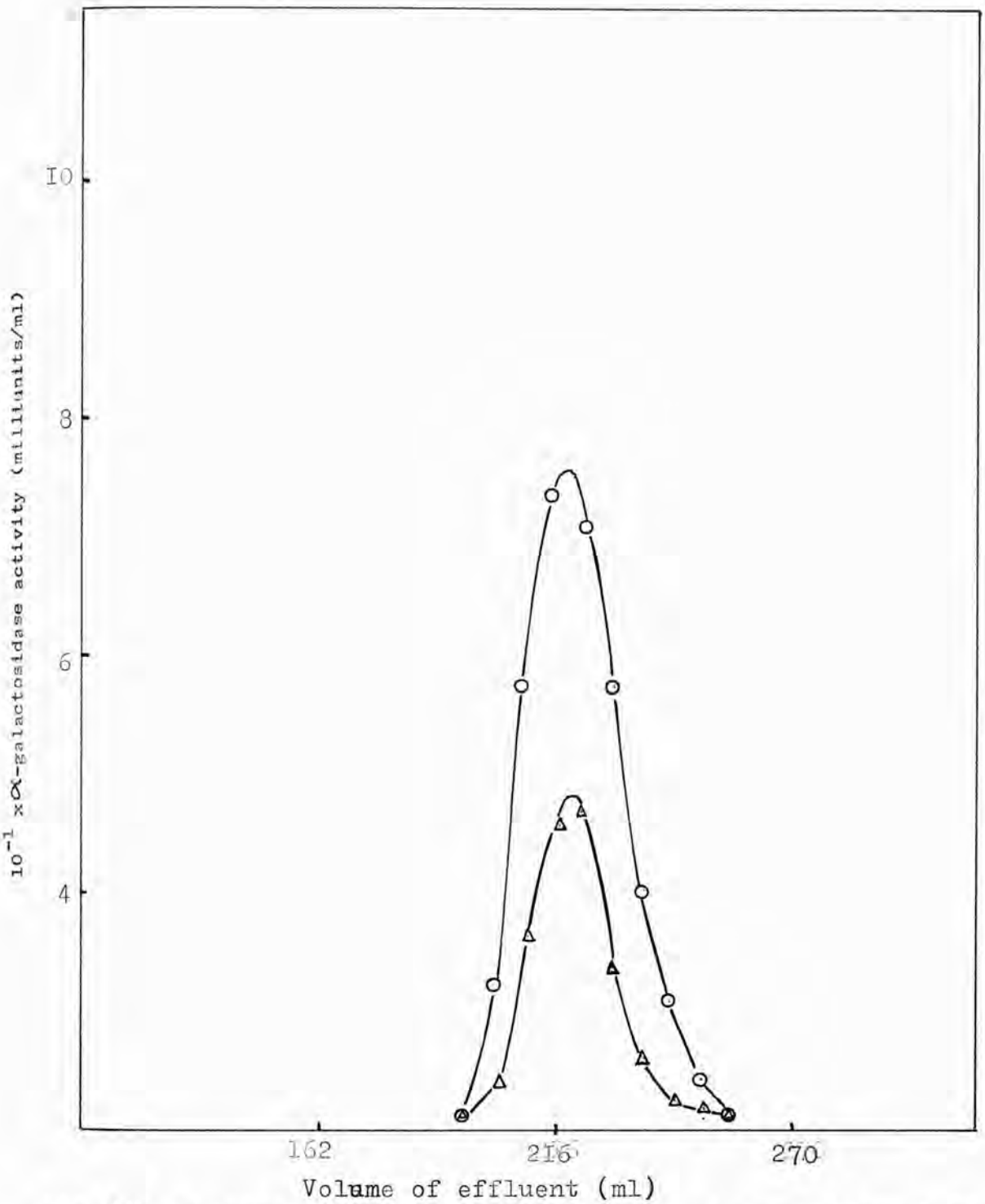


Fig. 10. Sephadex G-100 gel chromatography of an artificial mixture of Enzyme II^1 and II^2 from mature Vicia faba seeds. The enzymes were mixed in different activity ratio and passed separately through the column (25cmX86cm). The method is described in the text, p. 42 . o—o , enzymes II^1 and II^2 in a 4:I ratio; Δ—Δ , in I:4 ratio.

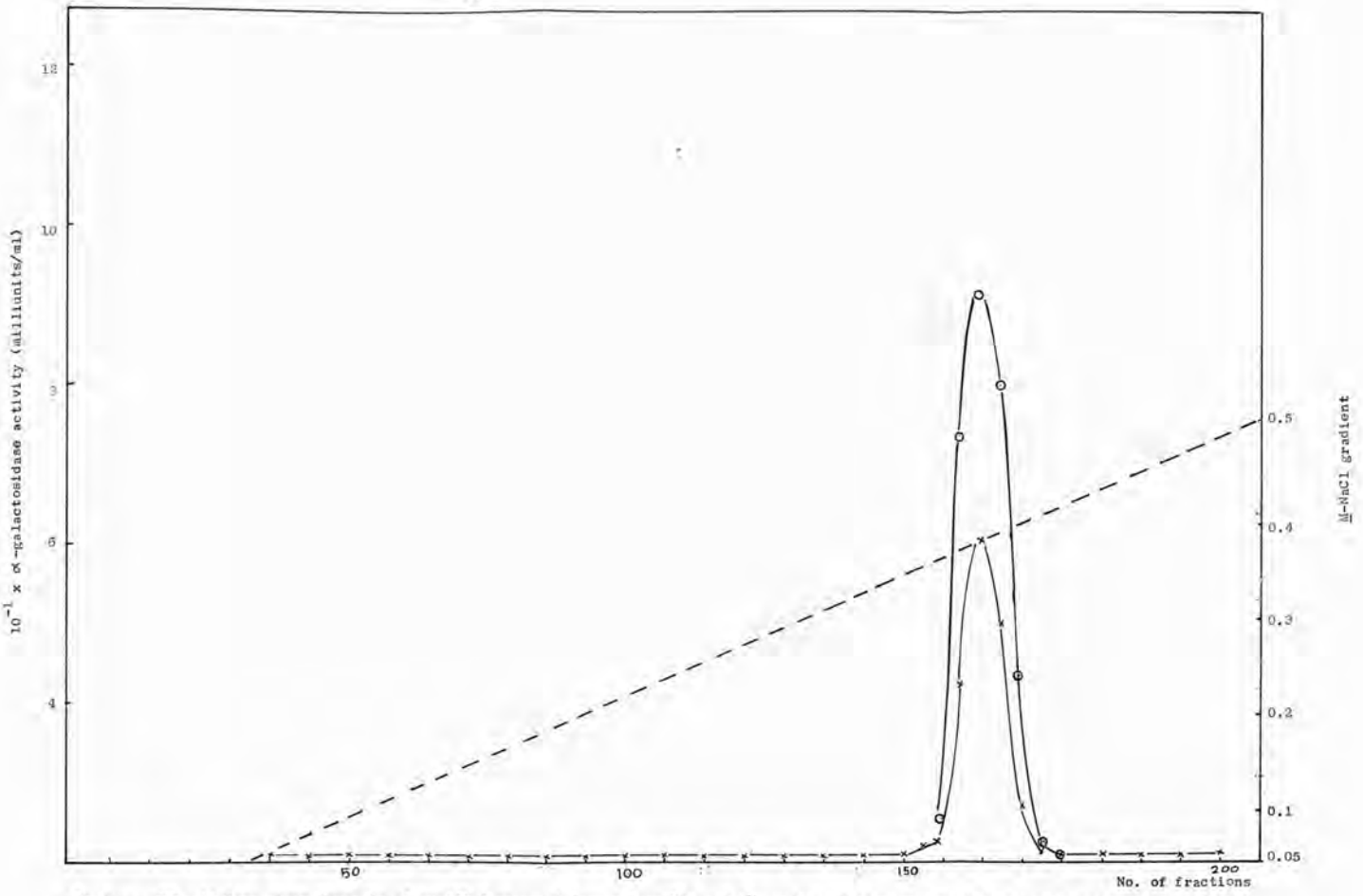


Fig.11 . CM-cellulose chromatography of purified enzyme I from mature Vicia faba seeds. Elution was carried out using a linear salt (NaCl) gradient ranging from 0.05M - 0.5M. The procedure is described in the text p.42. o-o, α-galactosidase activity; x-x, protein.

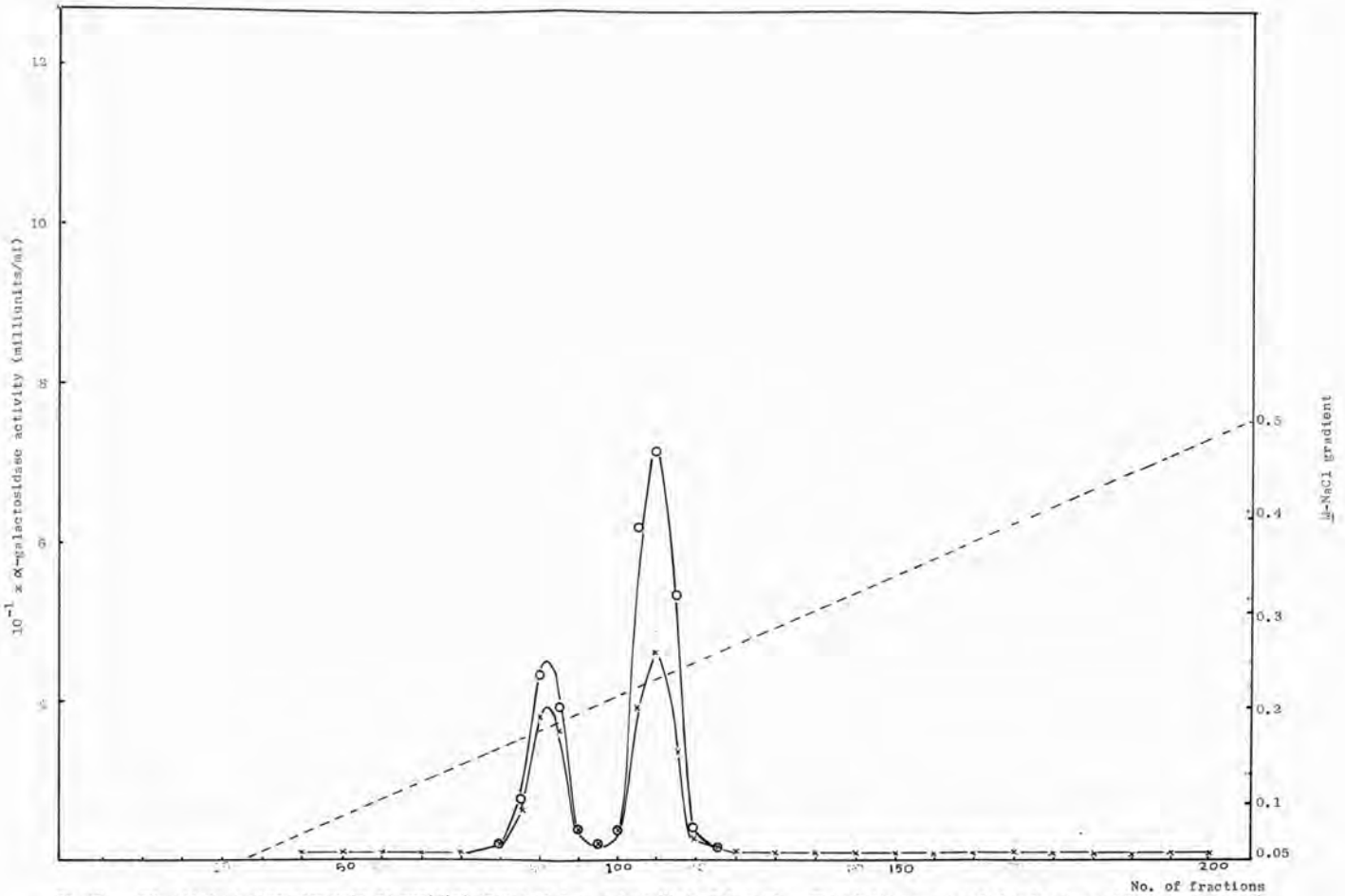


Fig.12 . CM-cellulose chromatography of purified enzyme II from mature Vicia faba seeds. The elution was carried out using a linear salt (NaCl) gradient ranging from 0.05 to 0.5M. The procedure is described in the text p.42. o-o α-galactosidase activity; x—x, protein.

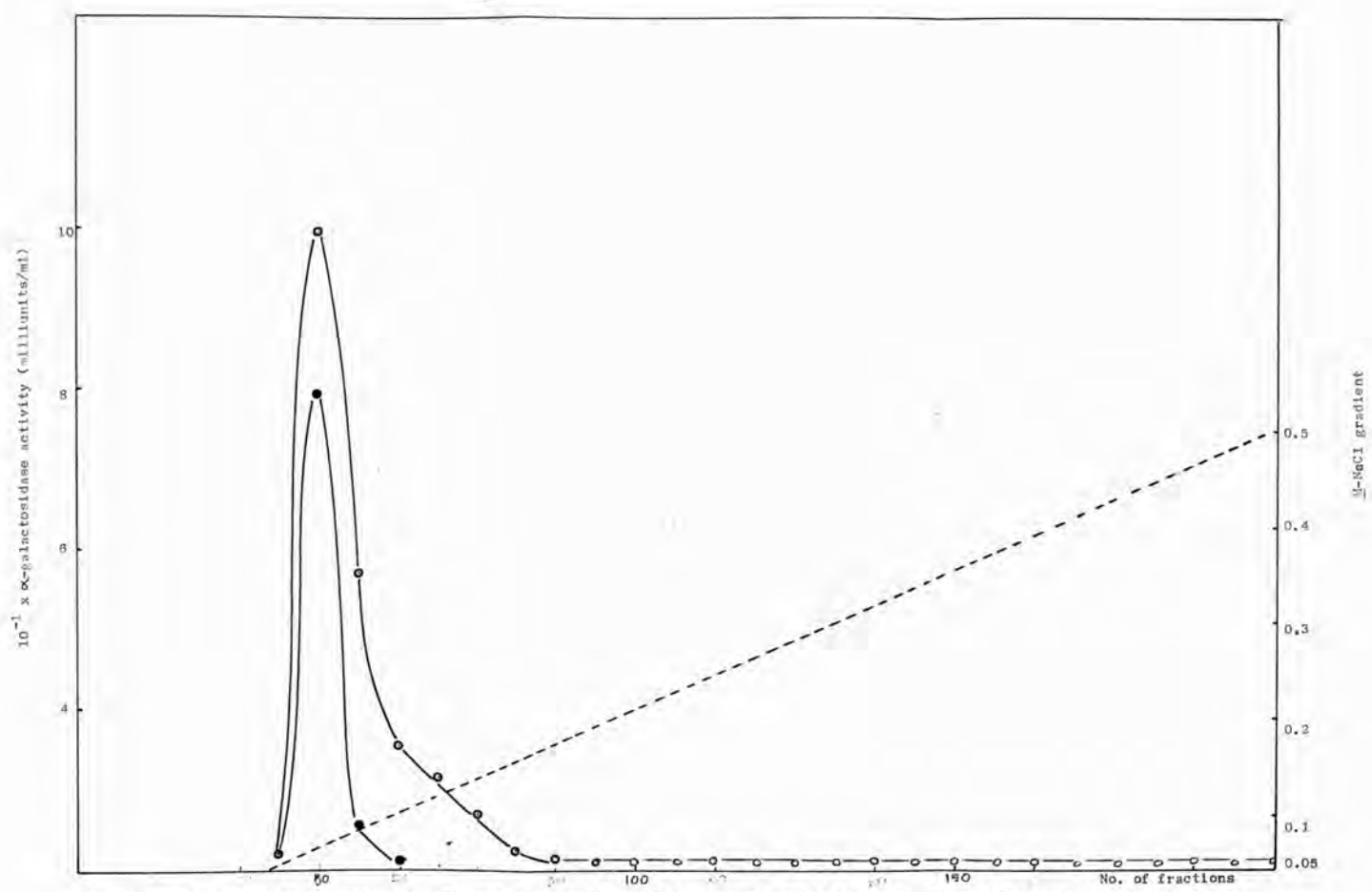


Fig.13 . DEAE cellulose chromatography of purified enzyme I and II from mature Vicia faba seeds. The elution was carried out by linear salt (NaCl) gradient ranging from 0.05M to 0.5M. The procedure is described in the text, p. 42. ○-○, ∝-galactosidase I; ●—●, ∝-galactosidase II.

2. Homogenity and Molecular Weights of α-galactosidases

The symmetry and coincidence of protein and activity peaks obtained from Sephadex and CM-cellulose columns (see Fig.7,8,9.) for enzymes I, II, II^1 and II^2 strongly suggested that these preparations were homogeneous. In addition, 96-98% of the activity could be recovered from these columns starting with enzymes purified to stage 8.

Calculation of apparent molecular weights of the preparations using the gel filtration method of Andrews (30) confirmed the values obtained by Dey and Pridham (25) for enzymes I and II (see table 5). The present studies also showed that the molecular size of enzyme II and its two components, II^1 and II^2 were approximately the same.

All preparations after stage 8 of purification were passed through Sephadex columns and then examined with an analytical ultracentrifuge using the sedimentation equilibrium method. The complete data for this study is given in tables 6, 7, 8, and 9. α-Galactosidase I, II, II^1 and II^2 all sedimented as single components. A computer analysis of the data by Dr. S.P. Spragg, showed that there was a linear relationship between Log_{10} of the vertical displacement of the fringe and the radial distance in each case. The weight, number and zeta averages calculated for each form of α-galactosidase were found to lie within a very narrow deviation range thus indicating the homogenity of the preparations. The molecular weights (weight average molecular weights) of the various forms of α-galactosidase are compared in Table10 (see also Tables 6, 7, 8, 9) with the data obtained by gel-filtration. The errors quoted for molecular weight determinations by gel-filtration are $\pm$10% in the case of non-conjugated proteins (30). If this is taken into account and the gel-filtration values then compared with

Table 5

Molecular weight of various forms of α-galactosidase from dormant Vicia faba seed; obtained by Sephadex G-100 gel filtration method (30).

Molecular forms of α-galactosidase	Molecular Weight
I	209,000
II	38,000
II^1	30,000
II^2	30,000

Table 6

Molecular Weight of α-galactosidase I (dormant *Vicia faba* seed). Computer data from ultra-centrifuge studies.

RUN 1890 J.PRIDHAM A-GALACTOSIDASE PH5. E1 CELL1 P03

SPEED = 0.8225E+04 GIVING OMEG**2 = 0.7420E+06
P. SP. VOL = 0.7500E+00 SOLUTION DENSITY = 0.1030E+01 EXPTL. TEMP = 0.2120E+02

MEAN MENISCUS = 0.6945E+01 WITH S.E. = 0.1916E-01

OPTICAL DENSITY FOR THIS SCAN = 0.4452E+00

MEAN RADIUS= 0.7060E+01

DATA FITS QUADRATIC WITH FOLLOWING COEFFICIENTS
-0.1451E+02 -0.7755E-02 0.5531E-02
AND STANDARD ERRORS
0.1780E+01 0.7410E-03 0.7114E-03

WT. AVERAGE MOLECULAR WEIGHTS FROM DERIVATIVE OF ABOVE REGRESSION EQUATION, FOR EDITING PURPOSES ONLY

OPTICAL DENSITY	MOL WT	ST ERROR		Z AVERAGE		NUMBER AVERAGE
0.1371E+00	0.1528E+06	0.1997E+05	0.1589E+06	0.1700E+08	0.1496E+06	0.7393E+04
0.1478E+00	0.1532E+06	0.2003E+05	0.1593E+06	0.1352E+08	0.1499E+06	0.7699E+04
0.1594E+00	0.1537E+06	0.2009E+05	0.1597E+06	0.1074E+08	0.1502E+06	0.8020E+04
0.1720E+00	0.1541E+06	0.2015E+05	0.1602E+06	0.8532E+07	0.1505E+06	0.8355E+04
0.1856E+00	0.1546E+06	0.2021E+05	0.1606E+06	0.6770E+07	0.1508E+06	0.8706E+04
0.2004E+00	0.1551E+06	0.2026E+05	0.1611E+06	0.5369E+07	0.1511E+06	0.9073E+04
0.2165E+00	0.1555E+06	0.2032E+05	0.1615E+06	0.4255E+07	0.1514E+06	0.9457E+04
0.2339E+00	0.1560E+06	0.2038E+05	0.1620E+06	0.3371E+07	0.1518E+06	0.9859E+04
0.2528E+00	0.1564E+06	0.2044E+05	0.1624E+06	0.2670E+07	0.1521E+06	0.1028E+05
0.2733E+00	0.1569E+06	0.2050E+05	0.1629E+06	0.2116E+07	0.1525E+06	0.1072E+05
0.2956E+00	0.1574E+06	0.2056E+05	0.1633E+06	0.1678E+07	0.1529E+06	0.1118E+05
0.3199E+00	0.1578E+06	0.2062E+05	0.1637E+06	0.1332E+07	0.1533E+06	0.1166E+05
0.3462E+00	0.1583E+06	0.2068E+05	0.1642E+06	0.1059E+07	0.1537E+06	0.1217E+05
0.3748E+00	0.1588E+06	0.2074E+05	0.1646E+06	0.8450E+06	0.1541E+06	0.1270E+05
0.4060E+00	0.1592E+06	0.2080E+05	0.1651E+06	0.6769E+06	0.1545E+06	0.1326E+05
0.4399E+00	0.1597E+06	0.2086E+05	0.1655E+06	0.5453E+06	0.1549E+06	0.1384E+05
0.4768E+00	0.1602E+06	0.2092E+05	0.1660E+06	0.4425E+06	0.1553E+06	0.1445E+05
0.5169E+00	0.1606E+06	0.2098E+05	0.1665E+06	0.3624E+06	0.1557E+06	0.1509E+05
0.5606E+00	0.1611E+06	0.2104E+05	0.1669E+06	0.3000E+06	0.1561E+06	0.1576E+05

DATA FITS STRAIGHT LINE OF SLOPE 0.5527E+00 AND STANDARD ERROR 0.9824E-02

SLOPE CORRECTED FOR SPEED = 0.7448E-06 S.E. = 0.1324E-07

WEIGHT AVERAGE MOLECULAR WEIGHT = 0.1604E+06 AND STAADARD ERROR = 0.2850E+04

ABSORBTION CORRECTED ASSUMING FIRST DATA POINT IS BASE LINE
OVERFLOW SET - CLEARED

Table 7

Molecular Weight of α-galactosidase II (dormant Vicia faba seed). Computer data from ultra-centrifuge studies.

RUN 1912 A-D GALACTOSIDASE EIIO P02

SPEED = 0.8225E+04 GIVING OMEG**2 = 0.7420E+06

>> SP. VOL = 0.7300E-00 SOLUTION DENSITY = 0.1000E+01 EXPTL. TEMP = 0.2120E+02

MEAN MENISCUS = 0.6905E+01 WITHS.E. = 0.9093E-02

OPTICAL DENSITY FOR THIS SCAN = 0.7034E+00

MEAN RADIUS= 0.7035E+01

DATA FITS QUADRATIC WITH FOLLOWING COEFFICIENTS
-0.4179E+01 0.2860E-02 0.1739E-02
AND STANDARD ERRORS
0.1883E+01 0.7100E-03 0.7737E-03

WT. AVERAGE MOLECULAR WEIGHTS FROM DERIVATIVE OF ABOVE REGRESSION EQUATION, FOR EDITING PURPOSES ONLY

OPTICAL DENSITY	MOL WT	ST ERROR	Z AVERAGE		NUMBER AVERAGE	
0.9063E+00	0.5355E+05	0.2346E+05	0.6013E+05	0.1644E+07	0.5022E+05	0.2234E+05
0.9337E+00	0.5375E+05	0.2355E+05	0.6030E+05	0.1586E+07	0.5032E+05	0.2276E+05
0.9621E+00	0.5394E+05	0.2364E+05	0.6047E+05	0.1529E+07	0.5042E+05	0.2318E+05
0.9916E+00	0.5414E+05	0.2372E+05	0.6064E+05	0.1475E+07	0.5052E+05	0.2362E+05
0.1022E+01	0.5434E+05	0.2381E+05	0.6081E+05	0.1422E+07	0.5063E+05	0.2407E+05
0.1054E+01	0.5453E+05	0.2390E+05	0.6099E+05	0.1372E+07	0.5075E+05	0.2453E+05
0.1086E+01	0.5473E+05	0.2399E+05	0.6116E+05	0.1323E+07	0.5086E+05	0.2500E+05
0.1120E+01	0.5493E+05	0.2407E+05	0.6133E+05	0.1275E+07	0.5098E+05	0.2548E+05
0.1156E+01	0.5512E+05	0.2416E+05	0.6151E+05	0.1229E+07	0.5110E+05	0.2597E+05
0.1192E+01	0.5532E+05	0.2425E+05	0.6168E+05	0.1185E+07	0.5122E+05	0.2648E+05
0.1230E+01	0.5552E+05	0.2434E+05	0.6186E+05	0.1143E+07	0.5135E+05	0.2699E+05
0.1269E+01	0.5572E+05	0.2443E+05	0.6204E+05	0.1101E+07	0.5147E+05	0.2752E+05
0.1310E+01	0.5592E+05	0.2452E+05	0.6221E+05	0.1061E+07	0.5160E+05	0.2806E+05
0.1353E+01	0.5612E+05	0.2460E+05	0.6239E+05	0.1023E+07	0.5174E+05	0.2862E+05
0.1397E+01	0.5632E+05	0.2469E+05	0.6257E+05	0.9856E+06	0.5187E+05	0.2918E+05
0.1442E+01	0.5652E+05	0.2478E+05	0.6275E+05	0.9496E+06	0.5201E+05	0.2976E+05
0.1490E+01	0.5672E+05	0.2487E+05	0.6292E+05	0.9147E+06	0.5215E+05	0.3036E+05
0.1539E+01	0.5692E+05	0.2496E+05	0.6310E+05	0.8811E+06	0.5230E+05	0.3096E+05
0.1590E+01	0.5712E+05	0.2505E+05	0.6328E+05	0.8486E+06	0.5244E+05	0.3159E+05

DATA FITS STRAIGHT LINE OF SLOPE 0.1710E+00 AND STANDARD ERROR 0.1644E-01

SLOPE CORRECTED FOR SPEED = 0.2811E-06 S.E. = 0.2703E-07

WEIGHT AVERAGE MOLECULAR WEIGHT = 0.5434E+05 AND STAADARD ERROR = 0.5225E+04

Table 8

Molecular Weight of α-galactosidase II[1] (dormant Vicia faba seed). Computer data from ultra-centrifuge studies.

RUN 1881 J.PRIDHAM B_GALACTOSIDASE PH 5, E 11 ONE CELL 3 POU

SPEED = 0.9341E+04GIVING OMEG**2 = 0.9570E+06
P. SP. VOL = 0.7500E+00SOLUTION DENSITY = 0.1000E+01EXPTL. TEMP = 0.1870E+02

MEAN MENISCUS = 0.6964E+01 WITHS.E. = 0.2721E-01

OPTICAL DENSITY FOR THIS SCAN = 0.7950E+00

MEAN RADIUS= 0.7112E+01

DATA FITS QUADRATIC WITH FOLLOWING COEFFICIENTS
-0.5003E+02 -0.3542E-01 0.1922E-01
STANDARD ERRORS
0.5339E+01 0.3739E-02 0.2171E-02

AVERAGE MOLECULAR WEIGHTS FROM DERIVATIVE OF ABOVE REGRESSION EQUATION.FOR EDITING PURPOSES ONLY

OPTICAL DENSITY	MOL WT	ST ERROR		Z AVERAGE		NUMBER AVERAGE
0.3694E-03	0.4402E+05	0.5081E+05	0.4456E+05	0.2981E+15	0.4377E+05	0.1003E+04
0.5285E-03	0.4419E+05	0.5099E+05	0.4472E+05	0.1190E+15	0.4390E+05	0.1172E+04
0.7186E-03	0.4435E+05	0.5117E+05	0.4487E+05	0.4731E+14	0.4404E+05	0.1372E+04
0.9786E-03	0.4451E+05	0.5135E+05	0.4503E+05	0.1871E+14	0.4419E+05	0.1606E+04
0.1335E-02	0.4467E+05	0.5153E+05	0.4519E+05	0.7362E+13	0.4434E+05	0.1883E+04
0.1824E-02	0.4483E+05	0.5171E+05	0.4535E+05	0.2883E+13	0.4449E+05	0.2208E+04
0.2496E-02	0.4499E+05	0.5189E+05	0.4551E+05	0.1123E+13	0.4464E+05	0.2593E+04
0.3423E-02	0.4515E+05	0.5207E+05	0.4567E+05	0.4353E+12	0.4480E+05	0.3046E+04
0.4700E-02	0.4531E+05	0.5226E+05	0.4583E+05	0.1679E+12	0.4496E+05	0.3582E+04
0.6465E-02	0.4547E+05	0.5244E+05	0.4599E+05	0.6444E+11	0.4512E+05	0.4216E+04
0.8909E-02	0.4564E+05	0.5262E+05	0.4614E+05	0.2460E+11	0.4528E+05	0.4967E+04
0.1230E-01	0.4580E+05	0.5280E+05	0.4631E+05	0.9346E+10	0.4545E+05	0.5855E+04
0.1700E-01	0.4596E+05	0.5299E+05	0.4647E+05	0.3532E+10	0.4561E+05	0.6909E+04
0.2355E-01	0.4612E+05	0.5317E+05	0.4663E+05	0.1328E+10	0.4577E+05	0.8159E+04
0.3267E-01	0.4629E+05	0.5335E+05	0.4679E+05	0.4968E+09	0.4594E+05	0.9643E+04
0.4540E-01	0.4645E+05	0.5354E+05	0.4695E+05	0.1849E+09	0.4610E+05	0.1141E+05
0.6321E-01	0.4661E+05	0.5372E+05	0.4711E+05	0.6845E+08	0.4627E+05	0.1351E+05
0.8815E-01	0.4678E+05	0.5391E+05	0.4727E+05	0.2521E+08	0.4643E+05	0.1600E+05
0.1231E+00	0.4694E+05	0.5409E+05	0.4743E+05	0.9239E+07	0.4660E+05	0.1898E+05

DATA FITS STRAIGHT LINE OF SLOPE 0.2240E+00 AND STANDARD ERROR 0.1505E-01

SLOPE CORRECTED FOR SPEED = 0.2340E-06S.E. = 0.1573E-07

WEIGHT AVERAGE MOLECULAR WEIGHT = 0.4573E+05 AND STAADARD ERROR = 0.3073E+04

Table 9

Molecular Weight of α-galactosidase II[2] (dormant Vicia faba seed). Computer data from ultra-centrifuge studies.

RUN 1904 P03 E112

SPEED = 0.9341E+04 SPIN OMEGA**2 = 0.9570E+06
V. SP. VOL = 0.7500E+00 SOLUTION DENSITY = 0.1000E+01 EXPTL. TEMP = 0.2040E+02

MEAN MENISCUS = 0.6777E+01 WITHS.E. = 0.6261E-01

OPTICAL DENSITY FOR THIS SCAN = 0.8585E+00

MEAN RADIUS= 0.7000E+01

DATA FITS QUADRATIC WITH FOLLOWING COEFFICIENTS
-0.4761E+01 -0.5815E-02 0.1601E-02
AND STANDARD ERRORS
0.6691E+00 0.4267E-03 0.2752E-03

** AVERAGE MOLECULAR WEIGHTS FROM DERIVATIVE OF ABOVE REGRESSION EQUATION. FOR EDITING PURPOSES ONLY

OPTICAL DENSITY	MOL WT	ST ERROR		Z AVERAGE		NUMBER AVERAGE
0.3946E+00	0.4064E+05	0.6440E+04	0.4595E+05	0.2607E+07	0.3795E+05	0.4046E+04
0.4059E+00	0.4079E+05	0.6463E+04	0.4608E+05	0.2404E+07	0.3802E+05	0.4110E+04
0.4176E+00	0.4094E+05	0.6486E+04	0.4621E+05	0.2217E+07	0.3810E+05	0.4191E+04
0.4296E+00	0.4109E+05	0.6509E+04	0.4634E+05	0.2047E+07	0.3818E+05	0.4266E+04
0.4421E+00	0.4124E+05	0.6532E+04	0.4647E+05	0.1891E+07	0.3826E+05	0.4343E+04
0.4550E+00	0.4139E+05	0.6555E+04	0.4660E+05	0.1749E+07	0.3835E+05	0.4422E+04
0.4684E+00	0.4154E+05	0.6578E+04	0.4673E+05	0.1619E+07	0.3843E+05	0.4502E+04
0.4823E+00	0.4169E+05	0.6601E+04	0.4686E+05	0.1500E+07	0.3852E+05	0.4584E+04
0.4966E+00	0.4184E+05	0.6624E+04	0.4699E+05	0.1392E+07	0.3861E+05	0.4668E+04
0.5114E+00	0.4199E+05	0.6647E+04	0.4713E+05	0.1293E+07	0.3871E+05	0.4753E+04
0.5268E+00	0.4214E+05	0.6670E+04	0.4726E+05	0.1203E+07	0.3880E+05	0.4841E+04
0.5427E+00	0.4229E+05	0.6693E+04	0.4739E+05	0.1121E+07	0.3890E+05	0.4931E+04
0.5592E+00	0.4245E+05	0.6716E+04	0.4753E+05	0.1046E+07	0.3899E+05	0.5022E+04
0.5762E+00	0.4260E+05	0.6740E+04	0.4766E+05	0.9773E+06	0.3909E+05	0.5116E+04
0.5939E+00	0.4275E+05	0.6763E+04	0.4780E+05	0.9148E+06	0.3920E+05	0.5212E+04
0.6122E+00	0.4290E+05	0.6786E+04	0.4793E+05	0.8576E+06	0.3930E+05	0.5310E+04
0.6312E+00	0.4306E+05	0.6810E+04	0.4807E+05	0.8053E+06	0.3941E+05	0.5410E+04
0.6509E+00	0.4321E+05	0.6833E+04	0.4820E+05	0.7575E+06	0.3951E+05	0.5513E+04
0.6713E+00	0.4336E+05	0.6857E+04	0.4834E+05	0.7136E+06	0.3962E+05	0.5618E+04

DATA FITS STRAIGHT LINE OF SLOPE 0.1775E+00 AND STANDARD ERROR 0.5764E-02 INTERCEPT -0.9133E+01
SLOPE CORRECTED FOR SPEED = 0.2392E-05 S.E. = 0.7768E-08 ERRER 0.2841E+00
WEIGHT AVERAGE MOLECULAR WEIGHT = 0.4339E+05 AND STAADARD ERROR = 0.1409E+04

Table 10

Molecular weights of various forms of α-galactosidase, from dormant Vicia faba seeds, obtained by Sephadex G-100 gel filtration and by the sedimentation equilibrium method.

Molecular Form of α-galactosidase	Carbohydrate Content (%)	Molecular Weight by Sedimentation Equilibrium	Molecular Weight by Sephadex G-100 gel filtration
I	8.0	160,400 ± 2,850	209,000
II	7.8	54,340 ± 5,225	38,000
II^1	4.2	45,730 ± 3,073	38,000
II^2	4.3	43,390 ± 1,409	38,000

the ultra-centrifuge data it is apparent that the molecular weights obtained by both methods may be similar for enzymes II^1 and II^2 and that the molecular weight of these forms is of the order of 40,000. This value in turn appears to be similar to that for enzyme II as calculated from gel-filtration studies. There is, however, a discrepancy in the data obtained by the two methods in the case of enzymes I and II. The ultra-centrifuge measurement gives a higher value than gel-filtration for enzyme II and, in the case of enzyme I, the molecular weight obtained by gel-filtration was much higher than that given by the ultra-centrifuge study. With the latter enzyme, Dr. S.P. Spragg reported that there was some indication that this protein possessed a sub-unit structure that could associate and dissociate depending on the 'age' of the preparation and pH (on two occasions molecular weight values of $365,300 \pm 587^5$ and $44,140 \pm 1730$ were obtained). Some support for this observation is given later (see page 103 and Fig. 31). No evidence of association/dissociation phenomenon has ever been observed when enzyme I was examined on Sephadex columns although treatment of the enzyme with urea (Dey and Pridham ref. 25) did suggest that it might possess a sub-unit structure.

In the case of glycoprotein, peroxidase, Andrews (30) observed that gel-filtration gave a 20% higher value than other methods. Although all the Vicia faba α-galactosidases contain carbohydrate (see page 68) the result reported by Andrews does not easily help to explain the anomalies as in one instance (enzyme I) the gel-filtration study gave a higher value than the ultra-centrifuge study where as with enzyme II the reverse was true although both enzymes contain the same amount of carbohydrate. It is, of course, possible that the carbohydrate in enzyme I occupies a different position in the molecule from that in enzyme II.

3. Amino Acid Analysis

All the purified α-galactosidases from dormant beans showed an absorption maximum at 280 nm (Fig. 14, 15, 16, 17). The E 280/260 ratio of enzymes I, II, II^1 and II^2 were 1.37, 1.43, 1.42 and 1.37, respectively. The $E^{1\%}$ 280 values were 18, 21, 19 and 18, respectively.

The amino acid compositions of the four α-galactosidase preparations from dormant Vicia faba seeds were analysed by the standard two column technique as described in the experimental section. The same seventeen amino acids were present in each form: any tryptophan present would, of course, have been destroyed during the hydrolytic process (see Table 2 and Experimental Section p.44). The total recoveries of amino acids from enzymes I, II, II^1 and II^2 were 71.5, 90.1, 81.7 and 90.3%, respectively. All enzyme proteins showed a high content of aspartic acid, glutamic acid, serine and glycine but in view of the differing yields of amino acids no firm quantitative comparisons of the total analysis can be made. However, the compositions of enzyme II^1 and II^2 would appear to be similar (see Fig. 18 and Table 2 , p.44) with the possible exception of the histidine, cysteine and methionine contents. The difference in the acidities of these two proteins, as revealed by the CM-cellulose study, may be due to differences in the environments of the charged groups rather than absolute differences in the amounts of acidic and basic amino acids present. As enzymes II^1 and II^2 are components of enzyme II it is not surprising to find that when the latter enzyme is hydrolysed it also produces a similar amino acid pattern to enzymes II^1 and II^2 (Fig. 18 and Table 2). In addition Fig. 18 (see also Table 2 p. 44) shows that, with the exception of proline (which is difficult to analyse, 203), there is a close relationship

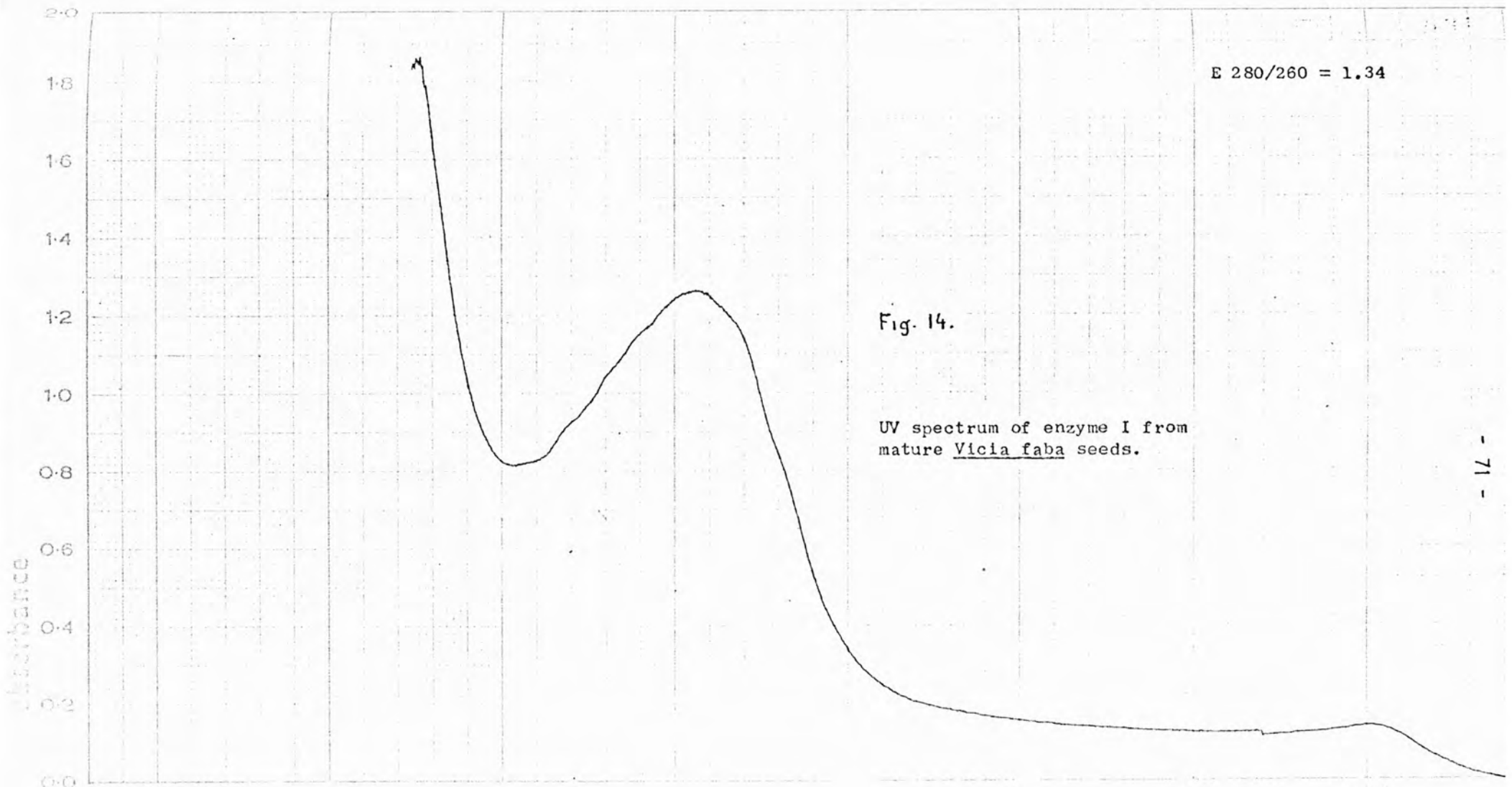


Fig. 14.

UV spectrum of enzyme I from mature Vicia faba seeds.

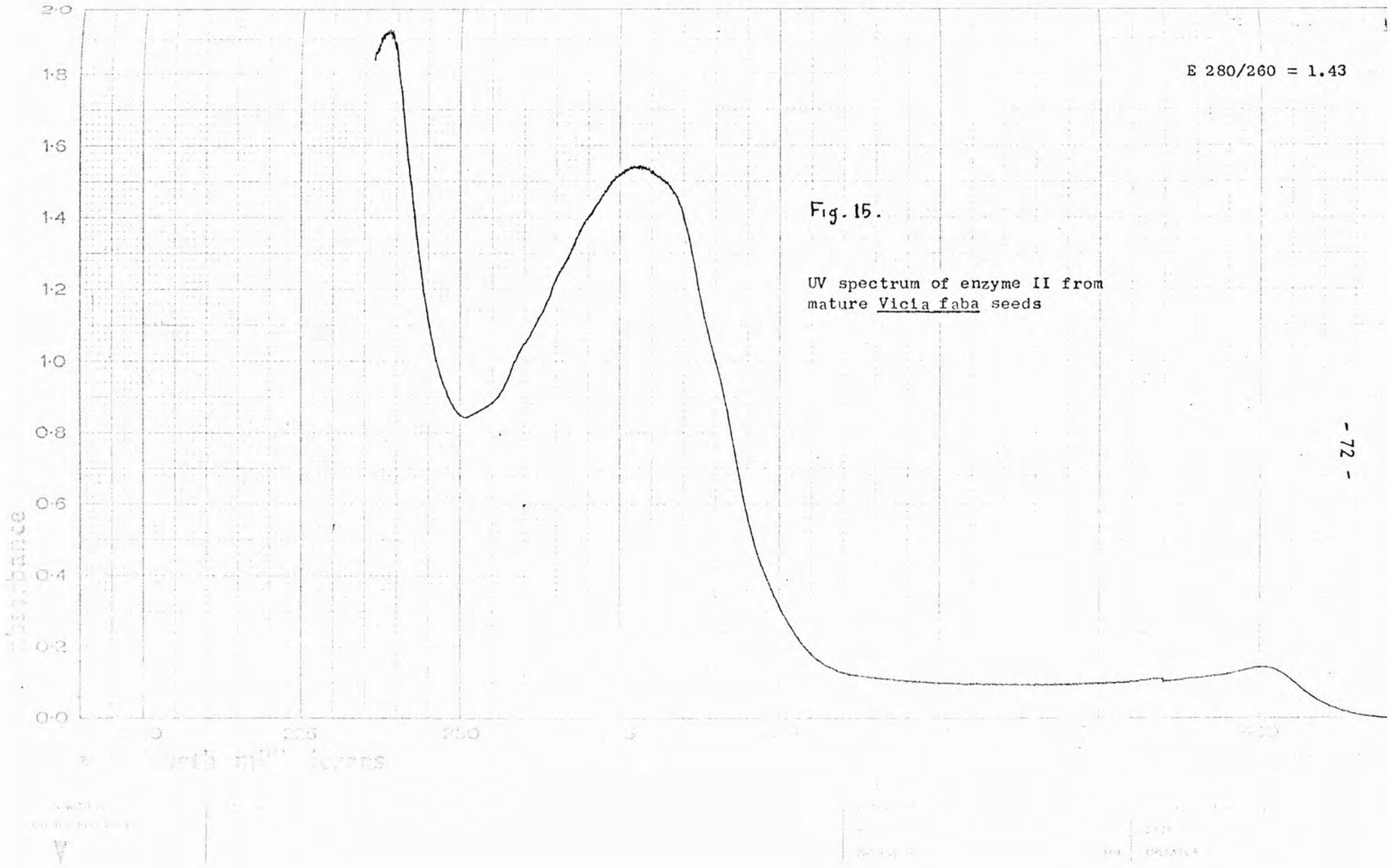
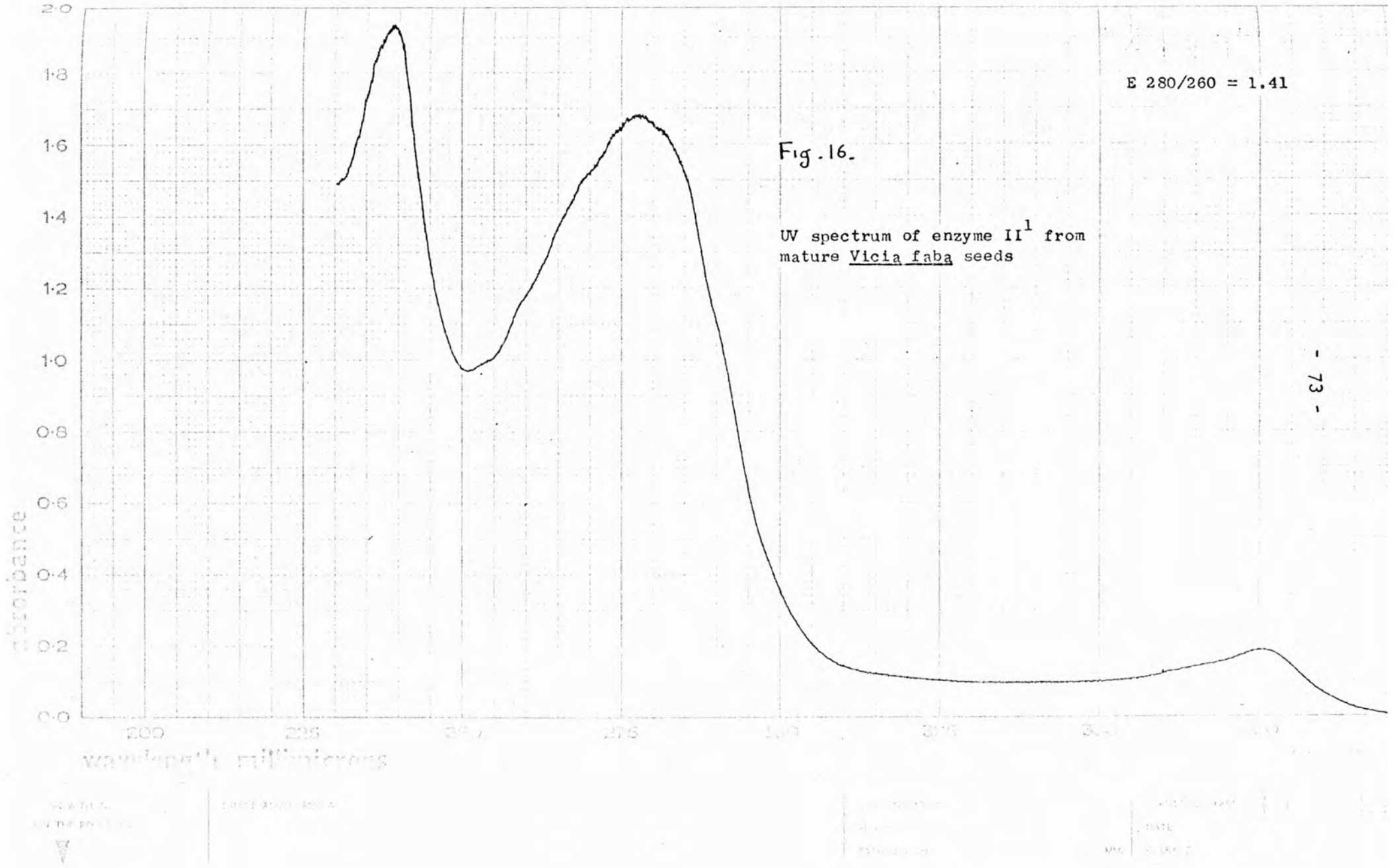


Fig. 15.

UV spectrum of enzyme II from mature Vicia faba seeds



E 280/260 = 1.41

Fig. 16.

UV spectrum of enzyme II[1] from mature Vicia faba seeds

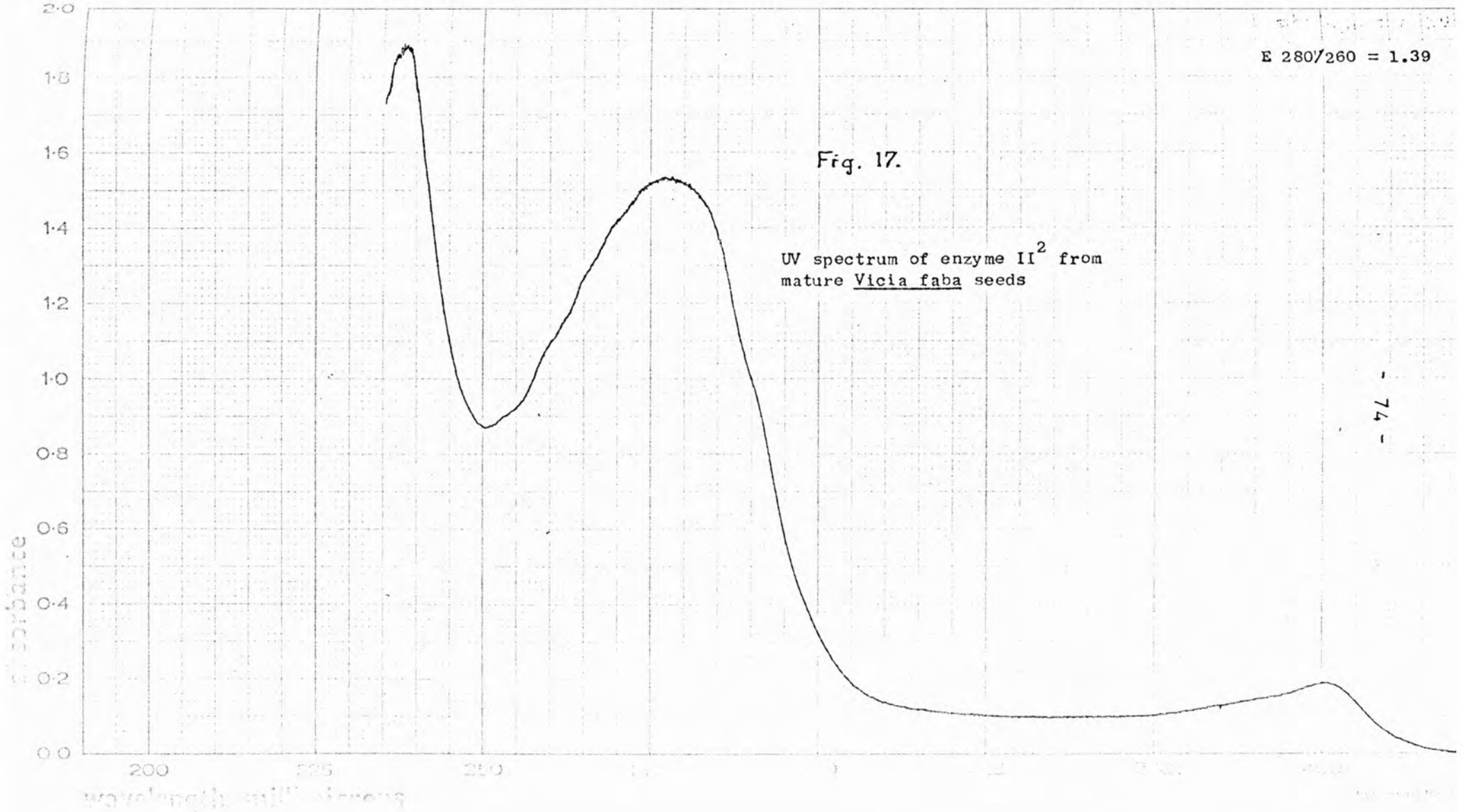


Fig. 17.

UV spectrum of enzyme II^2 from mature Vicia faba seeds

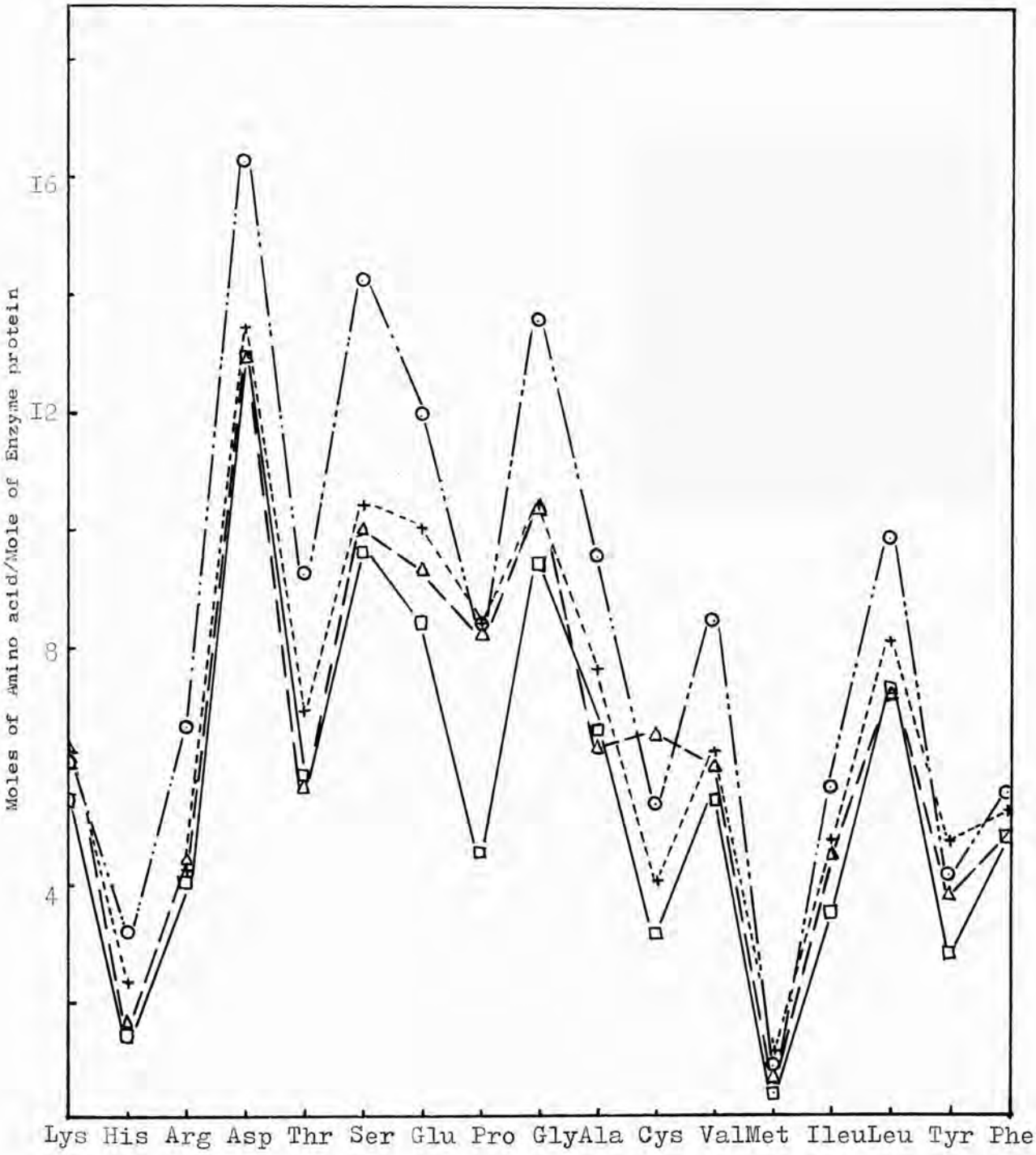


Fig. 18 . Pattern of amino acid composition of mature seed α-galactosidases. □—□, enzyme I /4 ; o—o , enzyme II ; Δ—Δ, enzyme II^{I}; +—+, enzyme II^{2}

between the amino acid contents of enzyme I (Fig. 18) and the other forms when calculations are based on the assumption that enzyme I has a molecular weight of ca 160,000.

In conclusion, the simplest working hypothesis which can be put forward on the basis of the molecular weight and amino acid data available is as follows: (1) that enzyme II contains equimolar amounts of the proteins II^1 and II^2; which are of similar size but which can be readily separated because of their differing acidities; although other enzymically active components, all of mol. wt. 40,000, are also probably present (see p.57): (2) that a number of units of enzyme II can associate to form enzyme I although at the present stage of work the number is uncertain.

The nature of the carbohydrate associated with the α-galactosidases (Table10) is not known. It may form part of the glycoprotein molecules on the other hand, in view of the large amount of soluble polysaccharide present in the seeds, some of this could be physically bound to the proteins. It is of interest to note, however, that enzymes I and II have almost identical total carbohydrate contents (which tends to support the association theory) and this is exactly double the quantity present in II^1 and II^2. If the hypothesis alone is correct then some or all of the inactive proteins detected by CM-cellulose chromatography of enzyme II may well possess very high carbohydrate contents

4. Catalytic Properties of the dormant Seed Enzymes

Effect of pH on activities

Following the purification, molecular weight determination and amino acid analysis the general enzymic properties of the various forms of α-galactosidase were examined.. The initial velocities of PNPG hydrolysis by the α-galactosidases were studied over the pH range of 1.5 - 7.0 using McIlvaine buffer and where necessary addition of a KCl - HCl mixture. In Fig. 18 the pH activity profiles show that all forms of the Vicia faba α-galactosidases have two pH optima. Enzyme I showed a minor optimum at pH 2.0 and main peak at pH 5.7. The activity at pH 2.0 is only 36% of that at pH 5.7. Enzyme I^1 shows a similar pH activity profile with two pH optima at 2.0 and 5.5. Unlike enzyme I, enzyme II is equally active at the two optima. Enzyme II^1 from dormant seeds showed pH optima at 3.0 and 5.5. However, enzyme II^2 exhibited optimal activity at pH 2.0 and 5.5. Again both enzyme II^1 and II^2 were equally active at their respective pH optima.

Specificity: hydrolysis of galactomannans

Previous studies had shown that both α-galactosidase I and II from Vicia faba hydrolysed various alkyl and aryl galactosides and oligosaccharides possessing terminal, non-reducing α-galactosyl groups (44). Specificity studies have now been extended to an investigation of the hydrolysis of galactomannans, such as Locust bean, Tara and Guar gums, by the various enzyme forms at pH 5.5. It was initially observed that the higher molecular weight enzyme (I) hydrolysed Locust bean and Tara gums with the liberation of galactose. Guar gum was unaffected by this enzyme. None of the lower molecular weight forms of the enzyme were active towards any of the galactomannans.

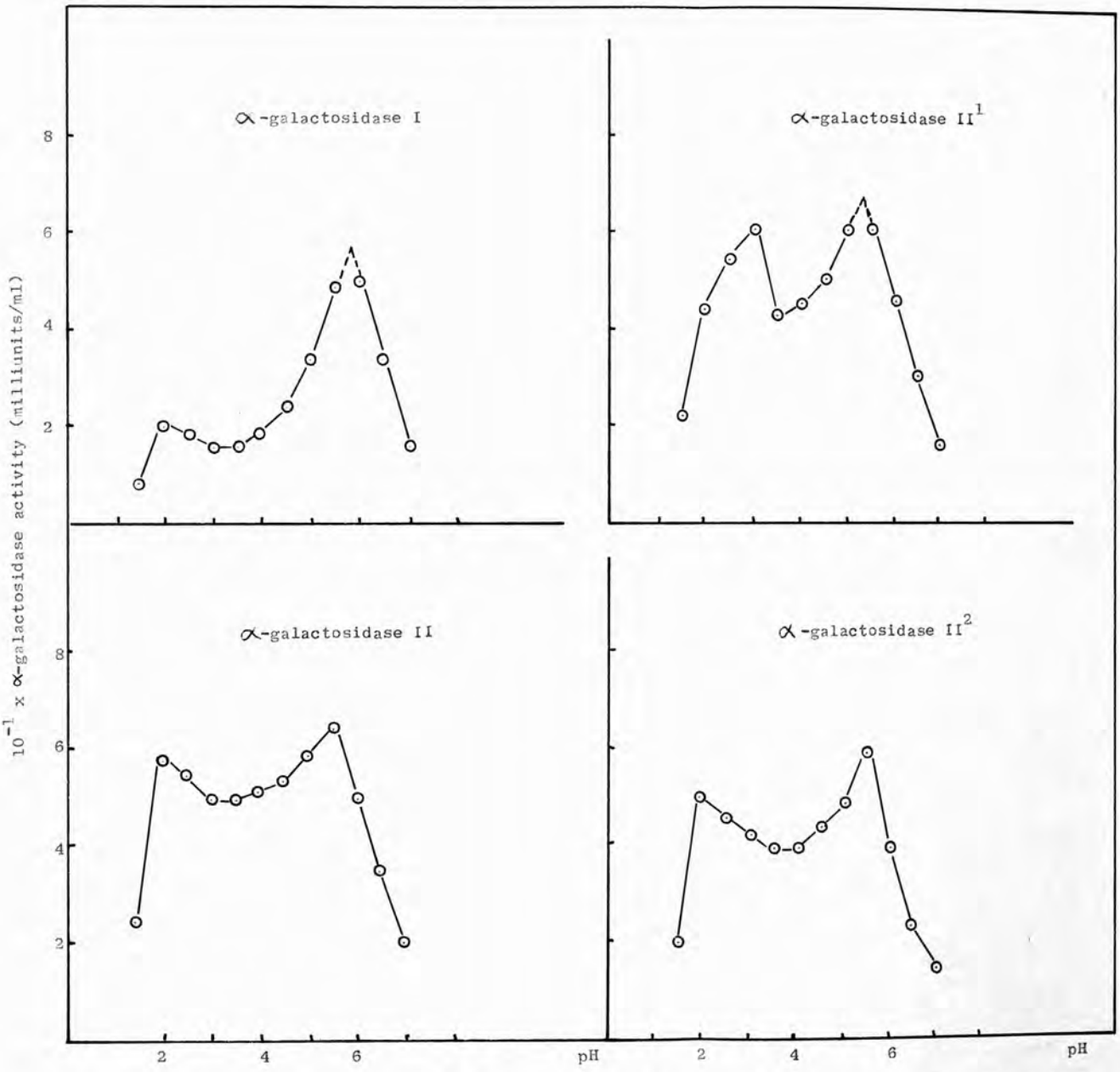


Fig.19 . Effect of pH on activities of α-galactosidases from mature Vicia faba seeds. PNPG substrate.

The three gums are polysaccharides composed of a β-(1→4)-linked mannopyranose backbone carrying galactopyranose units joined by α-(1→6)-linkages: they differ in their degree of branching (3). In case of Locust bean and Tara gums, on average, one galactose moiety in linked to every third or fourth residue of the mannopyranose chain. On the other hand, galactose units are attached to every alternate mannose residue in Guar gum. The galactose contents of Locust bean, Tara and Guar gums are 23%, 25% and 36% respectively. Quantitative estimation of galactose liberated by enzyme I from Locust bean gum and Tara gum was measured at 15 minute intervals for a period of 1 hour. This showed that enzyme I liberated 16.5% of the total galactose from the Locust bean gum in 45 minutes and then hydrolysis ceased under the particular experimental conditions used (Fig.20). Enzyme I required 1 hour to remove 16% of the galactose from Tara gum hydrolysed under identical conditions (Fig.20).

Examination of the residual polysaccharide products resulting from the treatment of galactomannans with enzyme I was also carried out by the method of specific optical rotation, by Dr.I.C.M. Dea (Unilever Research Laboratory, Sharnbrook, Bedford). The enzyme-treated Locust bean gum and Tara gum showed depletion of galactose and accompanying increases in the proportions of mannose (Table11).

Effect of substrate concentration:-

The Vmax and Km values for all the purified preparations of enzymes, at the two pH optima in each case, using PNPG as substrate are shown in Table12 . P-Nitrophenyl α-D-galactoside was inhibitory at higher concentration with all forms of the enzymes (see Fig.21). The Km and Vmax values were determined from the linear parts of the Lineweaver-Burk plots. At the higher pH optima

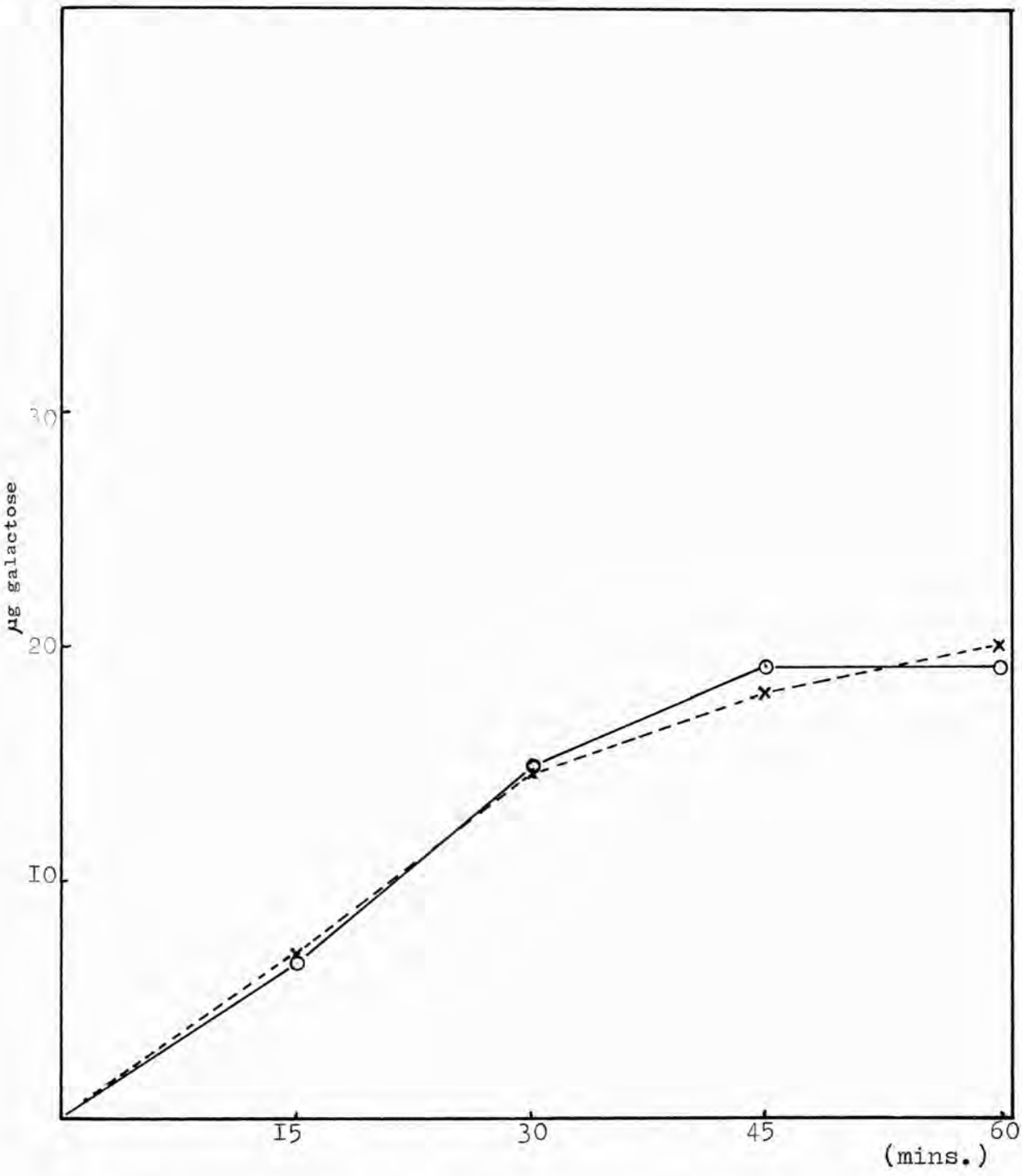


Fig. 20 . Hydrolysis of galactomannans by purified ∝-galactosidase I from mature Vicia faba seeds. Assay mixture contain 0·Iml. galactomannan (5mg/ml), 0·8 ml. McIlvaine buffer pH 55 and 0·Iml enzyme I (6650 milli units) . Temperature of assay 30°. Galactose liberated was estimated by the method of Nelson(I95). o—o , Locust bean gum; x----x, Tara gum.

Table 11 .

Analysis* of monosaccharide contents of galactomannans before and after treatment with α-galactosidase I.

Galactomannans	Treated		Untreated	
	Mannose(%)	Galactose (%)	Mannose (%)	Galactose (%)
Locust bean gum	83	17	77	23
Tara gum	81	19	75	25

*Analysis carried out at the Unilever Research Laboratory using an optical rotation technique.

Table 12

Properties of α-galactosidases from dormant *Vicia faba* seeds

Enzyme Forms	pH Optima*	Vmax* (μ mol/min/mg)	Km* (mM)
I	5.5	24.15	0.44
	2.0	14.62	0.44
II	5.5	2.93	0.54
	2.0	2.95	0.48
II^1	5.5	1.61	0.35
	3.0	1.72	0.34
II^2	5.5	0.97	0.33
	2.0	0.91	0.34

*p-Nitrophenyl α-D-galactoside as substrate.

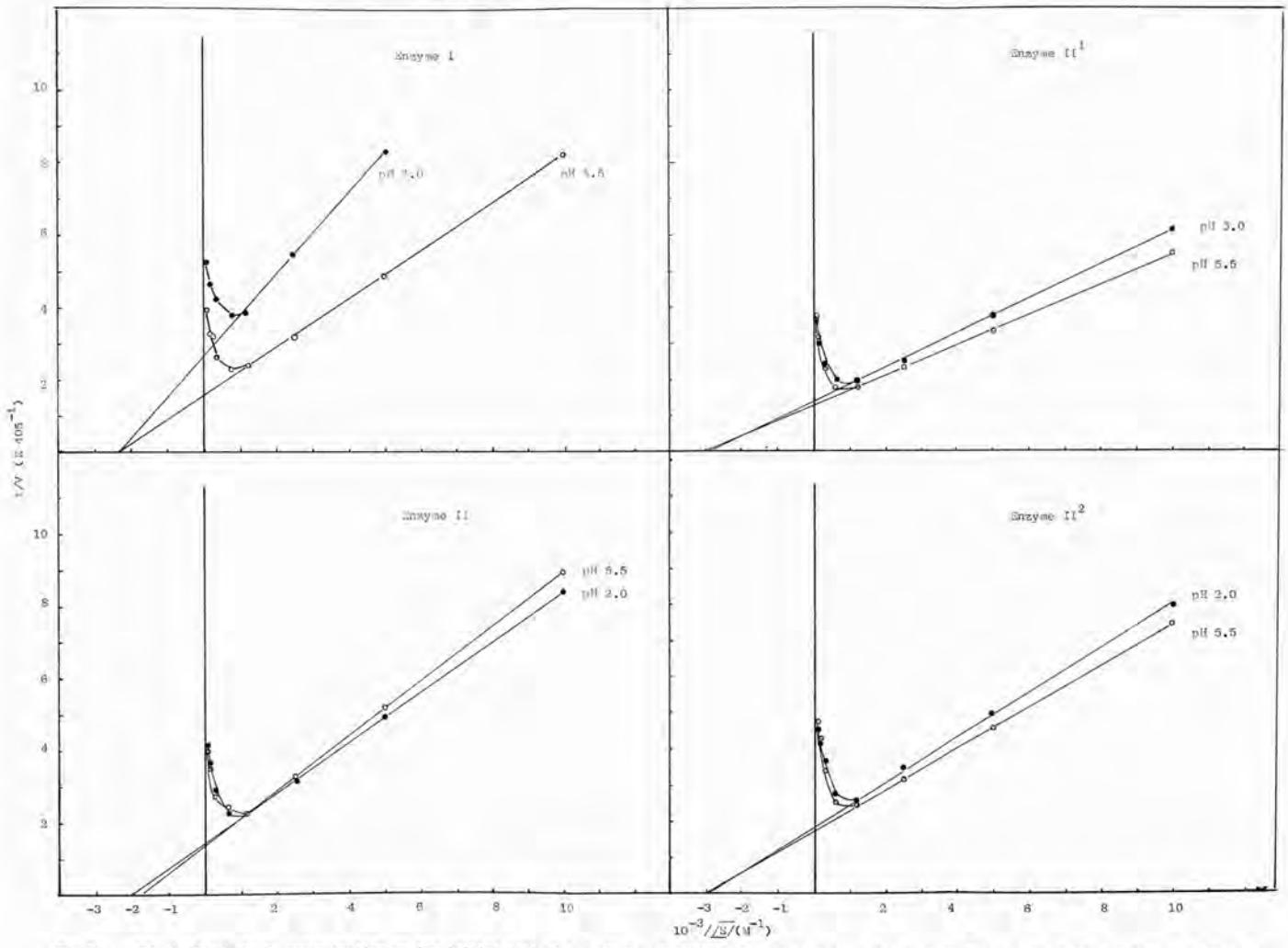


Fig.2| . Effect of substrate concentration on the initial rate of hydrolysis of PNPG by α-galactosidases from mature Vicia faba seeds in McIlvaine buffer at their respective pH optima.

some comparisons of the various forms of the enzyme can be made. Enzyme II^1 and II^2 have similar Km values which are lower than for enzyme II. The Vmax value for enzyme I is eight times higher than that of enzyme II and the latter enzyme possesses a higher Vmax value than either enzyme II^1 or II^2. Hence it appears that the combination of enzyme II^1 and II^2 in enzyme II produces a synergistic effect with increasing catalytic activity. The higher Vmax value for enzyme I obtained at pH 5.5 than at pH 2.0 should be noted and compared with the related values for enzymes II, II^1 and II^2 where there is not the large difference between activities at the low pH optima.

Effect of phenolic compounds and metal ions on the activity of α-galactosidases.

During the purification of α-galactosidase by the method recommended by Dey and Pridham (25) it was noted, in this present study, that at the Sephadex G-100 gel filtration stage approximately 200% recovery of the enzyme activity could be obtained. It was thought that a natural inhibitor might by present in the crude enzyme preparation which was being removed by the gel. One possibility was β-(3,4-dihydroxyphenyl)-L-alanine (DOPA) which is present in broad bean tissue in high concentration (204, 205). The effect of this phenolic compound and its oxidation products on the activity of α-galactosidase was, therefore, examined by incubating enzyme preparations (enzymes I and II) after Sephadex fractionation with a 1% solution of DOPA. Incubation was carried out for periods up to six hours to allow accumulation of DOPA quinone and other oxidation products but no noticeable inhibition of either enzyme

was observed. A solution of DOPA through which air had been bubbled also proved to be an ineffective inhibitor.

In order to look for other natural inhibitors which might have been present in the crude enzyme preparation and removed during gel filtration, all the fractions from a column were individually tested for inhibitory properties. In this experiment a dialysed enzyme preparation (after the ammonium sulphate fractionation stage) was passed through a Sephadex G-100 column and about 200 fractions (3 ml) were collected using McIlvaine buffer, pH 5.5, containing 0-1M-KCl as eluent. The Vt of the column was 430 ml). A pure preparation of enzyme I(0.4ml; $O.D_{405}=0.5$) was then added to samples (0.3 ml) of every alternate fraction and the tubes allowed to stand for 12 hours at 4^{o}. The fractions were assayed for enzyme activity (Fig.23). The experiment was repeated to observe the effect of the column fractions on pure enzyme II (Fig.24). In both cases no inhibitor could be detected in any of the fractions instead a synergestic activation when the fractions containing enzyme II were incubated with pure enzyme I was observed. This was later thought to be due to the conversion of enzyme II to enzyme I which has a higher specific activity (see page 103).

α-Galactosidase from E. coli (206) was reported to be activated by NAD in the presence of Mn^{2+} ions and that these components were essential for stabilization of the enzyme when in diluted solution. α-Galactosidase from V. faba, on the other hand, was found to be unaffected by such treatment and dilution, unlike the case of the E. coli enzyme, had no effect on the activity. However, in view of the failure to detect endogenous inhibitors in crude α-galactosidase preparations it was considered that activation was probably a result of a purification stage possibly involving

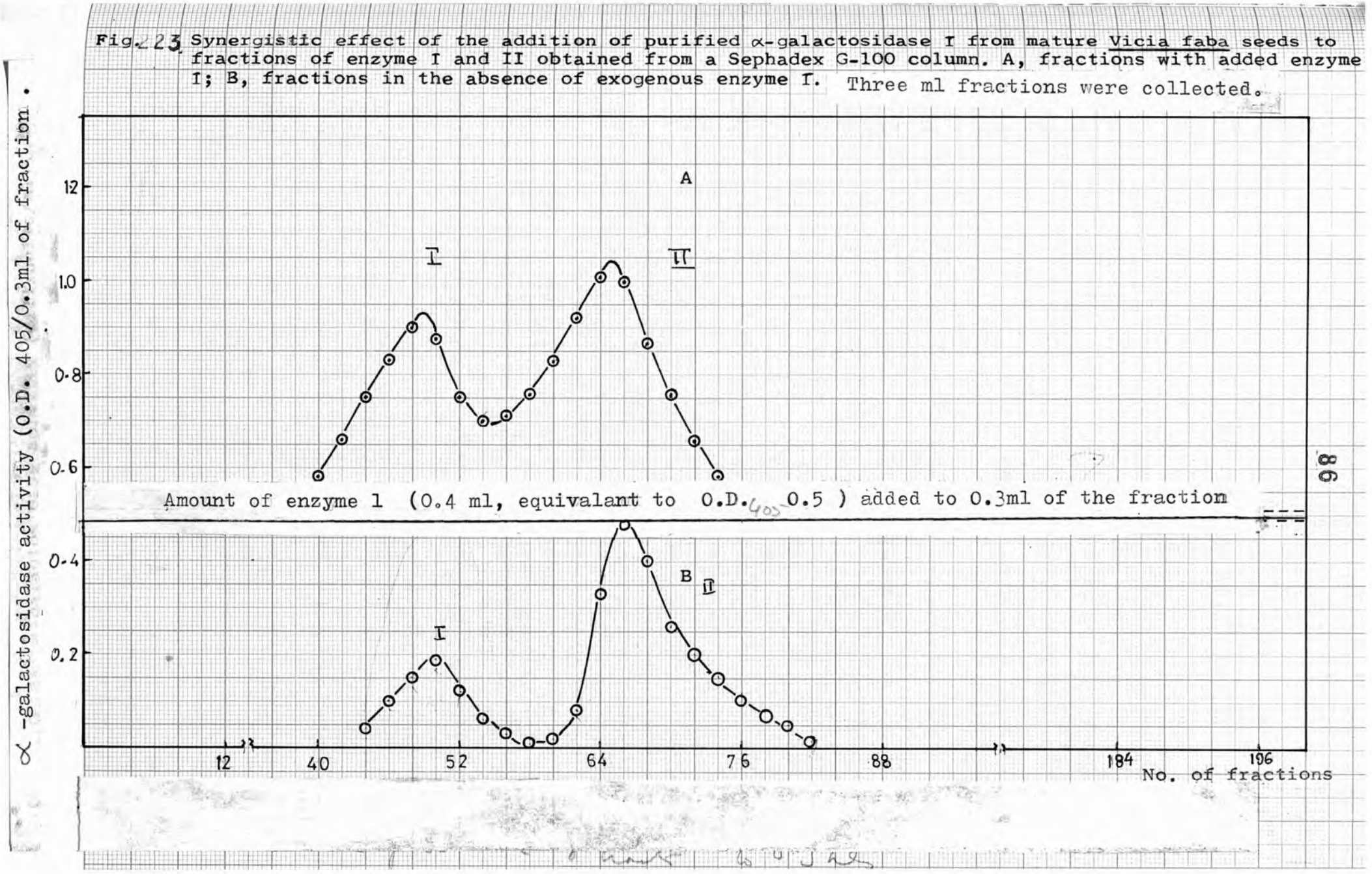


Fig. 23 Synergistic effect of the addition of purified α-galactosidase I from mature Vicia faba seeds to fractions of enzyme I and II obtained from a Sephadex G-100 column. A, fractions with added enzyme I; B, fractions in the absence of exogenous enzyme I. Three ml fractions were collected.
α-galactosidase activity (O.D. 405/0.3ml of fraction .
A
I
II
B
II
I
1.2
1.0
0.8
0.6
0.4
0.2
Amount of enzyme 1 (0.4 ml, equivalant to O.D.405 0.5) added to 0.3ml of the fraction
12
40
52
64
76
88
184
196
No. of fractions
86

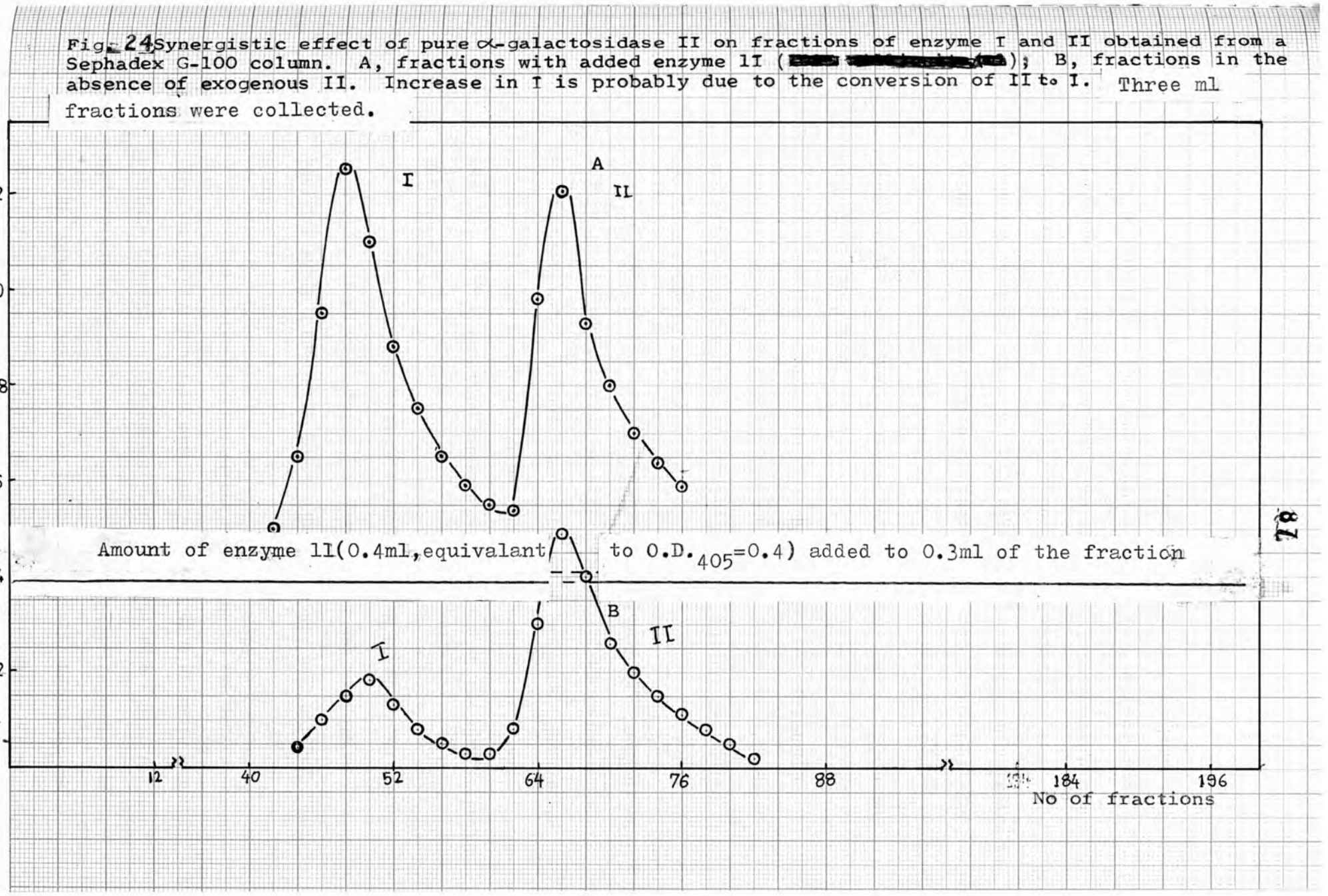


Fig. 24 Synergistic effect of pure α-galactosidase II on fractions of enzyme I and II obtained from a Sephadex G-100 column. A, fractions with added enzyme II ( ); B, fractions in the absence of exogenous II. Increase in I is probably due to the conversion of II to I. Three ml fractions were collected.
α-galactosidase activity (O.D. 405/0.3ml of fraction.
A
I
II
B
I
II
Amount of enzyme II(0.4ml, equivalant to O.D.405=0.4) added to 0.3ml of the fraction
No of fractions
12
40
52
64
76
88
184
196
0·2
0·4
0·6
0·8
1·0
1·2

addition of metal ion.

The gel filtration columns used in the later stages of enzyme purification were eluted with buffer containing 0.1M-KCl and hence it was thought that this salt might be responsible for the effect. In further experiments to corroborate this view, an ammonium sulphate fraction of V. faba α-galactosidase was applied to Sephadex G-100 column which was then eluted with McIlvaine buffer containing no Kcl. Two forms of the enzyme (I and II) were resolved but the recovery of the total activity was in this case only 100% (Fig. 25).

When an ammonium sulphate fraction of the enzyme was incubated with McIlvaine buffer containing 0.1M-KCl or 0.1M-KH_2PO_4 activation was observed and therefore the K^+ ion was considered to be the activating agent.

The effect of K^+ ion concentration was investigated using a carefully dialysed ammonium sulphate fraction of the enzyme which was incubated with various concentrations of Kcl at 4°, pH 5.5 for 1 hour. It was observed that maximum activation occurred with 0.12M-KCl (Fig. 26), and that higher concentrations of the salt inhibited the enzyme.

The effect of time of incubation with 0.12M-KCl on the activation is shown in Fig. 27. There was a 48% increase of activity after 2 hours but the maximum (60%) was only reached after 6 hours.

The action of other alkali metal ions on the activation of α-galactosidase was also examined. Samples of a dialysed ammonium sulphate fraction of the enzyme were incubated with LiCl, CsCl and RbCl (0.1M) for 2 hours prior to the α-galactosidase assay. Cs^+ and Rb^+ produced no activation (Table 13) but Li^+ increased the activity by 32%. The effect of Na^+ ion was examined by first

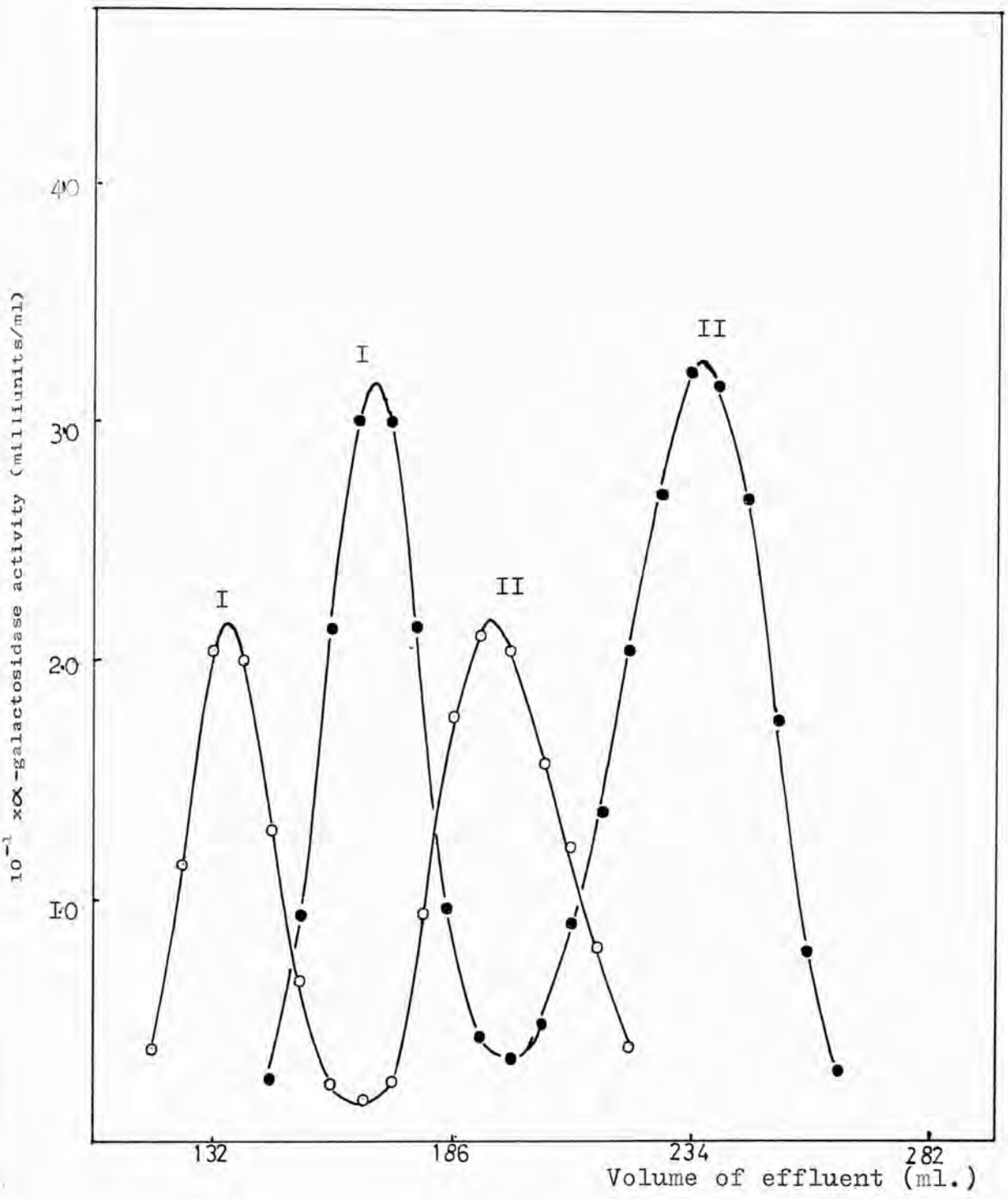


Fig. 25 Effect of 0·IM KCl in the Sephadex G IOO column eluent. The procedure is described in the text, p. 88 . ●—● , with 0 IM KCl in McIlvaine buffer PH 5 5 ; o—o without KCl.

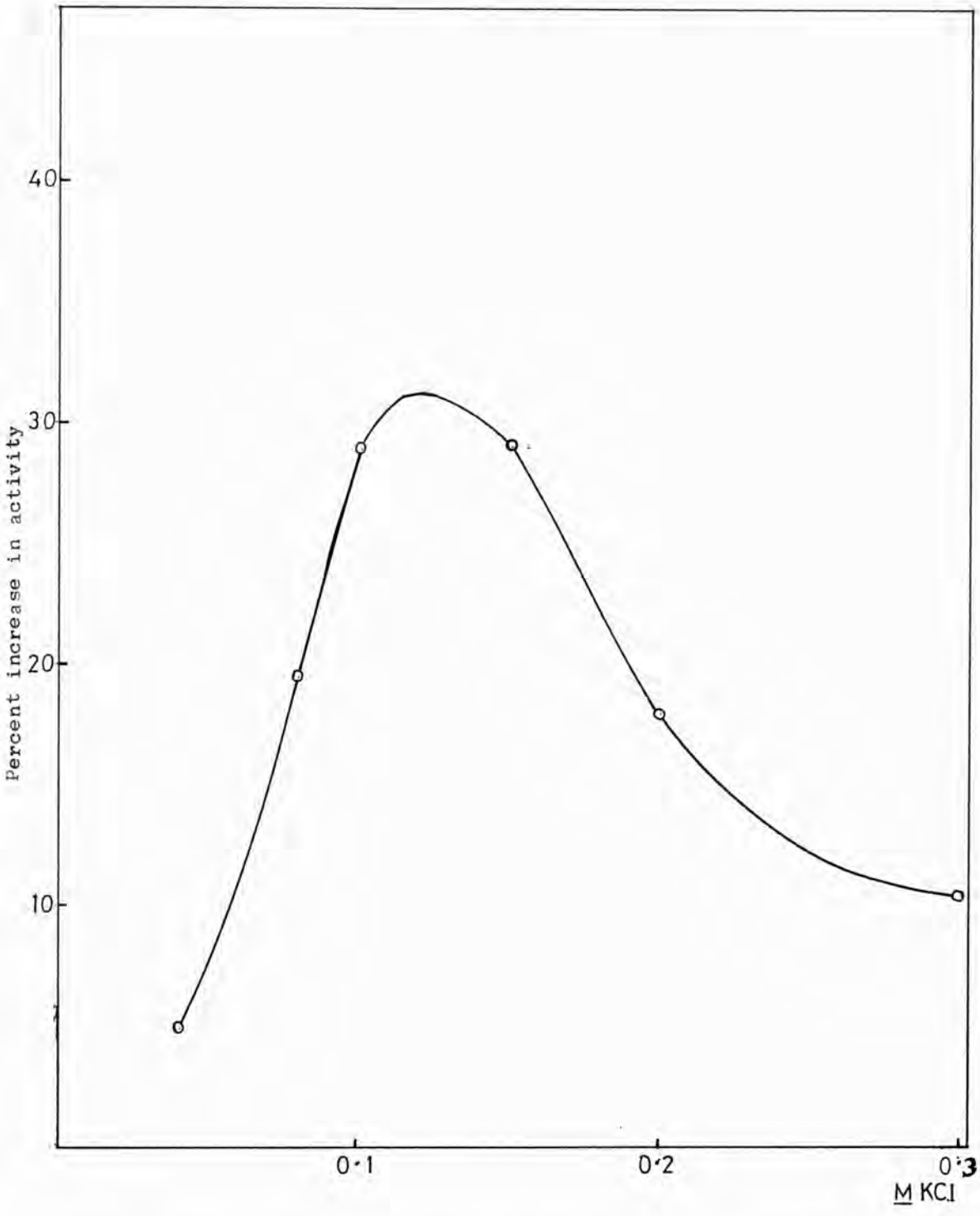


Fig. 26 Effect of KCl concentration on the hydrolysis of PNPG by 'Pre-Sephadex' purified ∝-galactosidase. The conditions are described in the text, p. 88 .

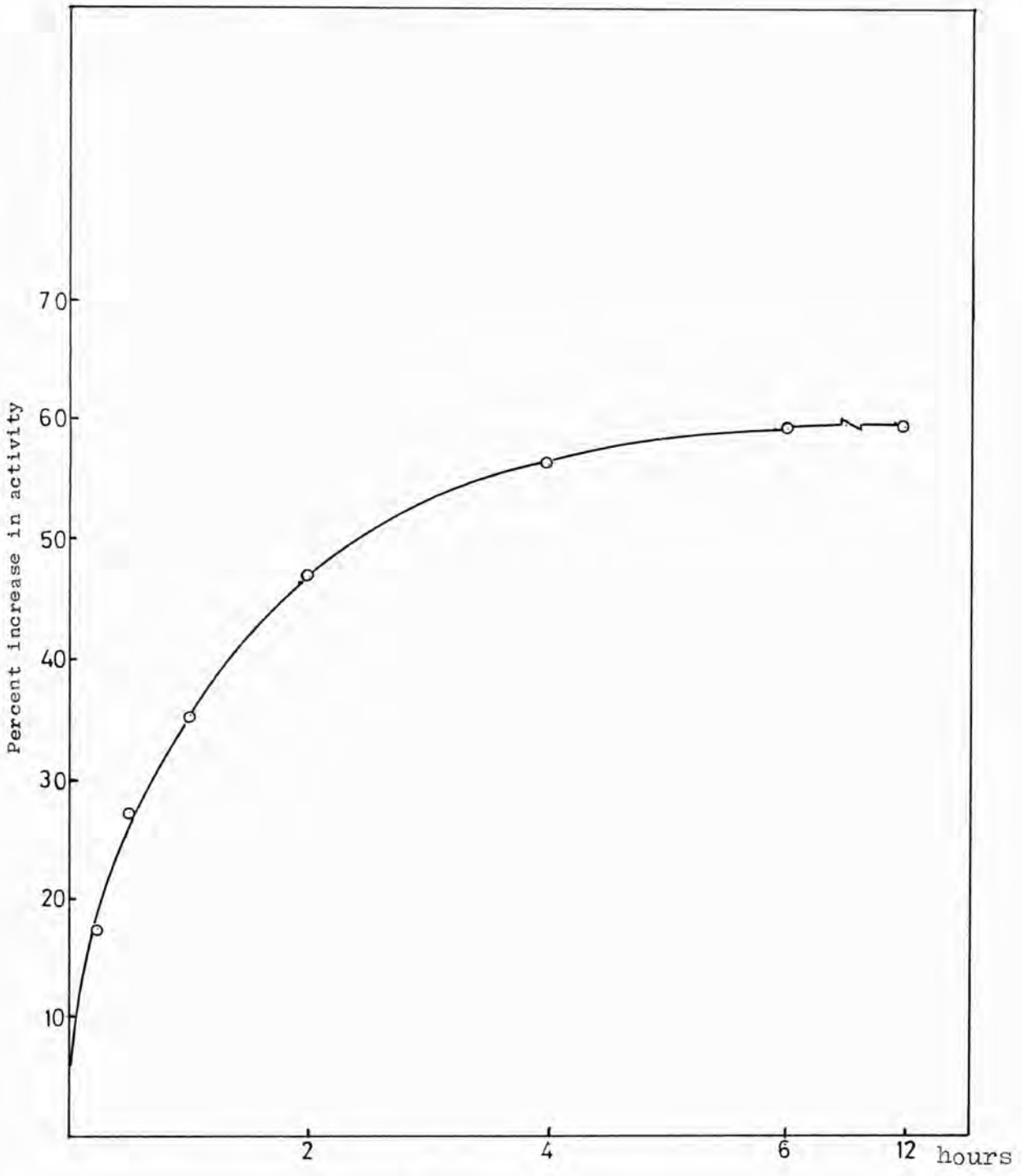


Fig. 27 Effect of 0·I2 M KCl on 'Pre-Sephadex' purified α- galactosidase(see Experimental Section, p.88).
PNPG Substrate.

Table 13

Effect of Monovalent Cations on α-galactosidase (I and II) activity.

Ion as Chloride (0.1M)	Hydrated Ionic Radii (A°)*	% Increase in Activity**
Li^+	10.03	32
Na^+	7.90	0
K^+	5.32	48
Rb^+	5.69	0
Cs^+	5.03	0
NH_4^+	5.37	28

* see ref. 207

** using 1 unit enzyme and incubating for 2 hours at 30°.

extracting the enzyme from dormant seeds with dionized water instead of McIlvaine buffer which contains Na^+ ions. The Na^+ ion had no effect on the activity of the ammonium sulphate fraction of this enzyme. This latter enzyme preparation was activated by K^+ ions. The effect of ammonium sulphate on α-galactosidase activity was also studied. An increase of 28% in the total activity was obtained when an enzyme preparation obtained after the protamine sulphate stage of purification was incubated with a 0-1M solution of this salt.

A detailed interpretation of the results obtained with the alkali metal (and NH_4^+) ions is difficult. Inspection of the ionic radii of the hydrated ions shows that the values are very similar for the activating ions, K^+ and NH_4^+ (5.32Å and 5.37Å - ref. 207) which in turn are different from those for Na^+, Rb^+ and Cs^+ (7.90Å, 5.69Å and 5.03Å respectively, ref. 207) which have no activating effect. Obviously, therefore, activation could depend on the ionic radius but the case of Li^+ which has a large hydrated ionic radius (10.03 Å) and appears to activate α-galactosidase cannot be reconciled with this theory. It should be noted, however, that the LiCl used in this experiment was the only salt which could not be obtained as an 'Analar' reagent. It is, therefore, conceivable that activation in this case could have been due to an impurity.

Kachmar and Boyer (207) have shown that pyruvate Kinase from muscle is activated by K^+, NH_4^+ and Rb^+ and that this activation is inhibited by Na^+ and Li^+. These authors suggest that the close relationship between the three activating ions in terms of ionic radii in crystals, ionic mobility and the size of the hydrated ion is the reason why they all affect the kinase in a similar manner. Happold and Beechey (208) studying the activation of tryptophanase by K^+ also believe that the ionic radius of the ion is an important

factor and Evans et al. (209) conclude that K^+ activates Acetic thiokinase by producing a conformational change in the enzyme protein which results in the exposure of buried active sites.

The investigations with alkali metal ions so far described were all with relatively crude enzyme preparations (i.e. pre-Sephadex stage of purification) containing a mixture of multiple forms of α-galactosidase. The effect of K^+ ions on individual forms of the enzyme was next studied. The reason for this study was that other workers had shown that the extent of metal ion activation is often changed during the course of enzyme purification. For example, Bach and Whitehouse (210) showed that partially purified arginase is activated by Co^{2+}, Mn^{2+} and Ni^{2+} (in descending order of effectiveness) but that the highly purified enzyme does not respond to activation by Co^{2+} and Ni^{2+}. In addition, crude phosphorylase kinase (211) is activated by Ca^{2+} and Mn^{2+} but not by Mg^{2+}, but the purified enzyme is activated by Mn^{2} and Mg^{2+} and not with Ca^{2+}. No explanation has been given for this phenomenon.

In the following experiments the basic kinetic properties of highly purified samples of all forms of the dormant bean α-galactosidase (i.e. post CM-cellulose stage, except enzyme II which was post Sephadex G-100 stage, (see p. 55) were examined.

The influence of K^+ ion on the pH optima of the α-galactosidase isozymes, using PNPG as substrate are shown in Fig. 28(a-d). In the case of enzyme I, the K^+ ion produced a shift in both optima i.e. the normal values of pH 2.0 and 5.5 were both increased by 0.5 pH units. The ratio of the activities at the two optima was not changed by addition of K^+. K^+ ion had no effect in the case of enzyme II but the two pH optima for enzyme II^1 were changed with the peak at pH 3.0 decreases to 2.0 and that at pH 5.5 increases to 6.0. With enzyme II^2 the first pH optima changed from pH 2.0 to 3.0 but the other shoulder remained unchanged. In view of these

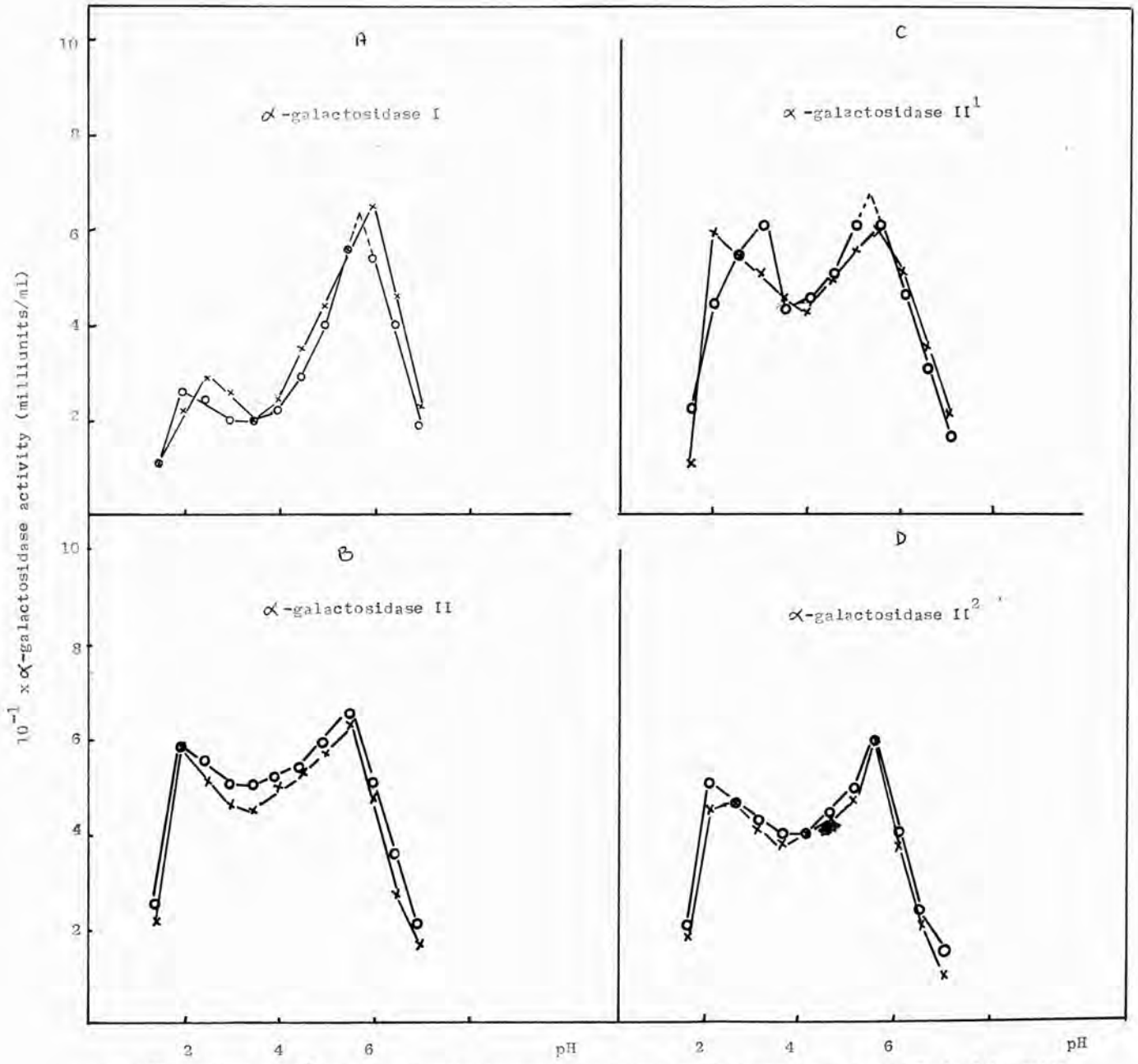


Fig.28. Effect of K^+ ion on pH activity profile of α-galactosidases from mature Vicia faba seeds. PNPG substrate. ×—×, with 0.12 M-KCl in the reaction mixture; o—o, control.

observed changes in the optima an attempt was made to show that K^+ ions altered the conformation of the protein in the case of enzyme I. In this experiment (carried out at Westfield College by Dr. P.M.Scoopes) the circular dichroism of the enzyme was measured in the presence of 0.12M-Kcl (Fig.29). It was observed that Kcl immediately decreased the absorbance value at 210 nm by 40% (Fig.29), but no further significant diminution occurred if the solution was allowed to stand for 18 hours (46%). The circular dichroism at 270-280 nm where aromatic amino acids absorb (212) was unaltered when K^+ was added to the enzyme in McIlvaine buffer, pH 5.5. The diminution of the absorbance at 210 nm probably indicates that some kind of disorder in the polypeptide chain of the protein (212) is produced by K^+ and indeed, the extent of this disorder could be the cause of the change of pH optima.

The effect of K^+ on Vmax and Km values of all the forms of α-galactosidase was also examined at the two optima in each case i.e. those optima observed in the presence of K^+ (see Table 14). Potassium ion had no effect on the Km values or substrate inhibition with any of the isozymes. The effect of K^+ ion on Vmax values was also negative. K^+ ions are unable to activate enzymes I and II, after these proteins are passed through Sephadex G-100. One possible explanation of this phenomenon is that in the case of the purified enzymes the K^+ produces a rapid marked change in conformation resulting in a change in the pH optima without altering the kinetic properties. With the relatively crude mixture of enzymes I and II (pre-Sephadex treatment) there may also be present a modifying factor (possibly a protein) which allows only a slow and different reaction of K^+ with the enzyme which in this case changes the Vmax values. Further work is obviously required to substantiate this

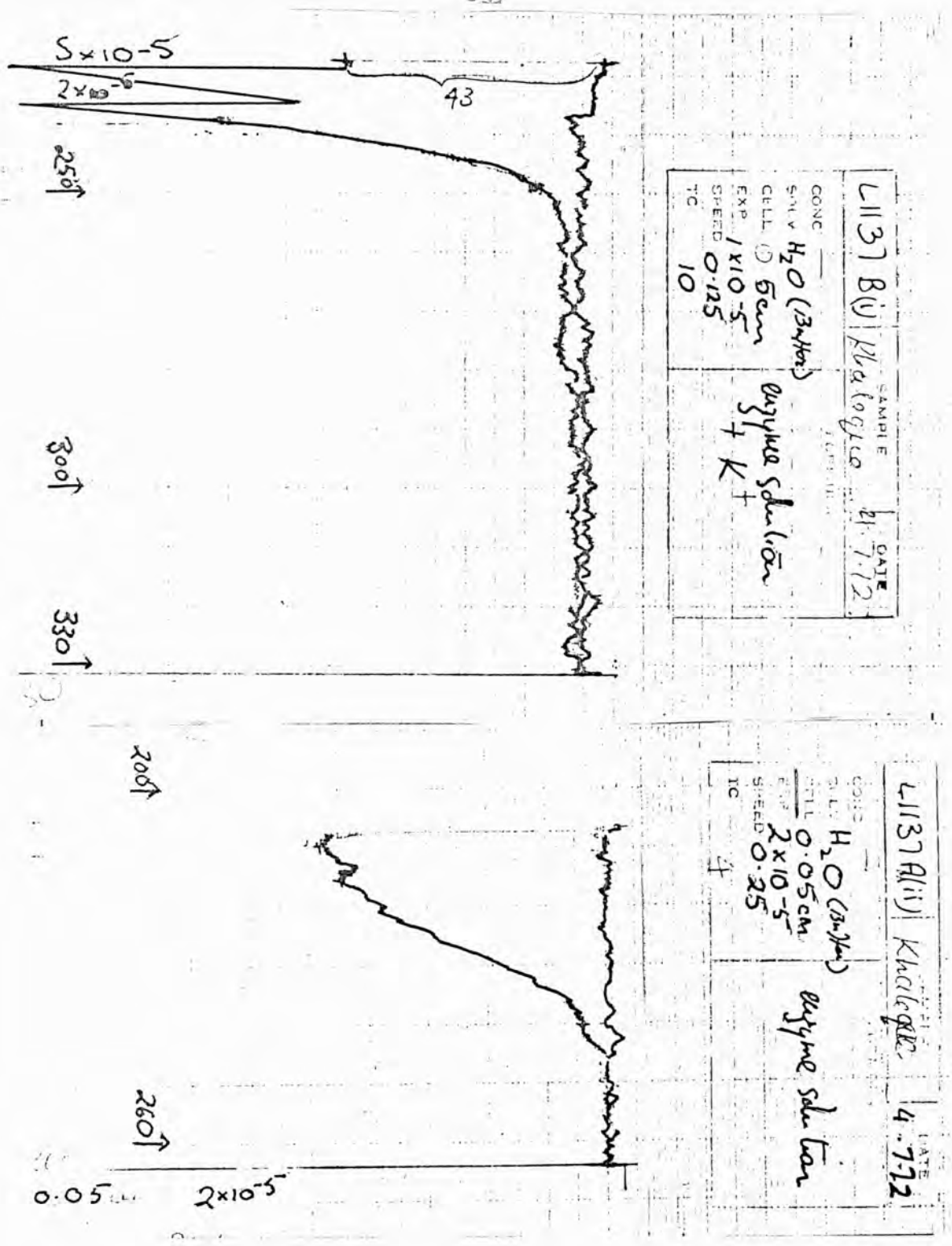


Fig. 29 . Effect of K^+ ion on the Circular dichroism of purified α-galactosidase I from mature Vicia faba seeds. The procedure is described in the text, p. 43 .

Table 14

Effect of K^+ on Vmax and Km of various forms of α-galactosidase from dormant Vicia faba seeds.

Enzyme Form	pH Optima	Vmax* μ mol/min/mg (Control)	Vmax* μ mol/min/mg (with 0.12M KCl)	Km * (mM) (Control)	Km * (mM) (with 0.12M KCl)
I	5.5	24.15	27.42	0.44	0.44
	2.0	14.62	16.15	0.44	0.42
II	5.5	2.93	4.22	0.54	0.51
	2.0	2.95	3.15	0.48	0.46
II^1	5.5	1.61	2.20	0.35	0.33
	3.0	1.72	1.75	0.34	0.33
II^2	5.5	0.97	1.18	0.33	0.33
	2.0	0.91	0.95	0.34	0.34

*p-Nitrophenyl α-D-galactoside as substrate.

R6

theory. It would be difficult to carry out a useful kinetic analysis of this action of K^+ on the pre-Sephadex mixture of enzymes: a search in the Sephadex column fractions could possibly be made for this hypothetical 'modifying factor'.

One further observation of interest is the measurement of bound K^+ in the various purified dormant seed α-galactosidases which were all prepared in the absence of exogenous K^+ (Table 15), shows that enzymes II^1 and II^2 possess a very low K^+ content in comparison with the other forms. A comparison of the K^+ contents of enzymes I and II from dormant seeds is of special interest as the former contains four times as much of the ion as the latter (all attempts to remove the bound K^+ from the enzymes by dialysis have failed). In this connection it should be remembered that the ultracentrifuge data suggests that enzyme I is a tetramer of enzyme II and hence the possibility that K^+ is involved in the association of monomer units and the increase in specific activity should be considered.

Table 15

Potassium content of various forms of α-galactosidases from dormant and germinated Vicia faba seeds.

Molecular Form of α-galactosidase	Potassium Content meq/mg protein
I (Dormant seed)	40.0
I (Obtained from II *in vitro*)	35.0
II (Dormant seed)	10.0
II (Germinated seed)	9.5
II^1 (Dormant seed)	3.0
II^2 (Dormant seed)	0.5

B. CHANGES IN THE α-GALACTOSIDASE PATTERN DURING SEED MATURATION

The α-galactosidase content of extracts of maturing V. faba seeds have also been examined. Buffered extracts of the tissues were partially purified by lowering the pH to 2.5 (stage 2, p.45) and then applied to Sephadex G-100 columns. This procedure showed that young green bean seeds contain only the lower molecular weight form of the enzyme (II) at low levels (Fig.30). The fully grown green seeds, still remaining in the pod, also showed a similar pattern. The higher molecular weight enzyme (I) begins to appear if the pod is left on the plant to dry out after the seeds have reached their full size. The appearance of enzyme I was also observed when the pods were removed from the plant and dried in the labbratory at room temperature. The increase in the level of activity of enzyme I parallels the decrease in the activity of enzyme II during maturation (Fig.30). The activity profile of enzyme II^1 and enzyme II^2 (components of enzyme II, which are resolved on CM-cellulose) remains similar during the maturation period and the patterns in turn closely resemble those obtained with the fully dormant seed (Fig.30).

The changes in the α-galactosidase pattern occurring during maturation almost certainly reflect a complex underlying series of biochemical reactions. It is possible that the changes in the level of enzyme I and II are not directly related i.e. that independent synthesis of enzyme I occurs whilst enzyme II is being degraded. A further explanation could be that the enzymes are present in the maturing tissues at an early stage are in inactive forms and that inhibitors and/or activators are synthesized which in turn control

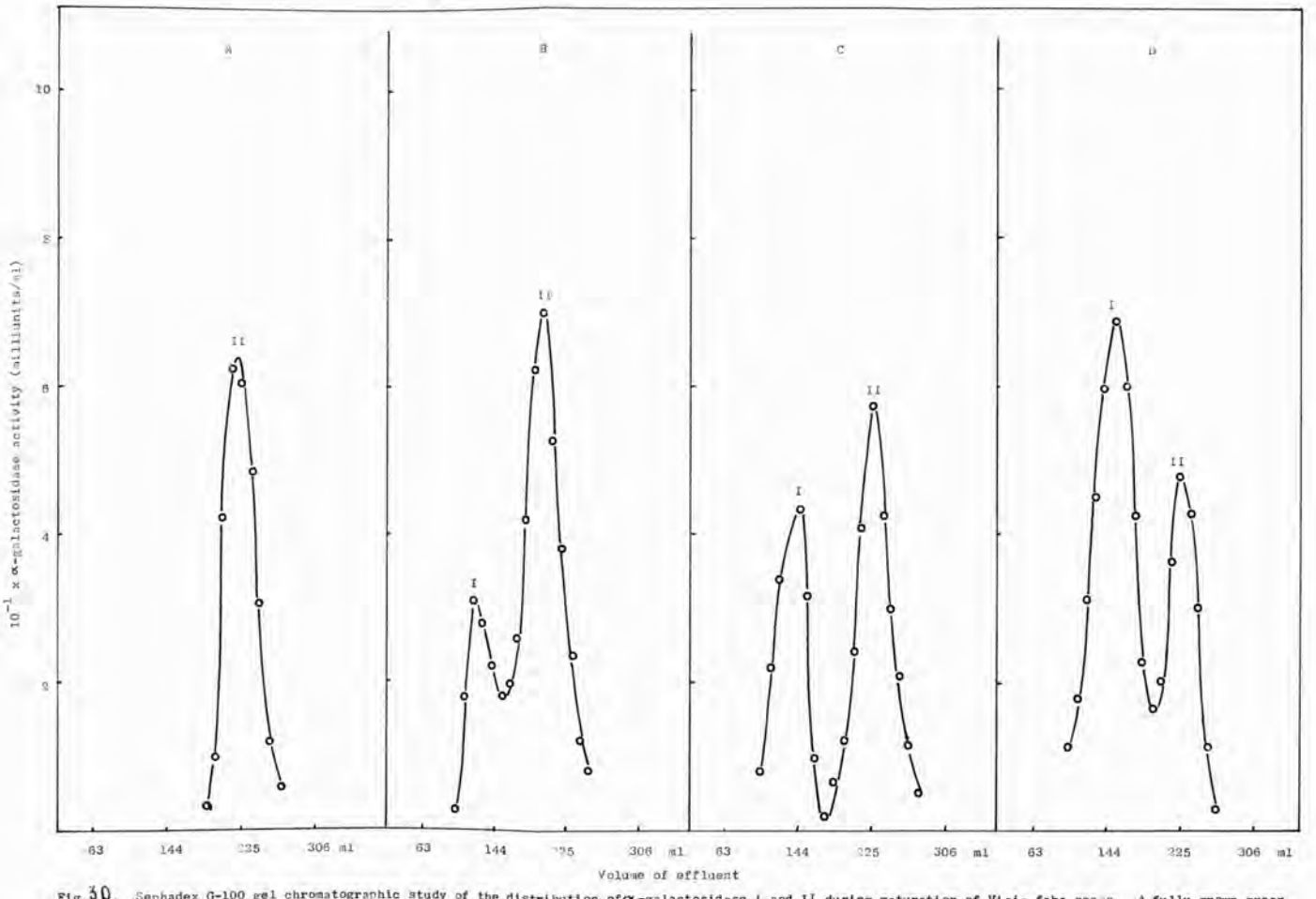


Fig.30. Sephadex G-100 gel chromatographic study of the distribution of α-galactosidase i and II during maturation of Vicia faba seeds. A, fully grown green seed in the pod. B, appearance of α-galactosidase I while dried in the laboratory at room temperature; C and D, different stages of seed maturation.

the levels of enzymes I and II. A third possibility, which is partly supported by the results of the study on the nature of the enzymes from dormant seeds, is that enzyme I is directly derived from association of units of enzyme II. A combination of all these phenomenon may also of course, exist.

C. THE CONVERSION OF ENZYME II TO I

The hypothesis that enzyme I is derived from enzyme II during maturation was investigated further.

α-Galactosidase II was extracted from a batch of fully grown immature seeds and purified as far as the ammonium sulphate fractionation (stage 5; see p. 46). When this preparation was stored at 4°, pH 5.5, for 7 days it was observed that there was an increase in the specific activity. Examination of this 'aged' enzyme by Sephadex G-100 gel filtration, showed that two forms (I and II) of the enzyme were now present. A further examination of the enzyme over a period of 10 weeks was then made, Fig. 31, shows the changes in the patterns of enzyme I and enzyme II during this time and Table 16 shows the increasing specific activity. The gradual appearance of enzyme I (which has a specific activity 10 times greater than enzyme II) can be correlated with the increase in specific activity of the enzyme solution. It appeared, therefore, that enzyme I could be formed <u>in vitro</u> from enzyme II. It was interesting to observe, however, that if enzyme II was further purified by passage through Sephadex G-100 it could not be converted to enzyme I. Further experiments were then carried out in an attempt to accelerate the conversion and the effects of pH, temperature and protein

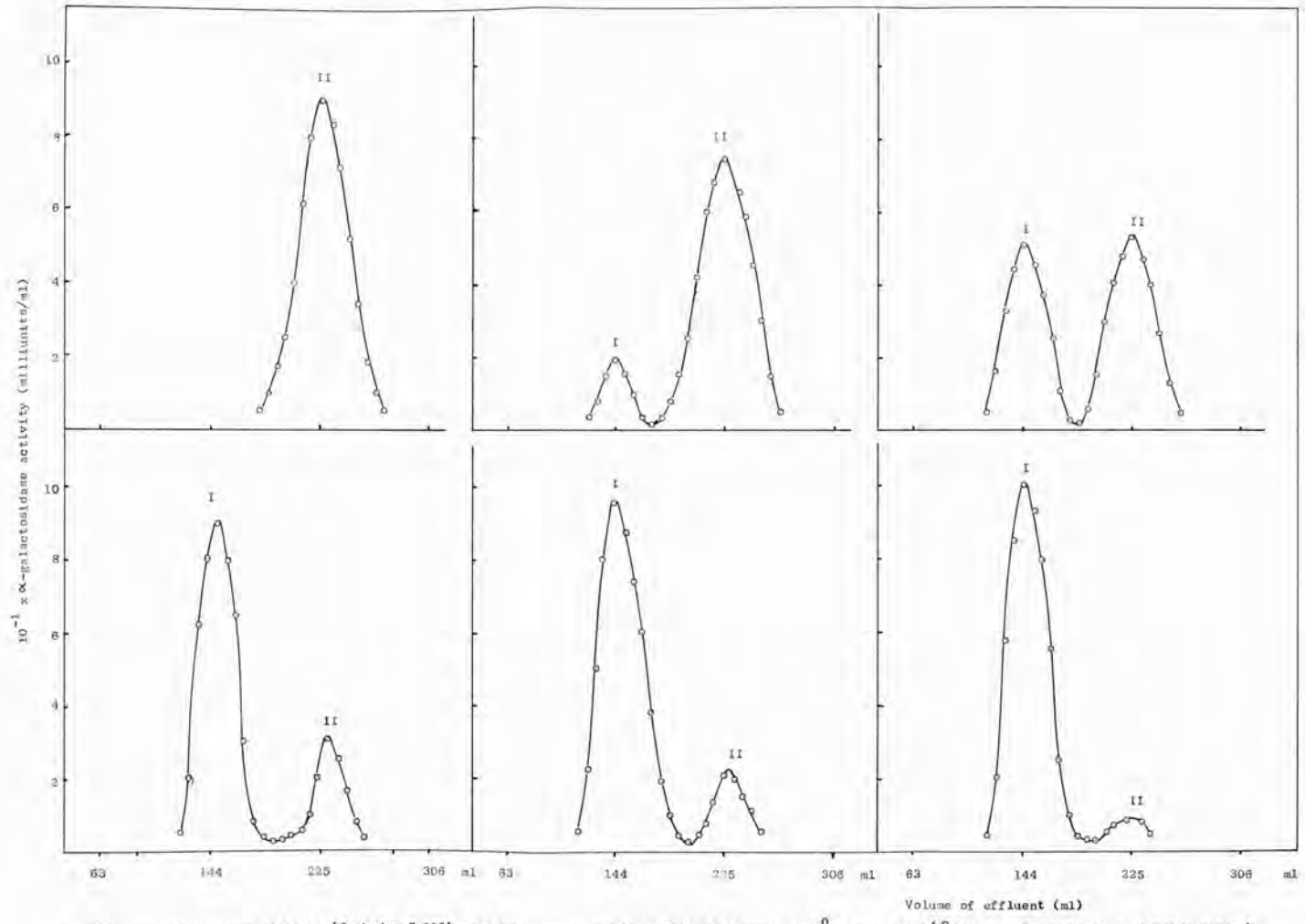


Fig. 31. Changing enzyme patterns (Sephadex G-100) on storing enzyme II from Vicia faba seeds at 4°. (see Table 16 for specific and total activity values).

Table 16

Conversion of α-galactosidase II from Vicia faba seed to I on storage at 4°. Enzymes were fractionated as Sephadex G-100 columns.

Storage Time (days)	Total Enzyme milli-Units	Total Sp. Activity (milliunits/mg Protein)	% of milli-Units as Enzyme I	% of milli-Units as Enzyme II
Original	1,100	40	0	100
14	1,800	88	10	90
28	2,820	157	40	60
42	3,710	226	72	28
56	2,430	266	90	10
70	1,900	402	92	8

concentration were examined. The results are shown in Table 17, and in all cases increase in specific activities were shown to be accompanied by the formation of enzyme I. The different protein concentrations examined appeared to have no appreciable effect on the conversion but the rate was increased by raising the pH to 7.0 and the temperature to 25°. An incubation of enzyme II for 12 hours under these optimal conditions was used to prepare a sample of enzyme I for further investigation and comparison with enzyme I from dormant seeds. The α-galactosidase I thus obtained was purified (8 stages; see p.46) and the molecular weight, amino acid composition and kinetic properties were examined. The results compared with those for enzyme I from dormant seeds are given in Table 18. Both the enzymes were eluted from G-200 columns as single, symmetrical peaks of activity (Ve = 190 ml). The protein peaks coincided exactly with the activity peaks (Fig 32). The elution patterns of both enzymes from CM-cellulose column were also identical (Fig. 8). The specific activity of the enzyme I, derived from enzyme II, was lower than that of enzyme I from dormant seeds but still 5 times higher than the value for enzyme II. The potassium content and the U.V. spectra of the enzymes were approximately the same (Table 15 and Fig. 33) and the amino acid compositions showed a close similarity (Fig. 34 ; Table 2). With regard to specificity, both preparations of enzyme I, unlike enzyme II, were shown to be able to liberate galactose from galactomannans (Fig 35). As shown in the Table 18 , the kinetic properties of the two preparations were comparable: both were inhibited at high substrate concentration (p-nitrophenyl α-D-galactoside). The significant differences between the two preparations of enzyme I were the carbohydrate contents and the

Table 17

Examination of the formation of Enzyme I during incubation of 9,223 milliunit samples of Enzyme II from dormant Vicia faba seeds at various protein concentrations, pH values and temperatures.

Temperature	pH	Initial Protein Concentration (mg/ml)	Final Total Enzyme Recovery (m.u./ml)	% Milliunits as Enzyme I	% Milliunits as Enzyme II
		40	10,100	15	85
	7.0	20	10,800	25	75
		10	11,800	25	75
		40	9,112	0	100
30°	5.5	20	9,220	0	100
		10	9,220	0	100
		40	9,050	0	100
	2.0	20	8,988	0	100
		10	8,690	0	100
		40	16,800	30	70
	7.0	20	17,500	35	65
		10	17,500	32	68
		40	11,120	10	90
25°	5.5	20	10,900	8	92
		10	10,770	10	90
		40	8,450	0	100
	2.0	20	9,110	0	100
		10	8,860	0	100
		40	15,600	18	82
	7.0	20	15,400	18	82
		10	14,800	16	84
		40	11,800	5	95
20°	5.5	20	9,250	0	100
		10	9,225	0	100
		40	8,990	0	100
	2.0	20	8,670	0	100
		10	8,880	0	100

Enzymes I and II were fractionated by Sephadex G-100 chromatography

Table 18

Properties of ∝-galactosidase I obtained from Enzyme II *in vitro*.

Properties		Enzyme I (From Dormant Seed)	Enzyme I (from Enzyme II *in vitro*)
Specific Activity (milliunits/mg of protein; substrate p-nitrophenyl ∝-galactoside).		65,631	53,240
Elution Volume (ml) (Sephadex G-200 column; 86 x 2.5 cm; Fig. 32)		190	190
Elution pH (CM-cellulose chromatography; Fig. 38)		4.5	4.5
Molecular Weight	Sephadex Gel filtration method	209,000	209,000
	Sedimentation Equilibrium method (Table 19)	160,000	118,000
Carbohydrate Content (as µ gm glucose/ mg protein)		80	58
E 280/260 (Fig. 33)		1.34	1.56
E 1%/280		18	21
Potassium Content (meq/mg protein)		40	35
pH Optima (Fig. 36)		2.0; 5.5	2.0; 5.5
Km (mM) (p-nitrophenyl ∝-D-galactoside as substrate)	pH 2.0	0.44	0.40
	pH 5.5	0.44	0.39
Vmax µ mol/min/mg (p-nitrophenyl ∝-D-galactoside as substrate)	pH 2.0	14.62	15.65
	pH 5.5	24.15	27.11
Action on locust bean (see Fig. 35) and Tara (see Fig. 35) galactomannans		Hydrolysed	Hydrolysed

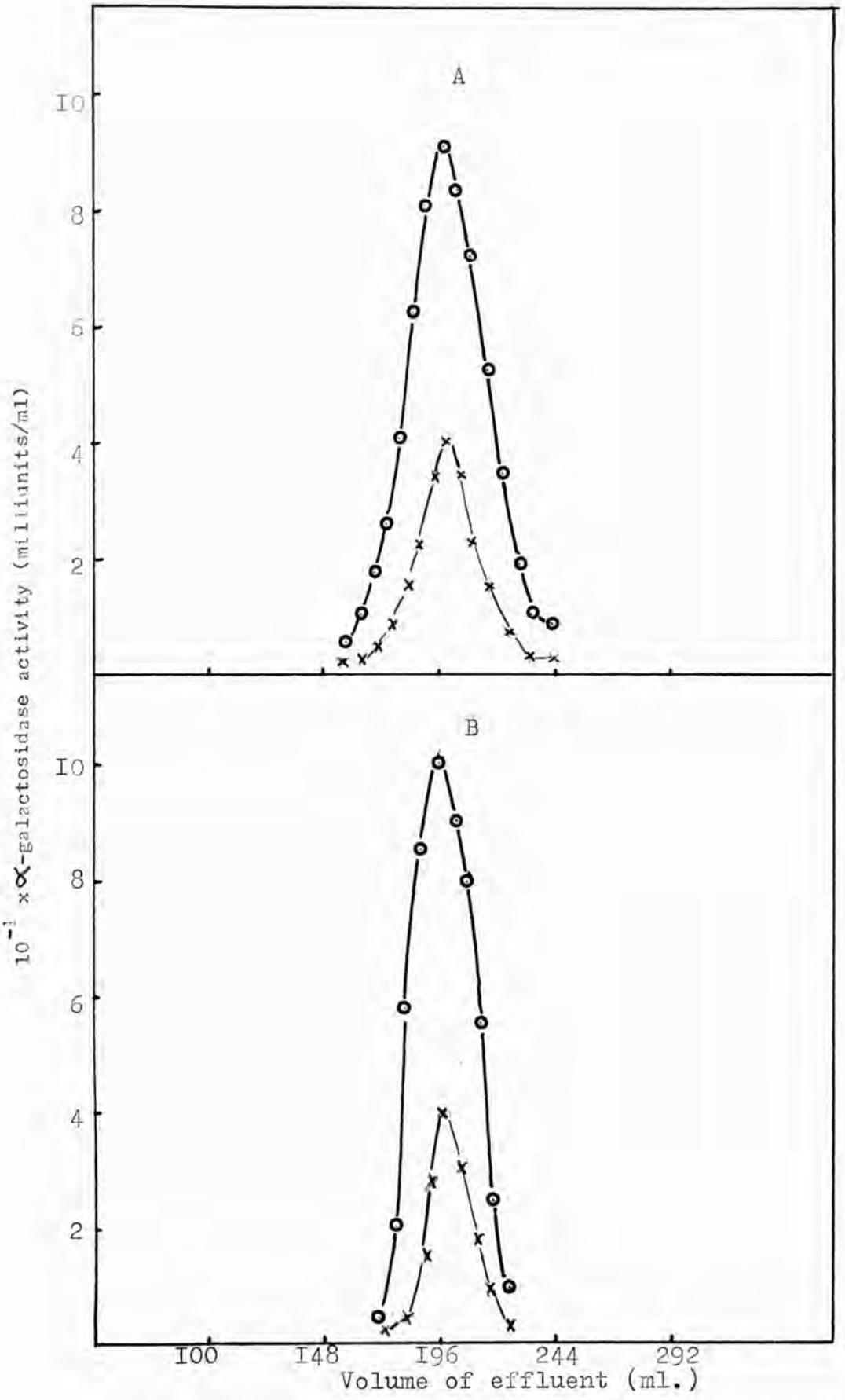


Fig. 32 . Comparison of Sephadex G-200 gel - filtration of ∝-galactosidase I from mature Vicia faba seeds (A) and enzyme produced from II at 25° pH 7·0 (B). o—o, ∝-galactosidase activity; x— x , protein.

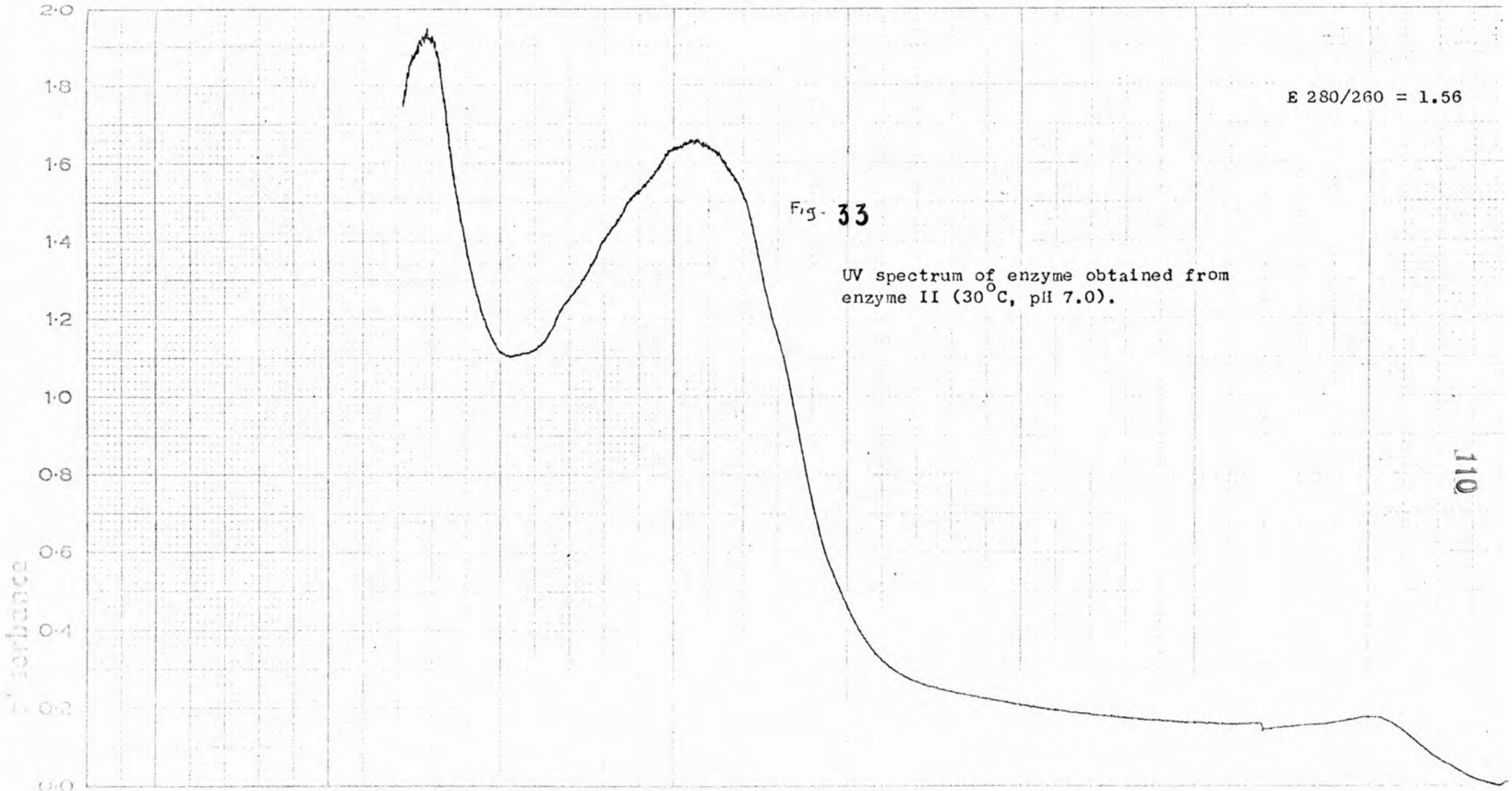


Fig. 33

UV spectrum of enzyme obtained from enzyme II (30°C, pH 7.0).

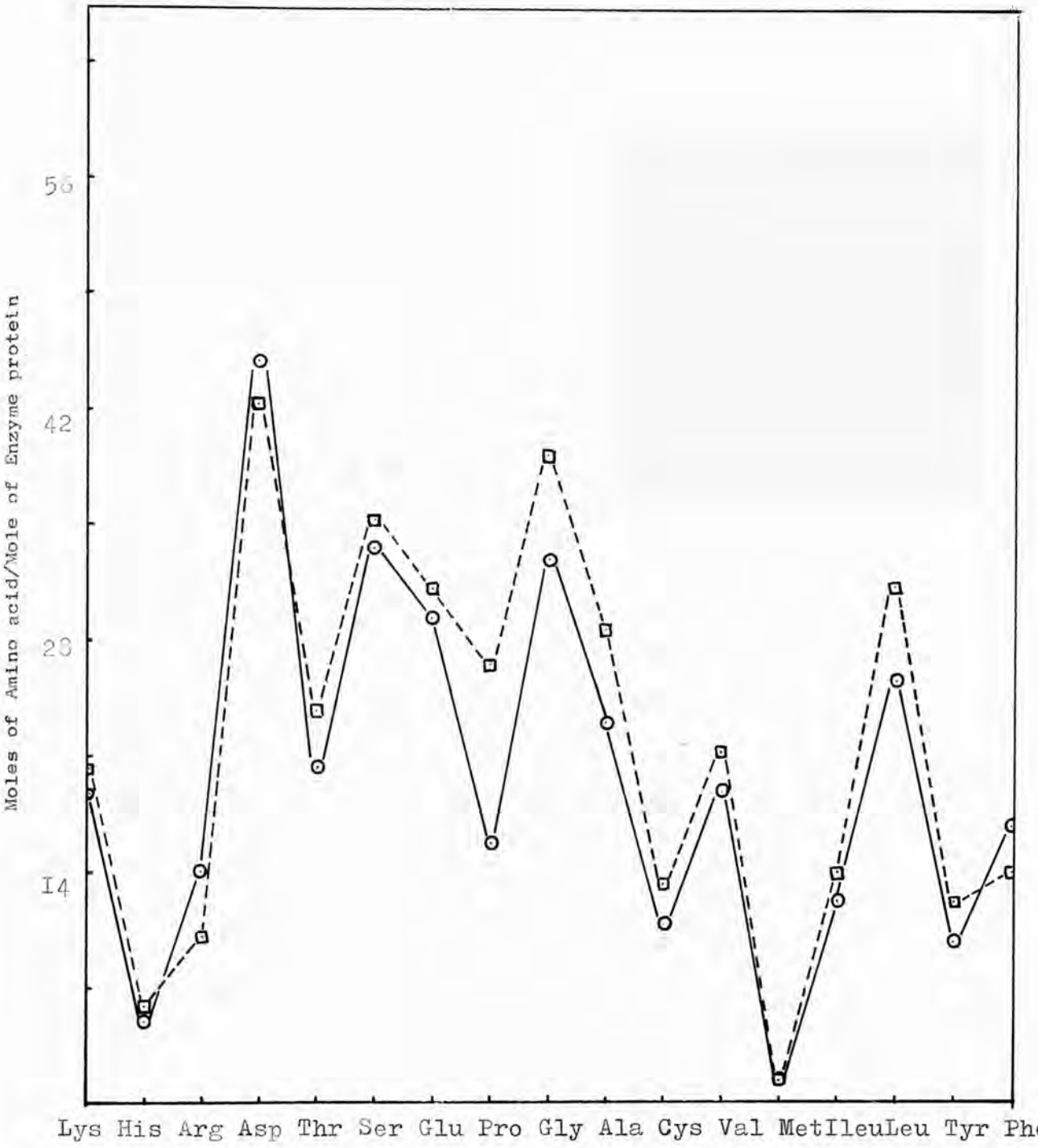


Fig. 34. Pattern of amino acid composition of mature V.faba seed α-galactosidaseI and enzyme formed from α-galactosidase II in vitro at 29 pH 70. o—o , enzyme I ; □—□ , enzyme produced from II. Ultracentrifuge mol. wt. used for these calculations.

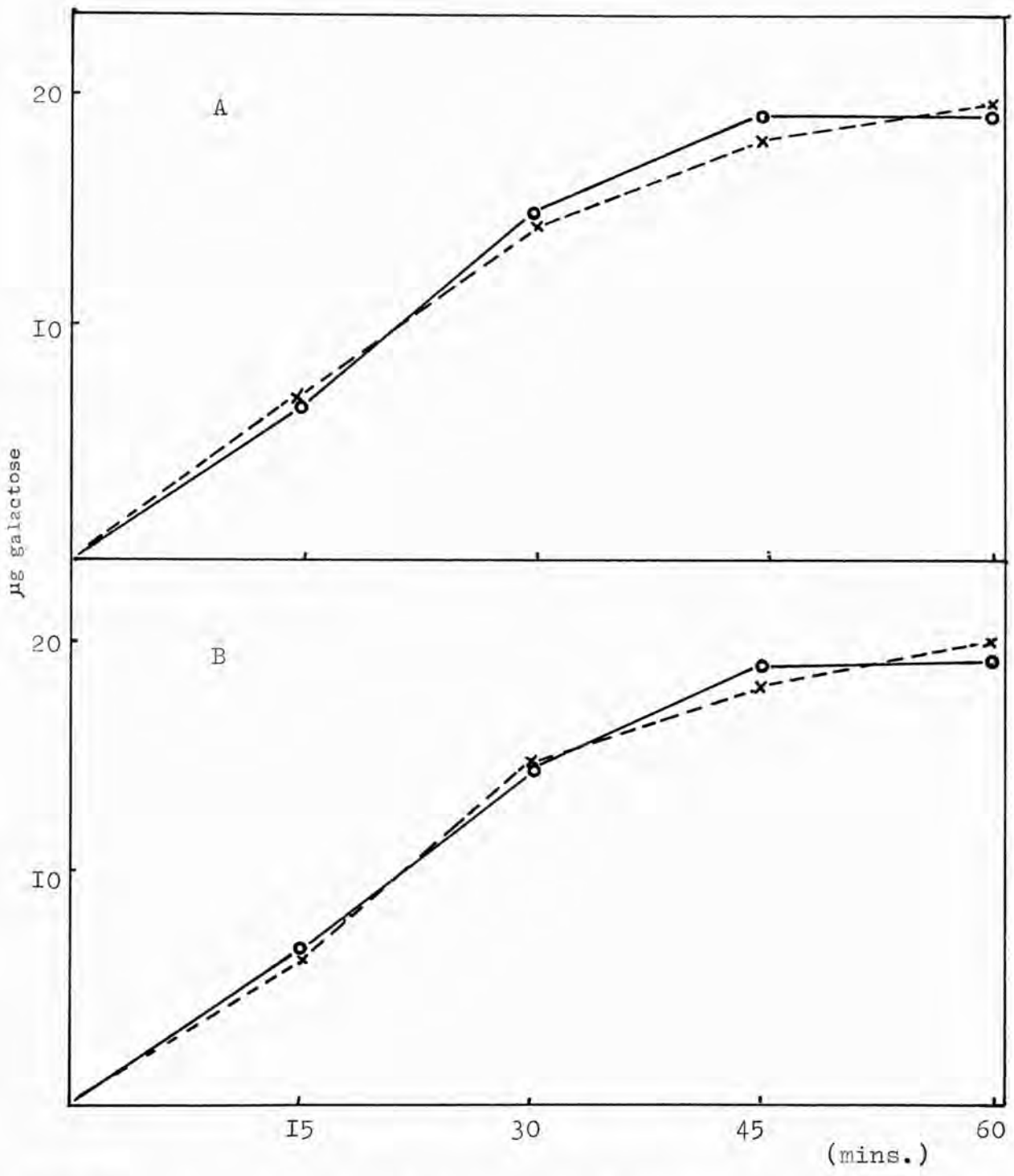


Fig. 35 . Hydrolysis of galactomannans by purified ∝-galactosidase I from mature Vicia faba seeds (A) and by enzyme produced from II in vitro at 25° pH 7·0 (B). The conditions of assay and estimation of galactose are same as described in Fig. •o—o , Locust bean gum; ×---× , Tara gum .

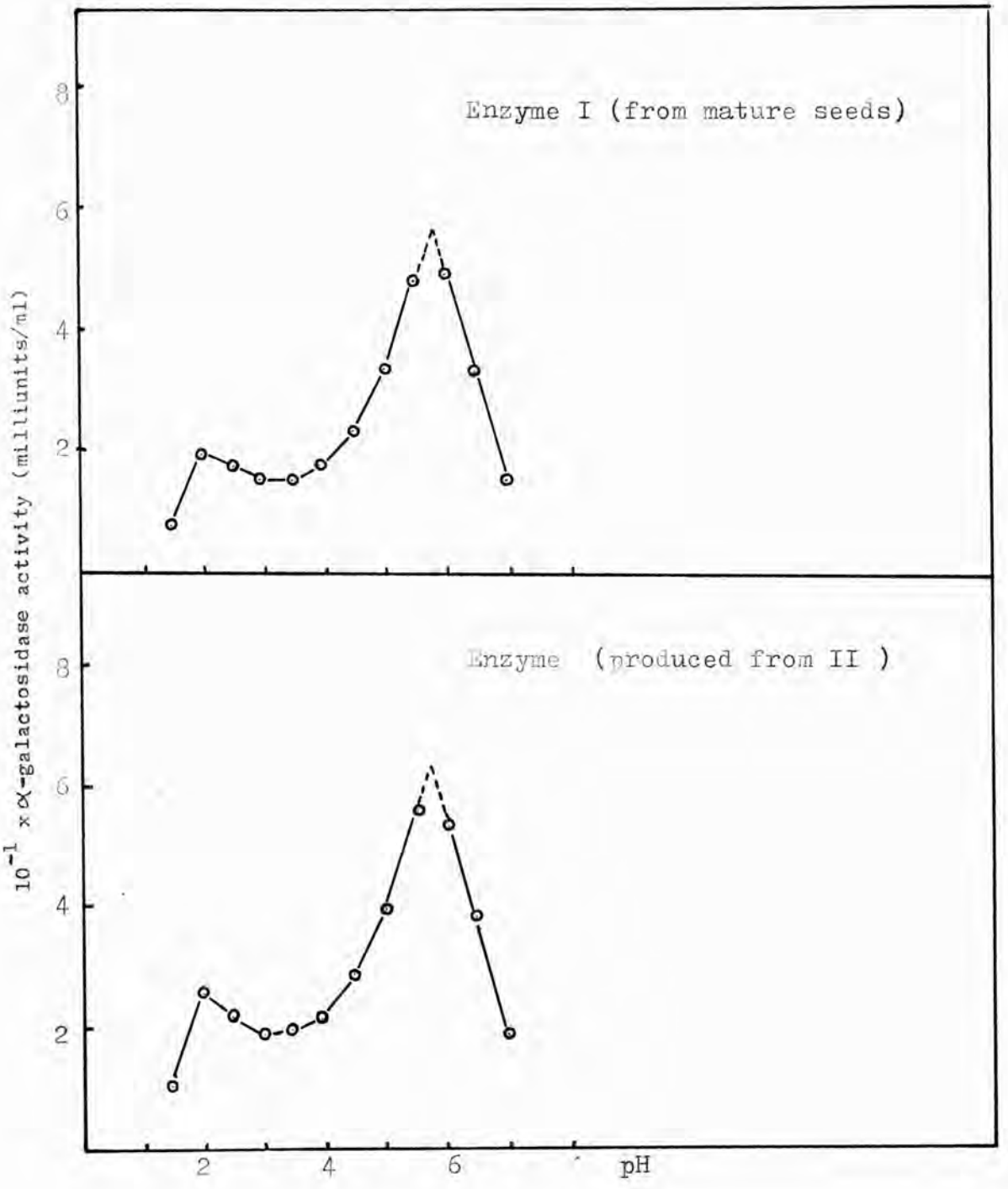


Fig. 36. Comparison of pH optima of α-galactosidase I from mature seeds and the enzyme produced from enzyme II in vitro at 25°, pH 7.0.

molecular weights. No explanation can be given except that these values may reflect different degrees of carbohydrate contamination. The problem regarding molecular weights is similar to that with enzyme I from dormant seeds i.e. that the value obtained by gel-filtration is different from that calculated from ultra-centrifuge measurements. In addition, the molecular weights obtained by ultra-centrifugation are different for the dormant seed enzyme (160,000) and enzyme I derived from enzyme II (118,000). It is possible here that the two values represent different associations of the common monomer unit (II): it was stated earlier (p.69) that some evidence for an association/dissociation phenomenon was obtained in case of enzyme I from dormant seeds. It should be noted once more, however, that no such evidence is provided by gel-filtration studies.

In conclusion, the two forms of enzyme I appear to be closely related with respect to the kinetic properties and amino acid composition. However, the anomalous results obtained by the two methods of molecular weight determination remain a dilemma which must be solved before it can be stated with certainty that enzyme I, found in dormant seeds, can be formed from enzyme II either *in vitro* or *in vivo*.

Interconversion of low and high molecular weight isozymes is not uncommon. *Streptococcus lactis* was reported to contain two forms of β-galactosidase (213 214). However, when the partially purified enzyme preparation was incubated for 18 hours 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 (215). It has also been shown that β-galactosidase from *E. coli* is made up of 4 sub-units (216,217) and temperature dependent association and diesociation can occur *in vivo* (218)

Table 19

Molecular Weight of α-galactosidase I obtained from enzyme II in vitro.

RUN 1890 J.PRIDHAM B-GALACTOSIDASE PHS.EIC CELL3 P01

SPEED = 0.8225E+04GIVING OMEG**2 = 0.7420E+06
P. SP. VOL = 0.7500E+00SOLUTION DENSITY = 0.1030E+01EXPTL. TEMP = 0.2120E+02

MEAN MENISCUS = 0.6944E+01 WITHS.E. = 0.1998E-01

OPTICAL DENSITY FOR THIS SCAN = 0.6247E+00

MEAN RADIUS= 0.7074E+01

DATA FITS QUADRATIC WITH FOLLOWING COEFFICIENTS
-0.1060E+02 -0.2536E-02 0.4054E-02
AND STANDARD ERRORS
0.5896E+00 0.2550E-03 0.2329E-03

WT. AVERAGE MOLECULAR WEIGHTS FROM DERIVATIVE OF ABOVE REGRESSION EQUATION,FOR EDITING PURPOSES ONLY

OPTICAL DENSITY	MOL WT	ST ERROR		Z AVERAGE		NUMBER AVERAGE
0.2817E+00	0.1128E+06	0.6538E+04	0.1189E+06	0.2659E+07	0.1097E+06	0.3470E+04
0.2978E+00	0.1132E+06	0.6557E+04	0.1192E+06	0.2243E+07	0.1099E+06	0.3579E+04
0.3150E+00	0.1135E+06	0.6577E+04	0.1195E+06	0.1892E+07	0.1101E+06	0.3691E+04
0.3332E+00	0.1138E+06	0.6596E+04	0.1198E+06	0.1594E+07	0.1103E+06	0.3808E+04
0.3526E+00	0.1142E+06	0.6615E+04	0.1202E+06	0.1344E+07	0.1105E+06	0.3928E+04
0.3732E+00	0.1145E+06	0.6635E+04	0.1205E+06	0.1132E+07	0.1108E+06	0.4053E+04
0.3951E+00	0.1149E+06	0.6654E+04	0.1208E+06	0.9535E+06	0.1110E+06	0.4183E+04
0.4183E+00	0.1152E+06	0.6674E+04	0.1211E+06	0.8032E+06	0.1112E+06	0.4317E+04
0.4431E+00	0.1155E+06	0.6694E+04	0.1214E+06	0.6769E+06	0.1115E+06	0.4456E+04
0.4695E+00	0.1159E+06	0.6713E+04	0.1218E+06	0.5707E+06	0.1117E+06	0.4600E+04
0.4975E+00	0.1162E+06	0.6733E+04	0.1221E+06	0.4815E+06	0.1120E+06	0.4749E+04
0.5273E+00	0.1166E+06	0.6752E+04	0.1224E+06	0.4068E+06	0.1122E+06	0.4903E+04
0.5591E+00	0.1169E+06	0.6772E+04	0.1227E+06	0.3442E+06	0.1125E+06	0.5064E+04
0.5930E+00	0.1172E+06	0.6792E+04	0.1231E+06	0.2919E+06	0.1128E+06	0.5230E+04
0.6291E+00	0.1176E+06	0.6811E+04	0.1234E+06	0.2482E+06	0.1130E+06	0.5402E+04
0.6675E+00	0.1179E+06	0.6831E+04	0.1237E+06	0.2118E+06	0.1133E+06	0.5581E+04
0.7084E+00	0.1183E+06	0.6851E+04	0.1241E+06	0.1814E+06	0.1136E+06	0.5766E+04
0.7521E+00	0.1186E+06	0.6871E+04	0.1244E+06	0.1562E+06	0.1139E+06	0.5959E+04
0.7987E+00	0.1190E+06	0.6891E+04	0.1247E+06	0.1352E+06	0.1142E+06	0.6158E+04

DATA FITS STRAIGHT LINE OF SLOPE 0.4069E+00 AND STANDARD ERROR 0.1325E-01

SLOPE CORRECTED FOR SPEED = 0.5484E-06S.E. = 0.1785E-07

WEIGHT AVERAGE MOLECULAR WEIGHT = 0.1180E+06 AND STAADARD ERROR = 0.3844E+04

A similar phenomenon was reported with a proteinase from Streptococcus lactis (219) and α-chymotrypsin from pancreatic juice (220). In the latter case, the conversion was dependent on protein concentration. The interconversion of two forms (A and B) of α-galactosidase obtained from human leukocytes, by gel electrophoresis, was reported by Kint (176). The author observed that when the mixture of isozymes was incubated with neuraminidase at pH 5.0, the activity of form A decreased slowly whilst B increased in activity: the total activity remained fairly constant. The author suggested that isozyme A, which possesses an acidic isoelectric point contains neuraminic acid and that removal of this acid produces the isozyme B with a neutral isoelectric point.

D. α-GALACTOSIDASE FROM GERMINATED V. FABA SEED

Dormant V. faba seeds contain two active forms of α-galactosidase, I and II, which can be resolved by Sephadex G-100 gel-filtration (Fig. 6) with enzyme I as the predominant activity in the tissues. Dey et al.(221) have observed a change in the isozyme pattern when the seeds are germinated; the activity of enzyme I decreases and that of enzyme II increases. This changing pattern was only examined by Sephadex gel chromatography and it was, therefore, necessary to check the identity of the activity which increased during germination and to see if this corresponded with enzyme II from dormant seeds.

Hence, V. faba seeds were allowed to germinate on moist cotton wool for 24 hours and the α-galactosidase was then extracted and purified according to the procedure described earlier (p.45 ; see Table 20). The enzyme was then subjected to a detailed

Table 20

Purification of α-galactosidase II from germinated Vicia faba seed.

Purification Stage	Volume (ml)	Enzyme Activity (milliunits/ml)	Protein (mg/ml)	Sp. Activity (milliunits/mg Protein)	Recovery (%)
1. Extraction	1,400	161	18.5	14.1	
2. Acidification (pH 2.5)	2,150	142	3.8	37.0	
3. Protamine Sulphate Treatment	2,200	134	2.5	52.5	
4. Acetone Fractionation	60	4342	29.9	145.5	
5. Ammonium Sulphate Fractionation	18	12,236	38.0	322	
	I II	I II	I II	I II	
6. Sephadex G-100 gel Filtration and Concentration	- 5	Trace 40,975	- 4.20	- 9,756	
7. Recycling through Sephadex G-100 Column and Concentration	- 5	Trace 38,620	- 3.29	- 11,738	

examination, as in the case of the dormant seed enzymes, and these results are presented in Table 21, fig. 37 together with those obtained with enzyme II from dormant seeds, for comparison. It can be seen that the properties of enzyme II from both dormant and germinated seeds are very similar. Both enzymes behave similarly in the ultra-centrifuge and on Sephadex columns. With the latter procedure, however, lower molecular weight values were obtained than with the former (see p. 68). The main difference between the enzymes from the two types of tissues is the carbohydrate content which appears to be somewhat higher in the α-galactosidase II from dormant seeds. The significance of this is not clear: it could reflect different glycoprotein structure or merely different levels of contaminating polysaccharides (see p. 68).

On CM-cellulose column the germinated seed enzyme was resolved into two active components (identical to II^1 and II^2) and small amounts of four other inactive proteins similar to those obtained from dormant seed enzyme II.

In conclusion, it is very probable that the enzyme from germinated seeds is identical to enzyme II from dormant seeds.

E. PHYSIOLOGICAL IMPORTANCE OF α-GALACTOSIDASES

The precise role of α-galactosidases in seed metabolism is still not clear but the investigation described in this thesis does suggest that it is complex and involves interconversion of isozymes at different physiological stages.

The seed maturation process in Vicia faba would normally appear to be accompanied by a build-up of the low molecular weight

Table 21

Comparison of Properties of α-galactosidase II obtained from dormant and germinated *Vicia faba* seeds

Properties		Enzyme II (From Dormant Seed)	Enzyme II (from germinated seed)
Specific Activity (milliunits/mg of protein; substrate: p-nitrophenyl α-D-galactoside.		14,300	11,738
Elution Volume (ml) (Sephadex G-100 column; 86 x 2.5 cm; Fig.38)			
Molecular Weight	Sephadex gel filtration method	38,000	38,000
	Sedimentation equilibrium method (Table22)	54,300	47,600
Carbohydrate Content (as μ gm glucose/mg of protein)		78	54
E 280/260 (Fig.39)		1.43	1.47
E 1%/280		18	18
Potassium Content (meq/mg of protein)		10	9.5
pH Optima (two in each case; Fig.40)		2.0; 5.5	2.0; 5.5
Km (mM) (p-nitrophenyl α-D-galactoside as substrate)	pH 2.0	0.48	0.49
	pH 5.5	0.54	0.51
Vmax μ mol/min/mg (p-nitrophenyl α-D-galactoside as substrate)	pH 2.0	2.95	2.61
	pH 5.5	2.93	2.74

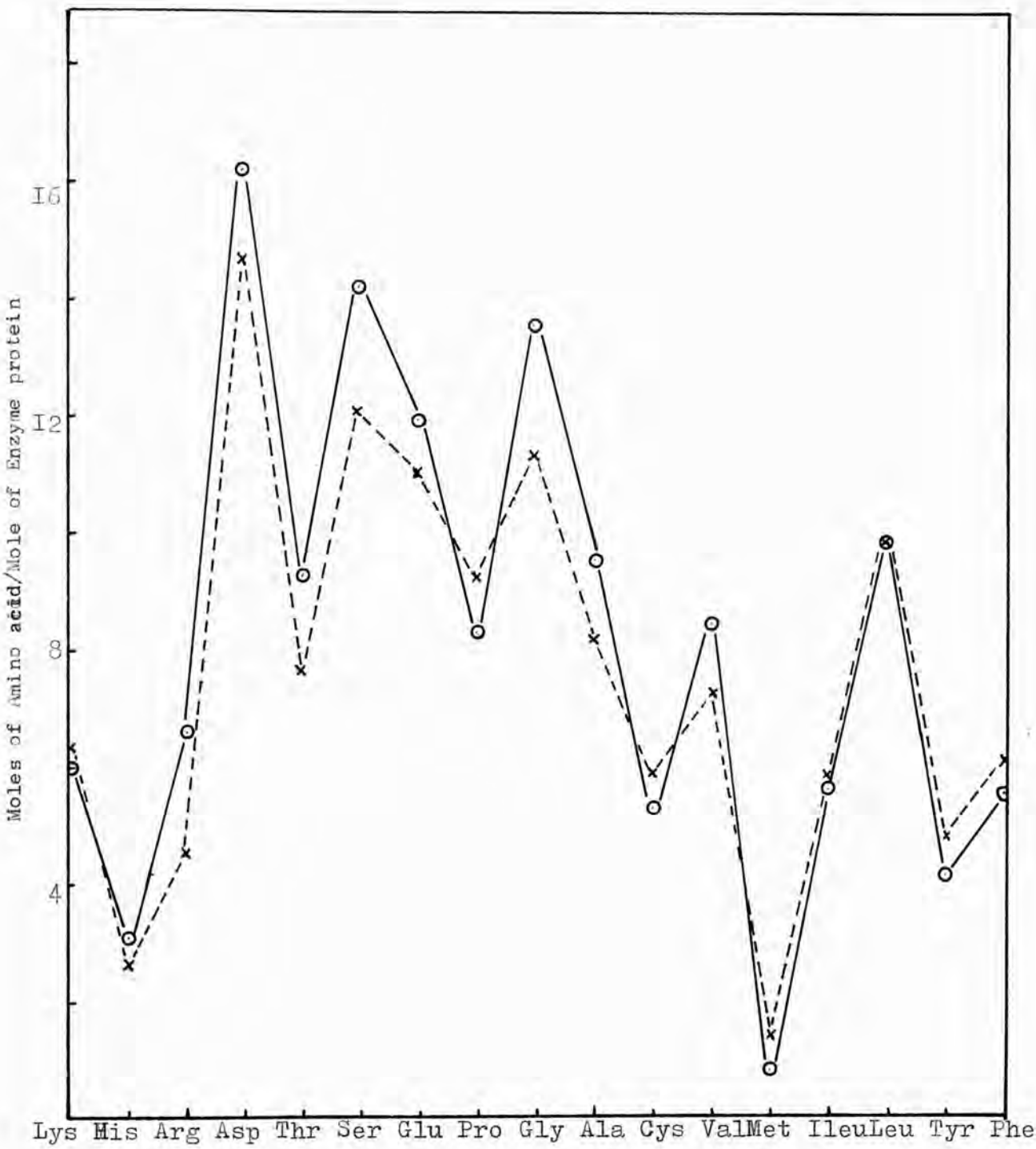


Fig. 37. Pattern of amino acid compositions of α-galactosidase II from mature and germinated Vicia faba seeds. o—o , enzyme from mature seed; ×——× , enzyme from germinated seed.

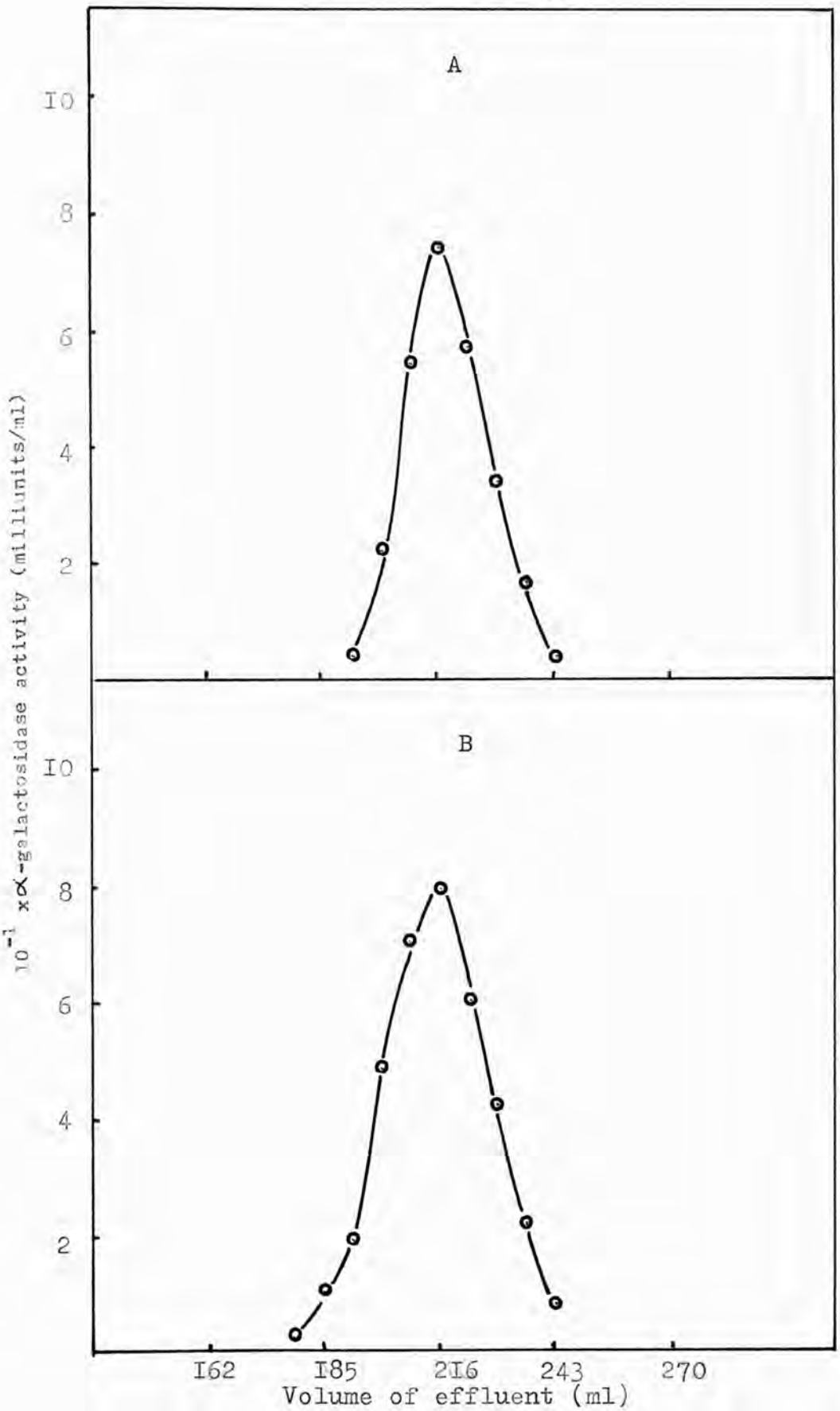


Fig. 38 . Comparison of Sephadex G-100 gel-filtration of purified α-galactosidase II from mature Vicia faba seeds(A) and α-galactosidase II from germinated seeds(B).

Table 22

Molecular Weight of α-galactosidase II obtained from germinated Vicia faba seeds.

RUN 1907 P03 E11C

SPEED = 0.1239E+05GIVING OMEG**2 = 0.1684E+07
P. SP. VOL = 0.7500E+00SOLUTION DENSITY = 0.1000E+01EXPTL. TEMP = 0.1725E+02

MEAN MENISCUS = 0.6884E+01 WITHS.E. = 0.2539E-02

OPTICAL DENSITY FOR THIS SCAN = 0.5242E+00

MEAN RADIUS= 0.7037E+01

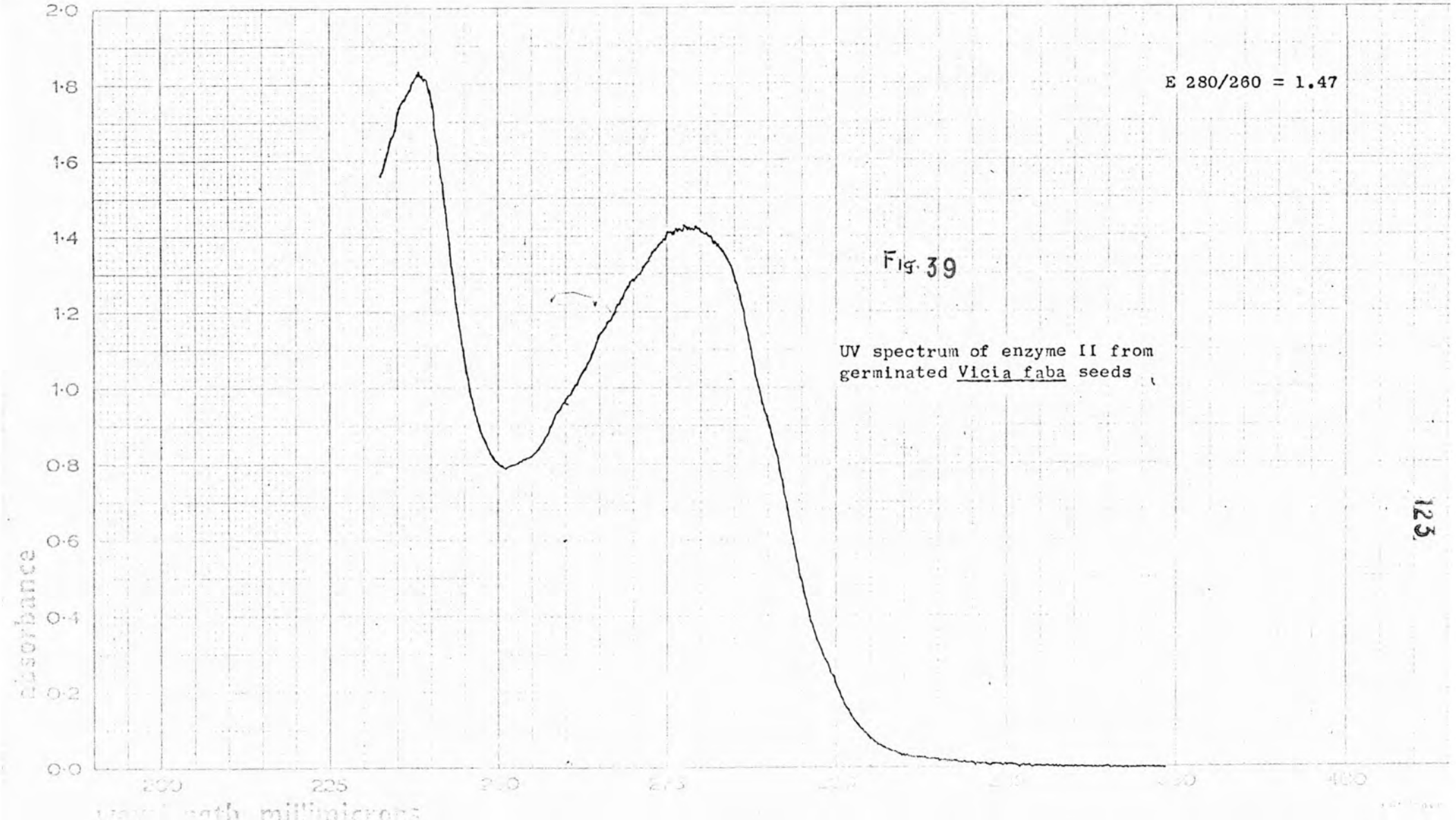
DATA FITS QUADRATIC WITH FOLLOWING COEFFICIENTS
-0.4939E+02 -0.1656E-01 0.1933E-01
AND STANDARD ERRORS
0.3346E+01 0.2306E-02 0.1359E-02

WT. AVERAGE MOLECULAR WEIGHTS FROM DERIVATIVE OF ABOVE REGRESSION EQUATION.FOR EDITING PURPOSES ONLY

OPTICAL DENSITY	M.L WT	ST ERROR		Z AVERAGE		NUMBER AVERAGE
0.2859E-02	0.4485E+06	0.3189E+05	0.4537E+06	0.7486E+12	0.4459E+06	0.1705E+04
0.3860E-02	0.4500E+06	0.3200E+05	0.4552E+06	0.3038E+12	0.4471E+06	0.1988E+04
0.5220E-02	0.4516E+06	0.3210E+05	0.4567E+06	0.1227E+12	0.4485E+06	0.2319E+04
0.7069E-02	0.4531E+06	0.3221E+05	0.4582E+06	0.4936E+11	0.4499E+06	0.2708E+04
0.9589E-02	0.4547E+06	0.3232E+05	0.4598E+06	0.1975E+11	0.4513E+06	0.3165E+04
0.1303E-01	0.4562E+06	0.3243E+05	0.4613E+06	0.7870E+10	0.4528E+06	0.3702E+04
0.1773E-01	0.4578E+06	0.3254E+05	0.4629E+06	0.3121E+10	0.4543E+06	0.4333E+04
0.2416E-01	0.4593E+06	0.3265E+05	0.4644E+06	0.1232E+10	0.4558E+06	0.5075E+04
0.3298E-01	0.4609E+06	0.3276E+05	0.4659E+06	0.4837E+09	0.4574E+06	0.5949E+04
0.4508E-01	0.4624E+06	0.3287E+05	0.4675E+06	0.1891E+09	0.4589E+06	0.6979E+04
0.6173E-01	0.4640E+06	0.3298E+05	0.4690E+06	0.7358E+08	0.4605E+06	0.8195E+04
0.8467E-01	0.4656E+06	0.3309E+05	0.4706E+06	0.2849E+08	0.4621E+06	0.9629E+04
0.1163E+00	0.4672E+06	0.3320E+05	0.4722E+06	0.1098E+08	0.4637E+06	0.1132E+05
0.1600E+00	0.4687E+06	0.3331E+05	0.4737E+06	0.4212E+07	0.4653E+06	0.1333E+05
0.2205E+00	0.4703E+06	0.3342E+05	0.4753E+06	0.1609E+07	0.4668E+06	0.1570E+05
0.3044E+00	0.4719E+06	0.3354E+05	0.4768E+06	0.6139E+06	0.4684E+06	0.1850E+05
0.4209E+00	0.4735E+06	0.3365E+05	0.4784E+06	0.2378E+06	0.4700E+06	0.2183E+05
0.5828E+00	0.4751E+06	0.3376E+05	0.4800E+06	0.1017E+06	0.4716E+06	0.2577E+05
0.8085E+00	0.4766E+06	0.3387E+05	0.4815E+06	0.6042E+05	0.4732E+06	0.3045E+05

DATA FITS STRAIGHT LINE OF SLOPE 0.1925E+01 AND STANDARD ERROR 0.1203E+00INTERCEPT -0.9730E+02
SLOPE CORRECTED FOR SPEED = 0.2594E-05S.E. = 0.1621E-06 ERRER 0.5967E+01

WEIGHT AVERAGE MOLECULAR WEIGHT = 0.4706E+06 AND STAADARD ERROR = 0.2941E+05



E 280/260 = 1.47

Fig. 39

UV spectrum of enzyme II from germinated Vicia faba seeds

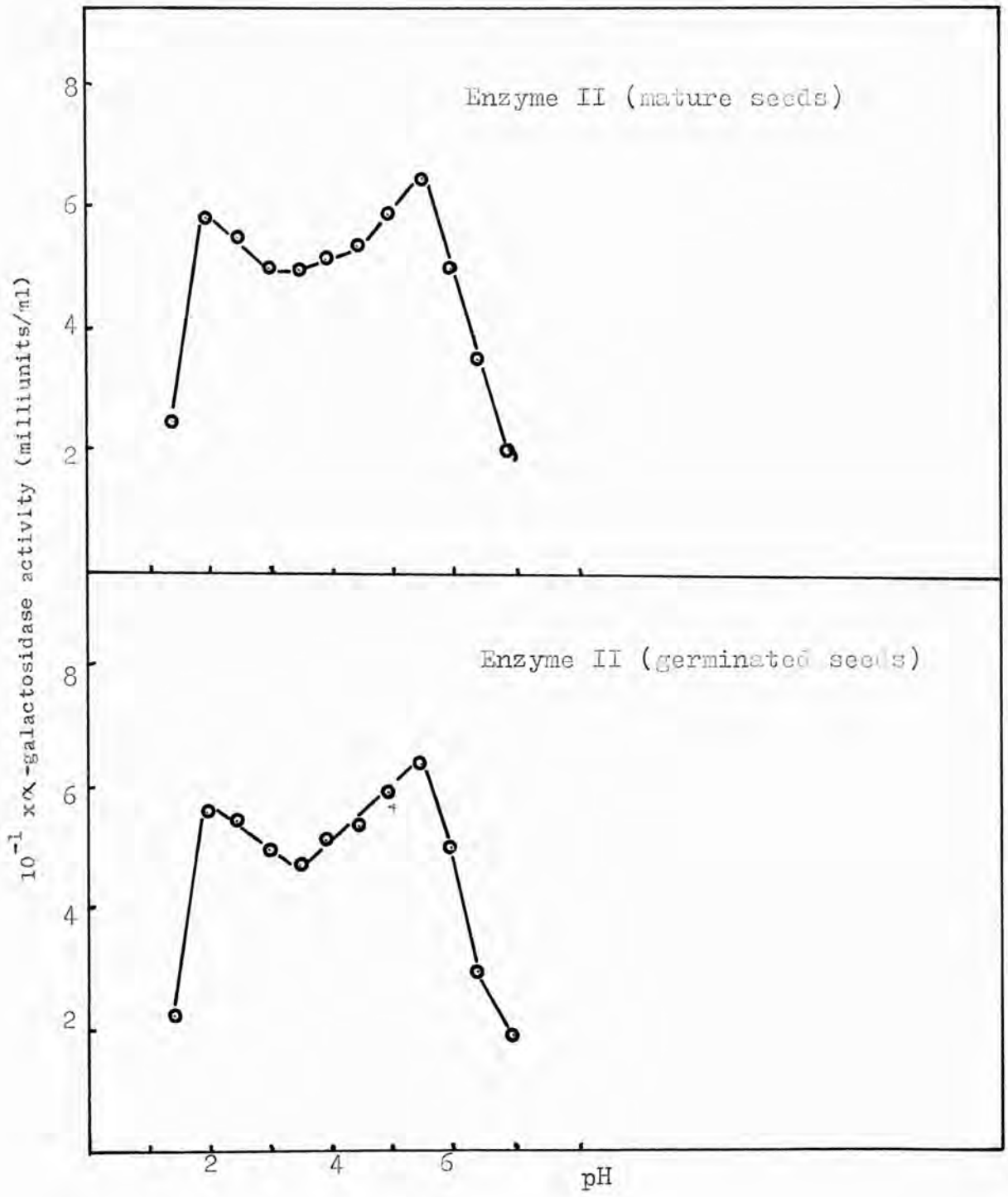


Fig. 40 . Comparison of pH optima of ∝-galactosidase II from mature Vicia faba seeds and enzyme II from germinated seeds.

enzyme II (a mixture of proteins) followed by a decrease in the activity of this form and the appearance of the high molecular weight isozyme I with a further increase in total activity. Some kind of 'compartmentalization' must be envisaged during this increase in activity as it parallels the storage of galactosylsucrose derivatives (see p.20) which are presumably the natural substrates for α-galactosidase. Whether this involves a physical barrier between enzymes and oligosaccharides or whether the activity is rendered latent by some other mechanism, such as the presence of an inhibitor, lack of K^+, or the presence of some other unfavourable microenvironment is not known.

Several factors suggest that enzyme I is produced during seed development from enzyme II. In particular, the amino acid compositions suggest this kind of relationship as does the study of the conversion of form II to form I in vitro. The number of units of II involved in the formation of I is, at present, not clear owing to the different molecular weight data obtained by gel-filtration and ultra-centrifuge procedures.

It is of interest to note that the fully developed seed possesses more of form I, with a high specific activity, than of II (the isozyme with low specific activity[44]). This presumably allows the seed to mobilize its galactose containing oligosaccharide reserves at a high rate during the early stages of germination when it is probable that the metabolism of soluble sugars is of more importance than that of starch.

A further observation of possible physiological significance is that α-galactosidase I but not II, will hydrolyse galactomannans. This could also be of importance at the onset of germination as a further source of energy for seedling development.

The process of germination with respect to α-galactosidase patterns, at a superficial level at least, appears to be a reversal of seed maturation. The level of enzyme I decreases rapidly and the activity of II increases: this is accompanied by a decrease in total α-galactosidase activity. It is not known whether this is a dissociation of I into units of II or whether the phenomenon is of a more complex nature. Dey *et al*. (221) have shown that the change is probably under matabolic control as cycloheximide interferes with the normal germination pattern and allows the enzyme complement to revert to that found in the dormant tissue.

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